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Jnk1 deficiency in hematopoietic cells suppresses macrophage apoptosis and increases atherosclerosis in LDL-receptor null mice

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Abstract

Objective—The c-Jun NH_2-terminal kinases (JNK) are regulated by a wide variety of cellular stresses and have been implicated in apoptotic signaling. Macrophages express two JNK isoforms, JNK1 and JNK2, which may have different effects on cell survival and atherosclerosis.

Approach and Results—To dissect the impact of macrophage JNK1 and JNK2 on early atherosclerosis, $Ldlr^{-/-}$ mice were reconstituted with wild type (WT), $Jnk1^{-/-}$ and $Jnk2^{-/-}$ hematopoietic cells and fed a high-cholesterol diet. $Jnk1^{-/-}\rightarrow Ldlr^{-/-}$ mice have larger atherosclerotic lesions with more macrophages and fewer apoptotic cells than mice transplanted with WT or $Jnk2^{-/-}$ cells. Moreover, genetic ablation of JNK to a single allele ($Jnk1^{+/-}/Jnk2^{-/-}$ or $Jnk1^{-/-}/Jnk2^{+/-}$) in marrow of $Ldlr^{-/-}$ recipients further increased atherosclerosis compared to $Jnk1^{-/-}\rightarrow Ldlr^{-/-}$ and WT$\rightarrow Ldlr^{-/-}$ mice. In mouse macrophages, anisomycin-mediated JNK signaling antagonized Akt activity, and loss of *Jnk1* gene obliterated this effect. Similarly, pharmacological inhibition of JNK1, but not JNK2, markedly reduced the antagonizing effect of JNK on Akt activity. Prolonged JNK signaling in the setting of ER stress gradually extinguished Akt and Bad activity in WT cells with markedly less effects in $Jnk1^{-/-}$ macrophages, which were also more resistant to apoptosis. Consequently, anisomycin increased and JNK1 inhibitors suppressed ER stress-mediated apoptosis in macrophages. We also found that genetic and pharmacologic inhibition of phosphatase and tensin homolog (PTEN) abolished the JNK-mediated effects on Akt activity, indicating that PTEN mediates crosstalk between these pathways.

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Conclusions—Loss of *Jnk1*, but not *Jnk2*, in macrophages protects them from apoptosis increasing cell survival and this accelerates early atherosclerosis.

Keywords

Atherosclerosis; Macrophages; JNK signaling; apoptosis; Akt pathway

Introduction

Macrophages play central roles in the development of atherosclerosis through modulation of cholesterol homeostasis, the immune-inflammatory response, and plaque cellularity [1]. Macrophage activation and survival are crucial determinants of atherosclerotic lesion development [2]. In addition, macrophages contribute to the integration of immune and metabolic responses, and their dysfunction contributes to chronic metabolic disorders such as obesity, type 2 diabetes and cardiovascular disease [3].

The c-Jun NH_2-terminal kinases (JNK) belong to the stress-activated protein kinase family, which are activated by a variety of environmental (radiation, osmotic and redox stress), and metabolic stresses, cytokines, and growth factors[45]. JNK plays an important role in inflammatory signaling, and its activation is crucial for programmed cell death [6]. In mammals, the JNK protein kinases are encoded by three genes: *Jnk1*, *Jnk2* and *Jnk3*, which transcribe several alternatively spliced isoforms [7]. *Jnk1* and *Jnk2* genes are expressed ubiquitously, whereas the *Jnk3* gene is restricted to the brain, cardiac smooth muscle, pancreatic islets and testis[4]. The targeted disruption of the *Jnk1* or *Jnk2* genes revealed that they compensate for each other's activity and are functionally redundant [8], but each isoform also exhibits distinct roles [9]. For example, activation of $CD8^+$ T cells is impaired in *Jnk1* knockout mice but enhanced in *Jnk2* null mice [10]. Loss of *Jnk1*, but not *Jnk2*, suppresses obesity and improves insulin sensitivity in mice [11]. JNK1, but not JNK2, activation plays an important role in the pathogenesis of insulin resistance [12–14]. Examination of cell types involved in metabolic functions of JNK illustrated contributions from many stromal cell types including neuronal cells, adipocytes and hepatocytes [14, 15]. Several studies also demonstrated the involvement of macrophage JNK activity at varying degrees in obesity and insulin resistance [8, 12, 14]. Ricci et al. [16] have shown that apoE null ($apoE^{-/-}$) mice lacking *Jnk2* ($apoE^{-/-}$/$Jnk2^{-/-}$ mice) develop less atherosclerosis than $apoE^{-/-}$ or $apoE^{-/-}$/$Jnk1^{-/-}$ mice. The impact of loss of *Jnk2* on atherosclerosis was attributed to reduced scavenger receptor A expression and foam cell formation by macrophages [16]. However, the role of macrophage JNK isoforms on apoptosis in the setting of atherosclerosis was not assessed and additional studies are needed to evaluate the role of individual macrophage JNK isoforms in atherogenesis[5].

JNK signaling has been implicated in apoptosis in response to a variety of stress stimuli [4, 6]. Though both JNK1 and JNK2 are involved in apoptotic signaling, only JNK1 is considered to be essential for apoptosis[17]. Murine embryonic fibroblasts (MEF) lacking *Jnk1*, but not *Jnk2*, have reduced c-Jun phosphorylation and UV-induced cell death [18]. Loss of both *Jnk1* and *Jnk2* in MEF produces a defect in death signaling and protects them from apoptosis [19]. Interestingly, the role of JNK in apoptosis depends on the activity of other cellular signaling

pathways, including the pro-survival phosphatidylinositol-3-kinase (PI3K/Akt) [20, 21]. Aikin and coauthors [22] were the first to report cross-talk between the PI3K/Akt and JNK pathways that protects islet cells from apoptosis. In addition, Sunayama and co-workers [23] have shown that JNK signaling antagonizes Akt activity in mammalian cells making them more susceptible to apoptosis. Similarly, JNK inhibition significantly suppresses pancreatic β-cell death [24] and decreases macrophage apoptosis [25]. Interestingly, phosphatase and tensin homolog (PTEN) may play a key role in the cross-talk between the PI3K/Akt and JNK pathways and PTEN deficiency impairs negative feedback regulation of PI3K in cancer cells [26]. However, the precise role of JNK signaling in apoptosis depends on cell type and the nature of the death stimulus [6, 17]. It is unclear whether JNK antagonizes Akt activity in mouse macrophages, or whether this cross-talk is mediated via PTEN with consequent suppression of cell survival that affects atherogenesis.

Here we used genetic loss-of-function and pharmacologic inhibition approaches to investigate the impact of JNK1 and JNK2 on Akt signaling in mouse macrophages and atherogenesis. Our data demonstrates the critical role of JNK1 signaling in macrophage apoptosis and development of early atherosclerosis.

