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BRCC3-mediated NLRP3 deubiquitylation promotes inflammasome activation and atherosclerosis in *Tet2* clonal hematopoiesis

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Abstract

Background: Clonal hematopoiesis (CH) has emerged as an independent risk factor for atherosclerotic cardiovascular disease (CVD) with activation of macrophage inflammasomes as a potential underlying mechanism. The NLRP3 inflammasome has a key role in promoting atherosclerosis in mouse models of *Tet2* CH, while inhibition of the inflammasome product IL-1 β appeared to particularly benefit patients with TET2 CH in CANTOS. TET2 is an epigenetic modifier that decreases promoter methylation. However, the mechanisms underlying macrophage NLRP3 inflammasome activation in TET2 deficiency and potential links with epigenetic modifications are poorly understood.

Methods: We used cholesterol-loaded TET2 deficient murine and embryonic stem cell derived isogenic human macrophages to evaluate mechanisms of NLRP3 inflammasome activation in vitro and hypercholesterolemic *Ldlr*^{-/-} mice modeling TET2 CH to assess the role of NLRP3 inflammasome activation in atherosclerosis.

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Results: *Tet2* deficiency in murine macrophages acted synergistically with cholesterol loading in cell culture and with hypercholesterolemia in vivo to increase JNK1 phosphorylation and NLRP3 inflammasome activation. The mechanism of JNK activation in TET2 deficiency was increased promoter methylation and decreased expression of the JNK-inactivating dual specificity phosphatase, *Dusp10*. Active Tet1-deadCas9 targeted editing of *Dusp10* promoter methylation abolished cholesterol-induced inflammasome activation in *Tet2*-deficient macrophages. Increased JNK1 signaling led to NLRP3 deubiquitylation and activation by the deubiquitinase BRCC3. Accelerated atherosclerosis and neutrophil extracellular trap formation (NETosis) in *Tet2* CH mice were reversed by holomycin, a BRCC3 deubiquitinase inhibitor, and also by hematopoietic deficiency of *Abro1*, an essential scaffolding protein in the BRCC3-containing cytosolic complex. Human *TET2*^{-/-} macrophages displayed increased JNK1 and NLRP3 inflammasome activation, especially following cholesterol loading, with reversal by holomycin treatment, indicating human relevance.

Conclusions: Hypercholesterolemia and TET2 deficiency converge on a common pathway of NLRP3 inflammasome activation mediated by JNK1 activation and BRCC3-mediated NLRP3 deubiquitylation with potential therapeutic implications for the prevention of CVD in TET2 CH.

Keywords

Atherosclerosis; hypercholesterolemia; clonal hematopoiesis; NLRP3 inflammasome; neutrophil extracellular traps; phosphorylation; deubiquitylation; neutrophils

Subject Codes:

TOC category – Basic; TOC subcategory - Atherosclerosis

Introduction:

Cardiovascular disease (CVD) is the leading cause of death worldwide and is increasing in the US again after decades of decline.¹ CVD usually arises from atherosclerosis of coronary, cerebral or peripheral arteries.² Atherosclerosis is initiated by the accumulation of cholesterol-rich lipoproteins in arteries, and their modification and uptake by macrophages, which triggers an inflammatory response. Recent clinical trials employing anti-inflammatory therapies, notably IL-1 β antibodies,³ or colchicine,^{4,5} have shown a reduction in CVD, indicating a central role of the inflammatory response, and specifically involving the macrophage inflammasome.⁶ However, there was an increased risk of infection and pneumonia in these clinical trials, emphasizing the need for more precise targeting of anti-inflammatory treatments in relevant patient populations for prevention of CVD.

Clonal hematopoiesis (CH) arises from somatic mutations that provide a fitness advantage to hematopoietic stem cells and lead to outgrowth of clones of blood cells. CH commonly involves variants in genes mediating epigenetic modifications (*TET2*, *DNMT3A*, *ASXL1*) or cytokine signaling (*JAK2*). Recent studies showed that CH increases in frequency with ageing and is present in > 10% of people aged > 70.⁷ CH has been identified as a major independent risk factor for CVD, with impact comparable to traditional risk factors such as smoking and elevated LDL.^{7,8} TET2 catalyzes the oxidation of 5-methylcytosine to 5-

hydroxymethylcytosine, leading to decreased methylation of DNA and thus TET2 deficiency leads to increased methylation and decreased activity at many gene promoters.⁹

NLRP3 inflammasome activation is a two-step process with priming events increasing the expression of inflammasome components and activation reflecting assembly of the inflammasome,¹⁰ binding of the adaptor ASC and activation of the effector, Caspase-1.¹¹ This leads to cleavage of IL-1 β , IL-18 and Gasdermin D (GSDMD), which forms membrane pores,¹² and permits the secretion of active IL-1 β and IL-18. In a post-hoc analysis of a subset of patients in the CANTOS, subjects with *TET2* CH treated with IL-1 β antibodies had improved outcomes compared to subjects with other forms of CH or non-CH subjects.¹³ In murine atherosclerosis studies, *Tet2* deficient macrophages exhibited a widespread increase in the expression of inflammatory genes, including *Il1b* and *Nlrp3*, indicating increased inflammasome priming,^{7,14} and the specific NLRP3 inhibitor MCC950 reversed accelerated atherosclerosis in *Tet2* CH.¹⁴ However, potential mechanisms of NLRP3 inflammasome activation independent of priming effects and the relevance of NLRP3 inflammasomes in human TET2 deficient macrophages have not been explored.

Post-translational modifications of NLRP3 by phosphorylation have been implicated as either increasing,^{15,16} or decreasing,¹⁷ the activation step. In this study, we discovered that a well characterized activating pathway involving JNK1-mediated phosphorylation and deubiquitylation of NLRP3 by BRCC3,^{16,18} is increased in TET2 deficient macrophages, especially when cholesterol loaded, suggesting potential relevance in the setting of atherosclerosis. We show that the specific BRCC3 inhibitor holomycin,¹⁹ or genetic deficiency of *Abro1* a scaffolding protein with an essential role in the deubiquitinase activity of BRCC3,¹⁸ reduce atherosclerosis in *Tet2* CH mice. Studies in human TET2 deficient macrophages indicate the translational relevance of NLRP3 inflammasome activation and the role of the BRCC3 pathway in this process.

Methods:

Upon reasonable request to the corresponding authors, the data, analytic methods, and study materials will be made available to other researchers. All supporting data are available within the article and the Expanded Methods in the Data Supplement.

Mice

All mice except *Abro1*^{-/-}, *Tet2*^{+/-}, *Tet2*^{+/-}*Abro1*^{-/-} and *Tet2*^{-/-}*Abro1*^{-/-} mice were purchased from Jackson Laboratories. *Tet2*^{+/-}, *Tet2*^{+/-}*Abro1*^{-/-} and *Tet2*^{-/-}*Abro1*^{-/-} mice were generated in our laboratory by crossbreeding *Tet2*^{-/-} mice (# 023359) with *Abro1*^{-/-} mice (kindly provided by Dr. Bin Wang, MD Anderson Cancer Center).²⁰ B6 CD45.1 (# 002014) and *Ldlr*^{-/-} mice (002207) were bought. Only female mice were used in experiments since they are more prone to developing atherogenesis. All mice used for these studies were on a C57BL/6J background and were housed in a specific pathogen-free facility under standard conditions of temperature (about 23 C) with a 12-h light dark cycle and food available ad lib (humidity was not noted). Cages and water were changed every 14–21 days. All mouse experiments were approved by Institutional Animal Care and Use Committee of

Columbia University and were conducted in accordance with the Institutional Animal Care and Use Committee of Columbia University guidelines.

