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## Interleukin 1 Receptor 1 and Interleukin 1 $\beta$ Regulate Megakaryocyte Maturation, Platelet Activation, and Transcript Profile During Inflammation in Mice and Humans

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### Abstract

**Objective**—Interleukin 1 Receptor 1 (IL1R1) and its ligand, IL1 $\beta$ , are upregulated in cardiovascular disease, obesity, and infection. Previously, we reported a higher level of IL1R1 transcripts in platelets from obese individuals of the Framingham Heart Study (FHS), but its functional effect in platelets has never been described. Additionally, IL1 $\beta$  levels are increased in atherosclerotic plaques and in bacterial infections. The aim of this work is to determine whether IL1 $\beta$ , through IL1R1, can activate platelets and megakaryocytes to promote atherothrombosis.

**Approach and Results**—We found that IL1 $\beta$ -related genes from platelets, as measured in 1819 FHS participants, were associated with increased body mass index, and a direct relationship was shown in wild-type mice fed a high-fat diet. Mechanistically, IL1 $\beta$  activated nuclear factor- $\kappa$ B and mitogen-activated protein kinase signaling pathways in megakaryocytes. IL1 $\beta$ , through IL1R1, increased ploidy of megakaryocytes to 64+ N by 2-fold over control. IL1 $\beta$  increased agonist-induced platelet aggregation by 1.2-fold with thrombin and 4.2-fold with collagen. IL1 $\beta$  increased adhesion to both collagen and fibrinogen, and heterotypic aggregation by 1.9-fold over resting. High fat diet-enhanced platelet adhesion was absent in IL1R1<sup>-/-</sup> mice. Wild-type mice infected with *Porphyromonas gingivalis* had circulating heterotypic aggregates (1.5-fold more than control at 24 hours and 6.2-fold more at 6 weeks) that were absent in infected IL1R1<sup>-/-</sup> and IL1 $\beta$ <sup>-/-</sup> mice.

**Conclusions**—In summary, IL1R1- and IL1 $\beta$ -related transcripts are elevated in the setting of obesity. IL1R1/IL1 $\beta$  augment both megakaryocyte and platelet functions, thereby promoting a

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#### Disclosures

None.

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prothrombotic environment during infection and obesity; potentially contributing to the development of atherothrombotic disease.

### Keywords

blood platelets; diet; high-fat; IL1R1 protein; human; infection; megakaryocytes

A proinflammatory environment attributable to either an acute bacterial infection or a chronic disease, such as obesity, has effects on multiple cell types and organs beyond the immune system and infected tissue. Obesity, considered a chronic inflammatory state, is associated with an increase in circulating proinflammatory cytokines, including interleukin 1 $\beta$  (IL1 $\beta$ ).<sup>1,2</sup> Some bacterial infections, including *Porphyromonas gingivalis* (*P. gingivalis*), are also associated with an increase in circulating IL1 $\beta$ .<sup>3,4</sup> Finally, increased levels of this cytokine have also been found in atherosclerotic plaques.<sup>5</sup> Strategies to reduce the expression or activity of IL1 $\beta$  or its receptor, interleukin 1 receptor 1 (IL1R1), have been shown to reduce plaque size in murine aortas.<sup>6-8</sup> An ongoing phase III clinical trial, Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS; NCT01327846), uses an antibody against IL1 $\beta$ , previously used in autoinflammatory diseases, to reduce recurrent cardiovascular events in individuals with a recent myocardial infarction and elevated C-reactive protein (CRP).<sup>9</sup> In the phase IIb trial, there was reduction in inflammation, as measured by IL6 and CRP, with no effect on lipids, in individuals given anti-IL1 $\beta$  antibody treatment.<sup>10</sup> Interestingly, fibrinogen levels also decreased compared with placebo.<sup>10</sup> New data from animal studies using IL1R1<sup>-/-</sup> apolipoprotein E<sup>-/-</sup> (ApoE<sup>-/-</sup>) mice show that although there was decreased plaques in certain regions, plaque stability and vessel remodeling were reduced in areas where hemorrhage was noted in the IL1R1<sup>-/-</sup> ApoE<sup>-/-</sup> mice.<sup>11</sup> The vessel wall instability was associated in part with matrix metalloproteinase 3 (MMP3) expression.<sup>11</sup> However, the presence of hemorrhage suggests that there could also be an alteration in hemostasis, such as a decrease in platelet function.

IL1R1 is expressed predominately on endothelial and immune cells.<sup>12</sup> Its ligand, IL1 $\beta$ , signals through myeloid differentiation primary response gene 88 (MYD88) to activate the nuclear factor (NF) $\kappa$ B pathway, increasing the expression of other immune-related cytokines, growth factors, and adhesion molecules.<sup>12</sup> Megakaryocytes were shown to respond to IL1 $\beta$  increasing platelet production,<sup>13-16</sup> through thrombopoietin<sup>17</sup> and transcription factors, including globin transcription factor-1<sup>17</sup> and NF-E2.<sup>17,18</sup> In addition, megakaryocytes were shown to secrete IL1 $\beta$ , which could act on the bone marrow to increase megakaryopoiesis.<sup>19</sup> Mice injected with IL1 $\beta$ , although showing an increase in the colony-forming units of the megakaryocytes,<sup>18,20</sup> had reduced numbers of circulating platelets, which could be avoided by splenectomy before treatment.<sup>20</sup>

Unlike megakaryocytes, platelets have only been shown to release IL1 $\beta$ . Previously, activated platelets were shown to express active IL1 $\beta$  on their cell surface, as measured by proliferation of T cells that require IL1 to respond to growth factors.<sup>21</sup> More recently, platelet-derived IL1 $\beta$  was shown to act on endothelial cells<sup>22,23</sup> and vascular smooth muscle cells<sup>24</sup> to stimulate inflammatory processes and promote adhesion.<sup>23</sup> This platelet-derived

IL1 $\beta$  was generated from pre-mRNA sequences in the platelet that were further processed and translated into protein upon activation.<sup>25,26</sup>

Specifically, how IL1 $\beta$  signals in the platelet and megakaryocyte is unknown. We hypothesized that IL1 $\beta$  and IL1R1 would regulate megakaryocyte maturation and platelet function. In the current study, we demonstrate that through known signaling pathways, megakaryocyte maturation and RNA production are altered by IL1 $\beta$  and IL1R1. Platelet function is enhanced through p38 mitogen activated protein kinase (MAPK) signaling pathway on IL1 $\beta$  stimulation. In particular, IL1 $\beta$  and IL1R1 increase platelet adhesion and heterotypic aggregate formation both in the setting of inflammation, high fat diet, and bacterial infection, that are reversed in IL1R1<sup>-/-</sup> and IL1 $\beta$ <sup>-/-</sup> mouse models. Therefore, IL1 $\beta$ , through IL1R1, promotes proinflammatory functions in both megakaryocytes and platelets that may contribute to the development of atherothrombotic diseases and is of immediate clinical relevance because this pathway has been targeted in a large ongoing clinical trial.

## Materials and Methods

Materials and Methods are available in the online-only Supplement.

