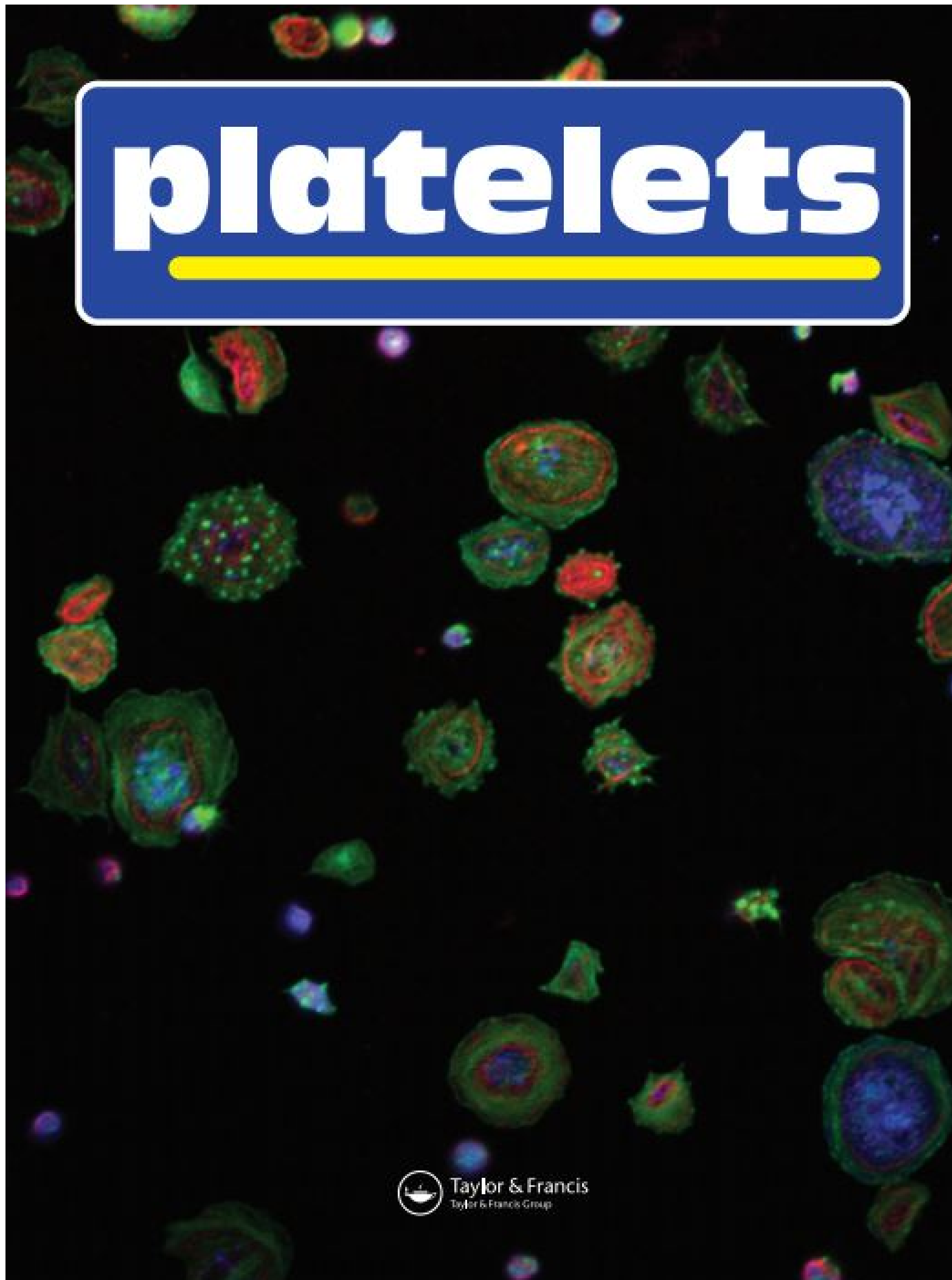

Plasma levels of the soluble form of the FcγRIIa receptor vary with receptor polymorphisms and are elevated in rheumatoid arthritis

Journal:	*Platelets*

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Plasma levels of the soluble form of the FcγRIIa receptor vary with receptor polymorphisms and are elevated in rheumatoid arthritis

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Running title: Soluble FcγRIIa levels and polymorphisms

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Abstract

Soluble forms of the low-affinity immunoglobulin receptor FcγRIIa (sFcγRIIa) lacking the cytoplasmic tail have been reported in plasma however the mechanism and functional consequences are unknown. This study aimed to evaluate mechanisms of FcγRIIa release compared to GPVI release from platelets, and examine whether genetic polymorphisms at positions 27 and 131 within FcγRIIa correlate with platelet FcγRIIa stability and function. Enzyme-linked immunosorbent assays (ELISAs) were used to measure plasma sFcγRIIa and sGPVI levels. FcγRIIa genotype at positions 27 and 131 was evaluated. sFcγRIIa levels were not significantly different between non-131HH and 131HH, but were significantly lower in 27W/131H than non-27W/131H. Treatment of platelets with aggregated immunoglobulin (Ig) G induced release of FcγRIIa and GPVI, but only sGPVI release was statistically significant, required functional FcγRIIa, and was blocked by inhibitors of signalling pathways and metalloproteinases. This indicated that sFcγRIIa was not released from platelets by metalloproteolysis. sFcγRIIa levels were not correlated with sGPVI levels in healthy individuals however levels of sFcγRIIa and sGPVI in plasma from patients with rheumatoid arthritis (RA) were significantly elevated above levels found in healthy individuals. Elevated level of sFcγRIIa in RA patients may reflect active immune-based arthritis and be predictive of active inflammation.

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Key words: FcγRIIa; polymorphism; platelet; GPVI; rheumatoid arthritis

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Introduction

FcγRIIa is the only IgG Fc-receptor expressed on the surface of platelets but is also found on neutrophils, monocytes, and macrophages [1, 2]. It is a low affinity receptor for Ig unique to humans and non-human primates, and composed of two extracellular Ig-like domains, containing the binding site for the Fc portion of IgG, a transmembrane domain and a cytoplasmic tail that contains a binding site for calmodulin and an immunoreceptor tyrosine-based activation motif (ITAM) domain that is essential for signal transduction and platelet activation. Both alleles of the gene encoding FcγRIIa are transcribed and translated, generating equal amounts of protein. A polymorphism, resulting from the amino acid substitution from arginine (R) to histidine (H) at position 131 in the second Ig-like domain of FcγRIIa has been previously shown to alter the binding affinity of the receptor for different IgG subclasses. FcγRIIa-H131 binds to human IgG2 with higher affinity than FcγRIIa-R131; however, FcγRIIa-R131 has a higher binding affinity for murine IgG1 than FcγRIIa-H131 [3-5]. Increasing evidence [6-9] demonstrated that the FcγRIIa polymorphism (H/R131) was associated with immune diseases due to different IgG binding affinity. A further polymorphism in FcγRIIa at position 27 (glutamine or tryptophan) was shown not to be linked with the polymorphism at position 131 and in isolation did not affect receptor function or IgG binding [10] but may have some impact on FcγRIIa signalling events [11].

