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Macrophage Inflammation, Erythrophagocytosis and Accelerated Atherosclerosis in *Jak2*^{V617F} Mice

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Abstract

Rationale: The mechanisms driving athero-thrombotic risk in individuals with *JAK2*^{V617F} (*Jak2*^{VF}) positive clonal hematopoiesis (CH) or myeloproliferative neoplasms (MPN) are poorly understood.

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AUTHORSHIP

Wei Wang, Wenli Liu, Ying Wang, Yang Tang, Brittany Woods, Bishuang Cai, Trevor Fidler, Carlos Silvestre-Roig, Carrie Welch, Nan Wang performed research and analyzed data. Wei Wang, Nan Wang, Alan R. Tall, Ross L. Levine, Ira Tabas, Yong-Guang Yang and Ding Ai, Andres Hidalgo, Oliver Soehnlein designed research, analyzed data or wrote the paper. The authors declare no conflict of interest.

DISCLOSURE

The authors disclose no conflict of interest.

Objective: The goal of this study was to assess atherosclerosis and underlying mechanisms in hypercholesterolemic mice with hematopoietic *Jak2*^{VF} expression.

Methods and Results: Irradiated low-density lipoprotein receptor knockout (*Ldlr*$^{-/-}$) mice were transplanted with bone marrow from WT or *Jak2*^{VF} mice and fed a high fat high cholesterol Western diet (WD). Hematopoietic functions and atherosclerosis were characterized. After 7 weeks of WD *Jak2*^{VF} mice showed increased atherosclerosis. Early atherosclerotic lesions showed increased neutrophil adhesion and content, correlating with lesion size. After 12 weeks of WD *Jak2*^{VF} lesions showed increased complexity, with larger necrotic cores, defective efferocytosis, prominent iron deposition and co-staining of erythrocytes and macrophages suggesting erythrophagocytosis. *Jak2*^{VF} erythrocytes were more susceptible to phagocytosis by WT macrophages and showed decreased surface expression of CD47, a “don’t eat me” signal. Human JAK2VF erythrocytes were also more susceptible to erythrophagocytosis. *Jak2*^{VF} macrophages displayed increased expression and production of pro-inflammatory cytokines and chemokines, prominent inflammasome activation, increased p38 MAP kinase signaling and reduced levels of MerTK, a key molecule mediating efferocytosis. Increased erythrophagocytosis also suppressed efferocytosis.

Conclusions: Hematopoietic *Jak2*^{VF} expression promotes early lesion formation and increased complexity in advanced atherosclerosis. In addition to increasing hematopoiesis and neutrophil infiltration in early lesions, *Jak2*^{VF} caused cellular defects in erythrocytes and macrophages, leading to increased erythrophagocytosis but defective efferocytosis. These changes promote accumulation of iron in plaques and increased necrotic core formation which, together with exacerbated pro-inflammatory responses, likely contribute to plaque instability.

Keywords

Atherosclerosis; efferocytosis; erythrophagocytosis; inflammasome activation; *JAK2*^{V617F}; myeloproliferation; hypercholesterolemia; coronary heart disease; clonal hematopoiesis

INTRODUCTION

Myeloproliferative neoplasms (MPNs) including essential thrombocytosis, polycythemia vera (PV) and primary myelofibrosis, present as clonal expansions of one or more myeloid lineages.[1] In 2005 several groups identified somatic *JAK2V617F* (*JAK2*^{VF}) mutations in ≈95% of PV patients and in ≈50–60% of essential thrombocytosis and primary myelofibrosis patients.[2–6] The mutation activates JAK2 (Janus kinase 2) and downstream signaling pathways[7, 8] leading to proliferation of hematopoietic stem and progenitor cells (HSPCs). MPN patients are at significantly increased risk of athero-thrombotic events, including cardiac ischemic events and thrombotic stroke.[9] More recently, DNA sequencing of subjects in the general population has shown that more than 10% of people aged 70 or more have clones of blood cells bearing mutations that have been associated with hematological malignancies, primarily loss of function variants in epigenetic modifiers *TET2*, *ASXL1*, *DNTM3A*, as well as *JAK2*^{VF}. While clonal hematopoiesis (CH) was associated with an increased risk of hematological malignancies, unexpectedly there was also a 2–3 fold increase in the risk of atherosclerotic cardiovascular disease (CVD), identifying CH as a major risk factor for CVD in the elderly. Moreover, the prevalence of

CH increases from age 40 onward and CH mutations increase the risk of early onset myocardial infarction (<50 years old) by 4 fold. Although less common than the epigenetic modifier variants, the increase in risk appears to be strongest for the *JAK2*^{VF} variant (12-fold increase in CVD). While the association of CH with atherosclerosis in human populations could be confounded by aging, a causal relationship between *TET2* deficiency and atherosclerosis was shown in mouse models with pan-hematopoietic or myeloid *TET2* deficiency, and increased macrophage inflammation was implicated as an underlying mechanism.[10, 11] Whether the same or different atherogenic mechanisms are involved in the effects of other CH mutations is not known. In this study we have assessed atherosclerosis in mice with hematopoietic *Jak2*^{VF} expression and explored the underlying mechanisms.

METHODS

The authors declare that all supporting data, analytical methods and materials developed from this group within the article and its online supplementary files are available.

A detailed description of methods and materials is provided in the Online Data Supplement.

RESULTS

Increased atherosclerosis in Jak2^{VF} mice.

To assess the impact of hematopoietic *Jak2*^{VF} expression on atherosclerosis, sub-lethally irradiated WT or *Ldlr*$^{-/-}$ mice were transplanted with WT or *Jak2*^{VF} expressing bone marrow (BM) cells.[12] To assess a possible interaction of the mutation with hypercholesterolemia, WT recipients were fed a chow diet (CD) and *Ldlr*$^{-/-}$ recipients were fed WD. While WT recipients fed the chow diet remained normocholesterolemic, WD feeding caused progressive hypercholesterolemia in *Ldlr*$^{-/-}$ recipients (Online Figure IA). However, the increase in plasma cholesterol caused by the WD was less pronounced in the *Ldlr*$^{-/-}$ mice receiving *Jak2*^{VF} BM (≈30% lower after 7 weeks, $p<0.01$), reflecting reduced VLDL+LDL cholesterol levels as shown elsewhere[13], which may reflect increased uptake of LDL by expanded myeloid cells[14] or inflammatory cytokine effects on hepatic production.[15] Plasma HDL cholesterol in WD-fed mice (Online Figure IB) or plasma total cholesterol in CD-fed mice (Online Figure IC) showed no change. Plasma triglyceride (TG) levels were also decreased in WD-fed *Jak2*^{VF} recipients (Online Figure ID). Relative to the WT recipients, *Jak2*^{VF} recipients displayed expansion of HSPCs, erythrocyte (ERP) and megakaryocyte (MKP) progenitors in BM (Online Figure IE) and marked erythrocytosis, thrombocytosis and neutrophilia (Online Figure IF through IJ), as reported[12], on both chow and WD diets. There was marked erythrocyte microcytosis and anisocytosis (Online Figure IIA and IIB). *Jak2*^{VF} also markedly increased platelet/monocyte and platelet/neutrophil aggregates (Online Figure IIC and IID), likely reflecting increased platelet and leukocyte counts and increased platelet activation as evidenced by increased surface P selectin presentation in the basal or PAR4 agonist (AYPGKF)-stimulated state (Online Figure IIE and IIF). The increases in HSPC counts (Online Figure IE), neutrophilia (Online Figure IJ), platelet/monocyte and platelet/neutrophil aggregates (Online Figure IIC and IID), platelet surface P-selectin (Online Figure IIE) in *Jak2*^{VF} were significantly more pronounced on the

WD ($p<0.05$) as assessed by 2-way ANOVA and Sidak's post-hoc test for multiple comparisons.

Despite the lower plasma cholesterol levels, atherosclerotic lesion size in the aortic root was increased by 1.6 fold in *Jak2*^{VF} recipients fed WD for 7 weeks (Figure 1A), indicating a potent pro-atherogenic impact of *Jak2*^{VF}. Consistent with the pronounced neutrophilia, there was a marked increase in neutrophils in early lesions of *Jak2*^{VF} recipients (shown by Ly6G staining; Figure 1B and 1C; Online Figure IIIA) while macrophage content was unchanged (Online Figure IIIB). Lesional MPO, another neutrophil marker which largely overlapped with the Ly6G marker in lesional cells (Figure 1B), was also markedly increased in early lesions of *Jak2*^{VF} recipients (Figure 1D) and correlated with lesion size (Figure 1E), consistent with a pro-atherogenic role of neutrophils in early atherogenesis.[16] Intravital fluorescence microscopy showed a marked increase in neutrophil rolling and firm adhesion on early carotid artery lesions in *Jak2*^{VF} recipients (Figure 2A through 2C); monocyte rolling but not adhesion was significantly increased (Online Figure IIIC and IIID). Neutrophils from WD-fed *Jak2*^{VF} recipients showed evidence of activation with increased expression of formyl peptide receptor 1 (FPR1) and adhesion molecule CD11b (Figure 2D and 2E). Neutrophils displayed increased adhesion to recombinant cell adhesion molecules (Figure 2F and 2G). Thus, in addition to neutrophilia, neutrophil activation likely contributed to increased adhesion and entry of neutrophils into early atherosclerotic plaques.