## Results

### IL1R1 Expression and Function on Megakaryocytes

The presence of IL1R1 on the cell surface of megakaryocytes was determined with intact Meg-01 cells using flow cytometry. As seen in Figure 1A, this human megakaryocytic cell line expressed IL1R1 on its surface. To determine whether this receptor is functional, Meg-01 cells were treated with 25 ng/mL IL1 $\beta$  for up to 1 hour. Activation of downstream signaling pathways of IL1R1 was measured through Western blotting for increased phosphorylation of each kinase. As seen in Figure 1B and 1C, there was a significant increase in NF $\kappa$ B p65 subunit phosphorylation after 5 minutes of IL1 $\beta$  treatment. MAPK pathways that are also activated downstream of IL1R1-tumor necrosis factor receptor-associated factor 6 (TRAF6), including extracellular signal-related kinase (ERK) and p38, showed significantly increased phosphorylation. ERK1/2 phosphorylation was increased significantly from 15 to 60 minutes of IL1 $\beta$  treatment. Phosphorylation of p38 was increased significantly at 30 minutes of IL1 $\beta$  treatment. Phosphorylation of Akt was increased, but not significantly (data not shown). The activation of these signaling pathways was confirmed to be through IL1R1 using wild-type (WT) and IL1R1<sup>-/-</sup> mice (Figure 1D). NF $\kappa$ B p65 and Akt phosphorylation was increased to 126% ( $\pm$  28.7) and 213.9% ( $\pm$  79.0), respectively, at 5 minutes in WT megakaryocytes. Both ERK and p38 phosphorylation were increased at 30 minutes to 210.1% ( $\pm$  111.0) and 156.9% ( $\pm$  54.1), respectively. There was no increase in NF $\kappa$ Bp65, Akt, and p38 phosphorylation in the IL1R1<sup>-/-</sup> megakaryocytes. However, there was an increase in ERK phosphorylation in the IL1R1<sup>-/-</sup> megakaryocytes that may be, in part, because of IL1 $\beta$  binding to IL1R2.<sup>18</sup> ERK signaling is, therefore, not completely specific to IL1R1, but is for IL1 $\beta$ , because the increase in phosphorylation occurs compared with control, which contains buffer. These data suggest IL1R1 is present on

megakaryocytes, and it can be activated by its ligand, IL1 $\beta$ , to signal through NF $\kappa$ B, Akt, and MAPK pathways.

IL1R1 is structurally similar to toll-like receptor 2 (TLR2). As previously reported, TLR2 binding and activation induced megakaryocyte maturation, as shown with an increase in adhesion and ploidy.<sup>27</sup> Treatment with 25 ng/mL IL1 $\beta$  significantly increased Meg-01 adhesion to fibrinogen by 286.7%  $\pm$  85.8 (Figure IA in the online-only Data Supplement) and with fibronectin by 236.3%  $\pm$  24.9 compared with No Treatment (Figure IB and IC in the online-only Data Supplement). IL1 $\beta$  (25 ng/mL) treatment *ex vivo* of mouse bone marrow for 3 days significantly increased ploidy in WT mouse megakaryocytes, particularly at the 4 N and 64+N population compared with control treatment (Figure 1E). Treatment with 50 ng/mL thrombopoietin (TPO), a regulator of megakaryocyte maturation, significantly increased 16 N and 32 N. IL1 $\beta$  treatment significantly reduced 16 N and 32 N compared with TPO treatment because of the movement of megakaryocytes into the more mature, higher ploidy 64+N that did not occur with TPO. This effect on megakaryocyte maturation was specific to IL1R1 because treatment of IL1R1<sup>-/-</sup> bone marrow *ex vivo* with IL1 $\beta$  did not yield the same results (Figure 1F). These data suggest that IL1R1 and IL1 $\beta$  increase megakaryocyte maturation to cells with a higher ploidy. The IL1R1<sup>-/-</sup> bone marrow cells did not respond like WT to TPO treatment, as previously seen with TLR2<sup>-/-</sup> megakaryocytes.<sup>27</sup> These data suggest that the presence of IL1R1 may be necessary for megakaryocyte maturation. To determine whether this maturation leads to altered platelet numbers, platelet counts were performed in WT and IL1R1<sup>-/-</sup> mice. In Table I in the online-only Data Supplement, there were more platelets in the IL1R1<sup>-/-</sup> mice compared with WT at baseline. However, after challenge with an oral pathogen, the WT mice had an increase in circulating platelets that did not occur in IL1R1<sup>-/-</sup> mice. Therefore, although at baseline, the lack of IL1R1 does not affect platelet production, when challenged, platelet production is delayed.

### IL1 $\beta$ Affects Megakaryocyte Inflammatory and Thrombotic Gene Expression

During megakaryocyte maturation, RNA production is increased for the production of platelets. Here, we tested whether IL1 $\beta$ /IL1R1-induced maturation could also affect gene expression. Several inflammatory and thrombotic genes, selected based on previous findings,<sup>27</sup> were measured in Meg-01 cells treated with IL1 $\beta$  for 3 hours. In Figure 2A through 2H, IL1 $\beta$  treatment significantly increased both inflammatory and thrombotic gene expression in the megakaryocytic cells. Two downstream targets of NF $\kappa$ B are cyclooxygenase 2 (COX2) and monocyte chemoattractant protein-1 (MCP-1). Both genes were upregulated with IL1 $\beta$  treatment to 123.1% $\pm$ 12.4 and 112.3% $\pm$ 3.5, respectively, compared with No Treatment (Figure 2A and 2B). Pretreatment with 50  $\mu$ M LY294002, an inhibitor of phosphoinositide 3-kinase (PI3K)/Akt pathway, reduced the effects of IL1 $\beta$  on MCP-1 gene expression (Figure 2B). U0126 (50  $\mu$ mol/L), an MEK1/2 inhibitor, and BAY 11-7082 (50  $\mu$ mol/L), an NF $\kappa$ B inhibitor, reduced the effects of IL1 $\beta$  on both COX2 and MCP-1 expression (Figure 2A and 2B). NF $\kappa$ B1 (p105/p50) was also upregulated by IL1 $\beta$  in the Meg-01 cells to 193.3% $\pm$ 35.9 compared with No Treatment (Figure 2C). Unlike COX2 and MCP-1, NF $\kappa$ B1 expression was upregulated by IL1 $\beta$  only through the NF $\kappa$ B pathway (Figure 2C). TLR2, a structurally similar receptor to IL1R1, was also upregulated by IL1 $\beta$  to

161.3%±36.0 compared with No Treatment, and its upregulation was signaled through PI3K, ERK, and NFκB pathways (Figure 2F). Finally, both IL1R1 and IL1β expressions were upregulated by IL1β treatment to 113.9%±5.6 and 165.7%±40.5 compared with No Treatment, respectively (Figure 2D and 2E). IL1β upregulated itself through NFκB, PI3K, and ERK pathways (Figure 2E). IL1R1 was upregulated by NFκB and PI3K pathways (Figure 2D).

Two thrombotic genes were significantly upregulated by IL1β in Meg-01 cells. CD41, also known as integrin α<sub>IIb</sub>, which binds to fibrinogen, was upregulated to 133.0%±16.4 compared with No Treatment (Figure 2G). This gene was upregulated by IL1β only through the PI3K pathway (Figure 2G). GP1b, part of the von Willebrand Factor receptor, was also upregulated by IL1β to 134.3%±18.7 compared with No Treatment (Figure 2H). GP1b was upregulated by IL1β through both the PI3K and ERK pathways (Figure 2H). Neither one of the thrombotic genes was upregulated by IL1β through the NFκB pathway.