Materials and Methods

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

Results

JNK deficiency in hematopoietic cells increases early stage atherosclerotic lesions

To examine the impact of hematopoietic cell *Jnk1*- and *Jnk2*-deficiency on atherosclerosis, 22-week-old male $Ldlr^{-/-}$ mice were lethally irradiated and transplanted with male wild type (WT; n=14), $Jnk1^{-/-}$ (n=11), or $Jnk2^{-/-}$ (n=13) bone marrow. After 4 weeks on a normal chow diet, mice were fed with the Western diet for another 8 weeks. No significant differences between the recipient groups were detected in body weight, serum total cholesterol and triglyceride levels on the chow and the Western diets (Table 1A). Size exclusion chromatography of serum revealed an accumulation of cholesterol in VLDL, LDL, IDL fractions in $Ldlr^{-/-}$ recipients with no differences between control and experimental groups in either experiment (data not shown). Mice reconstituted with WT, $Jnk1^{-/-}$ and $Jnk2^{-/-}$ marrow had similar levels of blood glucose (133.7±5.3, 139±6.8 and 137±6.7 mg/dl, respectively), erythrocytes (9.7±0.8, 9.9±1.1 and 9.7±0.9 $\times 10^6$/μl), platelets (649±66, 679±73 and 613±61 $\times 10^3$/μl) and white blood cells (7.8±06, 9.1±0.7 and 7.5±0.45 $\times 10^6$/ml). In contrast, the extent of atherosclerotic lesions in aortic sinus of the $Jnk1^{-/-} \rightarrow Ldlr^{-/-}$ mice was markedly increased (Figure 1A,B) compared to mice reconstituted with WT or $Jnk2^{-/-}$ marrow cells (Figure 1B; 241.6±38.1 vs. 110.8±13.4 and 95.8±17.6 $\times 10^3 \mu m^2$, respectively). Similarly, $Jnk1^{-/-} \rightarrow Ldlr^{-/-}$ mice had significantly increased size of atherosclerotic lesions in the distal aorta compared to WT$\rightarrow Ldlr^{-/-}$ and $Jnk2^{-/-} \rightarrow Ldlr^{-/-}$ mice (Figure 1C,D; 0.67±0.22 vs. 0.31±0.10% and 0.24±0.07%, respectively),

Next, examination of the cellular composition of atherosclerotic lesions in the aortic sinus of recipients showed that the proportion of smooth muscle, T and B cells in atherosclerotic

lesions did not differ significantly between the three groups (data not shown). The lesions predominantly consisted of macrophage-derived foam cells and *Jnk1*$^{-/-}$→*Ldlr*$^{-/-}$ mice had significantly bigger lesion area stained with MOMA-2 versus WT→*Ldlr*$^{-/-}$ and *Jnk2*$^{-/-}$→*Ldlr*$^{-/-}$ mice (Figure 1A; 167.1±29.4 vs. 82.4±10.3 and 76.4±4.6 ×10^{3}μm^2, respectively). The analysis of serial aortic sections stained with MOMA-2 and terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) revealed that *Jnk1*$^{-/-}$→*Ldlr*$^{-/-}$ mice contained significantly fewer numbers of apoptotic cells in macrophage-rich areas of lesions than WT→*Ldlr*$^{-/-}$ and *Jnk2*$^{-/-}$→*Ldlr*$^{-/-}$ mice (Figure 1E,F). Double staining of macrophages with MOMA-2 and cell nuclei with DAPI revealed increased (153%) numbers of nuclei per macrophage lesion area in *Jnk1*$^{-/-}$→*Ldlr*$^{-/-}$ mice compared to lesions of WT→*Ldlr*$^{-/-}$ and *Jnk2*$^{-/-}$→*Ldlr*$^{-/-}$ mice (Figure 1G). Together the data indicate that lack of *Jnk1* in hematopoietic cells increases the burden of early atherosclerotic lesions in the absence of changes in plasma lipid or glucose levels. The dramatic increase of macrophage numbers together with reduced apoptosis in atherosclerotic lesions of Jnk1$^{-/-}$→*Ldlr*$^{-/-}$ mice also suggested changes in viability of JNK1$^{-/-}$macrophages in vivo.

Genetic ablation to a single JNK allele further increases atherosclerosis

Peritoneal macrophages isolated from *Jnk1*$^{-/-}$→*Ldlr*$^{-/-}$ and *Jnk2*$^{-/-}$→*Ldlr*$^{-/-}$ mice exhibited a significant decrease in JNK protein content compared to WT cells (Figure 2A; 0.49±0.03 and 0.54±0.03 vs. 1.0±0.01, $p<0.05$ by One Way Analysis) and JNK kinase activity (Figure SI). They also had minimal residual expression of the knocked out isoform with compensatory increased expression of the other isoform (Figure 2B) indicating that maintaining total JNK activity is a vital for macrophages. Hence, to examine the impact of further genetic suppression of JNK signaling on atherosclerosis, we generated mice expressing a single allele of *Jnk1* or *Jnk2* in hematopoietic cells. Since the complete absence of both *Jnk1* and *Jnk2* causes early embryonic lethality, we intercrossed *Jnk1*$^{+/-}$/*Jnk2*$^{+/-}$ mice and collected FLC. Then, seventeen-week-old male *Ldlr*$^{-/-}$ mice were lethally irradiated and reconstituted with male WT(n=10), *Jnk1*$^{-/-}$(n=10), *Jnk1*$^{+/-}$/*Jnk2*$^{-/-}$(n=13) and *Jnk1*$^{-/-}$/*Jnk2*$^{+/-}$(n=12) FLC. Four weeks after transplantation, these mice were challenged with the Western diet for 8 weeks. Again, there were no differences between the recipient groups in body weight and plasma lipid levels either on the chow or the Western diets (Table 1B). Macrophages isolated from mice with a single JNK allele exhibited further decrease in JNK protein content compared to *Jnk1*$^{-/-}$ and WT cells (Figure SII). Remarkably, both *Jnk1*$^{+/-}$/*Jnk2*$^{-/-}$→*Ldlr*$^{-/-}$ and *Jnk1*$^{-/-}$/*Jnk2*$^{+/-}$→*Ldlr*$^{-/-}$ mice developed larger atherosclerotic lesions with increased macrophage MOMA-2-positive area in the proximal aorta (Figure 2E,F) than *Jnk1*$^{-/-}$→*Ldlr*$^{-/-}$ and WT$^{-/-}$→*Ldlr*$^{-/-}$ mice (Figure 2C,F,G; 183 and 172% vs. 131 and 100%, respectively). Similarly, the analysis of aorta en face demonstrated that these *Jnk1*$^{+/-}$/*Jnk2*$^{-/-}$→*Ldlr*$^{-/-}$ and *Jnk1*$^{-/-}$/*Jnk2*$^{+/-}$→*Ldlr*$^{-/-}$ mice had larger atherosclerotic lesions compared to *Jnk1*$^{-/-}$→*Ldlr*$^{-/-}$ and WT$^{-/-}$→*Ldlr*$^{-/-}$ mice (Figure 2H, I; 248 and 225% vs. 171 and 100%). Thus, genetic ablation of JNK to a single allele in hematopoietic cells resulted in further increases of atherosclerosis.