Previous studies *in vitro* demonstrated that engagement of either FcγRIIa or GPVI on platelets by their respective ligands induced both intracellular calpain-mediated cleavage of FcγRIIa and extracellular metalloproteinase-mediated ectodomain shedding of GPVI [12]. Increased levels of sGPVI are also observed in patients with immune thrombocytopenia [13, 14] and in patients with seropositive rheumatoid arthritis [15]. It is postulated that increased release of sGPVI could be induced by engagement of platelet receptor FcγRIIa by the Fc

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portion of anti-platelet autoantibodies in the plasma of ITP patients. The cytoplasmic domain of FcγRIIa binds calmodulin [12], a hallmark of many metalloproteolytically shed membrane receptors including GPVI [16], GPV [17] and L-selectin [18]. Two FcγRIIa transcripts (with or without the transmembrane domain encoding exon, termed FcγRIIa1 and FcγRIIa2 respectively), resulting from alternative RNA splicing, have been found in human platelets and a megakaryocytic cell line [19]. A third transcript, FcγRIIa3 with a 19-amino acid cytoplasmic insertion was identified in peripheral blood mononuclear cells [20]. Further, there are other transcript variants (X1 to X10) which are predicted by automated computational analysis. Soluble forms of FcγRIIa (FcγRIIa2) reported in serum samples from healthy individuals and patients with chronic lymphocytic leukaemia are released from thrombin-activated human platelets [21] and megakaryocyte cell lines [22]. Activated neutrophils from systemic lupus erythematosus patients demonstrated a serine proteinase-dependent cleavage of FcγRIIa that was correlated with markers of disease severity and complement activation [23]. However, the mechanism by which FcγRIIa is released from blood cells and whether ligand-induced release of FcγRIIa similar to that observed with GPVI shedding occurs *in vivo* has not been addressed.

This study aimed to evaluate the proteolytic mechanisms of FcγRIIa release compared to GPVI release and examine whether genetic polymorphisms at positions 27 and 131 within FcγRIIa correlate with platelet FcγRIIa stability and function.

Materials and Methods

Reagents

N-ethylmaleimide (NEM), GM6001 (a broad-range hydroxamic acid-based metalloproteinase inhibitor), calmodulin inhibitor W7 (N-(6-aminohexyl)-5-chloro-1-aphthalenesulfonamide)

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and the membrane permeable calpain inhibitor E64d (L-3-carboxy-*trans*-2, 3-epoxypropionyl-L-leucylamido-(3-methyl) butane) were from Calbiochem (La Jolla, CA, USA). The hydroxamate-based inhibitor GI254023 has been previously described and characterized for preferential inhibition of ADAM10 over ADAM17 [24]. The Syk inhibitor, 2-(7-(3,4-dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5-ylamino)-nicotinamide dihydrochloride (BAY61-3606) was from Enzo Life Sciences (Farmingdale, NY, USA).

Antibodies

Anti-FcγRIIa monoclonal antibody 8.26 recognising a non-ligand binding region and HRP-conjugated anti-FcγRIIa monoclonal antibody 8.7 recognising a ligand binding region within domain 2 have been described [25]. 14A2, which binds to tetraspanin CD151 and engages FcγRIIa via its Fc domain, has been previously described [26, 27]. The anti-FcγRIIa monoclonal antibody, IV.3 [28] was purified from hybridoma medium on protein A-Sepharose (Amersham, United Kingdom).

Measurement of sFcγRIIa by ELISA

The ELISA method for measuring sFcγRIIa was based on an established sandwich ELISA used to measure sGPVI in plasma [29]. Briefly, 96-well microtitre plates were coated with a “capturing” mouse monoclonal anti-FcγRIIa IgG 8.26 (2 μg/ml) in coating buffer (0.05 M bicarbonate, pH 9.6) overnight at 4ºC. Wells were washed six times with PBS (0.01 M sodium phosphate, 0.15 M sodium chloride, pH 7.4) containing 0.2% (v/v) Tween-20 (PBS-T), blocked with 1% (w/v) BSA in PBS for 1 h at room temperature, then washed six times with PBS-T, before addition of samples diluted 10% (v/v) in PBS in triplicate wells. After 1 h incubation at room temperature, wells were washed six times with PBS-T and the HRP-conjugated anti-FcγRIIa monoclonal antibody 8.7 was added at 1 μg/ml (100 μl/well). After further 1 h incubation, wells were washed six times, 100 μl of a one-half dilution of Super Signal ELISA

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3 Pico chemiluminescent substrate (Pierce Rockford IL, USA) was added and light emission
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5 (stable after 1 min) was measured using a Wallac-Victor2 luminescence plate reader.
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8 **Blood collection and platelet aggregation**
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11 Experiments were carried out with the approval of the Monash University Standing committee
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13 on Ethics in Research Involving Humans, and informed consent was obtained from all
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15 participants in accordance with the Declaration of Helsinki. Platelet-rich plasma (PRP) was
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17 obtained from blood collected into 3.2% (w/v) trisodium citrate and centrifuged for 20 min at
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19 160 x *g*. Platelet-poor plasma (PPP) was obtained by centrifuging PRP at 1,350 x *g* for 15 min.
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21 Washed platelets were prepared as previously described [30]. Platelet aggregation in citrated
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23 PRP was carried out at 37ºC in a ChronoLog lumiaggregometer (Havertown, PA, USA) stirred
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25 at 900 rpm using PPP as control. Donors were of broad ethnicity and included donors with
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27 Caucasian, Asian, and Middle Eastern heritage. The donors were not preselected and all
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29 experimental work was completed before the genotype of each sample was revealed.
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32 **DNA sequencing**
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35 Genomic DNA was sequenced using the BigDye Terminator v1.1 cycle kit (Applied
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37 Biosystems, CA, USA). The sequenced region of the FcγRIIa protein included the two
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39 extracellular Ig domains. The sequences of primer 1 for DNA sequencing (the first Ig domain)
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41 were as follows: Forward: 5'-CCCCCAAAGGCTGTGCTGAAACTTGAGC-3'; Reverse: 5'-
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43 TCATTGTTGTTGGCCTTGAACCTGTAG-3'. The sequences of primer 2 for DNA
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45 sequencing (the second Ig domain) were: Forward: 5'-CATCATGCTGAGGTGCCACAGC-
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47 3'; Reverse: 5'-GATGAGAACAGCGTGTAGCCTATGTTTC-3'. For FcγRIIa genotype
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49 displayed in this study, at amino acid position 27 (located within the first Ig domain), the
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51 polymorphic residues are Q: Glutamine, W: Tryptophan; at position 131 (within the second Ig
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domain) the polymorphic residues are H: Histidine, R: Arginine. For example QW/HR represents heterozygosity at both positions 27 (Q/W) and 131 (H/R) polymorphic sites.

Analysis of sFcγRIIa in immune-related disease

To compare levels of sFcγRIIa and sGPVI in plasma from individuals with circulating immune complexes, citrated plasma samples were obtained from consecutively recruited patients with an established diagnosis of RA, or healthy donors. The samples were not matched for age or gender. These samples were obtained with institutional ethics approval at the Mater Misericordiae University Hospital, Dublin, Ireland. People with a history of cardiovascular disease or who were receiving anti-platelet therapy or thromboembolic prophylaxis were excluded.