Expression of all the genes studied was confirmed to be regulated through IL1R1 by using WT and IL1R1<sup>-/-</sup> mice. As seen in Figure 2I, there was a significant increase in COX2, MCP-1, NFκB1, IL1β, IL1R1, TLR2, and GP1b compared with control in the WT mice. All of these genes were not increased in the IL1R1<sup>-/-</sup> mice (Figure 2J, note different scales for 2I and 2J), suggesting that the receptor is important for the effects of IL1β on gene expression. Baseline levels of each gene are comparable between WT and IL1R1<sup>-/-</sup> mice (Table II in the online-only Data Supplement), suggesting the loss of IL1R1 did not affect the baseline levels of the gene but the increase in gene expression. The only gene that was not consistent with the Meg-01 data was CD41, which could be related to differential effects seen between species.<sup>28</sup>

### IL1R1 Expression and Function on Platelets

As shown in Figure 3A, human platelets express IL1R1 on their surface, as determined by flow cytometry. Treatment of human platelets with various concentrations of IL1β did not cause washed platelet aggregation (data not shown), unlike what was shown with TLR2.<sup>29</sup> IL1β did enhance agonist-induced platelet aggregation. As shown in Figure 3B, pretreatment of IL1β increased the platelet response to collagen by ≈42% over collagen treatment alone. In the presence of increasing concentrations of thrombin, IL1β pretreatment also enhanced the response of the platelets by increasing aggregation on average 10.3% (13.3%–6.8%; Figure 3C). These data suggest that IL1β primes the platelets by activating signaling pathways to enhance their response to agonists.

Washed platelets from WT mice had an enhanced response to a low dose of thrombin (0.05 U/mL) by 18.5% when pretreated with 10 ng/mL IL1β as compared with thrombin alone (Figure 3D). This response was specific to IL1R1 because platelets from mice deficient in IL1R1 did not respond to IL1β pretreatment and had no enhanced response to thrombin (Figure 3E).

Megakaryocytes treated with IL1β resulted in the activation of multiple signaling pathways, including p38 MAPK. Platelets treated with IL1β leads to the phosphorylation of p38 (Figure IIA and IIB in the online-only Data Supplement). Pretreatment of platelets with IL1β

followed by treatment with either thrombin or collagen also leads to enhanced phosphorylation of p38 (Figure IIA and IIB in the online-only Data Supplement). To understand whether this pathway was involved in IL1 $\beta$  enhancement of aggregation, human platelets were pretreated with 0.2  $\mu$ mol/L SB203580, a selective p38 MAPK inhibitor, before treatment with IL1 $\beta$  and thrombin. As seen in Figure 3F, thrombin-stimulated aggregation was modestly enhanced with IL1 $\beta$  pretreatment by 8.1% over thrombin alone. In the presence of SB203580, IL1 $\beta$  was unable to enhance the platelet response to thrombin because aggregation returned to baseline level (86.4% $\pm$ 8.2 versus 86.9% $\pm$ 5.7, thrombin alone). Therefore, IL1 $\beta$ , through the activation of the p38 MAPK pathway, enhanced the response of platelets to thrombin.

Washed human platelets were labeled with calcein-AM for visualization purposes, and circulated over either collagen or fibrinogen. Unlike washed platelet aggregation, IL1 $\beta$  alone was able to induce adhesion to either substrate (Figure 3G). In addition, IL1 $\beta$  pretreatment enhanced thrombin-induced adhesion to either substrate, as seen in representative pictures in Figure 3G. These results suggest that IL1 $\beta$  directly activates and enhances platelet adhesion to various substrates.

A marker of platelet inflammatory response, heterotypic aggregate (platelet-positive neutrophils) formation was also tested using human whole blood. Treatment with IL1 $\beta$  (10 ng/mL) significantly induced the formation of heterotypic aggregates, as determined by flow cytometry (Figure 3H). This result was similar to what was shown with TLR2 and Pam3CysSerLys4 (Pam3CSK4),<sup>29</sup> which was used as a positive control. These findings suggest that IL1 $\beta$ , through IL1R1, promotes both thrombotic and inflammatory functions in platelets.

### IL1R1 Mediates Platelet Function In Vivo

Various disease processes induce the production of IL1 $\beta$  including obesity and infection. We have previously shown enhanced IL1R1 platelet expression associated with increased body mass index (BMI) in the Framingham Heart Study (FHS).<sup>30</sup> Obesity has also been associated with inflammation and an increase in circulating IL1 $\beta$  levels,<sup>1</sup> which could interact with platelets and affect their function. To test this hypothesis, WT and IL1R1<sup>-/-</sup> mice were placed on a high fat diet for 10 weeks. Both mouse models on the high fat diet gained weight compared with those on normal chow (Figure III in the online-only Data Supplement). WT mice gained 5.4 g on the high fat diet, whereas IL1R1<sup>-/-</sup> mice gained 8.7 g.

Platelets were isolated from these mice, and adhesion to collagen was tested. At baseline, platelets from WT mice adhere to collagen in the presence of 0.5 U/mL thrombin and 10 ng/mL IL1 $\beta$  (Figure 4A). Adhesion to collagen is enhanced in the presence of IL1 $\beta$  and thrombin, compared with thrombin alone. In platelets from IL1R1<sup>-/-</sup> mice, adhesion to collagen in the presence of thrombin or IL1 $\beta$  and thrombin is the same. IL1R1<sup>-/-</sup> platelets do not respond to IL1 $\beta$ . After being on a high fat or normal chow diet for 10 weeks, there was an increase in WT platelet adhesion in the presence of thrombin with a high fat diet compared with normal chow (Figure 4B), similar to baseline adhesion with IL1 $\beta$  and thrombin. There were fewer IL1R1<sup>-/-</sup> platelets adhering at baseline compared with WT.

With a high fat diet, unlike the WT mice, there was no increase in adhesion in the IL1R1<sup>-/-</sup> mice, suggesting that the effects of a high fat diet on platelet adhesion are partially mediated through IL1R1. When this receptor is not expressed on the platelets, the prothrombotic effects of the diet were diminished.

To determine the role of infection on IL1R1 and platelets, WT, IL1R1<sup>-/-</sup>, and IL1β<sup>-/-</sup> mice were challenged orally with the periodontal pathogen *P. gingivalis*. This bacterium has been previously shown to affect platelets<sup>29</sup> and circulating IL1β levels.<sup>3,4</sup> One day after the last oral challenge, there was a loss of the proform of IL1β into the active form in WT infected mice, but not in either the IL1R1<sup>-/-</sup> or IL1β<sup>-/-</sup> mice (Figure IVA and IVB in the online-only Data Supplement). A trend toward an increase in platelet counts with an acute infection in the WT mice (Table I in the online-only Data Supplement) was not seen in either knockout mouse model. WT mice (control) had a significant increase in platelet-positive neutrophils present in circulation (Figure IVC in the online-only Data Supplement) with an acute exposure to *P. gingivalis*, which did not occur in the IL1R1<sup>-/-</sup> or IL1β<sup>-/-</sup> mice. Six weeks after the last *P. gingivalis* challenge, there is again a loss of the proform of IL1β in the WT and IL1R1<sup>-/-</sup> infected mice, which does not occur in the IL1β<sup>-/-</sup> mice (Figure IVA and IVB in the online-only Data Supplement). Platelet counts increased slightly in the WT mice and to a lesser extent in the IL1β<sup>-/-</sup> mice, but there was a drop in platelets in the IL1R1<sup>-/-</sup> mice (Table I in the online-only Data Supplement). WT mice still had several circulating platelet-neutrophil aggregates (77.3%±6.7 versus 12.8%±2.2, Control; Figure IVD in the online-only Data Supplement). Again, both IL1R1<sup>-/-</sup> and IL1β<sup>-/-</sup> mice had no increase in circulating heterotypic aggregates compared with control. The least responsive of the mouse models was the IL1β<sup>-/-</sup> mice; however, ex vivo examination showed that platelets from these mice responded normally to both thrombin and IL1β in adhesion (Figure IVE and IVF in the online-only Data Supplement) and heterotypic aggregate (Figure IVG in the online-only Data Supplement) assays. Therefore, IL1R1 and the platelets were both responsive in the IL1β<sup>-/-</sup> mice, and the results seen in the infection model were attributable to the lack of IL1β production.