JNK1 signaling antagonizes Akt activity in macrophages

Next we investigated the mechanism(s) responsible for the increased macrophage numbers in atherosclerotic lesions of *Jnk1*$^{-/-}$→*Ldlr*$^{-/-}$ mice by focusing on Akt signaling, which is crucial for cell survival [20]. In macrophages Akt is constitutively activated, and inhibition of Akt signaling induces apoptosis [27, 28]. In addition, a recent report demonstrated that JNK activity antagonizes Akt signaling in some types of cells [23]. To examine whether JNK affects p-Akt in mouse macrophages, WT, *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ peritoneal macrophages were treated with insulin alone or together with anisomycin, a known activator of JNK signaling [23]. Insulin significantly (2–3-fold) activated phosphorylation of both Akt sites (p-AktS^{473} and T^{308}) in all types of cells (Figure 3A), whereas anisomycin suppressed Akt signaling activity in WT and *Jnk2*$^{-/-}$ macrophages, respectively, with no changes in total Akt or β-actin content (Figure 3A). Importantly, *Jnk1*$^{-/-}$ macrophages showed significantly less impact of JNK signaling on Akt activity than WT or *Jnk2*$^{-/-}$ cells (Figure 3B). The analysis of p-AktS^{473}/Akt and p-Akt T^{308}/Akt ratio in the same blot indicated a similar protective effect of *Jnk1* deficiency compared to WT or *Jnk2*$^{-/-}$ cells (Figure 3C,D). Direct comparison of WT and *Jnk1*$^{-/-}$ macrophages treated with insulin and anisomycin demonstrated a statistically significant inhibitory effect of JNK signaling in the p-Akt/β-actin ratio of WT, but not *Jnk1*$^{-/-}$ macrophages (Figure 3E,F). Thus, JNK1 is the isoform primarily responsible for JNK-mediated inhibition of Akt signaling in macrophages.

We also examined whether pharmacological inhibition of JNK can prevent the negative effects of JNK signaling on p-Akt. WT peritoneal macrophages were incubated with a mixture of insulin and anisomycin alone or in the presence of a JNK inhibitor. There was a 39% reduction of p-Akt levels in WT cells treated with anisomycin and a cell-permeable peptide inhibitor of JNK1, JNKI1, which preserved p-Akt levels in macrophages (Figure 4A,B). In contrast, treatment with a cell-permeable inhibitor IX, selective for JNK2 and JNK3 with little or no activity against JNK1, had no protective effects on Akt activity (Figure 4A,B). Taken together these data indicate that genetic ablation and pharmacological inhibition of JNK1, but not JNK2, eliminates the suppressive effects of JNK signaling on Akt activity.

JNK1 deficiency protects macrophages from apoptosis

JNK signaling has pro- or anti-apoptotic functions, depending on cell type, nature of the death stimulus, duration of its activation and the activity of other signaling pathways [17]. Taking into consideration the critical role of Akt in cell survival [20], we suggested that sustained JNK activation (1–6 hours) may promote apoptosis by exhausting anti-apoptotic Akt signaling and by subsequently reducing Bad S^{136} phosphorylation, which normally serves to inhibit apoptosis in macrophages [27, 29]. To test this hypothesis, we examined the impact of anisomycin on Akt signaling in WT and *Jnk1*$^{-/-}$ macrophages treated with palmitic acid (PA), a stress-mediated lipotoxic factor inducing ER stress and apoptosis[30]. The increased JNK signaling gradually suppressed p-Akt S^{473} in WT cells, whereas *Jnk1*$^{-/-}$ macrophages had higher p-Akt S^{473} levels and were more resistant to p-Akt suppression (Figure 4C,D). Similarly, the treatment progressively reduced p-Bad S^{136} levels in WT macrophages but there was less attenuation of p-Bad S^{136} in *Jnk1*$^{-/-}$ cells (Figure 4C). Thus, compared to WT cells, *Jnk1*$^{-/-}$ macrophages were able to preserve higher levels of Akt and

Bad phosphorylation, which are important protective and anti-apoptotic factors under conditions of ER stress [31].

In addition, to define the role of JNK signaling in macrophage apoptosis, WT, *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ macrophages were treated with BSA or PA. Treatment with BSA generated only a few apoptotic TUNEL-positive (TUNEL+) cells with no differences between cell types, whereas PA increased TUNEL+ cells 4-fold in WT and *Jnk2*$^{-/-}$ macrophages but not in *Jnk1*$^{-/-}$ cells (Figure 5A,B). The addition of anisomycin markedly (3-fold) increased the percentage of TUNEL+ cells to a similar degree in WT and *Jnk2*$^{-/-}$ cells, whereas apoptosis was significantly reduced (57% of WT cells) in *Jnk1*$^{-/-}$ macrophages (Figure 5C). In contrast, the selective inhibitor JNKI1 significantly (2-fold) reduced apoptosis in all types of cells but *Jnk1*$^{-/-}$ macrophages had less apoptosis than WT and *Jnk2*$^{-/-}$ cells (Figure 5D). When WT macrophages were treated with the specific inhibitors of JNK, JNKI1 and SP600125, they demonstrated similar levels apoptosis (Figure 5E). Importantly, when cells were loaded with human oxidized or acetylated LDL in combination with an ACAT inhibitor, *Jnk1*$^{-/-}$ macrophages generated significantly less apoptosis than WT and *Jnk2*$^{-/-}$ cells (Figure 5F). In addition, macrophages expressing a single Akt isoform (Figure SIII), Akt1 (*Akt2*$^{-/-}$/*Akt3*$^{-/-}$) or Akt3 (*Akt1*$^{-/-}$/*Akt2*$^{-/-}$) PA-BSA treatment induced a stepwise increase in apoptosis that was especially high in *Akt1*$^{-/-}$/*Akt2*$^{-/-}$ cells compared to WT cells. However, suppression of JNK signaling by the JNK inhibitor, SP600125, completely reversed the impact on cell survival with no differences between the groups (Figure SIV). Taken together our data indicate that JNK1 signaling regulates ER stress-mediated apoptosis in mouse macrophages and *Jnk1*$^{-/-}$ macrophages displayed clear resistance to apoptosis induced by different stimuli.

PTEN suppression impairs effects of JNK signaling on Akt activity

Recently Vivanco et al.[26] have shown that JNK regulates p-Akt via PTEN, and *Pten* null mouse embryonic fibroblasts exhibit an impaired negative feedback loop. To test whether PTEN plays a critical role in regulating this pathway in mouse macrophages, WT and *Pten*$^{-/-}$ cells were treated with insulin alone or together with anisomycin. In contrast to WT cells, which showed increased p-Akt S^{473} in response to insulin and reduced p-Akt S^{473} after treatment with anisomycin, *Pten*$^{-/-}$ macrophages had markedly increased basal p-Akt, which was not suppressed in response to anisomycin (Figure 6A,B). Similarly, treatment with BpV(pic), a potent PTEN inhibitor, with an IC_{50} about 10–100 fold lower than for other tyrosine phosphatases [32], decreased the inhibitory effects of JNK on p-Akt (Figure 6C,D). Taken together these results indicate that both genetic ablation and pharmacologic inhibition of PTEN effectively eradicated JNK-mediated inhibition of Akt phosphorylation in mouse macrophages.