In mice fed the WD for 12 weeks, plasma total cholesterol and TG levels were ≈50% lower in *Jak2*^{VF} recipients (Online Figure IVA and IVB). Despite the markedly reduced cholesterol and TG levels, lesion size showed a trend to be increased (1.3 fold, $p=0.07$) in the *Jak2*^{VF} recipients (Figure 3A). Necrotic core area, a well-established index of plaque instability[17], was significantly increased, both in absolute terms (area of necrotic core per section, 1.7 fold increase) or relative to lesion size (% of total lesion area, 1.4 fold increase) (Figure 3B though 3D) in *Jak2*^{VF} recipients compared to controls. Lesional macrophages (Figure 3E) but not neutrophils (Online Figure IVC) were increased in advanced lesions of *Jak2*^{VF} recipients. Unlike in early lesions, lesional neutrophil count showed no significant correlation with lesion area in advanced lesions (Online Figure IVD). Collagen content and fibrous cap thickness did not show differences between the genotypes in advanced lesions (Online Figure IVE).

Jak2^{VF} increases lesional erythrophagocytosis in advanced atherosclerosis.

Increased hematocrit as well as abnormalities in RBC morphology in *Jak2*^{VF} mice (Online Figure IF; Online Figure IIA and IIB) suggested a possible role of erythrocytes in atherosclerosis.[18] To assess this, we stained lesions for iron and erythrocytes. While WT recipients showed little lesional iron deposition, iron staining was clearly identified in the majority of *Jak2*^{VF} recipients in advanced lesions (Figure 4A, Online Figure IVF, $p<0.05$, Chi-Squared Test). Staining of Ter119, a specific erythrocyte marker, was also increased in advanced lesions of *Jak2*^{VF} recipients (Figure 4A, Online Figure IVG, $p<0.05$, Chi-Squared Test). Interestingly, when the Ter119 positive sections were stained with antibodies against the macrophage marker Mac-3, the two markers were largely co-localized (Figure 4A), suggesting erythrophagocytosis. Notably, macrophages containing erythrocyte markers were

primarily found on the periphery of necrotic cores (Figure 4A), particularly in 7 week early lesions (Online Figure VA).

To assess potential pathways for entry of RBCs into lesions, we assessed Von Willebrand Factor staining. However, there was little staining in advanced lesions and no difference between *Jak2*^{VF} and WT recipients (not shown). We did detect a marked increase in erythrocyte/neutrophil and erythrocyte/monocyte complexes in the circulation in *Jak2*^{VF} recipients (Online Figure VB), suggesting the possibility that erythrocyte/leukocyte complex formation facilitates RBC entry via the luminal surface of plaques.

Jak2^{VF} erythrocytes are more susceptible to erythrophagocytosis.

To gain insights into the mechanism of erythrophagocytosis in lesions, we incubated WT or *Jak2*^{VF} erythrocytes with WT or *Jak2*^{VF} macrophages and assessed erythrophagocytosis by fluorescence microscopy. *Jak2*^{VF} erythrocytes showed increased uptake by either WT or *Jak2*^{VF} macrophages (Figure 4B). Erythrophagocytosis rather than surface binding of erythrocytes by phagocytes was confirmed by reconstructed 3D images obtained by fluorescence confocal microscopy as well as overlay on bright field images (Online Figure VIA and VIB). To assess whether the findings of aberrant erythrophagocytosis with mouse *Jak2*^{VF} erythrocytes could be recapitulated with human erythrocytes, we obtained human blood samples from JAK2VF positive MPN patients and matched control human subjects. These patients were newly identified, non-treated or being treated with aspirin or phlebotomy but not with hydroxyurea or ruxolitinib. Incubation of human erythrocytes with human macrophages derived from peripheral blood mononuclear cells of healthy human subjects also resulted in robust erythrophagocytosis (Online Figure VIC). Quantification of erythrophagocytosis by flow cytometry indicated increased erythrophagocytosis of JAK2VF erythrocytes compared to control erythrocytes (Figure 4C and 4D).

Decreased CD47, a “don’t eat me signal” or increased calreticulin, an “eat me” signal, in Jak2^{VF} erythrocytes.

Accelerated erythrocyte aging has been proposed to explain increased erythrophagocytosis in some erythrocytosis models.[19] Increased erythrocyte band 4.1a/4.1b ratio has been used as a marker for erythrocyte senescence.[19] Band 4.1a/4.1b ratio in mouse *Jak2*^{VF} erythrocytes was not altered relative to the WT erythrocytes (Online Figure VID), suggesting no change of erythrocyte senescence. However, we noticed that band 4.2 was markedly decreased in *Jak2*^{VF} erythrocytes (Online Figure VID and VIE). Band 4.2 deficiency has been linked to accelerated erythrocyte clearance and marked reduction of CD47[20,21], a molecule protecting erythrocytes from fortuitous phagocytosis by macrophages.[22] We assessed erythrocyte surface CD47 levels by flow cytometry and found a significant reduction in *Jak2*^{VF} erythrocytes (Figure 5A and 5B). In contrast, human *Jak2*^{VF} erythrocytes did not show a decrease in surface CD47 (not shown) but rather displayed increased surface calreticulin (Figure 5C), consistent with a recent report.[23] Surface calreticulin counteracts CD47 signaling and promotes phagocytosis of erythrocytes.[24] These results suggest distinct mechanisms promoting erythrophagocytosis of human versus mouse *Jak2*^{VF} erythrocytes.

Defective efferocytosis in advanced lesions in Jak2^{VF} mice.

A body of work indicates that efficient efferocytosis of apoptotic cells by lesional macrophages is a key event limiting necrotic core formation in advanced atherosclerosis.[25–27] The increased necrotic core in advanced lesions of *Jak2^{VF}* mice led us to assess lesional efferocytosis. The ratio of free vs macrophage-associated apoptotic cells was markedly increased in advanced lesions of *Jak2^{VF}* mice (Figure 6A), indicating defective efferocytosis. Macrophage c-Mer tyrosine kinase (MerTK) serves as a cell surface receptor and signaling molecule mediating efferocytosis, and MerTK has a central role in promoting efferocytosis and decreasing necrotic core formation in atherosclerotic lesions.[25,28] We thus assessed surface MerTK levels *in vivo* in splenic macrophages and levels of soluble MerTK (sol-Mer), the product of surface MerTK cleavage, generated from the cultured macrophages of WT and *Jak2^{VF}* mice. This showed increased cleavage (inset, Figure 6B) and markedly decreased cell surface MerTK levels (Figure 6B) in *Jak2^{VF}* compared to WT macrophages, while plasma sol-Mer levels showed no change (Online Figure VIF). Importantly, MerTK levels in lesional macrophages were markedly decreased in advanced lesions of *Jak2^{VF}* mice (Figure 6C; Online Figure VIG and VIH). Next, we assessed the potential impact of erythrophagocytosis on efferocytosis *ex vivo*. Co-incubation of WT or *Jak2^{VF}* macrophages with erythrocytes and apoptotic Jurkat cells led to suppression of efferocytosis of Jurkat cells relative to incubation with Jurkat cells alone (Figure 6D). WT and *Jak2^{VF}* macrophages did not show difference in efferocytosis. In contrast, *Jak2^{VF}* erythrocytes caused a more pronounced suppression of efferocytosis in both WT and *Jak2^{VF}* macrophages (Figure 6D), likely reflecting the increased susceptibility of *Jak2^{VF}* erythrocytes to erythrophagocytosis. Together, these findings suggest that increased uptake of erythrocytes in combination with decreased uptake of apoptotic cells by lesional macrophages contributes to advanced lesion complexity including increased necrotic core formation in *Jak2^{VF}* mice.

Increased inflammatory activation of Jak2^{VF} macrophage.