### Inflammatory Genes Upregulated in Platelets from Mice on a High Fat Diet

Based on previously published results from the FHS,<sup>30</sup> platelet RNA from mice fed a high fat or normal chow diet was tested for inflammatory and thrombotic transcripts. At 8 weeks, 19 genes had altered expression with a high fat diet (Table 1). Intercellular adhesion molecule 1 (ICAM1), phospholipase A2 group VII (PLA2G7), and toll-like receptor 1 (TLR1) were highly upregulated in high fat diet fed mice compared with normal chow. Nine of the 19 genes upregulated with a high fat diet in mice were also increased in obese and overweight individuals of the FHS.<sup>30</sup> Two genes not upregulated in mice but increased in the FHS included interleukin 6 (IL6) and prostaglandin H2 synthase (PTGS2). Genes downregulated in mice fed a high fat diet included heparin-binding epidermal growth factor-like growth factor and tissue inhibitor of metalloproteinase 1 (TIMP1). These data suggest that at 8 weeks, the megakaryocytes are responding to the continued exposure to high fat and inflammation and increasing inflammatory and thrombotic transcripts.

## Platelet RNA Transcripts From the FHS

IL1R1 expression in platelets was previously shown to be significantly higher in individuals with increased BMI in the FHS<sup>30</sup> and in mice on a high fat diet (Table 1). To confirm the clinical relevance of our murine and in vitro findings, 6 additional inflammatory-related genes that are linked to IL1R1 and NF $\kappa$ B were examined. RNA from platelets isolated from participants of the FHS Offspring Cohort 8 was analyzed as previously described for white blood cell contamination.<sup>30</sup> As reported, there was a <1/50 000 white blood cells in the platelet samples, and platelet gene expression varied from that of white blood cells.<sup>30</sup> All data were normalized using 3 housekeeping genes,  $\alpha$ -actin (ACTB),  $\beta$ 2-microglobulin (B2M), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which were found to be highly correlated.<sup>30</sup> Clinical characteristics for the FHS participants are listed in Table 2. Of the 1819 participants, 51% were female, the average age was 67 $\pm$ 9 years, and the average BMI was 28.3 $\pm$ 5.3 kg/m<sup>2</sup>. The genes studied fell into 3 categories. Three genes were associated with IL1R1 and IL1 $\beta$  signaling, which included nucleotide-binding oligomerization domain, leucine rich repeat, and pyrin domain containing protein 3 (NLRP3),<sup>31</sup> MYD88,<sup>32</sup> and interleukin-1 receptor-associated kinase 1 (IRAK1).<sup>33</sup> There were genes whose expression was both regulated by IL1 $\beta$  and found to be upregulated with IL1 $\beta$  in experimental models, which included interleukin 18 (IL18)<sup>34,35</sup> and MMP9.<sup>4,36–38</sup> Relative expression values (Ct) were evaluated in multivariable regression models including terms for age, sex, and additional clinical factors listed in Tables III and IV in the online-only Data Supplement. Three of these genes were nominally significantly associated with BMI-score ( $P$ <0.05; Tables III and IV in the online-only Data Supplement): IL1 $\beta$  regression  $\beta$  coefficient=0.029 $\pm$ 0.015 ( $P$ =0.0490), MMP9 regression  $\beta$  coefficient=0.051 $\pm$ 0.014 ( $P$ =0.0003), and MYD88 regression  $\beta$  coefficient=0.030 $\pm$ 0.013 ( $P$ =0.0253). The association with MMP9 was the only one that survived correction for multiple comparisons. Of the other clinical covariates modeled, high-density lipoprotein level was associated with MMP9 expression, lipid treatment was associated with IL18, IL1 $\beta$ , and IRAK1 expression, and antihypertensive treatment was associated with IL1 $\beta$ , MYD88, and NLRP3 expression at  $P$ <0.05 (Table IV in the online-only Data Supplement); none of these additional associations survived correction for multiple corrections, however.

Gene expression values stratified by obesity status are presented in Figure 5. With the exception of IRAK1, all genes were upregulated in overweight or obese individuals in reference to normal weight individuals. The only associations statistically significant ( $P$ <0.05) were for MMP9, NLRP3, and MYD88 genes expression values contrasted between obese and normal weight individuals (results from full models are presented in Tables III and IV in the online-only Data Supplement).

Further testing was run on each platelet gene with a significant association with BMI and inflammatory biomarkers measured from plasma from the same visit (Offspring, eighth visit). In Table V in the online-only Data Supplement, associations between each biomarker, CRP, ICAM1, IL6, MCP-1, osteoprotegerin, P-Selectin, and TNFR, and each gene are listed. Each gene associated with 2 or more measured biomarkers. IL18 and MMP9 both had associations with biomarkers that were significant after corrections for multiple comparisons. Platelet MMP9 gene expression associated with the most inflammatory



biomarkers. To determine whether these biomarkers accounted for the MMP9–BMI association, additional analysis was performed adjusting for multiple corrections and various biomarkers (Table VI in the online-only Data Supplement). BMI was still significantly associated with MMP9 expression for all biomarkers, except CRP and IL6. These results further support that megakaryocytes respond to the increases in BMI and produce platelets with increased inflammatory transcripts, specifically related to IL1R1/IL1 $\beta$  signaling.

## Discussion

Increasing BMI has been shown to correlate with significant increases in inflammatory and immunity-related genes in platelets.<sup>30</sup> One transcript, IL1R1, although found to be significantly associated with obese and overweight individuals, had never been identified on platelets. Other IL1R1 and IL1 $\beta$ -related platelet transcripts were studied in the FHS and were shown to be increased with an increase in BMI (Figure 6A). These findings are consistent with previous observations that IL1 $\beta$  is increased in obese patients.<sup>1</sup> These changes, although small, are significant and are consistent with data previously published on alterations in platelet transcripts in FHS.<sup>30,39</sup> Mouse studies confirmed what has been shown in the FHS studies. TLR1, TLR2, and IL1R1 mRNA levels increased by 8 weeks in mice on a high-fat diet. ICAM1, PLA2G7, and Chemokine (C-C Motif) Ligand 2 all shown to be upregulated with an increase BMI in FHS, were also increased at 8 weeks in mice fed a high fat diet. MMP9 and MYD88, both shown in this study to be upregulated in the FHS, were also upregulated in mice on a high-fat diet. However, PTGS2 and IL6, which were both increased in the FHS, did not increase in the mice. The discrepancy between the mouse data and FHS may be because of the timing. It is possible that at 8 weeks, the mouse data do not precisely reflect what is occurring in humans, who have been exposed to a high fat diet for much longer.