Discussion

Numerous studies have linked macrophage or hematopoietic JNK1 activity to insulin resistance and abnormal glucose homeostasis in obesity[12, 33–35]. These studies targeting individual JNK isoforms have produced varying degrees of impact in different models, perhaps owing to interactions between isoforms and redundancies [8]. In fact, a recent report

using *Jnk1*-, *Jnk2*-combined deletion has shown that macrophage JNK promotes the establishment of obesity-induced insulin resistance and pancreatic islet dysfunction[12]. These findings suggest that macrophage JNK signaling may be crucial in other pathological conditions and warrants detailed studies of individual isoforms in cardiovascular disease models. Here, we examined the impact of *Jnk1* or *Jnk2* deficiency in hematopoietic cells on early stages of atherosclerosis using the *Ldlr*-deficiency model. Mice reconstituted with $Jnk1^{-/-}$ hematopoietic cells had significantly bigger atherosclerotic lesions compared to mice transplanted with WT or $Jnk2^{-/-}$ marrow with no differences in serum lipids. Genetic ablation to a single *Jnk* allele (either $Jnk1^{+/-}/Jnk2^{-/-}$ or $Jnk1^{-/-}/Jnk2^{+/-}$) in hematopoietic cells further increased atherosclerosis compared to $Jnk1^{-/-} \rightarrow Ldlr^{-/-}$ mice. We also found that JNK signaling antagonizes Akt activity in mouse macrophages acting mainly through JNK1. Therefore, $Jnk1^{-/-}$ macrophages had less suppression of p-Akt in response to sustained ER stress and were protected from apoptosis. Based on these data, we conclude that this resistance to apoptotic stimuli in *Jnk1* null macrophages increases lesion burden at the early stages of atherogenesis.

JNK signaling is over-expressed and activated in atherosclerotic lesions of cholesterol-fed rabbits[36]. Considering the role of JNK in inflammatory and metabolic responses, it is plausible that this stress-mediated JNK activation may impact macrophage viability and atherosclerosis. In fact, Ricci et al. were the first to report the involvement of JNK2 in atherosclerosis[16] showing that $Jnk2^{-/-}/apoE^{-/-}$ mice developed less atherosclerosis compared to control $apoE^{-/-}$ and $Jnk1^{-/-}/apoE^{-/-}$ mice. They analyzed a later stage of atherosclerosis with more severe lesions induced by a high-cholesterol (1.25%) diet for 14 weeks in total body JNK isoform deficiency in the *apoE*-deficienct model on a hybrid C57BL6/129SV background, whereas in the current study we explored early stage atherosclerosis using $Ldlr^{-/-}$ mice on C57BL/6 background reconstituted with hematopoietic cells null for JNK isoforms and fed with the Western diet (containing 21% milk fat and 0.15% cholesterol) for 8 weeks. The variation in genetic background of mice, stage-specific lesion burden, and *Jnk* deficiency in specific compartments are all important determinants of cholesterol absorption[37] and susceptibility to atherosclerosis[38], and they may underlie the apparent differences in our results.

In the current study, we observed a higher lesion burden in early atherosclerosis as a result of deficiency of *Jnk1*, but not *Jnk2*, in hematopoietic cells in the *Ldlr* null mice. Similar results were also obtained when combined deletion models (either $Jnk1^{+/-}/Jnk2^{-/-}$ or $Jnk1^{-/-}/Jnk2^{+/-}$) were used as donors to produce hematopoietic JNK-deficiency. These results may point to several possibilities. For example, it is possible that total JNK activity may be a more important determinant of the impact on macrophage apoptosis and atherogenesis than separate JNK isoforms. In the future, it would be highly informative to examine interactions between JNK isoforms in supporting total JNK activity *in vivo*. In this sense, our data are consistent with a recent report [39] indicating that loss of apoptosis signal-regulating kinase 1, which is upstream of JNK in certain contexts, in apoE null mice significantly reduced apoptosis and increased atherosclerosis by forming lesions enriched with macrophages. Due to the complexity of signaling upstream of JNK, multiple mechanisms may affect atherogenesis in a differential manner. For example, lack of mitogen-activated protein kinase phosphotase-1 protects apoE-null mice from atherosclerosis [40]; whereas genetic deletion of

Jnk1 reduces apoptosis in endothelial cells at atheroprone sites of the artery and thus diminishes atherosclerosis [41]. Similarly, the administration of anisomycin via osmotic minipump increased apoptosis and decreased the macrophage content in atherosclerotic lesions of rabbits [42]. In this scenario, prevention of macrophage death is likely a dominant feature of *Jnk*-deficiency, at least during early stages of atherosclerosis, supporting growth of vascular lesions enriched in macrophages. If this is the case, careful consideration of JNK's role in atherosclerosis and how it could be best utilized for therapeutic intervention would be well warranted. It is however equally likely that *Jnk1* deficiency and early preservation of macrophage death may yield favorable functional outcomes by ensuring plaque stability and preventing rupture, the predominant cause of morbidity and mortality due to atherosclerosis [43]. In fact, this would be quite reminiscent of the role of certain endoplasmic reticulum stress responses that are also related to macrophage death [2]. For example, CHOP-deficiency can prevent macrophage death and support the stability of vascular lesions and prevent rupture [44]. Finally, it is possible that isolated examination of hematopoietic JNK activity only may have limitations and may not reflect the complete role of JNK in the pathogenesis of atherosclerosis. Future studies should dissect these possibilities in additional models.

Next, to identify the mechanism(s) responsible for the actions of JNK signaling in macrophages, we focused on the fact that $Jnk1^{-/-}\rightarrow Ldlr^{-/-}$ mice had a dramatic decrease in apoptosis and increased numbers of macrophages in their atherosclerotic lesions compared to lesions of WT$\rightarrow Ldlr^{-/-}$ and $Jnk2^{-/-}\rightarrow Ldlr^{-/-}$ mice. These results suggested that *Jnk1* deficiency changes the balance between survival and pro-apoptotic signaling in macrophages at least in the setting they are examined. Indeed, our in vitro studies demonstrated that JNK signaling directly antagonizes Akt activity in mouse macrophages. This effect occurs within a short time (3–15min) and may be beneficial for inflammatory and stress responses by diverting energy sources from the synthetic Akt pathway [3]. In contrast, prolonged or sustained JNK activation suppresses Akt signaling and induces cell apoptosis [6]. Interestingly, this antagonizing effect is mediated mainly through JNK1, but not JNK2, and genetic ablation or pharmacological inhibition of JNK1 completely obliterated this effect. These data are consistent with the previous reports indicating that JNK signaling acts as a negative feedback loop that attenuates insulin action and insulin-induced PI3K activation [7, 12, 23, 45–47]. Together our data indicate that JNK1 signaling antagonizes and suppresses Akt activity in mouse macrophages.