To assess inflammatory responses we challenged WT or *Jak2^{VF}* macrophages with LPS, a stimulus relevant to toll-like receptor/Myeloid differentiation primary response 88 (TLR4/MyD88) and toll-like receptor/TIR-domain-containing adapter-inducing interferon-β (TLR4/TRIF) signaling pathways that are known to promote atherogenesis.[29,30] *Jak2^{VF}* macrophages showed increased expression of pro-inflammatory cytokines and chemokines i.e. IL-1β, IL-6, iNOS, Tnf-α and MCP-1 (Figure 7A; Online Figure IX; Online Figure XB; Online Figure XI). While IL-6 secretion from *Jak2^{VF}* macrophages was markedly increased, the increase in IL-1β secretion following 8 hour LPS stimulation was only moderate (Online Figure VIIA and VIIB), and less pronounced than the increase in IL-1β mRNA levels (Figure 7A). Prominent IL-1β secretion requires inflammasome activation.[31] Indeed, ATP-stimulated IL-1β production from *Jak2^{VF}* macrophages was increased more pronouncedly relative to wild type cells (Figure 7B), consistent with inflammasome activation. To evaluate the relevance *in vivo*, we examined inflammasome activation by assessing caspase 1 and caspase 11 cleavage in splenic CD11b^{+} or CD11b^{-} cells. This was markedly increased in *Jak2^{VF}* CD11b^{+} (Figure 7C) but not WT CD11b^{+} (Figure 7C) or *Jak2^{VF}* or WT CD11b^{-} cells (not shown), suggesting activation of both NLRP3 and non-canonical, Caspase-11 dependent macrophage inflammasomes.[32] Markedly increased IL-1β production from the freshly isolated splenic *Jak2^{VF}* CD11b^{+} cells, particularly in response to LPS, was also

consistent with inflammasome activation *in vivo* (Figure 7D). Additional evidence showing inflammasome activation came from the finding that plasma levels of IL-18, which production depends on and is considered as a marker of inflammasome activation *in vivo*[33], were markedly increased in *Jak2*^{VF} mice (Figure 7E).

While increased pro-inflammatory responses to LPS were consistently detected in briefly cultured concanavalin (ConA)-elicited mouse peritoneal macrophages (Figure 7A; Online Figure VIIC), the responses were less prominent in bone marrow derived macrophages (BMDM) cultured for 7 days (Online Figure VIID). ConA is known to induce T cell proliferation and interferon-γ (IFNγ) responses *in vivo*[34] and JAK2 has an essential role in mediating IFNγ signaling.[35] To explore the possibility that macrophages were primed for increased signaling in ConA-treated *Jak2*^{VF} mice, we assessed multiple molecules mediating IFNγ and JAK2 signaling. Levels of phosphorylated p38, JNK and AKT in the non-LPS treated basal state were significantly increased in ConA-elicited *Jak2*^{VF} macrophages, following a brief 6-hour culture *in vitro* (Online Figure VIIIA) and this was largely reversed after 48 hours in culture (Online Figure VIIIB). Total and phosphorylated STAT1 were markedly decreased, a finding consistent with the negative feedback regulation of STAT1 by IFNγ signaling.[36] P38 and JNK are critical in mediating TLR4 initiated pro-inflammatory responses in macrophage.[37,38] Inhibition of p38, JNK or combined inhibition of p38 and JNK partially or completely reversed the LPS-induced pro-inflammatory responses of ConA-elicited *Jak2*^{VF} macrophages (Online Figure IX), suggesting that increased priming had a major role in the enhanced inflammatory response to LPS. We also assessed the potential impact of altered endoplasmic reticulum (ER) stress or autophagy, which are known to regulate pro-inflammatory activation of macrophages.[39,40] CCAAT-enhancer-binding protein homologous protein (CHOP) expression, a marker of ER stress, showed no difference between WT and *Jak2*^{VF} macrophages either in the basal or tunicamycin-induced ER stress state (Online Figure XA). Rapamycin, an inducer of autophagy, decreased the expression of some cytokines in response to LPS but the effect was proportionate for WT and *Jak2*^{VF} macrophages (Online Figure XB). JAK1/2 inhibitor such as ruxolitinib has been approved as a treatment for JAK2VF positive MPN patients.[41] Notably, ruxolitinib reversed the increase in pro-inflammatory cytokine and chemokine expression in *Jak2*^{VF} macrophages, except for Tnf-α (Online Figure XI). In contrast, ruxolitinib failed to reverse the increased susceptibility of *Jak2*^{VF} erythrocytes to erythrophagocytosis (Online Figure XC).

DISCUSSION

Our study demonstrates that hematopoietic *Jak2*^{VF} expression in hypercholesterolemic mice results in accelerated atherosclerosis with features of plaque instability, consistent with the increase in athero-thrombotic cardiovascular disease seen in patients with JAK2VF-associated MPN or CH.[10,11,13,42] Increased neutrophil infiltration due to neutrophilia and neutrophil activation likely accounts for accelerated early lesion formation. In contrast, advanced atherosclerotic lesions displayed increased necrotic cores, increased macrophages, iron deposition and evidence of erythrophagocytosis: similar features have been associated with atherosclerotic plaque instability in humans.[18]

On a mechanistic level, *Jak2*^{VF} macrophages displayed increased cleavage and reduced surface levels of MerTK in association with defective efferocytosis in advanced lesions, likely contributing to increased necrotic core formation (Figure 8).[43,44] *Jak2*^{VF} macrophages showed increased inflammatory responses including p38 map kinase activation likely promoting MerTK cleavage.[45] There was marked inflammasome activation in *Jak2*^{VF} macrophages leading to increased IL-1β secretion and increased IL-18 plasma levels. Increased production of macrophage inflammatory cytokines could contribute to increased neutrophil production and activation and entry of leukocytes into lesions.[46,47] Augmented phagocytosis of RBC by macrophages likely reflected both increased RBC production as well as intrinsic RBC defects which were seen in both mice and humans. Erythrophagocytosis was shown to suppress efferocytosis, suggesting a mechanistic link between these two processes. Thus our studies demonstrate that the mechanisms underlying the pro-atherogenic effect of *Jak2*^{VF} are multifaceted, involving different hematopoietic lineages and their interactions (Figure 8).

Aberrant hematopoiesis and neutrophil infiltration of lesions were associated with increased early atherosclerosis in *Jak2*^{VF} mice, as reported in other models of neutrophil overproduction.[16] Consistent with a major role of neutrophils, increased rolling and firm adhesion of neutrophils was shown by intravital microscopy of carotid arteries in *Jak2*^{VF} mice. Platelet activation, neutrophil activation, platelet/neutrophil and platelet/monocyte aggregates which were prominently increased in *Jak2*^{VF} mice are known to promote recruitment of inflammatory leukocytes into lesions.[48,49] Importantly, several of these atherogenic propensities, such as basal platelet P-selectin exposure and platelet-monocyte aggregates, were augmented by an interaction of hypercholesterolemia with the *Jak2*^{VF} mutation. In addition, hypercholesterolemia interacted with the *Jak2*^{VF} to synergistically increase the bone marrow hematopoietic stem cell population, possibly reflecting cross-talk between JAK2VF signaling with signaling pathways that are activated by cholesterol accumulation in HSPCs.[50] This raises the possibility that hypercholesterolemia could promote the evolution of CH.

Erythophagocytosis has been described as a prominent feature in complex human atherosclerotic lesions and proposed to promote macrophage foam cell formation and lesional necrotic core formation.[18] Erythrocyte and macrophage markers co-localize in or around lesional necrotic cores in advanced human atherosclerotic lesions, suggesting that erythrophagocytosis may contribute to plaque instability.[18] Increased erythrophagocytosis appeared to involve different mechanisms in mouse versus human RBCs – with decreased levels of CD47, a don't eat me signal in mice and increased levels of calreticulin, a pro-phagocytic signal in human RBCs. The mechanisms responsible for increased RBC entry into lesions are uncertain but could involve the observed increase in formation of RBC-leukocyte aggregates which could carry RBCs from the arterial lumen into the subendothelial space. One limitation of the study is use of female *Ldlr*$^{-/-}$ mice only as recipients for atherosclerosis studies. Nevertheless, both male and female mice were used for *in vitro* assays of erythrophagocytosis, indicating that the altered erythrophagocytosis was not limited to females.