Work has shown the importance of TLRs in both platelets<sup>29,40</sup> and megakaryocytes.<sup>27</sup> However, little is known about IL1R1 and IL1 $\beta$  in regulating the function of both of these cells, in particular, in the setting of infection and obesity. Data presented here (summarized in Figure 6B) show that megakaryocytes express a functional IL1R1 that activates multiple signaling pathways, including NF $\kappa$ B, ERK MAPK, p38 MAPK, and PI3K/Akt pathways to increase both thrombotic and inflammatory gene expression. The changes in gene expression will result in altered transcript level in platelets. These transcripts can be translated into protein upon activation, as shown previously with IL1 $\beta$  transcripts in platelets,<sup>25,26</sup> or can be transferred to other cells, such as immune or endothelial cells, during thrombosis or inflammation.<sup>41</sup> Through IL1R1, IL1 $\beta$  regulates megakaryocyte maturation by increasing adhesion and ploidy. Baseline platelet counts in WT mice were slightly lower than in IL1R1<sup>-/-</sup> mice. When challenged with an infection, the *P gingivalis* infected WT platelet count rose, whereas the IL1R1<sup>-/-</sup> platelet count decreased. In response to an increase in circulating IL1 $\beta$ , megakaryocytes will produce more platelets that contain a higher amount of inflammatory and thrombotic genes, as seen in the FHS and mouse transcript data. Our data are similar to what was shown with TLR2.<sup>27</sup> Both TLR2 and IL1R1 can respond to infection, affecting platelet production. Because both IL1R1<sup>-/-</sup> and IL1 $\beta$ <sup>-/-</sup> mice have functional TLR2 on their megakaryocytes, the data presented here along with

what was previously published<sup>27</sup> suggest that both receptors are necessary to respond to inflammatory stimuli.

The work presented here (summarized in Figure 6B) also shows how IL1R1 functions in platelets. IL1 $\beta$  does not cause aggregation, as seen with TLR2<sup>29</sup>; however, it does enhance the response of platelets to both collagen and thrombin. This increased response is shown to be, in part, through p38 MAPK pathway. Interestingly, IL1 $\beta$  increased platelet adhesion to different substrates alone and in combination with thrombin and caused heterotypic aggregate formation. It is possible that p38 signaling could be involved in these functions as well. Recently, NADPH oxidase activity was shown to regulate collagen-induced platelet activation, through reactive oxygen species generation, and protein kinase C signaling.<sup>42</sup> These pathways could also be involved in the effects of IL1R1 and IL1 $\beta$  on platelets. IL1R1 promotes the inflammatory function in platelets, which could enhance atherosclerosis and thrombosis. A high fat diet enhanced platelet adhesion in WT mice, which was abrogated in the IL1R1<sup>-/-</sup> mice. Overall, these data suggest that platelets contribute to the development of cardiovascular disease during obesity through IL1R1 and IL1 $\beta$ . Inhibition of IL1R1 would be desirable to alter disease progression.

Some bacterial infections, including *P. gingivalis*, are associated with an increase in IL1 $\beta$ .<sup>3,4</sup> When WT mice are infected acutely or chronically with *P. gingivalis*, there is an increase in circulating heterotypic aggregates. Based on previous work,<sup>27,29,40</sup> it would be hypothesized that this bacteria is being recognized by TLR2 on the megakaryocytes and platelets. However, IL1R1<sup>-/-</sup> and IL1 $\beta$ <sup>-/-</sup> mouse platelets, which still express TLR2, did not respond to the bacteria and form heterotypic aggregates. It is concluded that *P. gingivalis* is recognized by TLR2 to increase IL1 $\beta$  levels, which, in turn, affects platelet–neutrophil interactions.

The work presented here uses 2 methods, epidemiological analyses and in vitro/in vivo experiments, to understand the role of IL1R1 and IL1 $\beta$  in megakaryocytes and platelets. There are some limitations of our approach. The FHS data were based on participants with a mean age of 67 $\pm$ 9 years. The conclusions drawn from this epidemiological study may not reflect what occurs in young obese/overweight individuals. Additionally, participants of the FHS only report if they are on medications, including lipid-lowering drugs and aspirin, and did not report the specific type of medication, the dosage, and if they were on it at the time of examination. Platelet transcript studies were performed through a targeted approach and not based on an array. Although biased, this approach allowed us to study gene expression on 1819 individuals, reducing any issues derived from multiple testing. Platelets collected from healthy human volunteers were used for functional studies and not for comparison. Mice used in experiments were total knockout models and not platelet/megakaryocyte-specific knockout models, which are not commercially available. However, studying isolated platelets ex vivo allows us to see how IL1R1/IL1 $\beta$  affects these cells specifically, without any contaminating immune cells.

Our observations that IL1R1 modulates platelet processes may be clinically relevant. Previous work suggests a role for IL1R1 and IL1 $\beta$  in the development of cardiovascular disease. Increased levels of IL1 $\beta$  have been found in atherosclerotic plaques,<sup>5</sup> and reduction

of the expression or activity of IL1 $\beta$  and IL1R1 decreases plaque size in murine aortas.<sup>6-8</sup> These results suggest that treatment that would reduce IL1 $\beta$  activity would be effective in reducing cardiovascular disease. Such a treatment could include the reduction in the levels or activation of IL1 $\beta$  or blockage of its receptor, including antibody therapy against IL1 $\beta$  or IL1R1. A clinical trial, CANTOS, has begun, which uses an antibody against IL1 $\beta$  to reduce cardiovascular events in individuals with coronary artery disease.<sup>9</sup> Data from a recent study using IL1R1<sup>-/-</sup> ApoE<sup>-/-</sup> mice<sup>11</sup> report that advanced atherosclerotic plaques are reduced in specific areas but are also showing signs of instability with increased intraplaque hemorrhage compared with IL1R1<sup>+/+</sup> ApoE<sup>-/-</sup> mice. These data raise the possibility that plaque hemorrhage may be because of impaired hemostasis, that is, altered platelet function. While our model firmly establishes a role for IL1R1 in platelet function and its transcript present in human platelets in a large observational study, we cannot directly infer that the use of an antibody directed against IL1 $\beta$  will have clinical implications such as bleeding or vessel instability.

In conclusion, we show that IL1R1 and IL1 $\beta$ , associated with the innate immune system, regulate both megakaryocyte and platelet functions in murine and human models. IL1 $\beta$  through IL1R1 enhances megakaryocyte maturation and alters the RNA profiles in the developing platelets to be proinflammatory and prothrombotic. We provide evidence that megakaryocytes and platelets become activated in association with obesity and infections through IL1R1 and IL1 $\beta$ . Thus, in the circulation, platelets react to IL1 $\beta$  through IL1R1, which enhances its activation and stimulates the inflammatory function of platelets.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

L.M. Beaulieu was involved in experimental design, performing experiments, data analysis, writing, and editing of the article. E. Lin and M. Koupenova were involved in performing experiments, data analysis, and editing of the article. E.O. Weinberg and C.D. Kramer were involved in performing experiments. C.A. Genco was involved in experimental design and editing of the article. E.J. Benjamin, E. Mick, and M.G. Larson were involved with Framingham Heart Study sample collection, analysis, and editing of the article. K. Tanriverdi and J.E. Freedman were involved in experimental design, data analysis, and editing of the article.