It is important to note that bone marrow transplantation may change every component of hematopoietic system in mice including monocyte-macrophages, T- and B cells and platelets. Several studies have shown that JNK is required for effector T-cell function [48]. JNK2 is important for T cell activation, apoptosis of immature thymocytes [49] and plays a role in control of $CD8^+$ T cell expansion in vivo, while JNK1 is involved in survival of activated T cells during immune responses [50]. Moreover, JNK1 is essential for platelet secretion and thrombus formation [51]. Therefore, we cannot exclude that these changes may also affect atherogenesis.

It is known that sustained JNK signaling restrains Akt activity, the major pro-survival signaling pathway that opposes apoptosis [20], suggesting a potential mechanism for impaired

macrophage viability. In our experiments, sustained JNK signaling under conditions of ER stress gradually extinguished Akt and Bad (S^{136}) activity in WT cells, whereas *Jnk1*$^{-/-}$ macrophages were much less affected (Figure 4C,D). Compared to WT cells, *Jnk1*$^{-/-}$ macrophages were also protected from apoptosis initiated by different stimuli. Moreover, JNK1 inhibition distinctly decreased ER stress-mediated apoptosis in macrophages. These results are consistent with the concept that chronically activated JNK1 signaling is crucial in type 2 diabetes and obesity [8, 11, 47, 52]. JNK-mediated phosphorylation of insulin receptor substrates 1 and 2 disrupts Akt signaling[33, 46] possibly by releasing Bad for translocation to the mitochondria[23] or association with Bcl-2/Bcl-xL and initiation of apoptosis. In addition, we examined whether macrophages utilize a natural brake of Akt signaling, PTEN, to suppress p-Akt. Given that PTEN has been reported to cooperate with JNK [53] to couple the PI3K/Akt and JNK signaling pathways [26], we examined whether PTEN mediates cross-talk between these pathways in mouse macrophages. Our results demonstrate that genetic and pharmacologic inhibition of PTEN virtually eradicates the JNK-mediated effect on p-Akt in macrophages. Thus, JNK signaling may also act via PTEN to antagonize Akt activity and suppress macrophage survival. Macrophage-derived foam cells are the predominant cell type of early atherosclerotic lesions, and loss of macrophages through increased apoptosis may reduce the size of early atherosclerotic lesions [54]. Together these data demonstrate that *Jnk1* deficiency significantly increases macrophage survival and this leads to cell accumulation in early stage atherosclerotic lesions. Importantly, JNK and PTEN signaling in macrophages can be altered pharmacologically with the use of their ligands or inhibitors, supporting these pathways as new potential therapeutic targets for the prevention of atherosclerosis and allowing for functional studies in a stage specific manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

JNK	c-Jun NH_2-terminal kinases
LDLR	LDL-receptor
FLC	fetal liver cells
PTEN	phosphatase and tensin homolog

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Highlights

- JNK1 signaling antagonizes pro-survival Akt activity in mouse macrophages
- *Jnk1* null macrophages were less affected by the stress factors and more protected from apoptosis than WT and *Jnk2* null macrophages
- Loss of *Jnk1*, but not *Jnk2*, in hematopoietic cells significantly increases early atherosclerosis
- Genetic ablation of JNK to a single allele ($Jnk1^{+/-}/Jnk2^{-/-}$ or $Jnk1^{-/-}/Jnk2^{+/-}$) in bone marrow recipients further increased atherosclerosis compared to mice reconstituted with WT or *Jnk*1 null bone marrow

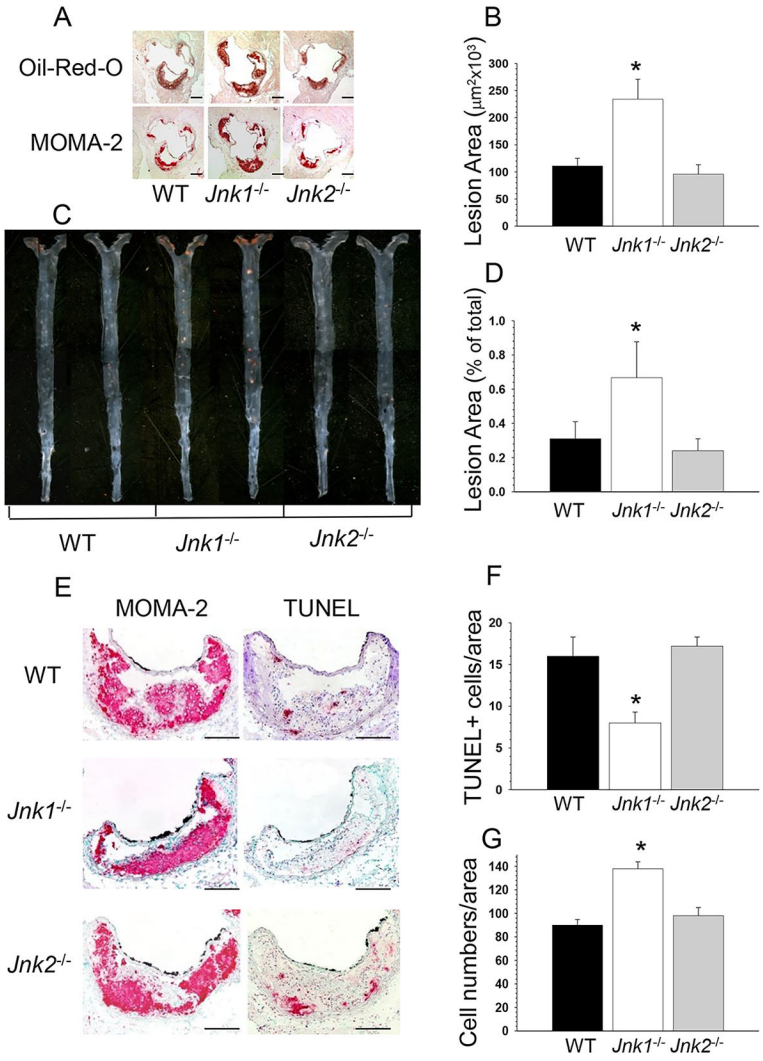

Figure 1. Loss of *Jnk1* in hematopoietic cells increases atherosclerosis

(**A,C**) Detection of atherosclerotic lesions in the aortic sinus and aortas pinned out en face in WT→*Ldlr*$^{-/-}$, *Jnk1*$^{-/-}$→*Ldlr*$^{-/-}$ and *Jnk2*$^{-/-}$→*Ldlr*$^{-/-}$ mice. Serial sections of the aortic sinus were stained with Oil-Red-O to detect neutral lipids or with the MOMA-2 antibody followed by biotinylated goat anti-rat IgG as the secondary antibody, avidin-biotin complex labeled with alkaline phosphatase and Fast Red TR/Naphthol AS-NX substrate to reveal macrophages. Aortas were pinned out and stained with Sudan IV. Scale bars, 200μm; a pin size, 10μm.