Statistical analysis

All data are presented as means + SEM. The number of mice included in the experiments can be found in the figure legends. The t-test was used to define differences between 2 datasets. For statistical analysis of IL-1 β secretion, LPS only or LPS/IFN- γ only conditions are excluded from analysis since IL-1 β secretion is very low or sometimes not detectable in LPS only or LPS/IFN- γ only conditions as well as we are interested in conditions where inflammasome is activated by ATP, Nigericin or oxLDL. For statistical analysis of in vitro MAPK phosphorylation such as JNK, ERK and p38 MAPK, No LPS conditions are excluded from analysis since phosphorylation of JNK, ERK and p38 MAPK is very low. The one-way ANOVA coupled with Tukey's test for multiple comparisons was used define differences between more than 2 datasets if there is only one variable. The two-way ANOVA (with Sidak's multiple comparison test) was conducted for where two variables (genotype vs treatment or diet or siRNA or virus). Two-way ANOVA results are shown next to dot plot with the exact p-value of genotype, exact p-value of treatment or diet or siRNA used and exact p-value of interactions between genotype and treatment or diet or siRNA used. The criterion for significance was set at **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. Statistical analyses were performed using GraphPad Prism 8 software (San Diego, CA).

Results:

Cholesterol loading and *Tet2* deficiency promote JNK and NLRP3 inflammasome activation

To address the mechanisms of NLRP3 inflammasome activation in *Tet2*^{-/-} macrophages, wild type (control) and *Tet2*^{-/-} bone marrow derived macrophages (BMDMs) were primed with 20 ng/ml lipopolysaccharide (LPS) and treated with 2 mM ATP or 10 μ g/ml Nigericin to activate the NLRP3 inflammasome.²¹ To mimic the state of macrophages in atherosclerotic lesions, i.e., as cholesterol-loaded foam cells, we tested the effect of macrophage cholesterol loading on inflammasome activation. Since native LDL does not efficiently load macrophages with cholesterol, BMDMs were incubated with modified LDL (acetyl-LDL, acLDL), a standard approach to inducing foam cell formation.²² AcLDL loading and *Tet2* deficiency led to increased IL-1 β secretion Caspase-1 and GSDMD cleavage with the largest effects in cholesterol loaded *Tet2* deficient macrophages (Fig 1A-C). Two-way ANOVA indicated significant interaction between genotype and acLDL treatment consistent with a synergistic interaction of cholesterol loading and *Tet2* deficiency (Figure 1A-B). In contrast, *Nlrp3* and *Il1b* mRNA expression were minimally increased in cholesterol loaded *Tet2*^{-/-} macrophages (Figure S1A-B), suggesting additional mechanisms promoting NLRP3 activation beyond the priming step. Because post-translational modifications such as phosphorylation and deubiquitylation may be required for full activation of NLRP3,²³ we investigated MAPK signaling. AcLDL loading plus *Tet2* deficiency resulted in greater JNK phosphorylation than either alone (Figure 1D), while ERK and p38 MAPK phosphorylation were unaffected or only increased by acLDL, respectively (Figure S1C-D). TET2 CH usually involves heterozygous loss of function mutations in the *TET2* gene in humans.²⁴ Both *Tet2*^{+/-} and *Tet2*^{-/-} BMDMs had elevated

JNK phosphorylation in response to LPS treatment and were further increased upon cholesterol loading (Figure 1E). Pre-treatment with SP600125 (a JNK inhibitor) before LPS priming partially reduced IL-1 β secretion in *Tet2*^{-/-} BMDMs bringing it to the same level as controls (Figure 1F-G) while having no effect on *Il1b* mRNA expression (Figure S1E) suggesting a specific role of increased JNK activity in the promotion of NLRP3 activation in *Tet2*^{-/-} cells. In a setting mimicking the inflammatory milieu of atherosclerotic lesions, JNK inhibition suppressed IL-1 β secretion induced by oxidized LDL (oxLDL) in macrophages activated by LPS/IFN- γ (Figure S1F) while having no effect on *Il1b* mRNA expression (Figure S1G). These findings indicate that *Tet2* deficiency promotes macrophage NLRP3 inflammasome activation via JNK, independent of increases in *Il1b* mRNA, and that treatment with acLDL or oxLDL enhances these effects.

To assess in vivo relevance, we prepared control or chimeric *Tet2* CH mice by transplanting 90% WT (CD45.1⁺)/10% *Tet2*^{+/+} (CD45.2⁺) or 90% WT (CD45.1⁺)/10% *Tet2*^{-/-} (CD45.2⁺) bone marrow into *Ldlr*^{-/-} recipients, then, after a 5-week recovery period, fed the mice chow or high fat/high cholesterol Western type diet (WTD) for 4 weeks. JNK phosphorylation and inflammasome activation (as assessed by Caspase-1 cleavage) were significantly elevated in Ly6G⁻CD11b⁺ splenic monocytes/macrophages in *Tet2* CH mice fed the WTD but not in *Tet2* CH mice fed the chow diet (Figure 1H). Similarly, caspase-1 cleavage and JNK phosphorylation were significantly increased in CD45.2⁺Ly6G⁻CD11b⁺ but not in CD45.1⁺Ly6G⁻CD11b⁺ monocyte/macrophages isolated from *Ldlr*^{-/-} mice that fed with WTD but not chow diet (Figure S1H). This indicates cell autonomous inflammasome activation in *Tet2* deficient macrophages under hypercholesterolemic conditions. In contrast, *Il1b* mRNA was slightly but significantly increased in both CD45.1⁺Ly6G⁻CD11b⁺ and CD45.2⁺Ly6G⁻CD11b⁺ monocyte/macrophages in WTD-fed *Tet2* CH mice (Figure S1I). The increase in *Il1b* mRNA in both mutant and WT macrophages may be explained by the ability of IL-1 β to increase its own gene expression.²⁵⁻²⁷ To further demonstrate dependence on cellular cholesterol content, we infused mice with a preparation of reconstituted HDL (rHDL, CSL111) that promotes efficient cholesterol efflux from macrophages.²⁸ This resulted in reversal of increased JNK phosphorylation and Caspase-1 cleavage in the *Tet2* CH mice (Figure 1I). These results indicate that cholesterol accumulation and TET2 deficiency act synergistically to promote NLRP3 inflammasome activation via JNK activation.

***Dusp10* promoter methylation drives JNK activation in *Tet2* deficiency**

Dual specificity phosphatases (DUSPs) regulate the magnitude and duration of signaling in MAPK pathways and are regulated at the gene expression level.²⁹ Thus, we assessed the effects of *Tet2* deficiency on expression of the seventeen DUSPs that have been associated with altered JNK activation.²⁹ Although several Dusps showed moderately reduced expression, only *Dusp10* mRNA expression was markedly decreased by TET2 deficiency in both murine *Tet2*^{-/-} BMDMs and isogenic human *TET2*^{-/-} embryonic stem cell (hESC)-derived macrophages compared to WT controls (Figure 2A-B). Similarly, *Dusp10* expression was decreased in *Tet2*^{+/+} BMDMs (Figure S2A) and in Ly6G⁻CD11b⁺ splenic monocytes/macrophages from WTD fed *Tet2* CH *Ldlr*^{-/-} mice compared to WTD fed *Ldlr*^{-/-} controls (Figure 2C).