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## Nonstandard Abbreviations and Acronyms

<b>BMI</b>	body mass index
<b>FHS</b>	Framingham Heart Study
<b>IL1<math>\beta</math></b>	interleukin 1 beta

<b>IL1R1</b>	interleukin 1 receptor 1
<b>IRAK1</b>	interleukin 1 receptor-associated kinase 1
<b>MCP-1</b>	monocyte chemotactic protein-1
<b>MMP</b>	matrix metalloproteinase
<b>MYD88</b>	myeloid differentiation primary response gene 88
<b>NLRP3</b>	nucleotide-binding oligomerization domain, leucine rich repeat, and pyrin domain containing protein 3
<b>TRAF6</b>	tumor necrosis factor receptor-associated factor 6
<b>TPO</b>	thrombopoietin
<b>WT</b>	wild-type

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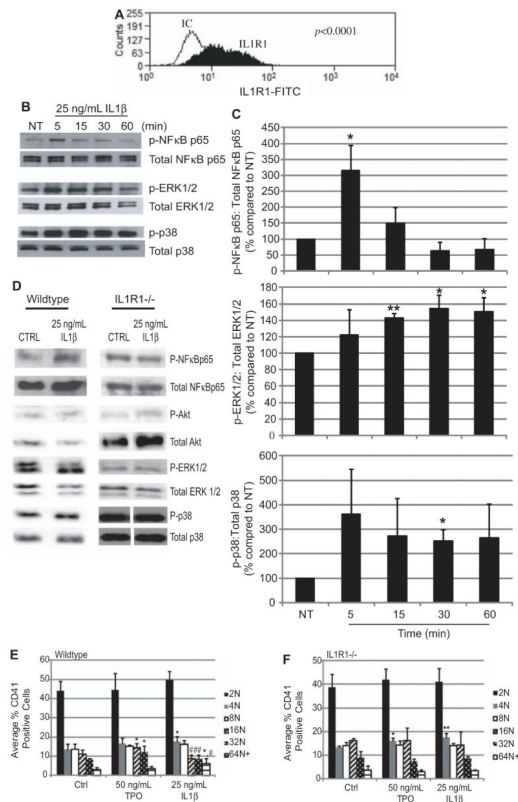
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### Significance

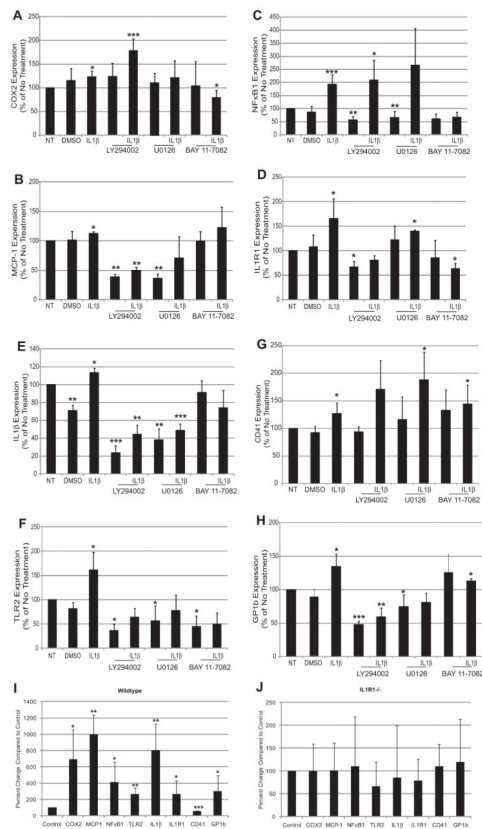
A proinflammatory environment attributable to either an acute bacterial infection or a chronic disease such as obesity is associated with an increase in circulating interleukin (IL)1 $\beta$ . A current phase III clinical trial, Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS), is investigating the use of an antibody against IL1 $\beta$  to reduce recurrent cardiovascular events in individuals with a recent myocardial infarction and elevated C-reactive protein. However, new data from animal studies using IL1R1<sup>-/-</sup> ApoE<sup>-/-</sup> mice show plaque stability and vessel remodeling were reduced in areas where hemorrhage was noted, suggesting that this pathway may have effects beyond the vessel wall. The work presented here shows that IL1R1 and IL1 $\beta$  can influence platelet function and potentially augment the development of disease. These results are of future relevance as inflammatory receptors found in the vasculature are being targeted for therapeutic intervention but are not well characterized in the platelet.