(**B,D**) The extent of atherosclerotic lesions in the proximal and distal aorta of *Ldlr*$^{-/-}$ mice reconstituted with WT(■), *Jnk1*$^{-/-}$(□), or *Jnk2*$^{-/-}$(■) bone marrow. Note, atherosclerotic lesions are bigger in *Jnk1*$^{-/-}$→*Ldlr*$^{-/-}$ than in WT→*Ldlr*$^{-/-}$ and *Jnk2*$^{-/-}$→*Ldlr*$^{-/-}$ mice. Graphs represent atherosclerotic lesion area (mean ± SEM) of the recipient *Ldlr*$^{-/-}$ mice (*p<0.05 compared to control group, WT→*Ldlr*$^{-/-}$ mice, by Kruskal-Wallis One Way Analysis of Variance on Ranks, Dunn's Method).

(**E**) Detection of macrophages by staining with anti-MOMA-2 antibodies and apoptotic cells by TUNEL in serial sections of the aortic sinus. Scale bars, 50μm

(**F,G**) Percent of TUNEL+ cells (F) and DAPI-stained nucleus numbers in MOMA-2+ area (G) in atherosclerotic lesions of WT→*Ldlr*$^{-/-}$, *Jnk1*$^{-/-}$→*Ldlr*$^{-/-}$ and *Jnk2*$^{-/-}$→*Ldlr*$^{-/-}$ mice (*p<0.05 compared to control group by One Way Analysis of Variance on Ranks).

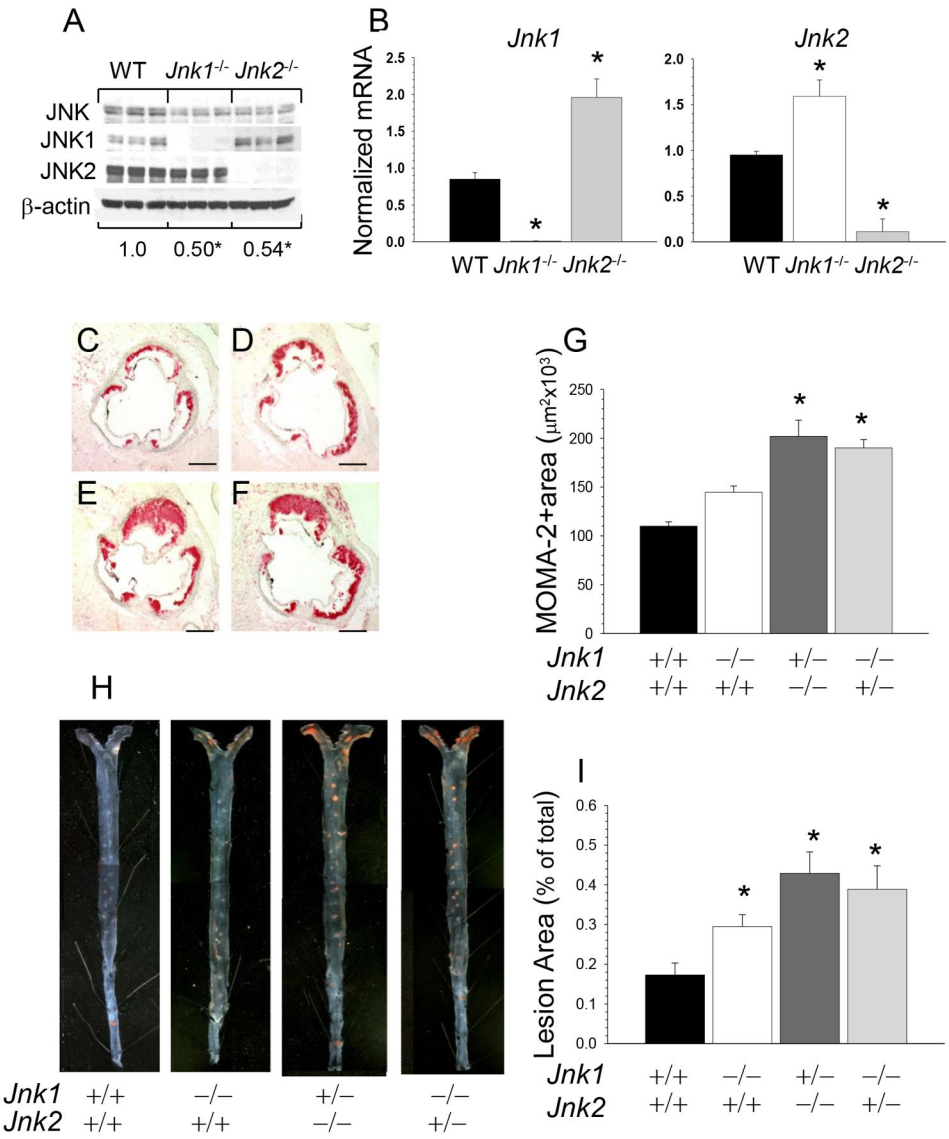

Figure 2. Genetic suppression of JNK signaling to a *Jnk* single allele further increases atherosclerosis

(**A**) JNK protein contents in WT, *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ macrophages (n=3/group); Proteins were isolated and JNK protein contents were analyzed by western blot, the ratio of JNK/β-actin is presented compared to WT cells (*p<0.05 by One Way ANOVA analysis).

(**B**) *Jnk1* or *Jnk2* gene expression levels in peritoneal macrophages from mice reconstituted with WT(■), *Jnk1*$^{-/-}$(□), or *Jnk2*$^{-/-}$(■) FLC; mRNA levels were analyzed by real-time PCR. Graphs represent data (mean ± SEM) with the same number (n=3) of mice per group (*p<0.05 by One Way ANOVA analysis).

(**C–F**) Detection of macrophages in the aortic sinus lesions of mice reconstituted with WT(C), *Jnk1*$^{-/-}$(D), *Jnk1*$^{+/-}$/Jnk2$^{-/-}$(E) or *Jnk1*$^{-/-}$/*Jnk2*$^{+/-}$(F) FLC. Sections were stained with MOMA-2; Scale bars, 50μm.

(**G**) The extent of macrophage lesion area in the proximal aorta of mice reconstituted with WT(■), *Jnk1*$^{-/-}$(□), *Jnk1*$^{+/-}$/*Jnk2*$^{-/-}$(■) or *Jnk1*$^{-/-}$/*Jnk2*$^{+/-}$(■) FLC (*p<0.05 by One way Analysis of Variance, multiple comparisons versus control group, Tukey Test).

(**H**) Atherosclerotic lesions in pinned out en face aorta of mice reconstituted with WT, *Jnk1*$^{-/-}$, Jnk1$^{+/-}$/Jnk2$^{-/-}$ or *Jnk1*$^{-/-}$/*Jnk2*$^{+/-}$ FLC; A pin size, 10μm.

(**I**) The extent of the atherosclerotic lesion area in *Ldlr*$^{-/-}$ mice reconstituted with WT, *Jnk1*, or *Jnk1*$^{+/-}$/*Jnk2*$^{-/-}$ or *Jnk1*$^{-/-}$/*Jnk2*$^{+/-}$ FLC (*p<0.05 by Kruskal-Wallis One Way Analysis of Variance on Ranks, Dunn's Method, versus control group, WT→*Ldlr*$^{-/-}$ mice).