The elevated expression of multiple pro-inflammatory cytokines and chemokines in *Jak2*^{VF} macrophage in response to LPS stimulation suggests that heightened inflammation also contributes to accelerated atherosclerosis in *Jak2*^{VF} mice. Increased lesional inflammation could trigger cleavage of cell surface MerTK in macrophages in *Jak2*^{VF} mice, leading to defective efferocytosis and increased necrotic core formation.[25] Cleavage of macrophage cell surface MerTK is primarily mediated by ADAM17 and this process can be up-regulated by Toll-like receptor 4 and p38 MAP kinase signaling[45] which was increased in *Jak2*^{VF} macrophages. Therefore, pro-inflammatory macrophage activation in *Jak2*^{VF} mice could exacerbate atherosclerosis by impaired efferocytosis via p38 MAP kinase and by pro-inflammatory cytokine and chemokine production. As previously reported[32] there was no detectable inflammasome activation in Western diet fed *Ldlr*$^{-/-}$ mice transplanted with wild type bone marrow. However, there was prominent inflammasome activation in splenic CD11b^{+} cells which includes macrophages in *Jak2*^{VF} mice. The increased production of IL-1β and possibly IL-18 could also contribute to neutrophilia and neutrophil infiltration detected in *Jak2*^{VF} mice.[46,47,51]

Another shortcoming of our study is that it involved pan-hematopoietic *Jak2*^{VF} and thus abnormalities in hematopoiesis may have contributed more prominently to atherogenesis than in a true CH model.[11] Although prospectively studied CH subjects did not have abnormal blood cell counts at baseline[52], qualitative changes in blood cell function, as well as development of myelo-proliferation in some patients[53], appear likely. In humans carrying mutations that cause CH and increased CVD risk, the single abnormality in blood cell phenotypes was an increase in erythrocyte anisocytosis[52] which was also prominent in *Jak2*^{VF} mice and is a known CVD risk factor in the general population.[54,55] We speculate that anisocytosis may be a marker of aberrant erythrocyte properties, possibly reflecting increased erythrocyte production or interactions between inflammatory myeloid cells and erythroblasts in the bone marrow[56], that predisposes to atherosclerosis, for example by stimulation of erythhrophagocytosis in atherosclerotic lesions.

The recommended treatment for low risk PV patients includes phlebotomy and low dose aspirin.[57,58] Current recommendations suggest that the hematocrit should be maintained below 45%[42], as higher hematocrits are associated with increased cardiovascular death and major thrombosis in PV patients.[42] Our findings suggest that *Jak2*^{VF} erythrocytes may have a direct role in promoting advanced atherosclerosis and plaque instability, raising the possibility that even lower levels of hematocrit may be desirable. Finally, our findings highlight the importance of increased myelopoiesis, pro-inflammatory macrophage activation, platelet activation and PLA formation in atherogenesis, suggesting the need for effective anti-platelet and cytoreductive therapies in MPNs and perhaps in CH. Since many of the underlying atherogenic mechanisms were aggravated by hypercholesterolemia in *Jak2*^{VF} mice, control of LDL cholesterol via statins and proprotein convertase subtilisin/kexin type 9 (PCSK9) mAbs may be particularly important in patients with JAK2VF-associated MPN or CH. Finally, the recent demonstration in the CANTOS trial that IL-1β antibodies reduced coronary heart disease opens a new vista on anti-inflammatory therapies as a treatment for atherosclerosis[59], and our findings suggest that patients with CH or MPN may particularly benefit from this or other anti-inflammatory therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms:

BM	Bone marrow
BMDM	Bone marrow-derived macrophage
CH	Clonal hematopoiesis
ConA	Concanavalin A
DAPI	4′,6-diamidino-2-phenylindole
HSPC	hematopoietic stem and progenitor cells
IFNγ	Interferon gamma
JAK	Janus kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MerTK	c-Mer tyrosine kinase
MPN	Myeloproliferative neoplasms
RBC	Red blood cell
WD	Western diet
WT	White type

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NOVELTY AND SIGNIFICANCE

What Is Known?

- Acquired activating mutations of *JAK2* notably $JAK2^{V617F}$ ($JAK2^{VF}$) drive development of clonal hematopoiesis (CH) and myeloproliferative neoplasms (MPN).
- $JAK2^{VF}$ positive CH and MPN are associated with increased athero-thrombotic risk, but the underlying mechanisms are poorly understood.

What New Information Does This Article Contribute?

- Hematopoietic $Jak2^{VF}$ expression in $Ldlr^{-/-}$ mice promotes neutrophil-enriched early lesion formation.
- Advanced lesions show increased necrotic cores, defective efferocytosis and prominent erythrophagocytosis
- $Jak2^{VF}$ macrophages displayed increased expression of pro-inflammatory cytokines and inflammasome activation, possibly driving neutrophil entry into lesions.
- $Jak2^{VF}$ erythrocytes undergo increased uptake by macrophages (erythrophagocytosis), leading to impaired uptake of apoptotic cells (efferocytosis), which together with increased cleavage of MerTK, promotes necrotic core formation and plaque instability.

$Jak2^{VF}$, a gain of function mutation that is commonly found in elderly patients with myeloproliferative neoplasms or clonal hematopoiesis, is associated with increased risk of athero-thrombotic diseases. In $Ldlr^{-/-}$ mice expressing $Jak2^{VF}$ in hematopoietic tissues, we showed accelerated early atherosclerosis and increased complexity of advanced lesions, despite lower levels of LDL cholesterol. Early lesions showed increased binding of neutrophils to endothelium and increased numbers of neutrophils in plaques. More advanced lesions showed increased necrotic cores, defective efferocytosis and erythrophagocytosis. Erythrophagocytosis refected a $Jak2^{VF}$ intrinsic red cell defect. Defective efferocytosis was linked to macrophage infammation and MerTK cleavage and to competition between red cells and apoptotic cells for macrophage uptake. These studies provide direct evidence that $Jak2^{VF}$ increases atherogenesis, involving different hematopoietic lineages and their interactions.

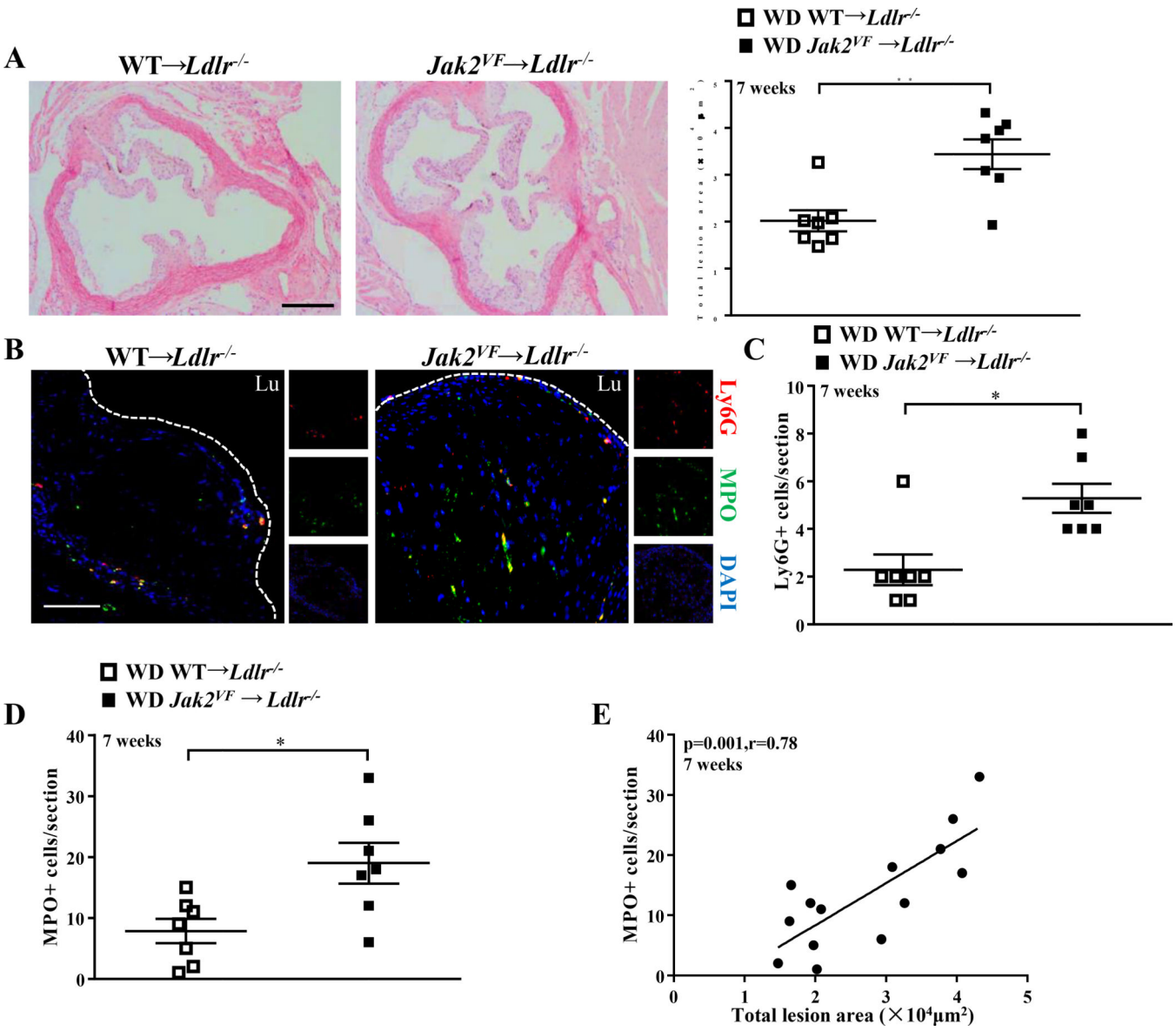

Figure 1: Increased early atherosclerotic lesions and neutrophil infiltration in *Jak2*^{VF} mice.