Statistical analysis

GraphPad Prism software (version 6.0) was used to apply a two-tailed student's unpaired t-test to compare sFcγRIIa or sGPVI levels between different groups of known FcγRIIa genotype and a two-tailed Pearson rank was used to analyse correlations between sFcγRIIa and sGPVI. $P < 0.05$ was considered to be statistically significant. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; ns: not significant.

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Results

sFcγRIIa measurement in plasma by ELISA

Evidence in the literature indicates that a soluble form of FcγRIIa (sFcγRIIa) exists in plasma [21]. To measure levels of sFcγRIIa in human plasma of healthy donors or patients with immune-related disease, an ELISA was established using antibodies against separate epitopes within the ectodomain. The ELISA output was reported in relative units, since purified or

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3 recombinant sFcγRIIa proteins of known concentration were not available to enable calibrating
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6 the signal to a standard concentration curve. Serial dilution of sample produced a linear
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8 concentration-dependent signal. Serial dilution (from 1 in 2 to 1 in 64) of samples from either
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10 normal plasma or supernatant from washed platelets showed a linear relationship with relative
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13 light emission (Relative Unit, RLU) (Figure 1), with linear regression analysis for plasma (r^2 =
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15 0.998) and supernatant sample (r^2 = 0.984) within an acceptable limit. sFcγRIIa levels in
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17 plasma from 24 healthy individuals was 0.743 ± 0.048 x 10^6 RLU (Mean ± SE) (data not
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20 shown), with a normal range from 0.11 to 1.19 x 10^6 RLU. The ELISA had a coefficient of
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22 variation of 3.8% and inter-assay variability of less than 10% indicating acceptable precision.
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24 Linear regression equations provided the best fit for the data.
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27 **Effect of FcγRIIa polymorphisms on plasma sFcγRIIa levels**
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30 In 24 healthy individuals with known genotype of FcγRIIa, the plasma sFcγRIIa level appeared
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33 to be dependent upon genotype (Figure 2A), however as three of the genotypes contained data
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35 from only one individual, conclusions from this cohort were limited [31]. Of note, the allele
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37 frequency of the W27 variant in this cohort was 0.3125, which was significantly higher than
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40 the minor allele frequency reported in global genetics reference databases (MAF 0.06 to 0.1)
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42 [32]. The reason for this was not clear but may be related to the heterogeneous ethnicity of the
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44 donor group. To improve the statistical power, in subsequent analyses, we grouped data based
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47 on occurrence of HH at position 131. Previous studies demonstrated that the amino acid at
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49 position 131 influenced binding affinity of FcγRIIa for IgG complexes [10]. FcγRIIa with a
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51 histidine (H) has a higher binding affinity to human IgG2 than FcγRIIa with an arginine (R) [4,
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54 5]. However, in this study, the sFcγRIIa level was not significantly different in non-131H and
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56 131H/H (Figure 2B), but was significantly lower in 27W than 27Q (Figure 2C), indicating that
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sFcγRIIa release may be distinct from ligand binding and that FcγRIIa polymorphisms at position 27 and 131 might influence release of sFcγRIIa.

The mechanism of FcγRIIa release from platelets

Soluble forms of FcγRIIa have been detected in human serum or plasma [21-23, 33, 34]. Platelets express FcγRIIa and are a potential source of plasma sFcγRIIa by either secretion of a soluble form of the receptor lacking the transmembrane domain, or proteolytic shedding of the ectodomain from the platelet surface. Previous studies demonstrated that sGPVI is generated from platelet GPVI by metalloproteinase-mediated ectodomain shedding [16] and that engagement of platelet FcγRIIa also triggered shedding of GPVI [12]. GPVI and FcγRIIa are structurally analogous members of the immunoreceptor family, and both receptors bind calmodulin within the cytoplasmic domain, a common feature of metalloproteinase-dependent shedding. To test the hypothesis that engagement of FcγRIIa, induces metalloproteinase-mediated ectodomain shedding and releases soluble fragment, human washed platelets were treated with aggregated-IgG (A-IgG) in the presence or absence of metalloproteinase inhibitor EDTA. Levels of sFcγRIIa or sGPVI were subsequently measured by ELISA. Treatment of washed platelets with A-IgG induced release of sFcγRIIa (Figure 3A), and sGPVI (Figure 3B) with a trend towards dose-dependency. This was consistent with previous studies showing engagement of FcγRIIa or GPVI by their respective ligands triggers both calpain-mediated intracellular cleavage of FcγRIIa and metalloproteinase-mediated ectodomain shedding of GPVI [12]. The release of sGPVI but not sFcγRIIa was statistically significant. Further, blockade of FcγRIIa by inclusion of IV.3 monoclonal antibody or inclusion of inhibitors of metalloproteinases, calpain or signaling molecules prevented GPVI shedding (Figure 3B), but did not inhibit the generation of sFcγRIIa. This strongly suggested that the mechanism of sFcγRIIa release was different from GPVI shedding (metalloproteinase-mediated). Taken

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together, these data demonstrate that the presence of sFcγRIIa could possibly be driven by a mechanism involving one or more unidentified sheddases, but not by an EDTA-inhibitable metalloproteinase.

FcγRIIa polymorphisms, FcγRIIa release and GPVI shedding

In addition to sharing similar structures, both FcγRIIa and GPVI/FcRγ contain an immunoreceptor tyrosine-based activation motif (ITAM) within the cytoplasmic tail involved in signaling transduction. In human platelets treated with agonists acting at either FcγRIIa or GPVI *in vitro*, there is both intracellular calpain-mediated cleavage of FcγRIIa (removing the ITAM signaling sequence) and extracellular metalloproteinase-mediated ectodomain shedding of GPVI [12]. In order to examine the relationship between generation of sFcγRIIa and sGPVI *in vivo*, the respective ELISA assays were used to measure sFcγRIIa and sGPVI in the same plasma samples obtained from healthy individuals. The observed lack of correlation between sFcγRIIa and sGPVI (Figure 4A) is not only consistent with the lack of effect of FcγRIIa polymorphisms on GPVI shedding (Figure 4B), but also suggests that sFcγRIIa could be derived from other vascular cells, since FcγRIIa is also expressed and shed from neutrophils and monocytes/macrophages [23]. Our previous studies demonstrated that coagulation induces platelet GPVI shedding as sGPVI level was markedly elevated in serum. To assess whether coagulation influences FcγRIIa release, blood was collected in different anti-coagulants or clotting tube and sFcγRIIa level was measured by ELISA with sGPVI level as a positive control. We showed that unlike elevated serum sGPVI level, sFcγRIIa level in serum is comparable with plasma level in citrate, acid citrate dextrose or EDTA anti-coagulants (data not shown), indicating that unlike GPVI, coagulation did not affect FcγRIIa release. All these results displayed above revealed that different mechanisms are likely to be involved in release of FcγRIIa, compared with GPVI release.