Since TET2 demethylates and activates some promoters,³⁰ we next assessed methylation of the *Dusp10* promoter using methylated DNA capture (MeDIP) and PCR amplification of CpG rich regions.³¹ *Dusp10* promoter methylation was increased by *Tet2* deficiency, but the effect was much more pronounced when macrophages were loaded with cholesterol (Figure 2D), paralleling the synergistic effects of TET2 deficiency and cholesterol loading on JNK phosphorylation and NLRP3 inflammasome activation shown above. Consistently, *Dusp10* knockdown significantly increased JNK phosphorylation and IL-1 β secretion in control but not *Tet2*^{-/-} macrophages (Figure S2B-D). Conversely, overexpression of human DUSP10 by lentiviral transduction (Figure S2E) reduced JNK (Figure 2E) but not ERK or p38 MAPK phosphorylation in *Tet2*^{-/-} macrophages (Figure 2E). In parallel to JNK phosphorylation, DUSP10 overexpression largely reversed the incremental increase in IL-1 β secretion in response to LPS+ATP or LPS+Nigericin in *Tet2*^{-/-} vs control macrophages (Figure 2F-G). DUSP10 overexpression also modestly increased *Il1b* mRNA expression (Figure S2F). To directly assess the contribution of increased *Dusp10* promoter methylation to reduced *Dusp10* expression and NLRP3 inflammasome activation, we used an approach,^{32,33} in which control and *Tet2*^{-/-} BMDMs were infected with lentiviruses expressing deadCas9-activeTet1 (dC-T) or deadCas9-catalytically inactive Tet1 (dC-dT) and with gRNAs targeting the *Dusp10* promoter region. As predicted, the active dC-T but not the control dC-dT combined with *Dusp10* gRNAs markedly elevated suppressed *Dusp10* mRNA expression in *Tet2*^{-/-} cells but not in control cells, with minor effects on other *Dusps* (Figure 2H). While promoter methylation was increased for both *Dusp10* and *Dusp6*, the differential effect of active vs inactive Tet1-dCas9 was much greater for *Dusp10* (Figure 2I). Importantly, active Tet1-dCas9 completely reversed the incremental increase in IL-1 β secretion observed in *Tet2*^{-/-} vs control macrophages while having no significant effect in control cells (Figure 2J-K).

While these findings show that decreased expression of *Dusp10* due to hypermethylation of its promoter is responsible for increased JNK and inflammasome activation in *Tet2*^{-/-} macrophages, they do not explain the effects of hypercholesterolemia and cholesterol loading. Since promoter methylation may be increased by DNMTs, we hypothesized that there might be a further increase in promoter methylation in cholesterol loaded *Tet2*^{-/-} cells due to upregulation of DNMTs. acLDL loading increased DNMT1 and DNMT3A but not DNMT3B levels specifically in *Tet2*^{-/-} BMDMs (Figure S3A). DNMT3A and DNMT3B levels were upregulated in Ly6G⁻CD11b⁺ splenic monocytes/macrophages from WTD-fed but not chow fed *Tet2* CH *Ldlr*^{-/-} mice (Figure S3B), suggesting increased DNMT3A might contribute to hypermethylation of the *Dusp10* promoter in cholesterol loaded *Tet2* deficient macrophages. Treatment of the cells with the DNMT inhibitor 5-azacytidine decreased *Dusp10* promoter methylation (Figure S3C) and IL-1 β secretion (Figure S3D) to a similar level in acLDL loaded control and *Tet2*^{-/-} macrophages. These findings are consistent with a cholesterol loading induced increase in DNMT3A acting in concert with TET2 deficiency to induce hypermethylation of the *Dusp10* promoter, potentially explaining the enhancement of inflammasome activation in *Tet2* deficient cells by cholesterol loading.

NLRP3 deubiquitylation promotes inflammasome activation in *Tet2*^{-/-} macrophages

JNK-1 has been reported to activate the NLRP3 inflammasome via specific serine phosphorylation of NLRP3 that leads to NLRP3 deubiquitylation by the cytosolic BRCC3 isopeptidase complex (BRISC) and active NLRP3 inflammasome assembly.^{16,18} We carried out experiments to assess the role of NLRP3 deubiquitylation by BRCC3 downstream of JNK1 activation,^{18,34} in cholesterol and TET2 deficiency mediated inflammasome activation. Paralleling the effects on JNK1 activation, acLDL loading+ *Tet2* deficiency resulted in greater NLRP3 deubiquitylation than either alone (Figure 3A). Pharmacological targeting of NLRP3 deubiquitylation by the specific BRCC3 inhibitor holomycin (HL),¹⁹ reduced IL-1 β secretion to the same level as controls in *Tet2* deficient macrophages (Figure 3B) while having no effect on *I11b* mRNA expression (Figure S4A). Similarly, HL treatment suppressed IL-1 β secretion induced by oxLDL in macrophages activated by LPS/IFN- γ (Figure S4B) while having no effect on *I11b* mRNA expression (Figure S4C). Furthermore, *Brc3* knockdown by siRNA (Figure S4D), largely reversed the incremental increase in IL-1 β secretion in *Tet2* deficient macrophages to a similar level as controls (Figure 3C-D). Finally, elevated IL-1 β secretion induced by LPS+ATP in *Tet2*^{-/-} macrophages was reversed by deficiency of *Abro1*, a scaffolding protein that is an essential component of the BRISC,¹⁸ (Figure 3E). Similar to *Tet2*^{-/-} BMDMs, HL decreased IL-1 β secretion in response to LPS+ATP or Nigericin in *Tet2*^{+/-} macrophages (Figure S4E-F). Moreover, elevated IL-1 β secretion in response to LPS+ATP or Nigericin in *Tet2*^{+/-} macrophages was reversed by the deficiency of *Abro1* (Figure S4G-H). Finally, *Abro1* deficiency decreased IL-1 β secretion induced by oxLDL in macrophages activated by LPS/IFN- γ (Figure S4I) while having no effect on *I11b* mRNA expression (Figure S4J). Together, these findings suggest that increased NLRP3 deubiquitylation mediated by BRCC3 is a key mechanism underlying NLRP3 mediated inflammasome activation in *Tet2*^{-/-} macrophages.

To test the human relevance of our findings, we used a human TET2 deficient embryonic stem cell hESC line (*TET2*^{-/-}), which was created by CRISPR/Cas9-mediated gene editing and the control isogenic *TET2*^{+/+} hESC line.³⁰ We used a macrophage differentiation protocol that we have shown leads to efficient generation of macrophages with minimal differences between WT and *TET2*^{-/-} cells (Figure 4A).³⁵ *TET2* deficiency increased IL-1 β secretion in response to LPS+ATP treatment, providing direct evidence for increased NLRP3 inflammasome activation in human cells (Figure 4B). Furthermore, similar to our findings in murine macrophages, IL-1 β secretion was further increased in cholesterol-loaded cells (Figure 4B). JNK phosphorylation was elevated in *TET2*^{-/-} hESC-derived macrophages (Figure 4C) and JNK inhibition decreased IL-1 β secretion to levels similar to those in isogenic *TET2*^{+/+} hESC-derived macrophages (Figure 4D). Moreover, inhibition of deubiquitinase activity by the isopeptidase inhibitor G5,³⁴ or the more specific BRCC3 inhibitor HL suppressed IL-1 β secretion in *TET2*^{-/-} hESC-derived macrophages (Figure 4E-F). These findings confirm similar findings in human and mouse TET2 deficient macrophages.