(A) Representative H&E-stained aortic root lesions and quantification of total lesion area of female *Ldlr*$^{-/-}$ recipients after 7 weeks of WD. Mann-Whitney *U* test. Scale bar, 500μm. **(B)** Representative immunofluorescence images of MPO (Green) and Ly6G (Red) with DAPI (Blue) of aortic root lesions from female mice fed WD for 7 weeks. Mann-Whitney *U* test. Scale bar,100μm. **(C)** Quantification of Ly6G positive cells (Mann-Whitney *U* test) and **(D)** MPO positive cells in the lesions. Unpaired *t* test. **(E)** Correlation between MPO^{+} neutrophils and total lesion size after 7 weeks of WD. *p<0.05, **p<0.01. Spearman correlation test.

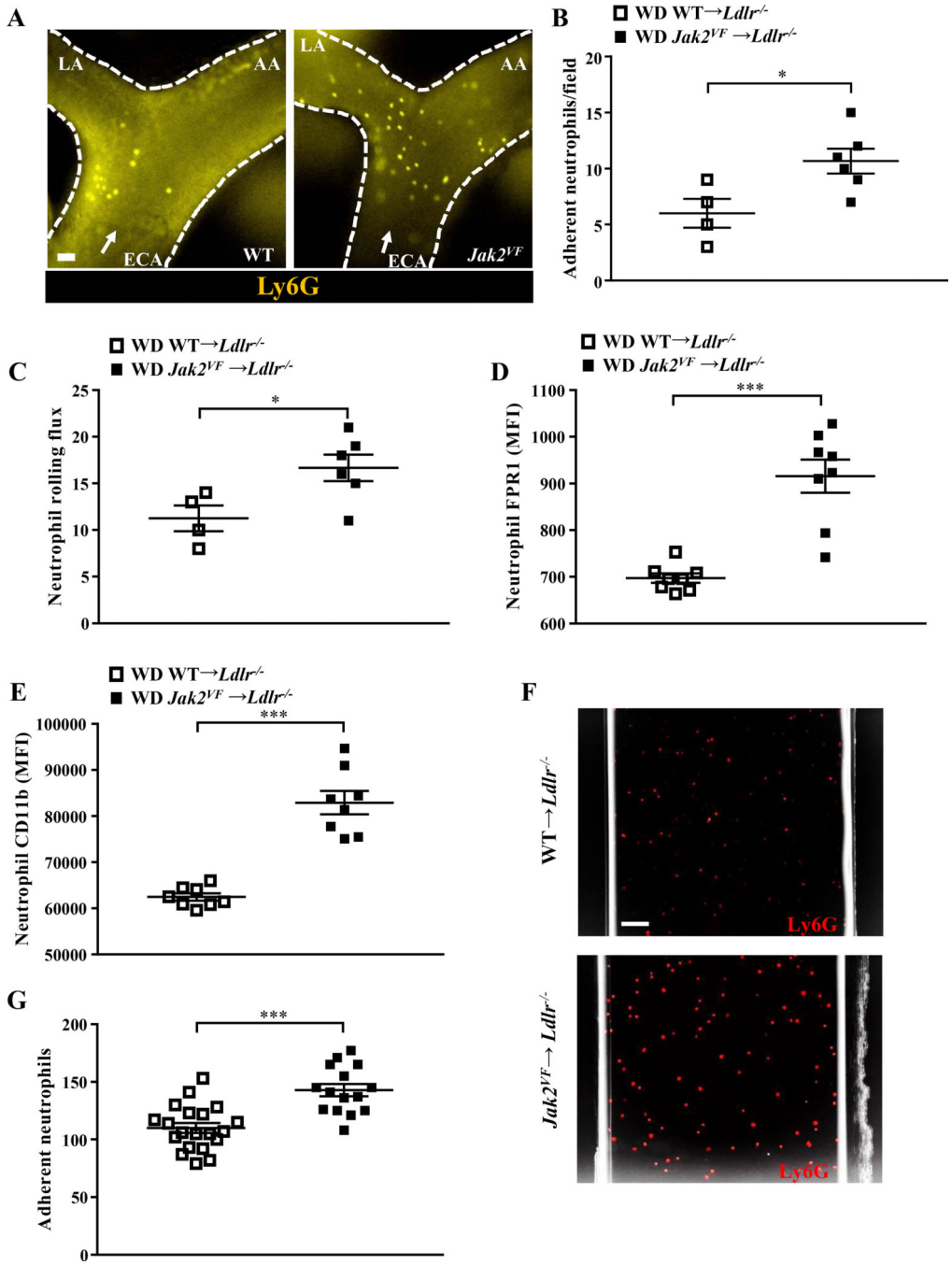

Figure 2: Increased rolling and adhesion of neutrophils in *Jak2*^{VF} mice.

Female *Ldlr*$^{-/-}$ recipients were fed WD for 5 weeks. **(A)** Representative image of epifluorescence intravital microscopy of the carotid artery showing interaction of Ly6G-stained neutrophils with the arterial vessel wall. ECA, external carotid artery; LA, lingual artery; AA, auricular artery. Arrow indicates flow direction. Scale bar, 50μm. **(B)** Quantification of Ly6G-stained neutrophil adhesion in the carotid artery by intravital microscopy. Mann-Whitney *U* test. **(C)** Neutrophil rolling flux was assessed by intravital microscopy. Expression of **(D)** FPR1 and **(E)** CD11b MFI on neutrophils were measured by

flow cytometry. Mann-Whitney *U* test. **(F,G)** Flow chamber assays for neutrophil adhesion using equal number of neutrophils. **(F)** Representative images and **(G)** quantification of adhesion. Mann-Whitney *U* test. Scale bar, 50μm. *$p<0.05$, ***$p<0.001$.