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FcγRIIa polymorphisms, release and platelet aggregation

14A2, a murine monoclonal antibody against CD151, induces platelet activation and aggregation *via* engagement of FcγRIIa [27]. Platelet aggregation in response to 14A2 contributed to identifying functional differences associated with FcγRIIa polymorphisms [35]. In the present study, 14A2 at a threshold concentration (1.0 µg/ml,) was used to investigate platelet aggregation and polymorphisms of FcγRIIa. Based on the time taken to achieve 50% aggregation, platelet aggregation to 14A2 was divided into quartiles (+++, ++, +, ---). Among 24 healthy individuals with known genotype of FcγRIIa (Table 1), platelet aggregation in response to 14A2 was heterogeneous, consistent with published studies [26, 35]. Standard appropriately powered statistical evaluation approaches could not be used to evaluate genotype and propensity to aggregate, due to three genotypes containing data from a single individual. However, in healthy individuals with HH at position 131 of FcγRIIa, platelet aggregation was completely impaired regardless of the polymorphism at 27, possibly due to the lower binding affinity to murine IgG (14A2) for FcγRIIa with HH [10]. However, non-responsiveness to 14A2 was also found in individuals with HR, indicating that other factors, such as copy number or surface density of FcγRIIa, or genetic variation of the protein tyrosine phosphatase CD148 that regulates ITAM receptor-mediated platelet activation could also contribute to the function of FcγRIIa [36]. The plasma level of sFcγRIIa under resting conditions was also heterogeneous in individuals with different platelet responses to 14A2.

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Analysis of sFcγRIIa in immune-related disease

There is increasing evidence showing the critical role of FcRs in auto-immune disease induced by immune complexes [1, 23, 37, 38], specifically the importance of activating FcγRIIa in the pathogenesis of RA [39]. Available plasma samples from RA patients, previously used to measure levels of sGPVI, provided an opportunity to apply the new ELISA to measure

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sFcγRIIa levels in the same samples. As seen in Figure 5A, the sFcγRIIa level in RA patients was significantly higher than that of healthy controls, indicating FcγRIIa may be a potential marker in the pathogenesis of RA, and consistent with previous studies [39]. Plasma sGPVI levels were also significantly higher in RA patients when compared with controls (Figure 5B), revealing platelets might also play a role in this disease. There was no correlation between sFcγRIIa and sGPVI in RA patients (Figure 5C), consistent with data obtained from healthy individuals. These results support the possibility that the release mechanisms for these two receptors are different, further supporting the *in vitro* studies of shedding mechanisms shown in Figure 3. Unfortunately, genotyping of the RA patients was not possible, and detailed clinical information on the progression and outcomes of disease in different individuals could not be obtained. Nevertheless, findings presented here provide pilot data which would justify expanded future studies of platelet activation and plasma sFcγRIIa analysis in RA using the ELISA and reference data described in this study.

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Discussion

FcγRIIa is the only IgG-Fc receptor with low affinity expressed on platelets and megakaryocytes. Three RNA splice variants of the *FCGR2A* gene have been described with FcγRIIa1 the most widely studied. FcγRIIa2 is secreted or released from human epidermal Langerhans cells [40], neutrophils [23, 34] or thrombin-activated human platelets or a megakaryocytic cell line [22]. Subsequent studies demonstrated that FcγRIIa2 lacking a hydrophobic segment of the transmembrane exonic sequence was present in human serum [21]. FcγRIIa3, is identical to FcγRIIa1 with the addition of a 19-amino acid insertion in the juxta-membrane region of the cytoplasmic tail. However, to date the levels of sFcγRIIa present in normal plasma or in immune-related disease states have not been widely analysed in terms of

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normal variability between individuals or how levels change in immune or other disease states. In this study, an ELISA was developed for quantifying relative levels of sFcγRIIa in human plasma, to analyse the mechanism of sFcγRIIa release from platelets, evaluate the effect of sFcγRIIa genotype on function, and increase understanding of sFcγRIIa as a biomarker and/or role in the pathology of immune-related platelet dysfunction.

Our previous *in vitro* study showed engagement of either GPVI or FcγRIIa on platelet by their respective ligands induced both extracellular metalloproteinase-mediated ectodomain shedding of GPVI and intracellular calpain-mediated proteolysis of FcγRIIa [12]. When platelets were treated with A-IgG, release of GPVI (sGPVI) and FcγRIIa (sFcγRIIa) was detected by ELISA in a dose-dependent manner. From our study, several lines of evidence suggest that the release of sFcγRIIa from human platelets is regulated by a different mechanism to that involved in shedding of GPVI. First, A-IgG-induced release of sFcγRIIa from platelets is not inhibited by metalloproteinase inhibitors or by inhibitors of signaling pathways involved in GPVI shedding, consistent with a recent study showing metalloproteinase inhibitor GM6001 and E64d did not affect FcγRIIa shedding from neutrophils after addition of TLR agonists. In this case, TLR agonist-induced FcγRIIa shedding was shown to be dependent on serine proteases, including the pro-protein convertase furin [23]. Second, coagulation induced pronounced shedding of GPVI [41], whereas plasma and serum levels of sFcγRIIa were comparable, consistent with a previous study showing serum and plasma sFcγRIIa levels were similar [21]. Third, there is no apparent correlation between levels of sGPVI and sFcγRIIa in normal healthy plasma, which might have been expected to correlate if common triggers or pathways were involved in regulating their release from platelets. Although in this regard, unlike sGPVI which is only derived from platelets, sFcγRIIa may be derived from platelets or other cell types.

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Previous studies demonstrated that the FcγRIIa polymorphism (H131R) was associated with some autoimmune diseases due to different IgG binding affinity [6, 7, 9] and another polymorphism at position 27 (W/Q) did not affect receptor function or IgG binding and was not linked with the polymorphism at position 131 [10]. Regarding the plasma sFcγRIIa level in individuals with different genotype, we showed the sFcγRIIa level was not significantly different between H131H and non-H131H groups, but is significantly lower in the 27W group, indicating sFcγRIIa release may not depend on ligand binding. Our work indicates that the genotype at positions 27 and 131 influence receptor stability of FcγRIIa in healthy individuals. These findings provide a potential mechanistic basis for altered responsiveness of platelets to antiplatelet antibodies, and may provide a suitable rationale justifying future studies to evaluate the link between genotype and immune-based platelet defects.

Consistent with previous studies showing FcγRIIa-R131 binds to murine IgG with higher binding affinity than FcγRIIa-H131 [6, 7] and that the amino acid at position 27 did not affect receptor function [10], platelet aggregation response to the threshold concentration of 14A2, a murine monoclonal antibody against CD151, was completely impaired in individuals with H131H, regardless of the amino acid at polymorphic position 27. However, impaired platelet aggregation was also observed in some individuals with H131R, suggesting apart from polymorphism, other factors, such as surface expression of FcγRIIa, genetic variation of the protein tyrosine phosphatase CD148 could also be involved in FcγRIIa-dependent platelet aggregation [36].