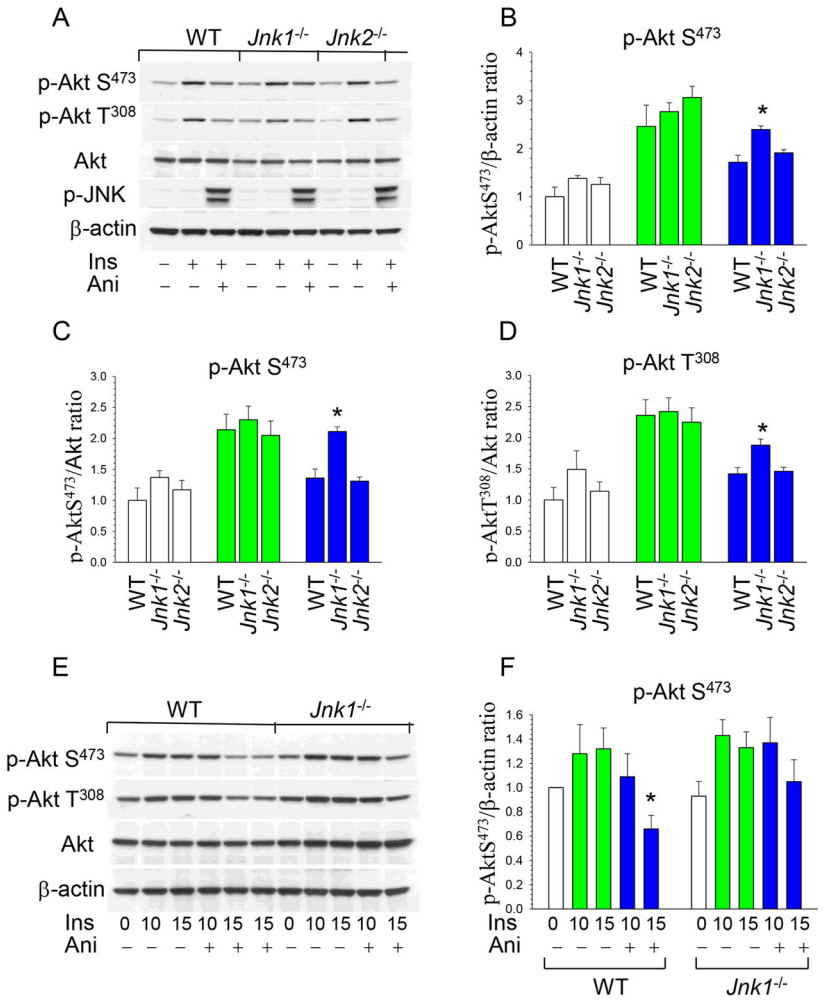

Figure 3. JNK signaling antagonizes p-Akt activity and loss of JNK1 obliterated this effect

(**A**) WT, *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ peritoneal macrophages were pre-incubated in serum-free media for 24 hours then untreated or treated with insulin (100nM) alone or together with anisomycin (10μg/ml) for 15min. Macrophage proteins were extracted, resolved by electrophoresis (50μg), and analyzed by Western blot.

(**B–D**) Ratio of p-AktS^{473}/b-actin, p-AktS^{473}/Akt and p-Akt T^{308}/Akt in untreated (white color) or treated with insulin (green color) or insulin plus anisomycin (blue color). Graphs represent data (mean ± SEM) of three experiments (*p<0.05 by One Way Analysis of Variance on Rank compared to control WT cells treated with insulin together with anisomycin).

(**E,F**) WT and *Jnk1*$^{-/-}$ macrophages were treated with insulin alone (green color) or together with anisomycin (blue color) for 10 and 15 min. Graphs represent data (mean ± SEM) of three experiments (*p<0.05 by One Way Analysis of Variance on Rank compared to WT cells treated with insulin).

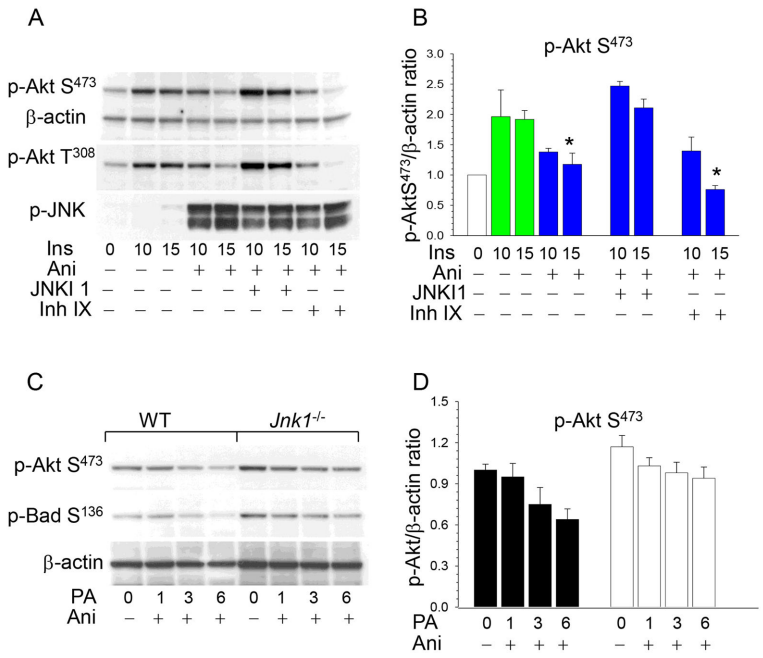

Figure 4. The JNK1 inhibitor, JNKI1, preserves Akt signaling, and *Jnk1*$^{-/-}$ macrophages are more resistant to ER stress than WT cells

(**A,B**) WT peritoneal macrophages were pre-incubated in serum-free media for 24 hours then treated with insulin alone (green color) or together with anisomycin (blue color) without or with the specific JNK inhibitor 1, JNKI1 (3μM) or specific JNK2 and JNK3 inhibitor, inhibitor IX (50nM) for indicated time. Macrophage proteins were extracted, resolved (60μg/well) and analyzed by Western blot with noted antibodies. Graphs represent data (mean ± SEM) of experiments with four mice/group (*$p<0.05$ compared to control WT cells treated with insulin for 15min by One Way Analysis of Variance on Ranks); (**C,D**) WT(■) and *Jnk1*$^{-/-}$(□)peritoneal macrophages were untreated or treated with 0.5mM PA-BSA and anisomycin (10μg/ml) for the indicated time. Graphs represent data (mean ± SEM) of three experiments.