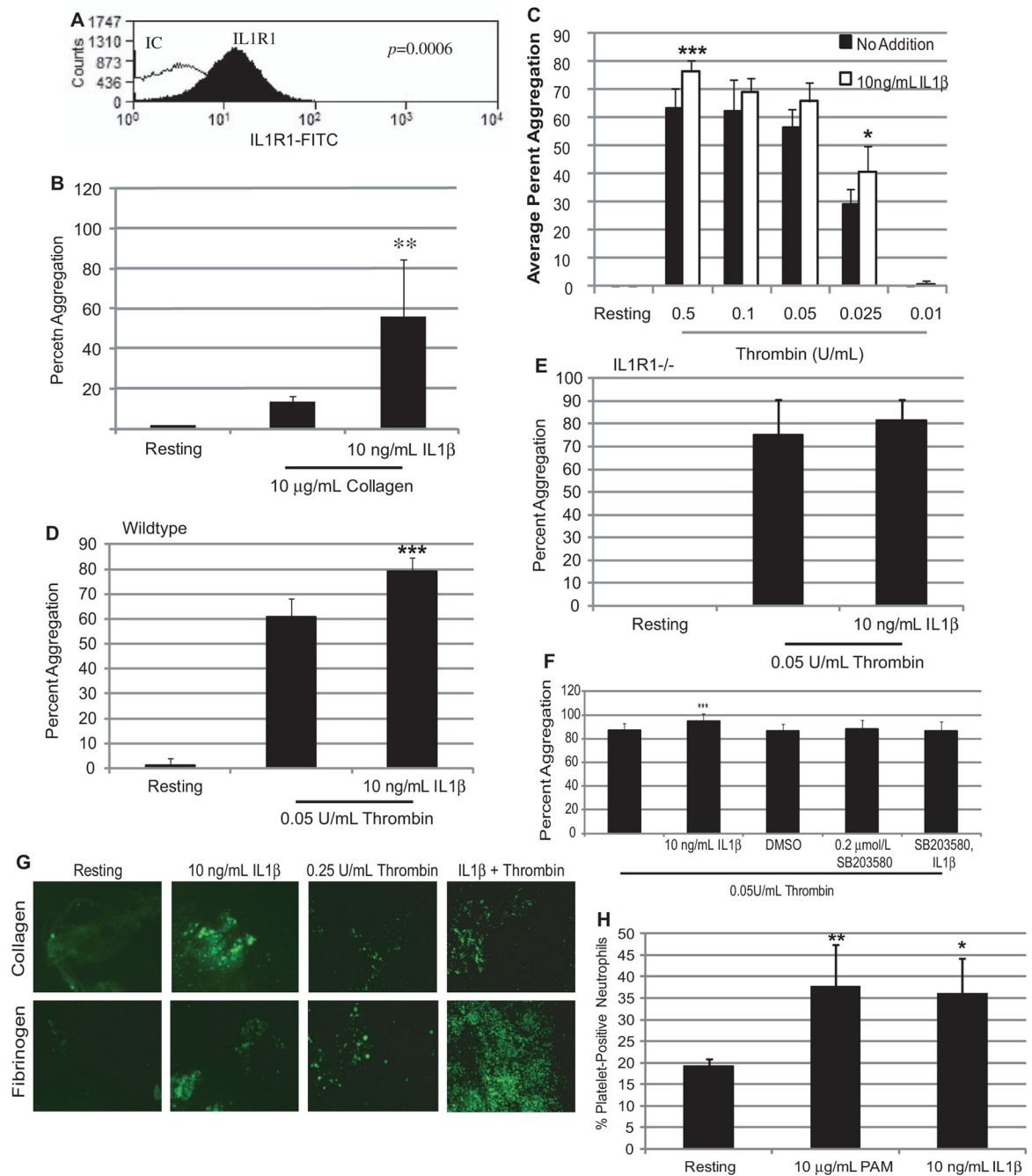
**Figure 1.**

Expression of interleukin (IL)1R1 and function on megakaryocytes. Representative histogram of Meg-01 cells (A) stained with anti-human IL1R1 fluorescein isothiocyanate (FITC); n=5; IC indicates isotype control. B, Representative Western blots probed for phospho- and total NFκB p65, phospho- and total ERK, and phospho- and total p38 in Meg-01 cells. C, Quantification of the increases in phosphorylation of NFκB p65, ERK, and p38 after treatment with IL1β. n=4; \* $P < 0.05$ , \*\* $P < 0.01$  compared with NT. D, Representative Western blots probed for phospho- and total NFκB p65, phospho- and total Akt, phospho- and total ERK, and phospho- and total p38 in isolated wild-type (WT) and IL1R1<sup>-/-</sup> mouse megakaryocytes; n=4. Isolated WT (E) or IL1R1<sup>-/-</sup> (F) mouse bone marrow cells were treated with vehicle (Control – Ctrl), thrombopoietin (TPO), or IL1β for 3 days. Megakaryocyte DNA content (2 N-64+N) was measured. n=18; \* $P < 0.05$ , \*\* $P < 0.01$  compared with Control, # $P < 0.05$ , ## $P < 0.01$  compared with TPO.



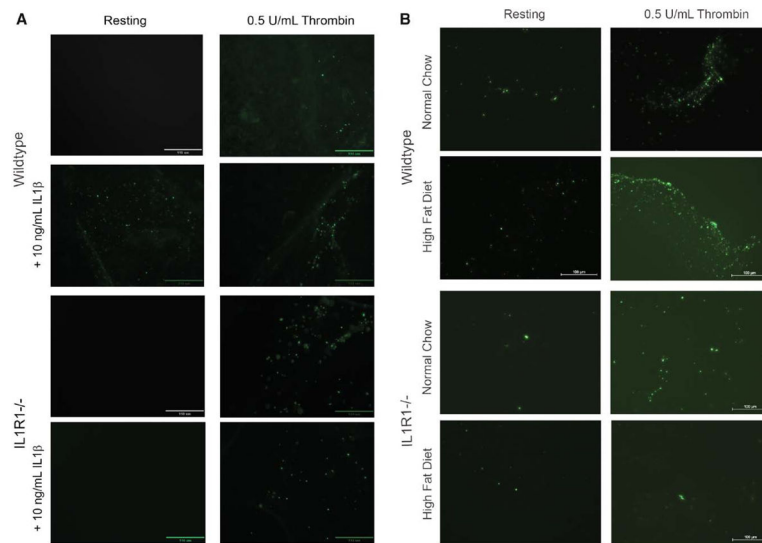


**Figure 2.** Interleukin (IL)1 $\beta$  alters megakaryocyte inflammatory- and thrombosis-related gene expression through NF $\kappa$ B, ERK, and PI3K/ Akt pathways. Gene expression of Meg-01 cells pretreated with 50  $\mu$ mol/L LY294002, 50  $\mu$ mol/L U0126, 50  $\mu$ mol/L BAY 11-7082, or dimethyl sulfoxide (DMSO), then treated with IL1 $\beta$ . COX2 (**A**; n=8), monocyte chemotactic protein-1 (MCP-1; **B**; n=5), NF $\kappa$ B1 (**C**; n=8), IL1R1 (**D**; n=3), IL1 $\beta$  (**E**; n=4), TLR2 (**F**; n=7), CD41 (**G**; n=10), and GP1b (**H**; n=7). \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 compared with NT (No Treatment). Gene expression in isolated wild-type (WT; **I**) and IL1R1<sup>-/-</sup> (**J**) mouse megakaryocytes treated with IL1 $\beta$ . n=3 for WT; n=3 for IL1R1<sup>-/-</sup>. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 compared with NT.

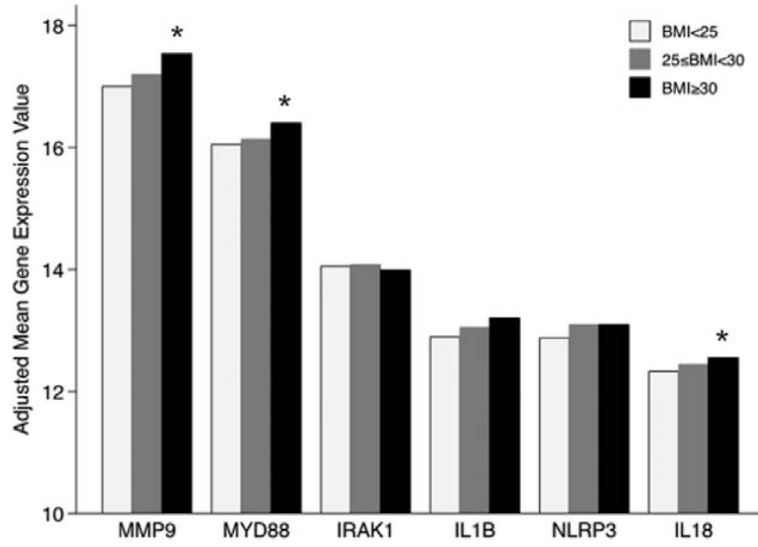


**Figure 3.** Interleukin (IL)1 $\beta$  affects platelet function. **A**, Representative histogram of isolated human platelets stained with anti-human IL1R1 FITC antibodies;  $n=5$ ; IC indicates isotype control. **B**, Isolated human platelets aggregation was measured for 10 minutes in the presence of collagen.  $n=4$ ;  $**P<0.01$  compared with collagen alone. **C**, Isolated human platelet aggregation was measured for 10 minutes in the presence of increasing concentrations of thrombin.  $n=10$ ;  $***P<0.001$ ,  $*P<0.05$  compared with thrombin alone. Isolated WT (**D**;  $n=4$ ) and IL1R1<sup>-/-</sup> (**E**;  $n=3$ ) mouse platelet aggregation was measured in the presence of