Finally, an ELISA for measuring sFcγRIIa in human plasma should ultimately enable the levels of sFcγRIIa to be evaluated in diseases involving elevated immune complexes. In this study, preliminary analysis of sFcγRIIa in healthy donors and patients with arthritis has shown that plasma levels of sFcγRIIa were significantly higher in RA patients when compared

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with control (healthy donors), indicating FcγRIIa was involved in the pathogenesis of RA, consistent with previous studies. sFcγRIIa level measured by ELISA may reflect active immune-based arthritis and be predictive of active inflammation. In addition, plasma sGPVI level was also elevated in patients with RA when compared with control, implying platelet and/or collagen receptor GPVI might also be involved in RA, consistent with inflammation amplified by platelet in arthritis via collagen/GPVI-dependent microparticle generation [42]. The elevated sGPVI level might be due to GPVI-dependent platelet activation induced by collagen binding in RA. Unfortunately there was no genomic information available for this RA cohort to evaluate the FcγRIIa polymorphisms. It is worth noting however that other studies have identified an association between the FCGR2A R allele and RA in Europeans (p=0.020), but not in East Asians populations [43], or in a Japanese cohort [44].

In conclusion, it appears that soluble FcγRIIa release results from secretion, possibly dependent on ligand engagement of FcγRIIa, rather than ectodomain shedding, consistent with FcγRIIa release from activated human platelets *in vitro*. Recombinant soluble FcγRIIa has been previously shown to inhibit Fc-mediated human platelet aggregation in a dose-dependent manner [22]. Therefore, soluble FcγRIIa released from platelets could be a down-regulation mechanism of platelet function activated *via* Fc binding in autoimmune disease.

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Author Contributions

JQ performed research and interpreted data; ED and BW developed key reagents and interpreted data; DK, GMM, PMH and KX provided key patient samples and reagents; RKA and EEG designed the research and interpreted the data; all authors co-wrote the manuscript.

Conflicts of Interest

All authors have no conflict of interest to declare.

References

1. Hogarth, P. M. and Pietersz, G. A. (2012) Fc receptor-targeted therapies for the treatment of inflammation, cancer and beyond. Nat Rev Drug Discov 11, 311-31.
2. Qiao, J., Al-Tamimi, M., Baker, R. I., Andrews, R. K., Gardiner, E. E. (2015) The platelet Fc receptor, FcγRIIa. Immunol. Rev. 268, 241-252.
3. Parren, P. W., Warmerdam, P. A., Boeije, L. C., Arts, J., Westerdaal, N. A., Vlug, A., Capel, P. J., Aarden, L. A., van de Winkel, J. G. (1992) On the interaction of IgG subclasses with the low affinity Fc gamma RIIa (CD32) on human monocytes, neutrophils, and platelets. Analysis of a functional polymorphism to human IgG2. J. Clin. Invest. 90, 1537-46.
4. Salmon, J. E., Edberg, J. C., Brogle, N. L., Kimberly, R. P. (1992) Allelic polymorphisms of human Fcγ receptor IIA and Fcγ receptor IIIB. Independent mechanisms for differences in human phagocyte function. J. Clin. Invest. 89, 1274-81.
5. Tate, B. J., Witort, E., McKenzie, I. F., Hogarth, P. M. (1992) Expression of the high responder/non-responder human FcγRII. Analysis by PCR and transfection into FcR-COS cells. Immunol. Cell Biol. 70 (Pt 2), 79-87.
6. Ali, M. M., Elghazali, G., Montgomery, S. M., Farouk, S. E., Nasr, A., Noori, S. I., Shamad, M. M., Fadlelseed, O. E., Berzins, K. (2007) FcγRIIa (CD32) polymorphism and onchocercal skin disease: implications for the development of severe reactive onchodermatitis (ROD). Am. J. Trop. Med. Hyg. 77, 1074-8.
7. Forthal, D. N., Landucci, G., Bream, J., Jacobson, L. P., Phan, T. B., Montoya, B. (2007) FcγRIIa genotype predicts progression of HIV infection. J. Immunol. 179, 7916-23.
8. Carlsson, L. E., Santoso, S., Baurichter, G., Kroll, H., Papenberg, S., Eichler, P., Westerdaal, N. A. C., Kiefel, V., van de Winkel, J. G. J., Greinacher, A. (1998) Heparin-

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induced thrombocytopenia: new insights into the impact of the FcγRIIa-R-H131 polymorphism. Blood 92, 1526-1531.
9. Rollin, J., Pouplard, C., Cheng Sung, H., Leroux, D., Saada, A., Gouilleux-Gruart, V., Thibault, G., Gruel, Y. (2015) Increased risk of thrombosis in FcγRIIA 131RR patients with HIT due to defective control of platelet activation by plasma IgG2. Blood 125, 2397-2404.
10. Warmerdam, P. A., van de Winkel, J. G., Vlug, A., Westerdaal, N. A., Capel, P. J. (1991) A single amino acid in the second Ig-like domain of the human Fcγ receptor II is critical for human IgG2 binding. J. Immunol. 147, 1338-1343.
11. Flinsenberg, T. W. H., Janssen, W. J., Herczenik, E., Boross, P., Nederend, M., Jongeneel, L. H., Scholman, R. C., Boelens, J.-J., Maas, C., van Gijn, M. E., van Montfrans, J. M., Leusen, J. H., Boes, M. (2014) A novel FcγRIIa Q27W gene variant is associated with common variable immune deficiency through defective FcγRIIa downstream signaling. Clin. Immunol. 155, 108-117.
12. Gardiner, E. E., Karunakaran, D., Arthur, J. F., Mu, F. T., Powell, M. S., Baker, R. I., Hogarth, P. M., Kahn, M. L., Andrews, R. K., Berndt, M. C. (2008) Dual ITAM-mediated proteolytic pathways for irreversible inactivation of platelet receptors: De-ITAM-izing FcγRIIa. Blood 111, 165-174.
13. Qiao, J., Schoenwaelder, S. M., Mason, K. D., Tran, H., Davis, A. K., Kaplan, Z. S., P., J. S., Kile, B. T., Andrews, R. K., Roberts, A. W., Gardiner, E. E. (2013) Low adhesion receptor levels on circulating platelets in patients with lymphoproliferative diseases prior to receiving Navitoclax (ABT-263). Blood 121, 1479-1481.
14. Gardiner, E. E., Al-Tamimi, M., Mu, F. T., Karunakaran, D., Thom, J. Y., Moroi, M., Andrews, R. K., Berndt, M. C., Baker, R. I. (2008) Compromised ITAM-based platelet receptor function in a patient with immune thrombocytopenic purpura. J. Thromb. Haemost. 6, 1175-1182.
15. Stack, J. R., Madigan, A., Helbert, L., Dunne, E., Gardiner, E. E., Andrews, R. K., Finan, R., Smyth, E., Kenny, D., McCarthy, G. M. (2017) Soluble glycoprotein VI, a specific marker of platelet activation is increased in the plasma of subjects with seropositive rheumatoid arthritis. PLoS One 12, e0188027.
16. Gardiner, E. E., Arthur, J. F., Kahn, M. L., Berndt, M. C., Andrews, R. K. (2004) Regulation of platelet membrane levels of glycoprotein VI by a platelet-derived metalloproteinase. Blood 104, 3611-3617.
17. Rabie, T., Strehl, A., Ludwig, A., Nieswandt, B. (2005) Evidence for a role of ADAM17 (TACE) in the regulation of platelet glycoprotein V. J Biol Chem 280, 14462-8.
18. Kahn, J., Walcheck, B., Migaki, G. I., Jutila, M. A., Kishimoto, T. K. (1998) Calmodulin regulates L-selectin adhesion molecule expression and function through a protease-dependent mechanism. Cell 92, 809-18.
19. Cassel, D. L., Keller, M. A., Surrey, S., Schwartz, E., Schreiber, A. D., Rappaport, E. F., McKenzie, S. E. (1993) Differential expression of FcγRIIA, FcγRIIB and FcγRIIC in hematopoietic cells: analysis of transcripts. Mol. Immunol. 30, 451-60.
20. Anania, J. C., Trist, H. M., Palmer, C. S., Tan, P. S., Kouskousis, B. P., Chenoweth, A. M., Kent, S. J., Mackay, G. A., Hoi, A., Koelmeyer, R., Slade, C., Bryant, V. L., Hodgkin, P. D., Aui, P. M., van Zelm, M. C., Wines, B. D., Hogarth, P. M. (2018) The rare anaphylaxis-associated FcγRIIa3 exhibits distinct characteristics from the canonical FcγRIIa1. Front. Immunol. 9, 1809.
21. Astier, A., Merle-Beral, H., de la Salle, H., Moncuit, J., Cazenave, J. P., Fridman, W. H., Hanau, D., Teillaud, J. L. (1997) Soluble Fcγ receptor, FcγRIIa2, is present in two