BRCC3-mediated NLRP3 deubiquitylation accelerates atherosclerosis in *Tet2* CH

To assess the impact of BRCC3 mediated inflammasome activation on *Tet2* CH atherogenesis, we lethally irradiated *Ldlr*^{-/-} mice and transplanted them with chimeric

Tet2^{-/-} bone marrow cells containing 10% CD45.2 *Tet2*^{-/-} and 90% CD45.1 wild type cells (*Tet2* CH). A control group was transplanted with mixed bone marrow from 10% littermate control CD45.2 *Tet2*^{+/+} and 90% CD45.1 wild type bone marrow cells. 5-weeks after bone marrow reconstitution and 2-weeks after commencing the WTD, control and *Tet2* CH mice were injected intraperitoneally with HL (1 mg/kg) or vehicle control (DMSO/PBS) every second day for 6 weeks. Mice then were sacrificed after a total of 8 weeks of WTD. *Tet2* deficient blood cells expanded from about 46 to 71 % during the course of the HL injections similar to previous reports;¹⁴ however, HL administration did not affect the expansion of *Tet2*^{-/-} blood cells (Figure S5A). There was a slight decrease in body weight in control mice (Figure S5B). Splenic weight was higher in *Tet2* CH mice without significant change after HL administration (Figure S5C). Hematopoietic *Tet2* deficiency or HL treatment did not alter plasma cholesterol (Figure S5D). HL treatment reduced the total white blood cells (WBC), and lymphocyte counts in both groups (Figure S5E-F). HL decreased monocyte counts in the *Tet2* CH group while having no effect on neutrophil counts (Figure S5G-H). Importantly, HL administration promoted NLRP3 deubiquitylation in splenic Ly6G⁻CD11b⁺ monocyte/macrophages (Figure S5I) and decreased active caspase-1 (p20) in the *Tet2* CH group (Figure 5A). In the *Tet2* CH group, caspase-1 cleavage was significantly increased in CD45.2⁺Ly6G⁻CD11b⁺ monocyte/macrophages and reversed by HL administration but was not detected in CD45.1⁺Ly6G⁻CD11b⁺ monocyte/macrophages (Figure S5J). The expression of inflammatory genes such as *Nlrp3*, *Il1b* and *Il6* was upregulated in Ly6G⁻CD11b⁺ splenocytes of *Tet2* CH group and reduced to control levels by HL administration (Figure S5K-M). Such changes could be secondary to changes in inflammasome activation and IL-1 β secretion.^{25-27,36} *Il1b* mRNA was elevated in both CD45.1⁺Ly6G⁻CD11b⁺ and CD45.2⁺Ly6G⁻CD11b⁺ monocyte/macrophages in the *Tet2* CH group and abrogated by HL treatment (Figure S5N). IL-1 β staining was significantly elevated in *Tet2* CH lesions and suppressed by HL administration (Figure 5B), consistent with inflammasome inhibition in plaques. These results suggest that NLRP3 deubiquitylation is key factor in NLRP3 inflammasome activation in *Tet2* CH mice. HL administration largely reversed the increase in plaque size of *Tet2* CH mice (Figure 5C). HL seemed to decrease necrotic core area in *Tet2* CH mice, but these trends were not significant (Figure 5C). HL increased fibrous cap thickness in *Tet2* CH but not control lesions (Figure 5D). Our recent studies have shown that macrophage inflammasome activation and IL-1 β secretion promotes NET formation in atherosclerotic plaques.³⁷ Accordingly, we showed increased NETosis in *Tet2* CH mice that was reversed by HL treatment, assessed by both MPO⁺/3HCit (Figure 5E) or Ly6G⁺/3HCit staining (Figure S5O). There was no effect of HL on MPO⁺ (Figure 5E) or Ly6G⁺ (Figure S5O) staining of neutrophils in plaques consistent with activation of neutrophils after entry into plaques.³⁷ Since NETs are formed secondary to NLRP3 activation in myeloid cells,^{37,38} this suggests a direct connection between cholesterol induced NLRP3 inflammasome activation via BRCC3 in macrophages and plaque NETosis.

To verify the impact of BRCC3 mediated inflammasome activation on *Tet2* CH atherogenesis, we also employed a genetic approach using deficiency of the essential BRCC3 co-factor ABRO1.¹⁸ To better simulate human CH, we also employed a group

with heterozygous *Tet2* deficiency (*Tet2*^{+/-} mice). Thus, we transplanted WT, 10% *Abro1*^{-/-}/90% WT, 10% *Tet2*^{+/-}/90% WT, 10% *Tet2*^{-/-}/90% WT, 10% *Tet2*^{+/-}*Abro1*^{-/-}/90% WT and 10% *Tet2*^{-/-}*Abro1*^{-/-}/90% WT BM into *Ldlr*^{-/-} mice followed by administration of WTD for 8 weeks. Since BRCC3 mutations have also been discovered as potential drivers of clonal hematopoiesis,³⁹ we wondered whether *Abro1* deficiency could affect the expansion of *Tet2* mutant cells. *Tet2*^{+/-} and *Tet2*^{-/-} deficient blood cells expanded as expected while *Abro1* deficiency modestly decreased the expansion of *Tet2*^{+/-} blood cells and increased the expansion of *Tet2*^{-/-} blood cells (Figure S6A). *Abro1* deficiency did not alter body weight (Figure S6B). Spleen weight was higher in *Tet2*^{-/-} CH and reduced by *Abro1* deficiency (Figure S6C). Hematopoietic *Tet2* or *Abro1* deficiency did not change plasma cholesterol or WBC or lymphocyte, monocyte, or neutrophil counts (Figure S6D-H). *Abro1* deficiency decreased active caspase-1 (p20) in Ly6G⁻CD11b⁺ splenic monocyte/macrophages in the *Tet2*^{+/-} CH group and decreased both active caspase-1 (p20) and active IL-1β (p17) in Ly6G⁻CD11b⁺ splenocytes of *Tet2*^{-/-} CH group (Figure 6A). Importantly, *Abro1* deficiency reduced plaque size in both *Tet2*^{+/-} and *Tet2*^{-/-} CH mice (Figure 6B). *Abro1* deficiency reduced necrotic core area (Fig. 6B) and improved fibrous cap thickness in *Tet2*^{+/-} CH lesions (Figure 6C). *Abro1* deficiency markedly decreased NETs measured by MPO⁺/3HCit (Figure 6D) or Ly6G⁺/3HCit (Figure S6I) co-staining as well as both MPO⁺ (Figure 6D) or Ly6G⁺ (Figure S6I) neutrophil content in lesions. Thus, *Abro1* deficiency clearly reduced lesion and necrotic core area and increased cap thickness in *Tet2*^{+/-} CH mice. The changes were similar although less statistically significant effects in the *Tet2*^{-/-} CH group, possibly reflecting opposite effects on clonal expansion of *Tet2* deficient blood cells. Overall, the *Abro1* deficiency experiments provide strong confirmation of the changes in atherosclerosis mediated by the BRCC3 inhibitor HL treatment in *Tet2* CH mice.

Discussion:

TET2 CH represents a common somatic genetic variation promoting atherosclerotic CVD especially in the elderly, similarly, affecting men and women and groups of different ancestry in the US.⁴⁰ Our study provides new insight into the mechanisms of NLRP3 inflammasome activation in TET2 deficient murine and human macrophages, and in *Tet2* CH mice, mediated by a JNK1/BRCC3 NLRP3 deubiquitylation pathway that increases NLRP3 inflammasome activation. This mechanism of NLRP3 inflammasome activation has not previously been implicated in accelerated atherosclerosis. Importantly, we were able to link activation of this pathway to epigenetic changes in the *Dusp10* promoter resulting from TET2 deficiency. Cholesterol loading of macrophages acted convergently with TET2 deficiency to increase methylation of the *Dusp10* promoter and thereby to increase JNK and NLRP3 activation in cell culture and in vivo. Mechanism-based interventions to suppress the BRCC3 pathway of NLRP3 deubiquitylation and activation led to reduced atherosclerotic lesion area with improved features of plaque stabilization and markedly reduced NETosis in *Tet2* CH mice. Our studies suggest new therapeutic approaches to the treatment of TET2 CH using BRCC3 inhibitors, and implicate the importance of control of hypercholesterolemia in TET2 CH.