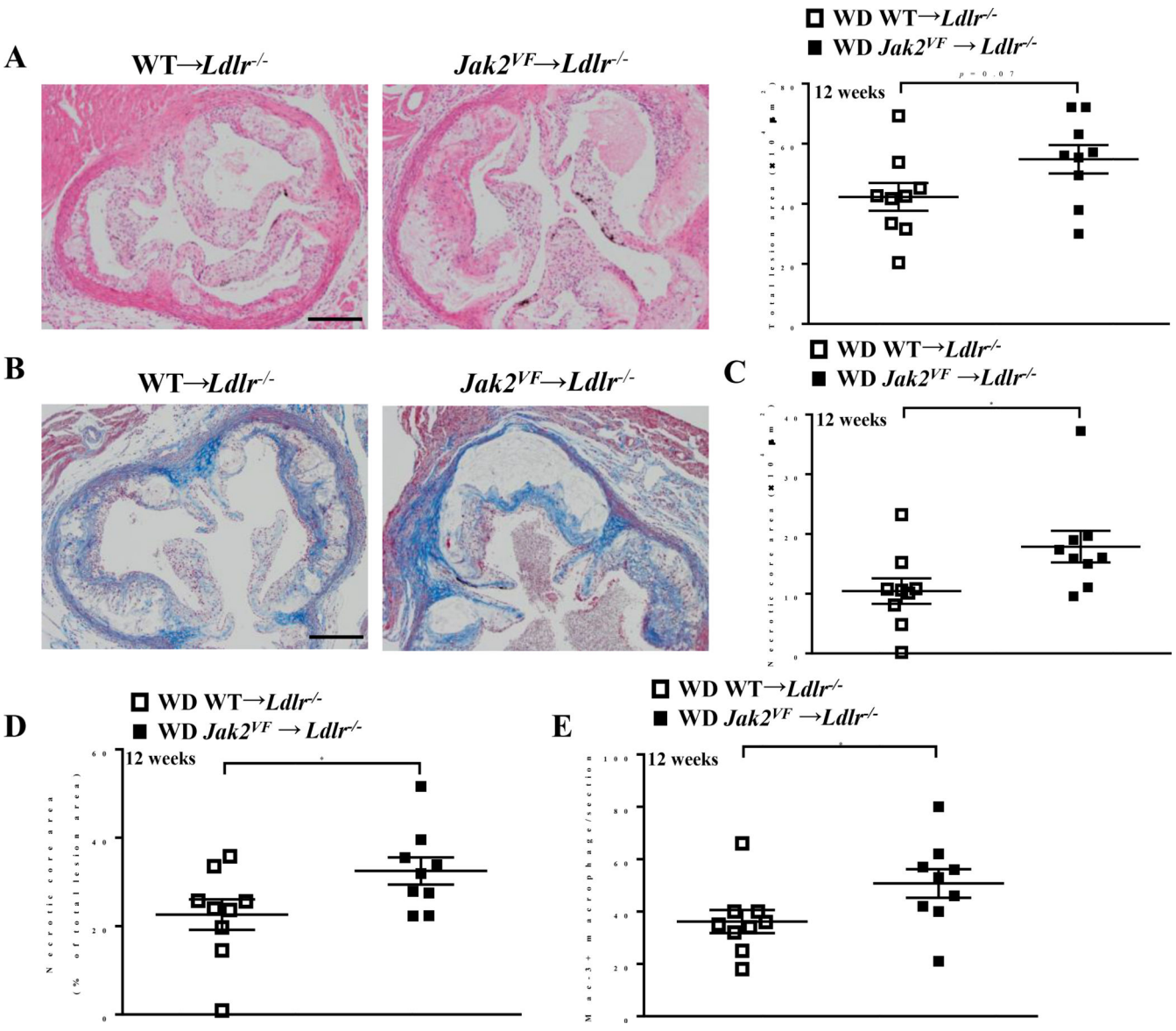

Figure 3: Increased necrotic core in advanced lesions of *Jak2*^{VF} mice.

(A) Representative H&E-stained aortic root lesions and quantification of total lesion area of female *Ldlr*$^{-/-}$ recipients after 12 weeks of WD. Unpaired *t* test. Scale bar, 500μm. **(B)** Representative massion trichrome stain images of lesions. Scale bar, 500μm. **(C)** Quantification of necrotic core area and **(D)** as a percentage of total lesion area of female *Ldlr*$^{-/-}$ recipients after 12 weeks WD. Mann-Whitney *U* test. **(E)** Quantification of Mac-3^{+} macrophage in the lesions of female mice WD-fed for 12 weeks. Mann-Whitney *U* test. **p*<0.05.

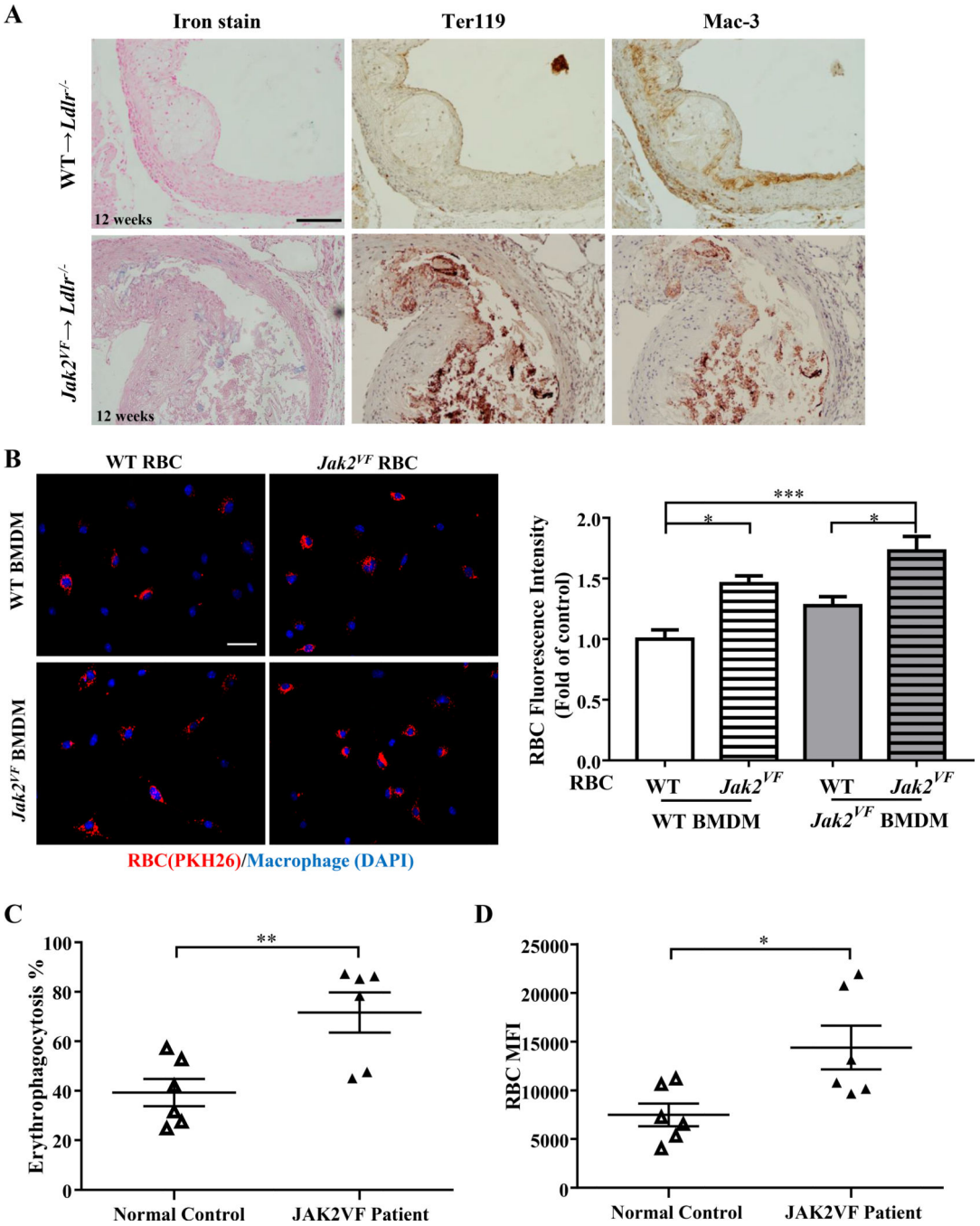

Figure 4: *Jak2*^{VF} mice displayed marked increase in erythrophagocytosis.

(A) Representative images of iron staining, immunohistochemistry staining of red blood cell marker Ter119 and macrophage marker Mac-3 in lesions of female *Ldlr*$^{-/-}$ recipients after 12 weeks WD. Scale bar, 100μm. **(B)** Bone marrow derived macrophages (DAPI, Blue) were incubated with 2 million PKH26-labeled erythrocytes (Red) overnight and quantification of relative red blood cells fluorescence intensity. Cells from both male and female mice were used for the assays. Pooled data from 5 independent experiments were used for analysis. Scale bar, 50μm. 2-way ANOVA. **(C)** Erythrophagocytosis rate (Mann-Whitney *U* test) and

(D) red blood cells MFI of human normal control and JAK2VF patients were measured by flow cytometry. Unpaired *t* test. $*p<0.05$, $**p<0.01$, $***p<0.001$.