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forms in human serum and is increased in patients: with stage C chronic lymphocytic leukemia. Leuk. Lymphoma 26, 317-26.

22. Gachet, C., Astier, A., de la Salle, H., de la Salle, C., Fridman, W. H., Cazenave, J. P., Hanau, D., Teillaud, J. L. (1995) Release of FcγRIIa2 by activated platelets and inhibition of anti-CD9-mediated platelet aggregation by recombinant FcγRIIa2. Blood 85, 698-704.
23. Lood, C., Arve, S., Ledbetter, J., Elkon, K. B. (2017) TLR7/8 activation in neutrophils impairs immune complex phagocytosis through shedding of FcγRIIA. J. Exp. Med. 214, 2103-2119.
24. Ludwig, A., Hundhausen, C., Lambert, M. H., Broadway, N., Andrews, R. C., Bickett, D. M., Leesnitzer, M. A., Becherer, J. D. (2005) Metalloproteinase inhibitors for the disintegrin-like metalloproteinases ADAM10 and ADAM17 that differentially block constitutive and phorbol ester-inducible shedding of cell surface molecules. Comb Chem High Throughput Screen 8, 161-71.
25. Ierino, F. L., Hulett, M. D., McKenzie, I. F., Hogarth, P. M. (1993) Mapping epitopes of human Fc gamma RII (CDw32) with monoclonal antibodies and recombinant receptors. J. Immunol. 150, 1794-1803.
26. Roberts, J. J., Rodgers, S. E., Drury, J., Ashman, L. K., Lloyd, J. V. (1995) Platelet activation induced by a murine monoclonal antibody directed against a novel tetra-span antigen. Br. J. Haematol. 89, 853-60.
27. Ashman, L. K., Aylett, G. W., Mehrabani, P. A., Bendall, L. J., Niutta, S., Cambareri, A. C., Cole, S. R., Berndt, M. C. (1991) The murine monoclonal antibody, 14A2.H1, identifies a novel platelet surface antigen. Br. J. Haematol. 79, 263-70.
28. Looney, R. J., Ryan, D. H., Takahashi, K., Fleit, H. B., Cohen, H. J., Abraham, G. N., Anderson, C. L. (1986) Identification of a second class of IgG Fc receptors on human neutrophils. A 40 kilodalton molecule also found on eosinophils. J Exp Med 163, 826-36.
29. Al-Tamimi, M., Mu, F. T., Moroi, M., Gardiner, E. E., Berndt, M. C., Andrews, R. K. (2009) Measuring soluble platelet glycoprotein VI in human plasma by ELISA. Platelets 20, 143-149.
30. Qiao, J., Arthur, J. F., Collecutt, M., Shen, Y., Mu, F. T., Berndt, M. C., Davis, A. K., Andrews, R. K., Gardiner, E. E. (2012) An acquired defect associated with abnormal signaling of the platelet collagen receptor glycoprotein VI. Acta Haematol 128, 233-41.
31. Winters, R., Winters, A., Amedee, R. G. (2010) Statistics: a brief overview. Ochsner J 10, 213-216.
32. The Genomes Project (2018) Database of single nucleotide polymorphisms (dbSNP). National Center for Biotechnology Information, National Library of Medicine, Bethesda, Maryland. https://www.ncbi.nlm.nih.gov/snp/rs139202723
33. Astier, A., de la Salle, H., de la Salle, C., Bieber, T., Esposito-Farese, M. E., Freund, M., Cazenave, J. P., Fridman, W. H., Teillaud, J. L., Hanau, D. (1994) Human epidermal Langerhans cells secrete a soluble receptor for IgG (FcγRII/CD32) that inhibits the binding of immune complexes to FcγR+ cells. J. Immunol. 152, 201-12.
34. Nagarajan, S., Venkiteswaran, K., Anderson, M., Sayed, U., Zhu, C., Selvaraj, P. (2000) Cell-specific, activation-dependent regulation of neutrophil CD32A ligand-binding function. Blood 95, 1069-77.
35. Mazurov, A. V., Vinogradov, D. V., Vlasik, T. N., Burns, G. F., Berndt, M. C. (1992) Heterogeneity of platelet Fc-receptor-dependent response to activating monoclonal antibodies. Platelets 3, 181-8.

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36. Rollin, J., Pouplard, C., Gratacap, M. P., Leroux, D., May, M. A., Aupart, M., Gouilleux-Gruart, V., Payrastre, B., Gruel, Y. (2012) Polymorphisms of protein tyrosine phosphatase CD148 influence FcγRIIA-dependent platelet activation and the risk of heparin-induced thrombocytopenia. Blood 120, 1309-16.
37. Takai, T. (2005) Fc receptors and their role in immune regulation and autoimmunity. J Clin Immunol 25, 1-18.
38. Pritchard, N. R., Cutler, A. J., Uribe, S., Chadban, S. J., Morley, B. J., Smith, K. G. (2000) Autoimmune-prone mice share a promoter haplotype associated with reduced expression and function of the Fc receptor FcγRII. Curr. Biol. 10, 227-30.
39. Mullazehi, M., Mathsson, L., Lampa, J., Ronnelid, J. (2006) Surface-bound anti-type II collagen-containing immune complexes induce production of tumor necrosis factor-α, interleukin-1β, and interleukin-8 from peripheral blood monocytes via Fcγ receptor IIA: a potential pathophysiologic mechanism for humoral anti-type II collagen immunity in arthritis. Arthritis Rheum. 54, 1759-71.
40. de la Salle, C., Esposito-Farese, M. E., Bieber, T., Moncuit, J., Morales, M., Wollenberg, A., de la Salle, H., Fridman, W. H., Cazenave, J. P., Teillaud, J. L., et al. (1992) Release of soluble FcγRII/CD32 molecules by human Langerhans cells: a subtle balance between shedding and secretion? J. Invest. Dermatol. 99, 15S-17S.
41. Al-Tamimi, M., Grigoriadis, G., Tran, H., Paul, E., Servadei, P., Berndt, M. C., Gardiner, E. E., Andrews, R. K. (2011) Coagulation-induced shedding of platelet glycoprotein VI mediated by factor Xa. Blood 117, 3912-20.
42. Boilard, E., Nigrovic, P. A., Larabee, K., Watts, G. F., Coblyn, J. S., Weinblatt, M. E., Massarotti, E. M., Remold-O'Donnell, E., Farndale, R. W., Ware, J., Lee, D. M. (2010) Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. Science 327, 580-3.
43. Lee, Y. H., Bae, S. C., Song, G. G. (2015) FCGR2A, FCGR3A, FCGR3B polymorphisms and susceptibility to rheumatoid arthritis: a meta-analysis. Clin. Exp. Rheumatol. 33, 647-54.
44. Kyogoku, C., Tsuchiya, N., Matsuta, K., Tokunaga, K. (2002) Studies on the association of Fcγ receptor IIA, IIB, IIIA and IIIB polymorphisms with rheumatoid arthritis in the Japanese: evidence for a genetic interaction between HLA-DRB1 and FCGR3A. Genes Immun. 3, 488-493.