Our study reveals a key role of the JNK1/BRCC3 pathway of NLRP3 activation in accelerated atherosclerosis caused by TET2 CH. The JNK1 pathway of NLRP3

phosphorylation and activation was discovered in a screen for NLRP3 inhibitors,¹⁶ and subsequently linked to a BRCC3 K63- deubiquitylation mechanism that decreases NLRP3 serine 195 phosphorylation but does not change NLRP3 abundance.¹⁸ This pathway has been shown to promote Cryopyrin mediated periodic syndromes and urate crystal mediated NLRP3 inflammasome activation but has not been previously implicated in atherosclerosis or metabolic diseases.¹⁹ Suspecting involvement of the BRCC3 pathway in TET2 deficient macrophages, we performed a screen of JNK related dual specificity phosphatases (Dusps) revealing decreased expression of *Dusp10*. The role of DUSP10 in inflammasome activation was verified by *Dusp10*, JNK and *Brcc3* knockdown or inhibition and by specific reversal of *Dusp10* promoter methylation using active TET1-dCas9. Myeloid *Dusp10* deficiency has been shown to increase JNK activation, inflammatory responses and autoimmunity in mice.⁴¹ Our findings extend these studies by linking increased *Dusp10* promoter methylation to increased JNK1 and NLRP3 inflammasome activation especially in the milieu of hypercholesterolemia and atherosclerosis.

Cholesterol loading and hypercholesterolemia acted convergently with TET2 deficiency in macrophages to increase *Dusp10* promoter methylation and NLRP3 inflammasome activation. This was associated with increased expression of DNMT1 and DNMT3A. DNMT3A-mediated *Dusp4* promoter methylation was recently shown to sustain ERK activation, enhance macrophage efferocytosis and decrease necrotic core formation in atherosclerotic lesions.⁴² Interestingly, *Tet2* CH was associated with increased atherosclerosis but not with increased necrotic core formation in this or an earlier study.⁴³ Increased DNMTs in cholesterol loaded *Tet2* deficient cells could perhaps be compensatory, improving some macrophage functions such as efferocytosis while worsening inflammasome activation. Although global methylome changes in DNMT3A or TET2 deficient cells have been studied,⁴⁴ the broader impact of TET2 or DNMT3A deficiency in specific metabolic settings such as hypercholesterolemia and atherosclerosis deserve further investigation.

Our study may have therapeutic implications. Despite some recent controversy,^{40,45,46} CH is increasingly recognized as an independent CVD risk factor.⁷ However, there are no guidelines or practical approaches to address screening for CH for example in the elderly with multiple risk factors. Our findings that suggest synergistic effects of cholesterol loading and *Tet2* deficiency in macrophages and of hypercholesterolemia and *Tet2* CH on inflammasome activation imply that *TET2* CH subjects could particularly benefit from LDL cholesterol lowering therapies. Moreover, our studies have uncovered a novel way to inhibit NLRP3 in atherosclerosis compared to earlier studies employing the NLRP3 inhibitor MCC950. MCC950 binds the NACHT domain of NLRP3 and has an essential role in NLRP3 activation while not affecting priming.⁴⁷ Although MCC950 reduced atherosclerosis in *Tet2* CH mice,¹⁴ clinical development of this drug was halted due to hepatotoxicity.⁴⁸ Other formulations or related molecules may still have potential in the clinic.⁴⁸ However, similar to IL-1 β inhibition, complete inactivation of NLRP3 may prove to be immunosuppressive. The discovery of the BRCC3 pathway of NLRP3 activation led to the development of specific inhibitors, such as Thiolutin and Holomycin.¹⁹ BRCC3 inhibition or *Abro1* knockdown reduced inflammasome activation in Cryopyrin disorders but did not completely reverse NLRP3 inflammasome activation.^{18,19} Similarly

in the present study JNK1 inhibitors, holomycin and *Abro1* deficiency primarily reduced incremental NLRP3 inflammasome activation mediated by TET2 deficiency without suppressing basal inflammasome activation. This suggests that BRCC3 inhibitors may be less immunosuppressive than direct NLRP3 or IL-1 β inhibitors and could provide a new precision medicine approach to reducing CVD risk in *TET2* clonal hematopoiesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

TET2	Tet methylcytosine dioxygenase 2
CH	Clonal hematopoiesis
CVD	Cardiovascular disease
NETs	Neutrophil extracellular traps
ASC	Apoptosis-associated speck-like protein containing a C-terminal caspase recruit-ment domain
IL	Interleukin
NLRP3	Nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing 3
GSDMD	Gasdermin D
BRCC3	BRCA1/BRCA2-containing complex subunit 3
LPS	Lipopolysaccharide
IFN	Interferon
oxLDL	Oxidized LDL
acetyl-LDL, acLDL	modified LDL

WT	Wild type
WTD	Western-type diet
rHDL	Reconstituted HDL
DUSP	Dual specificity phosphatase
ESC	Embryonic stem cell
DNMT	DNA methyltransferase
BRISC	BRCC3 isopeptidase complex
HL	holomycin
WBC	white blood cells
3HCit	Citrullinated histone

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CLINICAL PERSPECTIVES

What is new?

- Studies in *Tet2* CH mouse models and in human TET2 deficient macrophages reveal a novel pathway of NLRP3 inflammasome activation involving the deubiquitinase BRCC3.
- Targeting atherosclerosis risk using BRCC3 inhibitors may represent a new precision medicine approach to reducing CVD risk in individuals with TET2 CH.
- Hypercholesterolemia and TET2 deficiency converge on a common pathway of NLRP3 inflammasome activation, which suggests the importance of control of hypercholesterolemia in TET2 CH.

What are the clinical implications?

Targeting the JNK/BRCC3 pathway of NLRP3 inflammasome activation with deubiquitinase inhibitors or LDL lowering drugs may reduce atherosclerotic cardiovascular disease risk in patients with *TET2* CH.