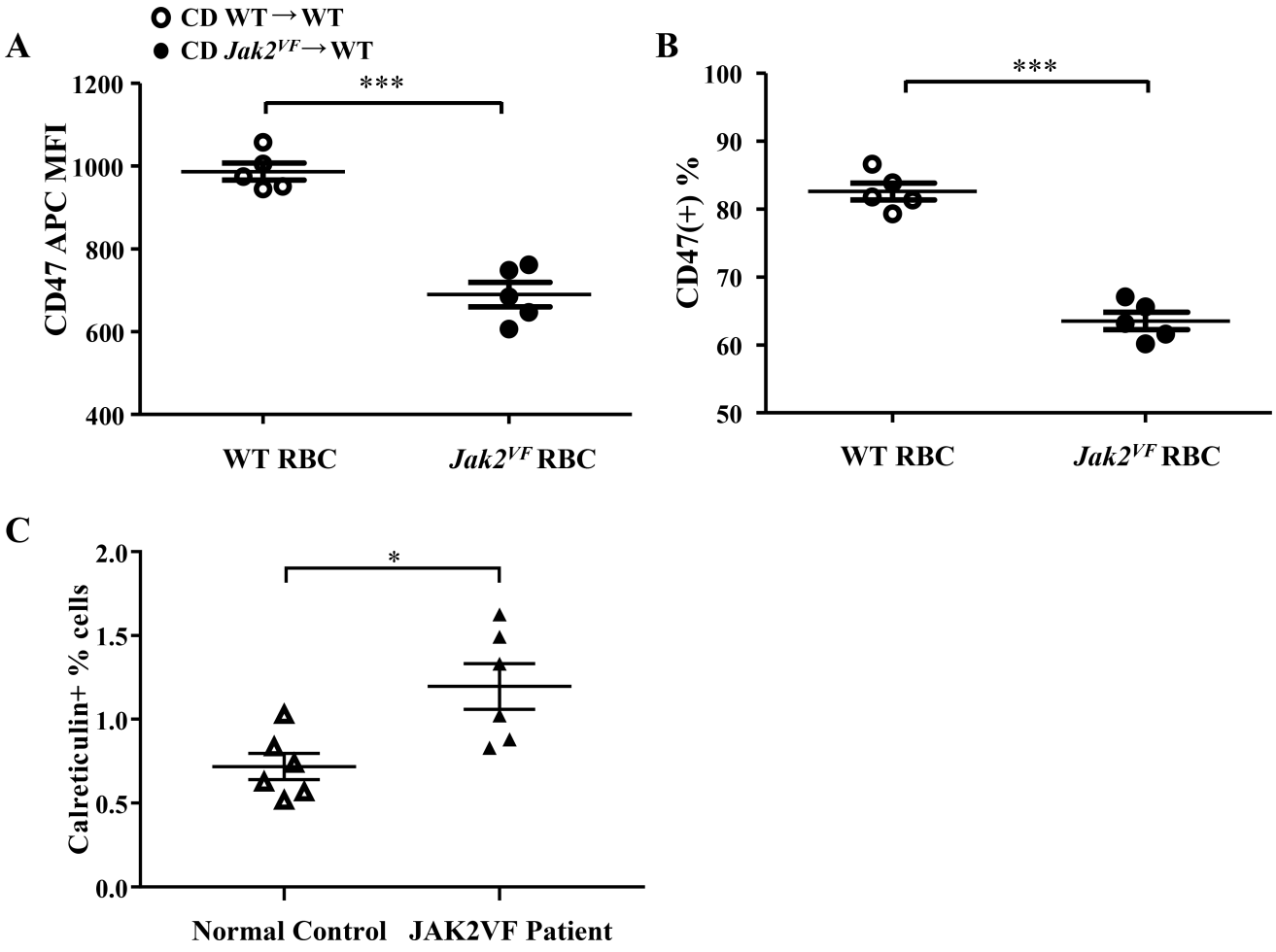

Figure 5: *Jak2*^{VF} mice showed reduced surface CD47 expression in erythrocytes.

Erythrocytes were from chow-fed female mice. **(A)** Mean fluorescence intensity of anti-CD47 antibody bound to erythrocytes (Unpaired *t* test) and **(B)** percentage of CD47^{hi} erythrocytes by flow cytometry. Unpaired *t* test. **(C)** Percentage of calreticulin positive erythrocytes in human normal controls and JAK2VF patients. Unpaired *t* test. *p<0.05, ***p<0.001.

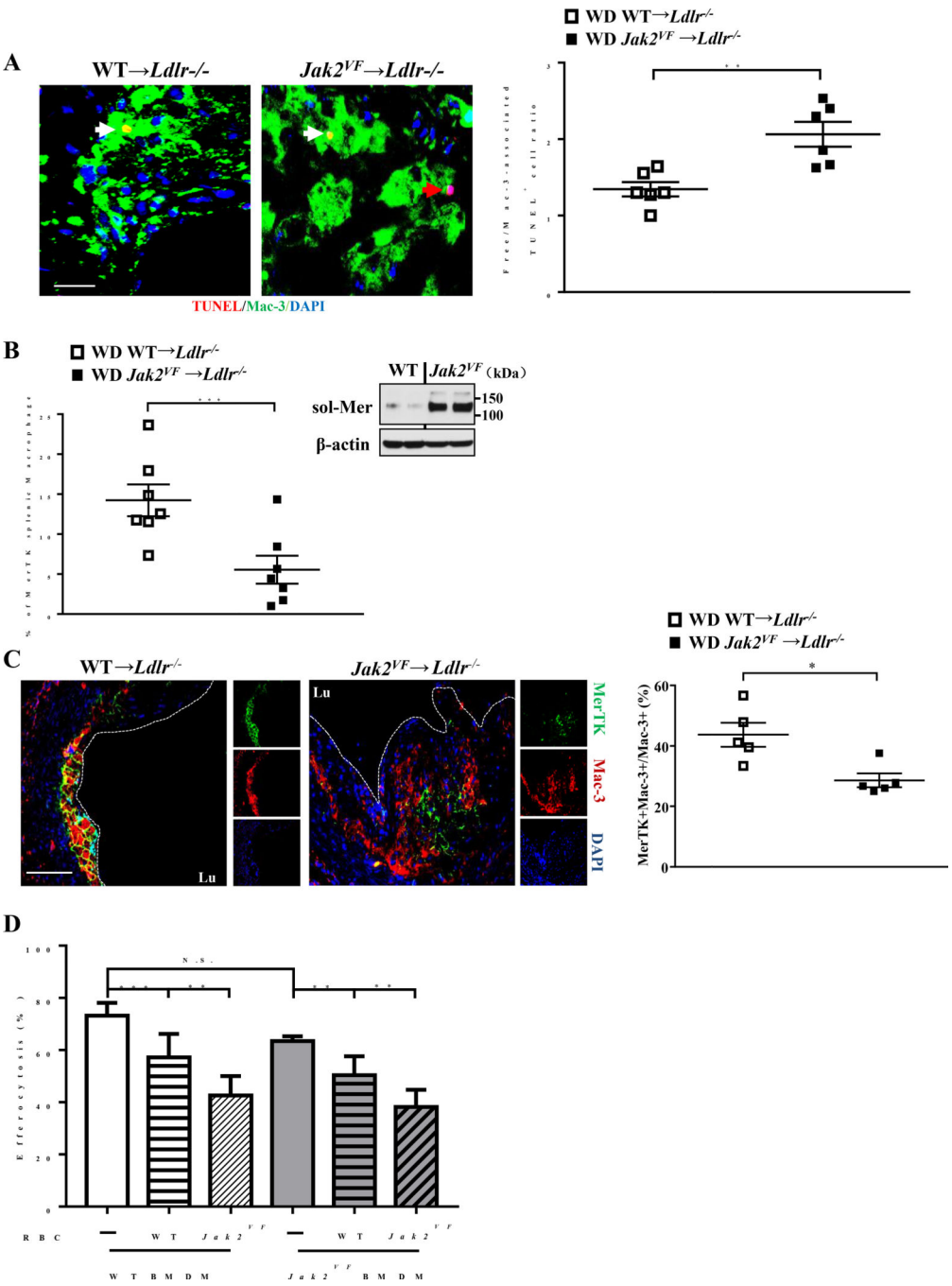

Figure 6: Defective efferocytosis was associated with decreased macrophage surface MerTK in *Jak2*^{VF} mice.

(A) Representative images of advanced lesions (12 weeks WD-fed) in which apoptotic cells were stained by TUNEL (Red), macrophages by Mac-3 (Green) and nuclei by DAPI (Blue). Efferocytosis was assessed as the ratio of free to macrophage-associated TUNEL positive cells. The red arrow depicts free apoptotic cells and the white arrow depicts macrophage associated apoptotic cells. Scale bar, 20μm. Unpaired *t* test**. (B)** Percentage of MerTK postive macrophages in total spleen cells as determined by flow cytometry and western blot

of soluble MerTK levels in cultured media of splenic macrophages. Unpaired *t* test**. (C)** Representative single (small panel) or merged fluorescence images (large panel) of Mac-3 (Red), MerTK (Green) or DAPI (Blue) and quantification of the ratio of MerTK/Mac-3 co-positive to Mac-3 positive macrophages. Scale bar, 100μm. Mann-Whitney *U* test. **(D)** Bone marrow derived WT or $Jak2^{VF}$ macrophages were treated with or without 5 million WT or $Jak2^{VF}$ erythrocytes in the presence of apoptotic Jurkat cells for 20 hours to assess efferocytosis by fluorescence microscope. Data were the representative of 5 independent experiments. 2-way ANOVA. *p<0.05, **p<0.01, ***p<0.001.