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Figure legends

Figure 1. Measurement of sFcγRIIa by ELISA assay. Serial dilution of plasma (A) or supernatant (S/N) from washed platelets (B) was used to measure the soluble level of FcγRIIa by ELISA as described in Methods.

Figure 2. sFcγRIIa levels in healthy individuals with different FcγRIIa genotype. Plasma or genomic DNA from 24 healthy individuals was used to measure sFcγRIIa level by ELISA and FcγRIIa genotype by DNA sequencing. (A) Six different alleles of FcγRIIa were identified. Plasma from healthy individuals with (B) non-HH and HH genotype or (C) 27Q and 27W genotypes of FcγRIIa were used for sFcγRIIa measurement. Unpaired student t-test was used to compare sFcγRIIa level with different genotypes. $^{**}P<0.01$; ns: not significant.

Figure 3. sFcγRIIa and sGPVI release from platelets. Washed platelets from healthy donors were treated with A-IgG in the presence or absence of EDTA (10 mM), IV.3 (10 μg/ml), GM6001 (100 μM), BAY-61 (5 μM), E64d (10 μM) (all final concentrations) for 3 h at room temperature and supernatant was isolated to measure soluble forms of (A) FcγRIIa and (B) GPVI by ELISA. The bars represent mean ± SEM for triplicate data points. Significant differences from the untreated sample were evaluated using 1-way ANOVA with Dunnett's multiple comparisons test. *$p<0.05$; **$p<0.01$; ***$p<0.005$.

Figure 4. sGPVI and sFcγRIIa level in healthy individuals. (A) Plasma obtained from 27 healthy individuals was used to evaluate any relationship between sGPVI and sFcγRIIa levels by ELISA. Plasma sGPVI level in healthy individuals with (B) non-HH and HH genotype or (C) non 27W/131H and 27W/131H genotype of FcγRIIa. Unpaired student t-test was used to compare sFcγRIIa level with different genotypes. ns: not significant.

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Figure 5. Levels of sFcγRIIa and sGPVI in plasma from RA patients or healthy donors.

Plasma from RA patients or healthy donors (control) was used to measure (A) sFcγRIIa or (B) sGPVI by ELISA. (C) A correlative analysis of sFcγRIIa and sGPVI in RA patients. $^{***}p < 0.001$; $^{**}p < 0.01$.

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Table 1 Summary of FcγRIIa genotypes in healthy individuals. FcγRIIa genotype at position 27 and 131, platelet aggregation in response to 14A2 and sFcγRIIa levels in plasma from 24 healthy donors. The ranges of times taken to achieve 50% aggregation are < 2 min (+++), 2-3min (++) and ≥ 3min (+) respectively. **---** indicated no platelet aggregation in response to 1 μg/ml 14A2 after 20 min. All data were obtained using PRP and were reproducible on different days (n=2-3).

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	FcγRIIa genotype		Aggregation (1.0 μg/ml 14A2)				Plasma soluble FcγRIIa (RLU x 10^6) (range)
n	**27**	**131**	+++	++	+	---	
3	QQ	HH				3	0.89-1.19
1	QW	HH				1	0.61
1	WW	HH				1	0.31
12	QQ	HR	1	4	2	5	0.49-1.19
6	QW	HR		4	2		0.32-0.80
1	QQ	RR		1			0.11

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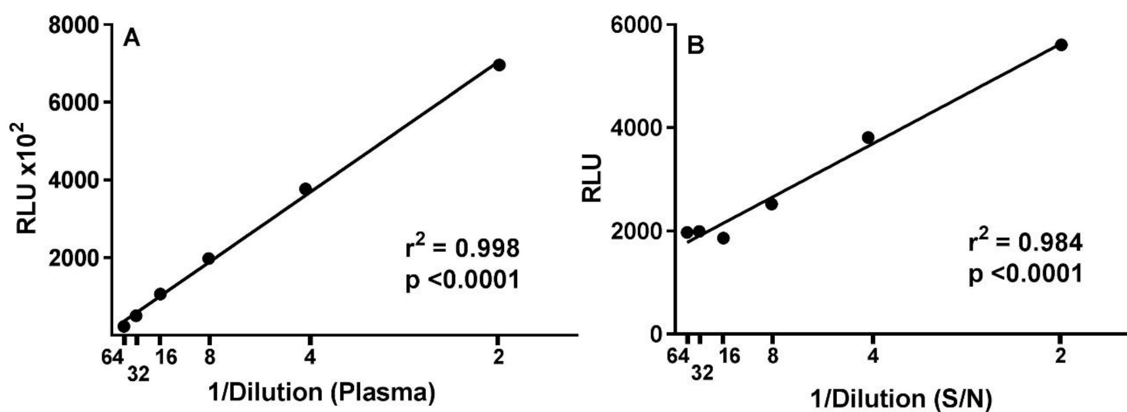
Figure 1



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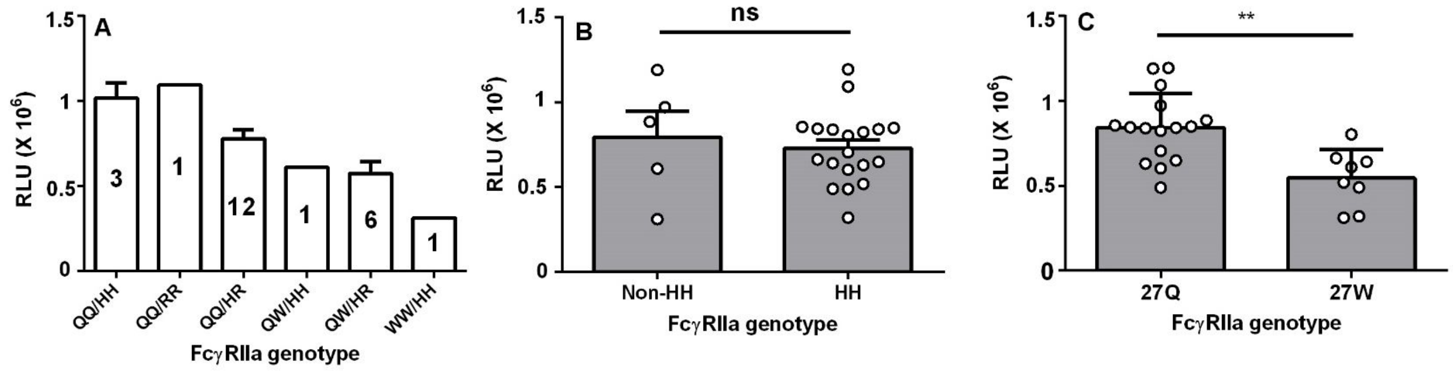