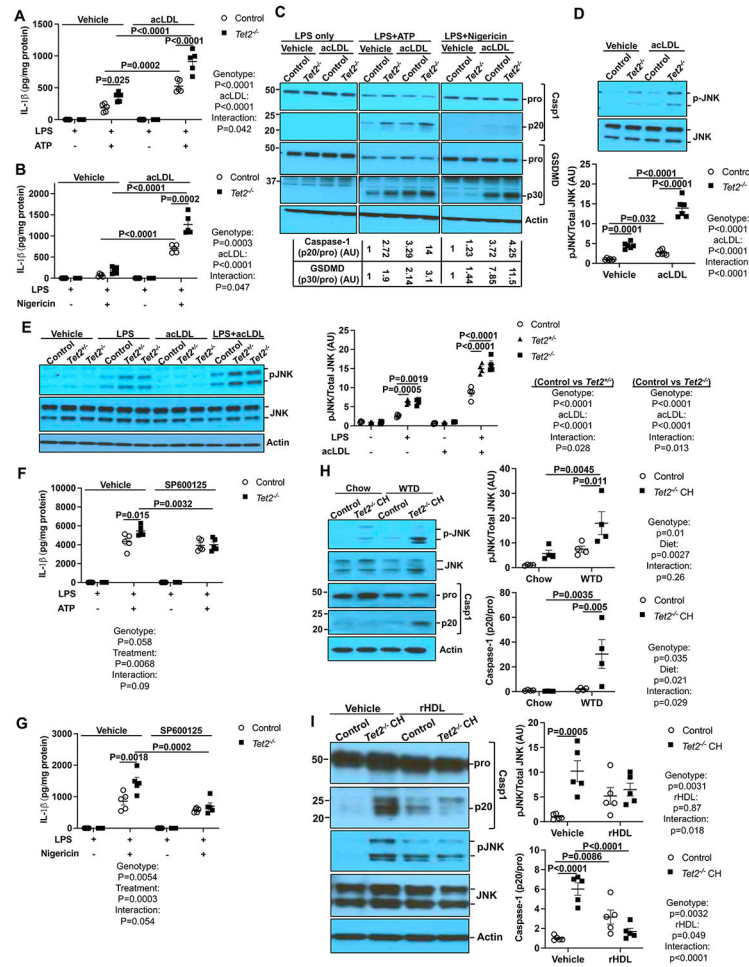


Figure 1. Elevated JNK activation drives inflammasome activation in *Tet2*^{-/-} macrophages. (A-C) Vehicle and acLDL loaded control and *Tet2*^{-/-} BMDMs were primed with LPS for 3h and treated with ATP or Nigericin for an additional 1h to induce inflammasome activation. (A-B) IL-1 β secretion from control and *Tet2*^{-/-} BMDMs that were treated with ATP (A) or Nigericin (B). (C) Immunoblot of intracellular Caspase-1 and GSDMD cleavage from (A-B). (D) Immunoblot analysis of JNK (Thr183/Tyr185) phosphorylation in vehicle and acLDL loaded control and *Tet2*^{-/-} BMDMs. (E) Immunoblot of JNK (Thr183/Tyr185) phosphorylation in vehicle and acLDL loaded control, *Tet2*^{+/-} and *Tet2*^{-/-} BMDMs upon LPS treatment for 15 min. (F-G) IL-1 β from control and *Tet2*^{-/-} BMDMs that were pretreated with JNK inhibitor SP600125 for 30 min and primed with LPS for 3h and treated with ATP (F) or Nigericin (G) for an additional 1h. (H) Immunoblot analysis of JNK (Thr183/Tyr185) phosphorylation and intracellular Caspase-1 cleavage in Ly6G⁻CD11b⁺ monocytes/macrophages isolated from *Ldlr*^{-/-} mice that were transplanted with bone marrow mixture of WT or 10% *Tet2*^{-/-}/90%WT and fed with chow or WTD for 4 weeks. (I) Immunoblot analysis of JNK (Thr183/Tyr185) phosphorylation and intracellular Caspase-1 cleavage in Ly6G⁻CD11b⁺ monocytes/macrophages isolated from *Ldlr*^{-/-} mice that were transplanted with bone marrow mixture of WT or 10% *Tet2*^{-/-}/90%WT and fed with WTD for 2 weeks and infused with vehicle or reconstituted HDL (rHDL, CSL111)

once at the end of the first week. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ by two-way ANOVA with Sidak's multiple comparison test.

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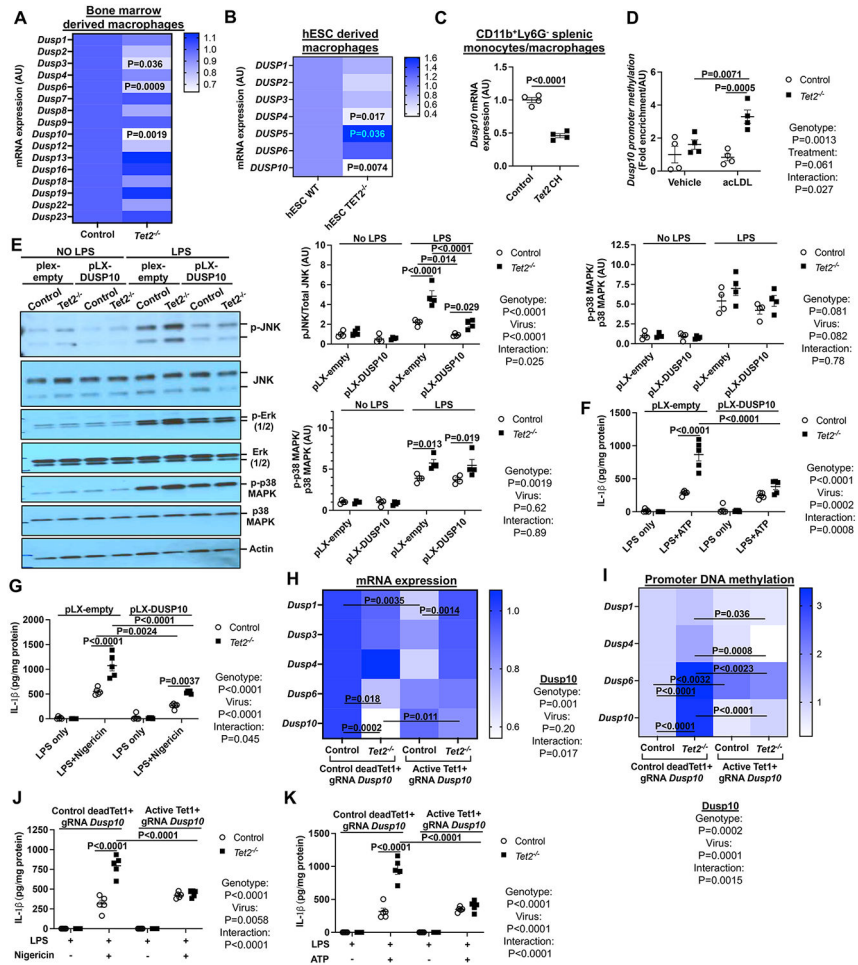


Figure 2. *Dusp10* downregulation due to elevated promoter methylation drives JNK and inflammasome activation in *TET2*^{-/-} macrophages. (A) qPCR analysis of dual phosphatases in control and *Tet2*^{-/-} BMDMs. (B) qPCR analysis of DUSP genes in WT and *TET2*^{-/-} human embryonic stem cell (hESC)-derived macrophages. (C) *Dusp10* mRNA expression in Ly6G⁻CD11b⁺ monocytes/macrophages isolated from *Ldlr*^{-/-} mice that were transplanted with bone marrow mixture of WT or 10% *Tet2*^{-/-}/90% WT and fed with chow or WTD for 4 weeks. (D) meDIP analysis of *Dusp10* promoter methylation in vehicle and acLDL loaded control and *Tet2*^{-/-} BMDMs. (E-G) Control and *Tet2*^{-/-} BMDMs were infected with control (pLX-empty) or DUSP10 lentiviruses for 72h. (E) Immunoblot analysis of JNK (Thr183/Tyr185), Erk (1/2) and p38 MAPK phosphorylation from cells primed with LPS for 15 min (F) IL-1β secretion from LPS+ATP. (G) IL-1β secretion from LPS+Nigericin. (H-K) Control and *Tet2*^{-/-} BMDMs were infected with lentiviruses expressing deadCas9-Tet1 (active dC-T) or deadCas9-a catalytically dead form of Tet1 (control dC-dT) with gRNAs targeting the *Dusp10* promoter region for 72 hours. (H) qPCR analysis of *Dusp* genes. (I) meDIP analysis of *Dusp* promoter methylation. (J) IL-1β secretion from LPS+Nigericin. (K) IL-1β secretion from LPS+ATP. **** *P*<0.0001, *** *P*<0.001, ** *P*<0.01, * *P*<0.05 by *t*-test (A-C) and two-way ANOVA with Sidak's multiple comparison test (D-K).