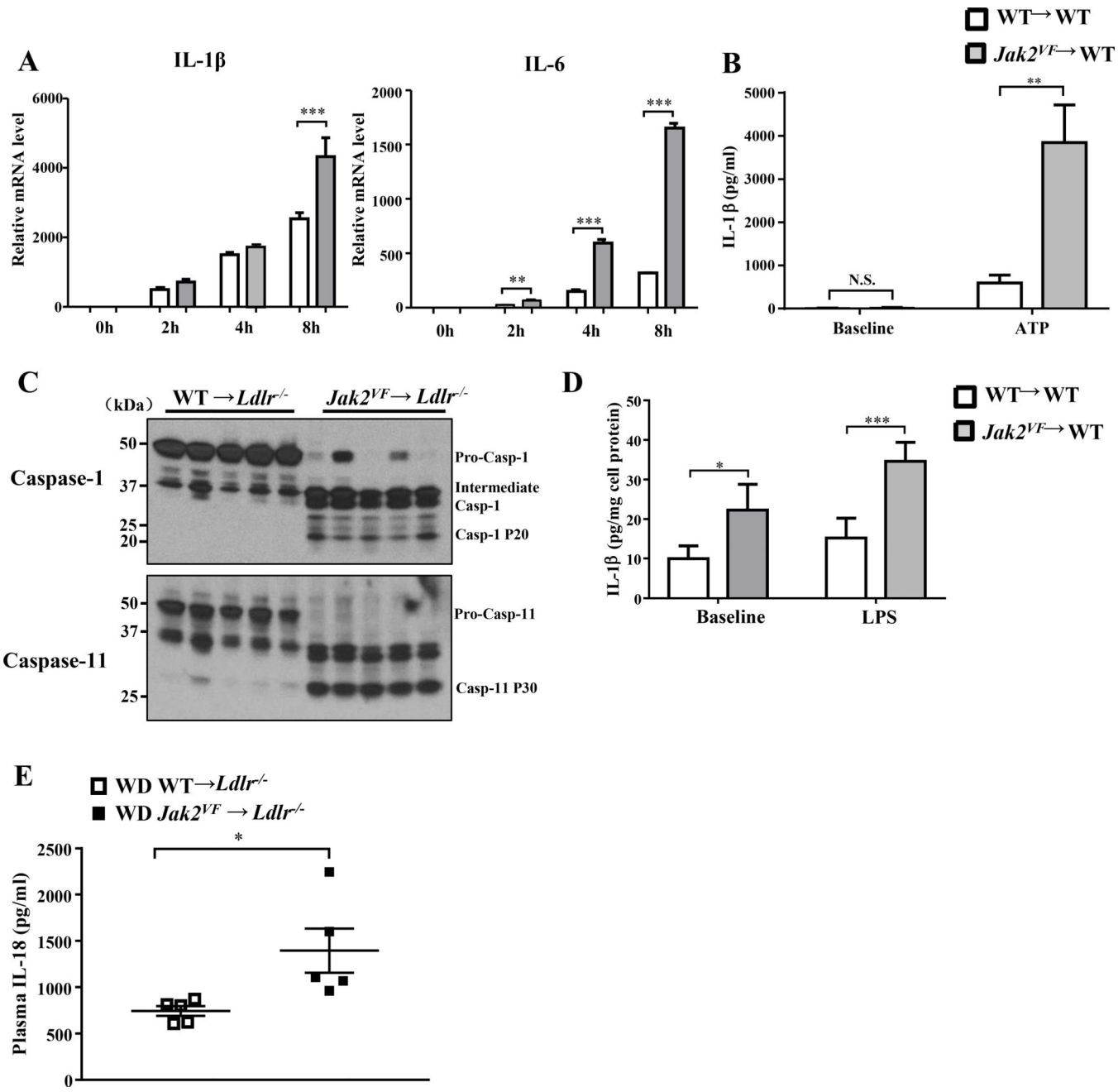

Figure 7: *Jak2*^{VF} myeloid cells displayed enhance inflammasome activation.

(A) Con-A induced peritoneal macrophages were challenged with or without 10ng/ml LPS for the indicated time and qPCR analysis of mRNA level of IL-1β and IL-6. Data were from 5 independent experiments. 1-way ANOVA. **(B)** ELISA of IL-1β in cultured medium of bone marrow derived macrophage challenged with 10ng/ml LPS for 1 hour followed by 1mM ATP for 3 hours. Baseline was LPS (10ng/ml) only for 4 hours. 1-way ANOVA. **(C)** Western blot of caspase-1 and caspase-11 cleavage in splenic CD11b^{+} cells from female recipients WD-fed for 7 weeks. **(D)** ELISA of IL-1β in cultured medium of CD11b^{+} cells from female recipients WD-fed for 7 weeks. Cells were treated with or without 1μg/ml LPS

for 8h. Data were the representative from 4 independent experiments. 2-way ANOVA. **(E)** ELISA of plasma IL-18 in female recipients WD-fed for 8 weeks. Unpaired *t* test. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

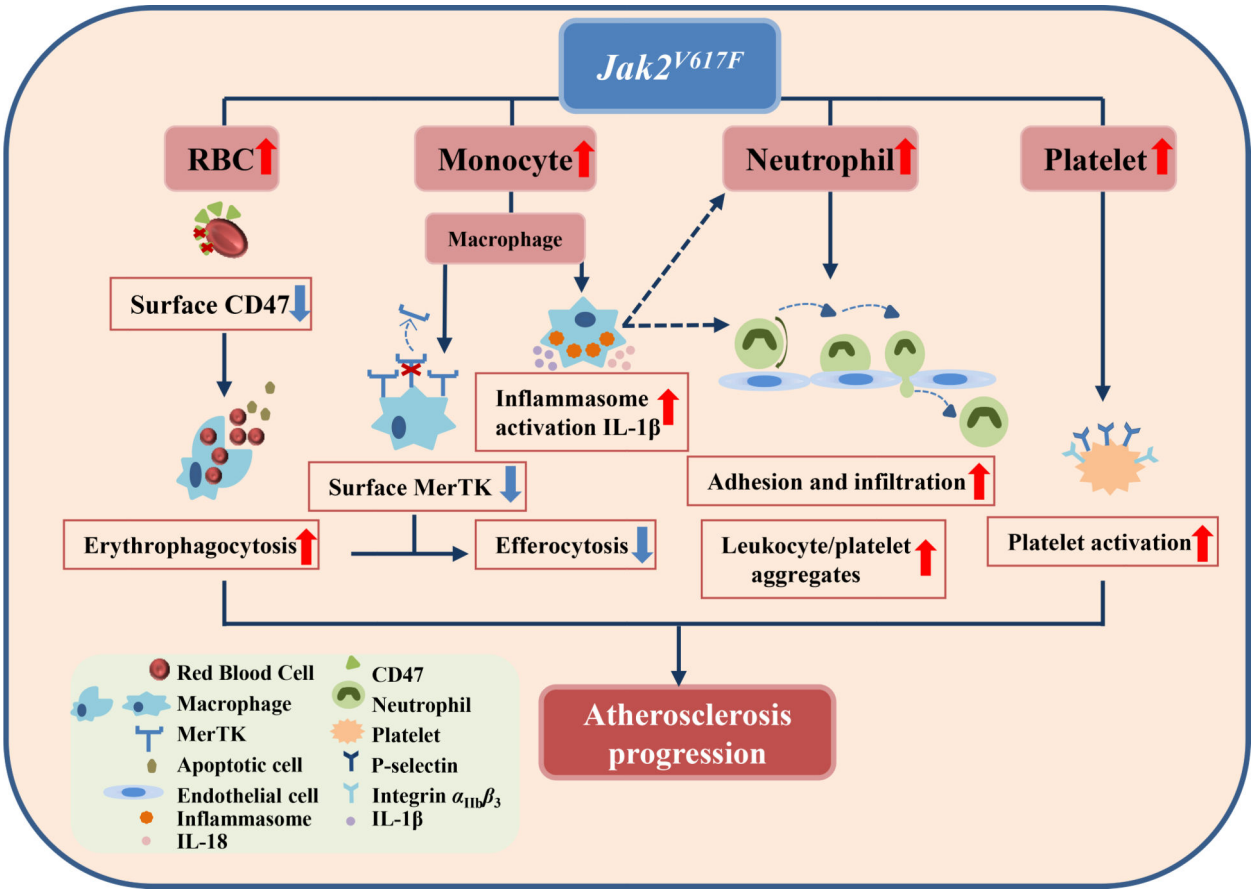

Figure 8: Schematic model.

Mechanisms underlying increased atherosclerosis in *Jak2*^{VF} mice.