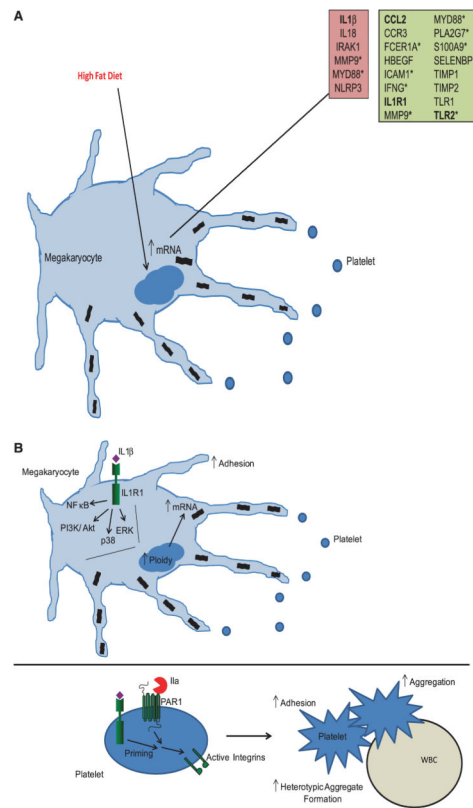
thrombin. \*\*\* $P < 0.001$  compared with thrombin alone. **F**, Isolated human platelet aggregation was measured in the presence of thrombin for 10 minutes.  $n = 15$ ; \*\*\* $P < 0.001$  compared with thrombin alone. **G**, Representative isolated human platelets treated with IL1 $\beta$  or thrombin, or IL1 $\beta$ , then thrombin adherent on collagen or fibrinogen;  $n = 3$ . **H**, The percentage of platelet-positive neutrophils in the presence of Pam3CSK4 (PAM) or IL1 $\beta$ .  $n = 6$ ; \* $P < 0.05$ , \*\* $P < 0.01$  compared with Resting.



**Figure 4.** Interleukin (IL)1 $\beta$  affects adhesion in the setting of high fat diet. **A**, Representative photographs of isolated wild-type (WT) and IL1R1<sup>-/-</sup> platelets treated with IL1 $\beta$ , thrombin, or both and adherent on collagen at baseline; n=5 to 6 mice in each group. **B**, Representative photographs of isolated WT and IL1R1<sup>-/-</sup> platelets treated with thrombin and adherent to collagen; n=3 to 4 mice in each group.



**Figure 5.** Platelet RNA expression in Framingham Heart Study (FHS) individuals associated with increased body mass index (BMI). The platelet RNA expression of IL1 $\beta$ -associated genes in FHS individuals stratified by BMI status adjusted by clinical covariates listed in Tables III and IV in the online-only Data Supplement. Relative expression values (Ct) corrected by the housekeeping genes (GAPDH, B2M,  $\alpha$ -actin [ACTB]) were rescored so that larger values represent increased expression. MMP9, NLRP3, and myeloid differentiation primary response gene (MYD88) were all significantly increased in obese individuals (BMI  $\geq$  30) compared with normal weight individuals (BMI<25); \* $P$ <0.05.



**Figure 6.** Schematic of the effects of interleukin (IL)1 $\beta$ /IL1R1 on megakaryocyte and platelet function. **A**, A high fat diet will cause megakaryocytes to produce platelets with an increase in both inflammatory and thrombotic genes. Red highlighted data represent new Framingham Heart Study (FHS); green highlighted data represent mouse data; \*confirmed in both human and mouse platelets; Bolded genes were confirmed to be upregulated in Meg-01 by IL1 $\beta$ . **B**, IL1 $\beta$  in circulation as a result of increased body weight will bind IL1R1 on megakaryocytes. This interaction leads to the activation of the nuclear factor (NF) $\kappa$ B, PI3K/Akt, and mitogen activated protein kinase (MAPK) (ERK and p38) pathways. As a result, there is an increase in megakaryocyte maturation, including increased adhesion, increases in ploidy, and increases in mRNA production of inflammatory and thrombotic genes. IL1 $\beta$  can also bind IL1R1 on platelets and either enhance aggregation induced by agonists or promote adhesion and heterotypic aggregate formation.

**Table 1**

Inflammatory and Thrombotic Genes in Murine Platelets Fed a High-Fat Diet Compared With Normal Chow

Gene	Name	Function	Changes in Gene Expression at 8 Weeks	
			CT (SD)	Fold Change
CCL2	Chemokine (C-C motif) ligand 2 (MCP-1)	Inflammatory chemokine	-0.90 (0.82)	1.96
CCR3	Chemokine (C-C motif) receptor 3	Receptor for various chemokines	0.29 (1.95)	1.39
FCER1A	IgE Fc receptor subunit alpha	Inflammatory receptor involved in allergies	0.32 (1.17)	0.95
FN1	Fibronectin 1	Extracellular matrix protein	-0.07 (1.49)	1.68
HBEGF	Heparin-binding EGF-like growth factor	Mitogen for fibroblasts, smooth muscle cells	1.40 (0.90)	0.42
ICAM1	Intercellular adhesion molecule 1	Ligand for leukocyte adhesion	-0.39 (2.25)	2.22
IFNG	Interferon, gamma	Inflammatory cytokine	0.59 (1.83)	1.09
IL1R1	Interleukin 1 receptor 1	Innate immune receptor	1.07 (2.52)	1.02
IL6	Interleukin 6	Inflammatory cytokine	3.68 (1.70)	0.13
MMP9	Matrix metalloproteinase 9	Breaks down type IV and V collagen	0.76 (1.48)	1.50
MYD88	Myeloid differentiation primary response gene (88)	TLR and ILR signaling mediator	0.17 (1.72)	1.16
PLA2G7	Phospholipase A2, group VII	Regulates platelet activating factor activity	-0.17 (1.60)	2.16
PTGS2	Prostaglandin H2 synthase	Converts arachidonate to prostaglandin	3.01 (1.40)	0.62
S100A9	S100 calcium-binding protein A9	Proinflammatory mediator of infections	0.07 (1.83)	1.75
SELENBP1	Selenium-binding protein 1	-	-0.23 (1.79)	1.78
TIMP1	Tissue inhibitor of metalloproteinase 1	Inactivates various MMPs	0.47 (1.70)	0.91
TIMP2	Tissue inhibitor of metalloproteinase 2	Inactivates various MMPs	0.02 (1.07)	1.30
TLR1	Toll-like receptor 1	Innate immune receptor	-0.34 (1.71)	2.59
TLR2	Toll-like receptor 2	Innate immune receptor	0.58 (2.74)	1.21

Inflammatory- and thrombotic-related gene expression levels in platelets from mice fed a high-fat diet were compared with mice on normal chow at 8 wk (high-fat diet n=14; normal chow n=14). EGF indicates epidermal growth factor; Fc, fragment, crystallizable; IgE, immunoglobulin E; and MCP-1, monocyte chemoattractant protein-1. SD=(standard deviation of target gene CT<sup>2</sup> + standard deviation of actin [ACTB] CT<sup>2</sup>)<sup>0.5</sup>).

**Table 2**

## Characteristics of the Framingham Offspring Study Sample Participants

<b>Variables</b>	<b>Mean±SD/Number (%)</b>
Sample size	1819
Female sex, n (%)	993(51)
Age, y	67±9
BMI, kg/m <sup>2</sup>	28.3±5.3
Lipid treatment, n (%)	798(44)
Total cholesterol, mg/100 mL	185±38
HDL cholesterol, mg/100 mL	57±19
Triglyceride, mg/100 mL	116±67
Antihypertensive treatment, n (%)	919(51)
Systolic blood pressure, mm Hg	129±17
Diastolic blood pressure, mm Hg	73±10
Glucose, mg/dL	107±25
Diabetes mellitus, n (%)	255(14)
Prevalent coronary heart disease, n (%)	199(11)
Aspirin (3 per week), n (%)	823(45)
Current hormone replacement therapy, n (%)	104(6)
Smoker, n (%)	155(8.5)

HDL indicates high-density lipoprotein.