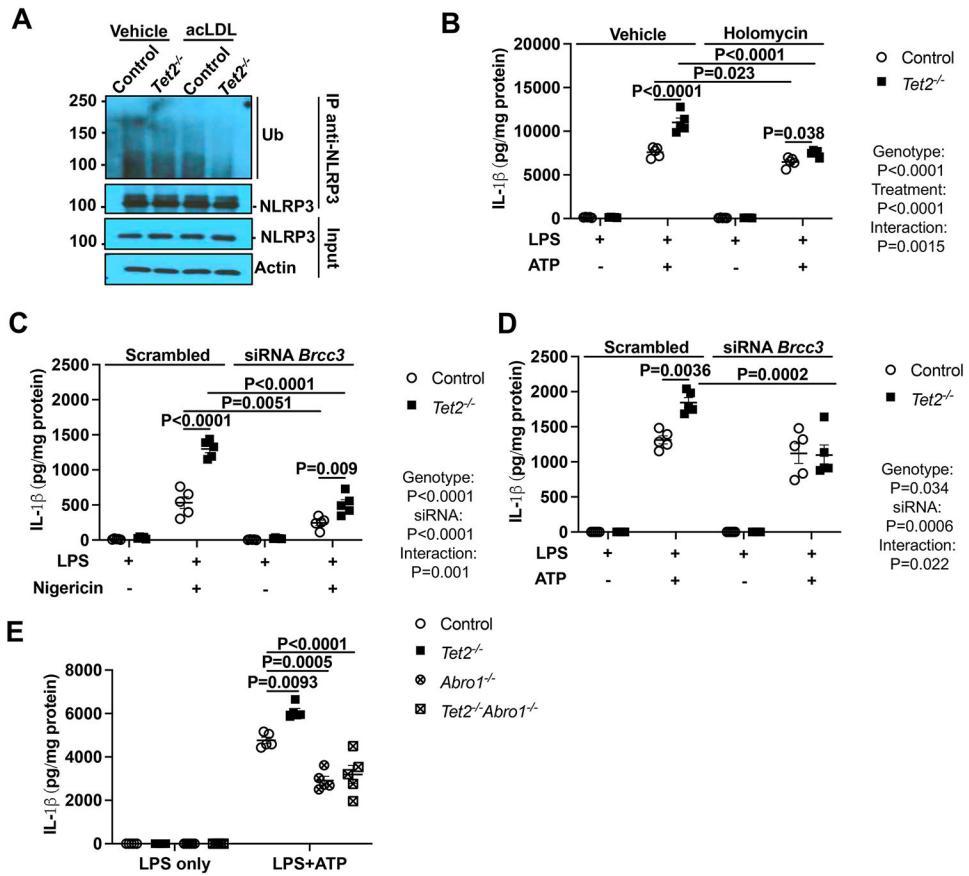


Figure 3. BRCC3-mediated NLRP3 deubiquitylation is essential for inflammasome activation in *Tet2* deficiency.

(A) Immunoblot of NLRP3 ubiquitylation in cell lysates immunoprecipitated with anti-NLRP3 in vehicle and acLDL loaded control and *Tet2*^{-/-} BMDMs primed with LPS for 3h (B) IL-1 β secretion from control and *Tet2*^{-/-} BMDMs were primed with LPS for 3h and treated with holomycin (HL) for 30 min and treated with ATP for additional 1h. (C-D) IL-1 β secretion from control and *Tet2*^{-/-} BMDMs transfected with control (Scrambled) or siRNA against *Brcc3* for 48h. After 48 hours of transfection, cells were primed with LPS for 3h and and treated with Nigericin (C) or ATP (D) for an additional 1h. (E) IL-1 β secretion from control, *Tet2*^{-/-}, *Abro1*^{-/-} and *Tet2*^{-/-}*Abro1*^{-/-} BMDMs that were primed with LPS for 3h and treated with ATP for an additional 1h. *****P*<0.0001, ****P*<0.001, ***P*<0.01, **P*<0.05 by one-way ANOVA coupled with Tukey's comparison test (E) and two-way ANOVA with Sidak's multiple comparison test (B-D).

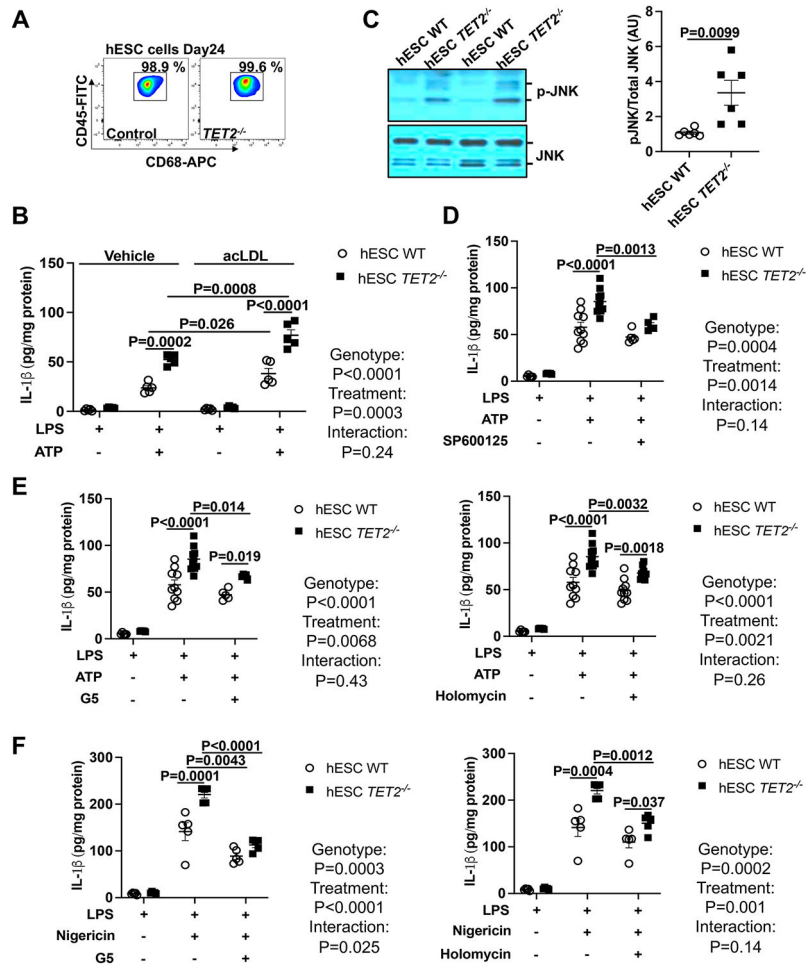


Figure 4. JNK1 mediated NLRP3 deubiquitylation drives NLRP3 inflammasome in human embryonic stem cell-derived *TET2*^{-/-} macrophages. (A) The verification of WT and *TET2*^{-/-} human embryonic stem cell (hESC)-derived macrophages via flow cytometry analysis of CD45 and CD68. (B) IL-1 β secretion from WT and *TET2*^{-/-} hESC-derived macrophages loaded with acLDL overnight and primed with LPS for 3h and treated with ATP for additional 1h. (C) Immunoblot of JNK (Thr183/Tyr185) phosphorylation in WT and *TET2*^{-/-} hESC-derived macrophages. (D) IL-1 β secretion from from WT and *TET2*^{-/-} hESC-derived macrophages with LPS for 3h in the absence or presence of SP600125 (JNK inhibitor) and treated with ATP for an additional 1h. (E-F) IL-1 β secretion from WT and *TET2*^{-/-} hESC-derived macrophages that were primed with LPS for 3h and treated with Holomycin or G5 for 30 min and treated with ATP (E) or Nigericin (F) for an additional 1h. *****P*<0.0001, ****P*<0.001, ***P*<0.01, **P*<0.05 by *t*-test (C) and two-way ANOVA with Sidak’s multiple comparison test (B, D-F).