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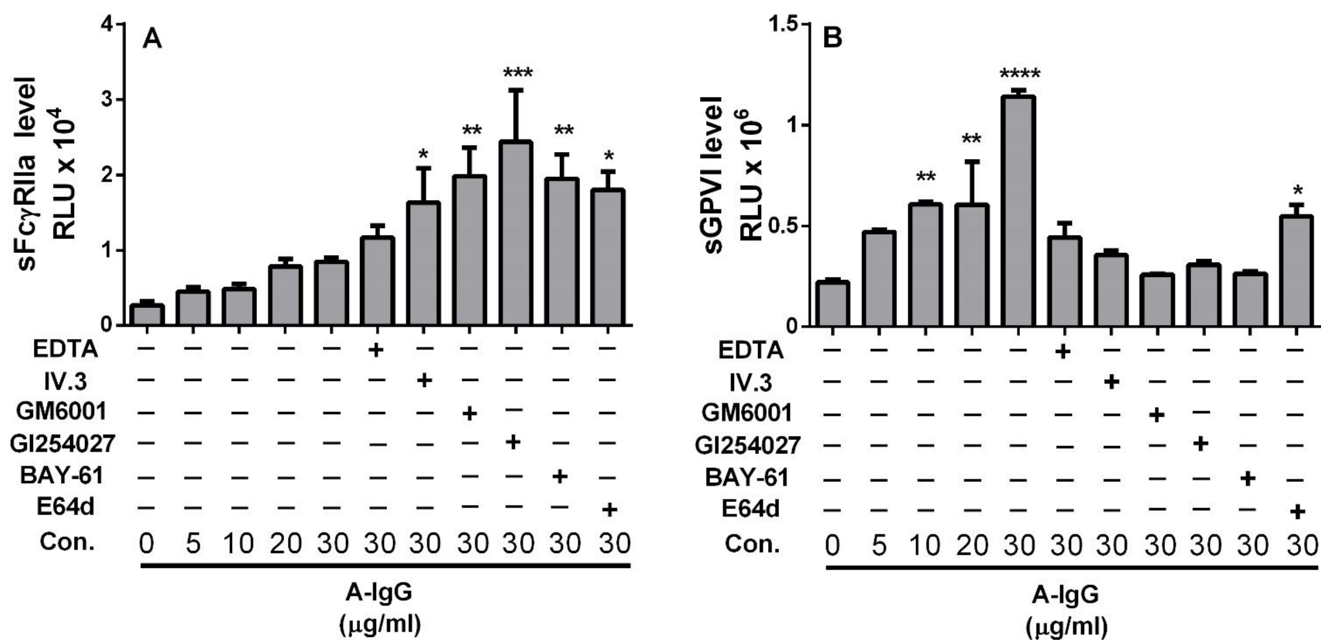

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Figure 4

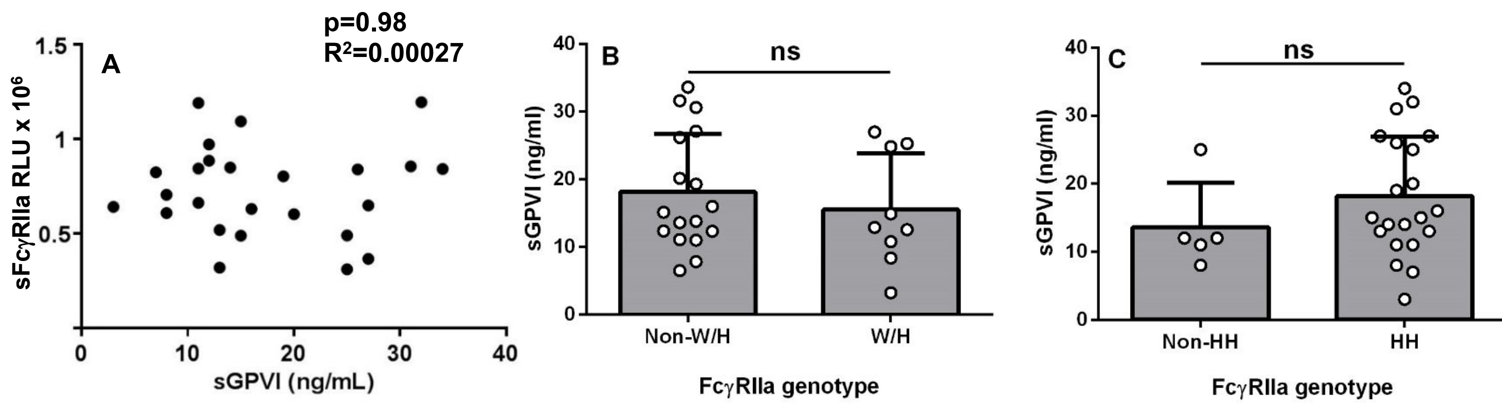

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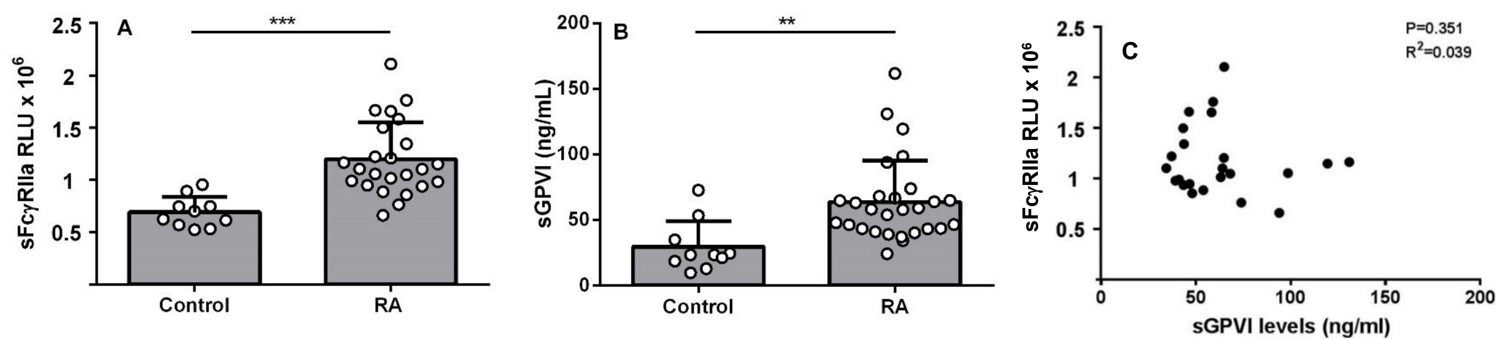
Figure 5



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