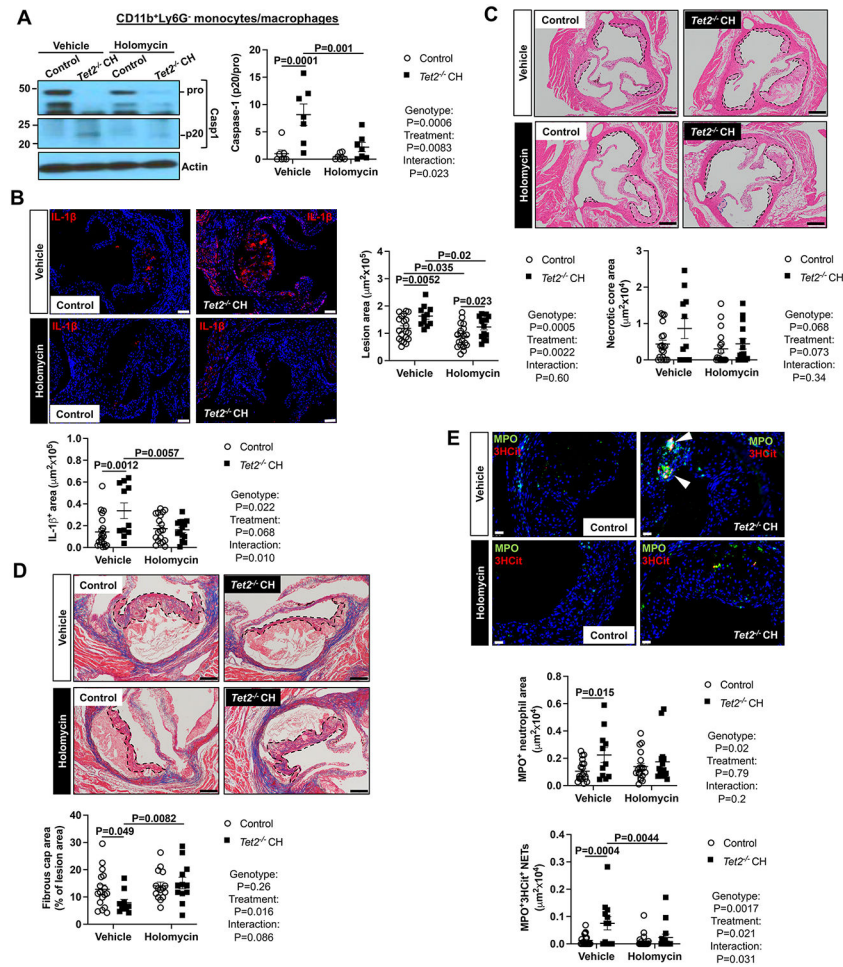


Figure 5. Inhibition of NLRP3 deubiquitylation decreases NETosis and atherosclerosis in *Tet2* CH mice.

Ldlr^{-/-} mice were transplanted with bone marrow mixture of WT or 10% *Tet2*^{-/-}/90% WT and fed WTD. After 2 weeks WTD feeding, mice were injected intraperitoneally with holomycin (1 mg/kg) or vehicle control (DMSO/PBS) for 6 weeks. Mice were sacrificed after a total of 8 weeks of WTD. **(A)** Immunoblot of intracellular Caspase-1 cleavage in Ly6G⁻CD11b⁺ splenic monocytes/macrophages. **(B)** Lesions were stained for IL-1β. **(C)** Atherosclerotic lesion area and necrotic core area in the aortic root. Representative images of hematoxylin and eosin (H&E)-stained sections are shown; atherosclerotic plaques are delineated by dashed lines. Scale bars, 200 μm. **(D)** Fibrous cap thickness analysis via Masson trichrome staining. **(E)** Neutrophils were stained in atherosclerotic lesions using MPO and MPO⁺ percentages of lesion size was quantified. Concomitantly, lesions were stained for 3HCit. To assess NETs, the overlap of MPO and 3HCit was quantified as percentage of the total lesion area. Representative pictures are shown. Scale bars: 200 μm for **(B)**, 75 μm for **(C)**, 100 μm for **(D)** and 25 μm for **(E)**. Each datapoint represents an individual mouse. **** *P*<0.0001, *** *P*<0.001, ** *P*<0.01, * *P*<0.05 by two-way ANOVA with Sidak's multiple comparison test.

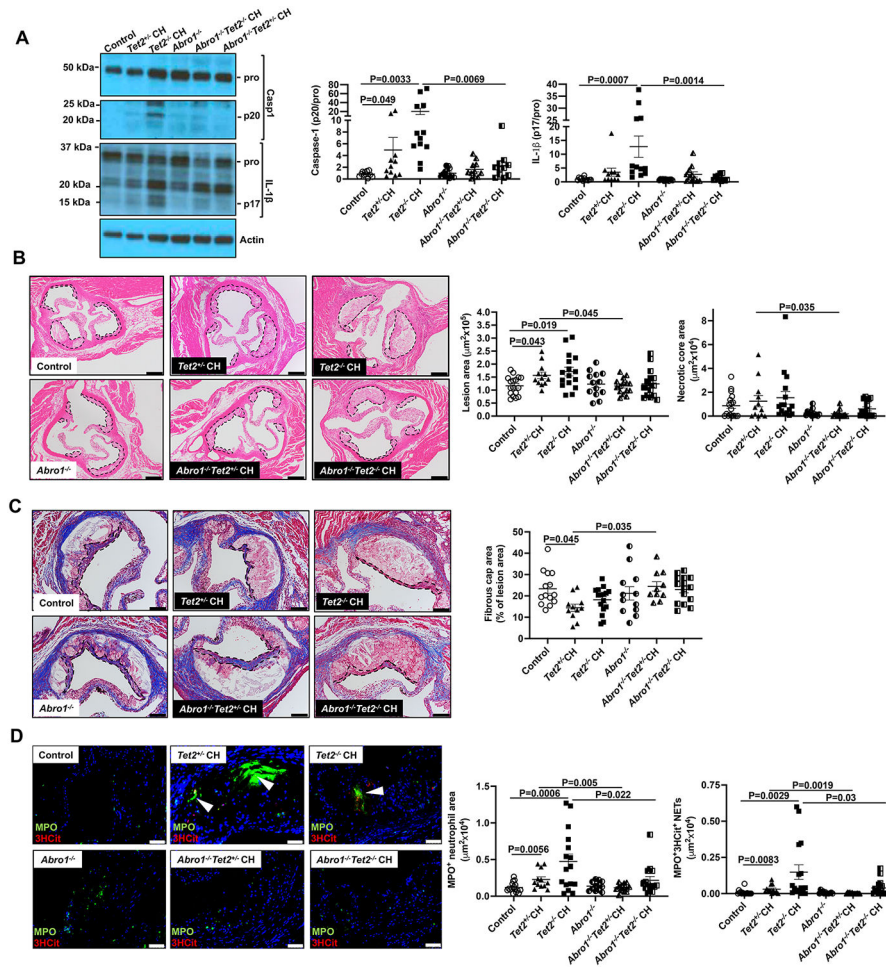


Figure 6. *Abro1* deficiency decreases NETosis and atherosclerosis in *Tet2* CH mice. *Ldlr*^{-/-} mice were transplanted with bone marrow mixture WT, 10% *Abro1*^{-/-}/90% WT, 10% *Tet2*^{+/-}/90% WT, 10% *Tet2*^{-/-}/90% WT, 10% *Tet2*^{+/-}*Abro1*^{-/-}/90% WT or 10% *Tet2*^{-/-}*Abro1*^{-/-}/90% WT. After 5 weeks of recovery time, they were fed with WTD and then were sacrificed after a total of 8 weeks of WTD. **(A)** Immunoblot of intracellular Caspase-1 and IL-1β cleavage in Ly6G⁻CD11b⁺ splenic monocytes/macrophages. **(B)** Atherosclerotic lesion area and necrotic core area in the aortic root. Representative images of hematoxylin and eosin (H&E)-stained sections are shown; atherosclerotic plaques are delineated by dashed lines. Scale bars, 200 μm. **(C)** Fibrous cap thickness analysis via Masson trichrome staining. **(D)** Neutrophils were stained in atherosclerotic lesions using MPO and MPO⁺ percentages of lesion size was quantified. Concomitantly, lesions were stained for 3HCit. To assess NETs, the overlap of MPO and 3HCit was quantified as percentage of the total lesion area. Representative pictures are shown. Scale bars: 200 μm for **(B)**, 100 μm for **(C)** and 50 μm for **(D)**. Each datapoint represents an individual mouse. *****P*<0.0001, ****P*<0.001, ***P*<0.01, **P*<0.05 by one-way ANOVA coupled with Tukey's comparison test for 4 groups (Control, *Abro1*^{-/-}, *Tet2*^{+/-}, *Tet2*^{-/-}*Abro1*^{-/-}) or (Control, *Abro1*^{-/-}, *Tet2*^{+/-} and *Tet2*^{-/-}*Abro1*^{-/-}).