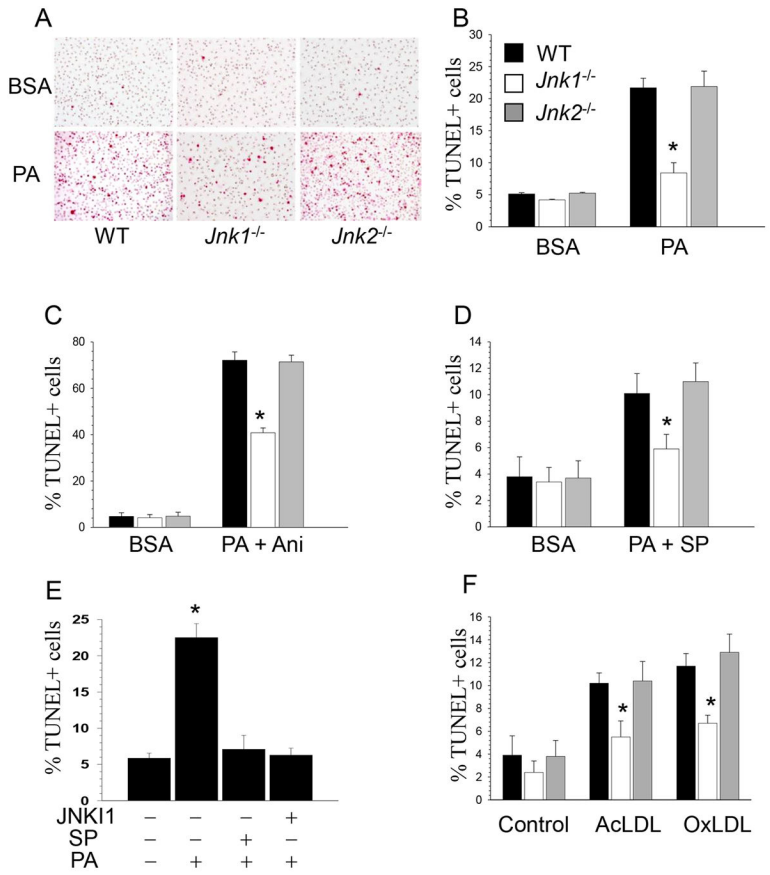

Figure 5. Jnk1$^{-/-}$ macrophages are protected from apoptosis and anisomycin increases, whereas JNK inhibition suppresses ER-mediated apoptosis

(**A**) Detection of apoptosis in WT, *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ macrophages treated with BSA (control) and 0.5mM PA-BSA for 24 hours by TUNEL assay. Note TUNEL-positive cells (red), nuclei counterstained with Mayer hematoxylin;

(**B**) Percent of TUNEL+ WT, *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ macrophages treated with BSA or PA-BSA (*p<0.05 by One Way Analysis of Variance on Rank compared to WT cells treated with PA-BSA);

(**C, D**) Percent of TUNEL+ WT, *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ macrophages treated with BSA or PA-BSA together with anisomycin (10mg/ml) or the JNK inhibitor, JNKI1 (3μM) for 24 hours (*p<0.05 by One Way Analysis of Variance on Rank compared to WT cells treated with PA +Ani or PA+SP);

(**E**) Percent of TUNEL+ cells in WT macrophages untreated or treated with 0.5mM PA-BSA alone or together with JNK inhibitors, SP600125 (100nM) or JNKI1 (3μM) for 24 hours (*p<0.05 by One Way Analysis of Variance on Rank compared to untreated WT cells);

(**F**) Percent of TUNEL+ in WT, *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ macrophages untreated (control) or treated with human acetylated LDL (100μg/ml) in the presence of the ACAT inhibitor CP-113,818 (2μg/mL) or human oxidized LDL (100μg/ml) for 48 hours (*p<0.05 compared to control WT cells treated with Ac-LDL by One Way Analysis of Variance on Rank).

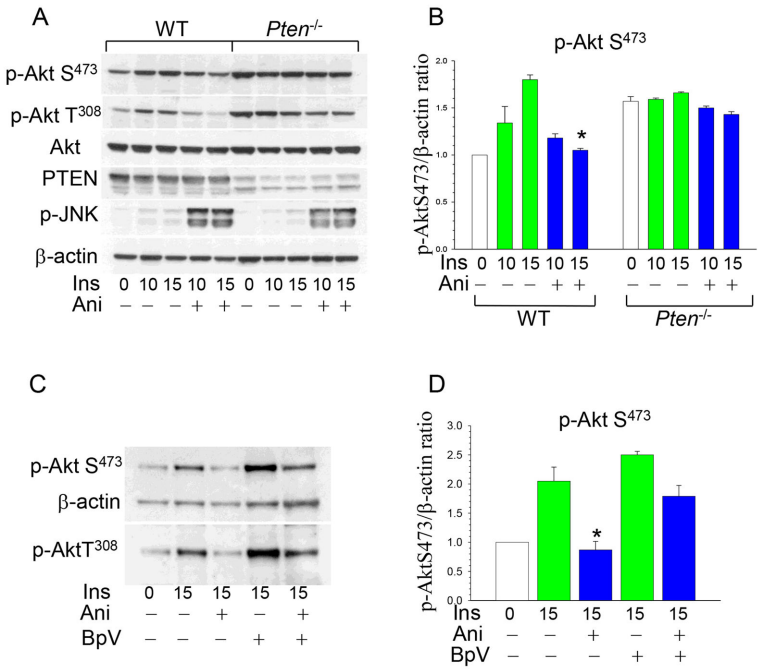

Figure 6. Genetic and pharmacologic inhibition of PTEN eradicates anisomycin-mediated suppression of p-Akt in macrophages

(**A,B**) Akt signaling in WT and *Pten*$^{-/-}$ macrophages treated with insulin and anisomycin. Cells were pre-incubated with serum-free media for 16 hours, then untreated or treated with insulin alone (green color) or together with anisomycin (blue color) for the indicated time. Graphs represent data (Mean ± SEM) of three experiments (*$p<0.05$ between untreated and treated cells by One Way ANOVA analysis).

(**C,D**) PTEN inhibitor bpV(pig) preserves p-Akt signaling in WT peritoneal macrophages treated with anisomycin. Cells were pre-incubated in serum-free media for 24 hours and treated with insulin alone (green color) or with anisomycin (blue color) with or without bpV(pig) (0.1μM) for 15min. Graphs represent data (mean ± SEM) of two (*$p<0.05$ by One Way Analysis of Variance on Rank compared to cells treated with insulin).

Table 1

Body weight (BW), total serum cholesterol (TC) and triglyceride (TG) levels in male *Ldlr*$^{-/-}$ mice reconstituted with WT, *Jnk1*$^{-/-}$, *Jnk2*$^{-/-}$, *Jnk1*$^{+/-}$/*Jnk2*$^{-/-}$ and *Jnk1*$^{-/-}$/*Jnk2*$^{+/-}$ hematopoietic cells on chow and high-fat diets

	Chow diet			High-fat diet		
Type of bone marrow reconstituted	**BW (g)**	**TC (mg/dl)**	**TG (mg/dl)**	**BW (g)**	**TC (mg/dl)**	**TG (mg/dl)**
A.						
WT (n=14)	29.3±0.6	242±11	125±4	32.3±0.7	1063±49	453±40
Jnk1$^{-/-}$ (n=11)	27.8±0.5	245±16	137±5	29.8±0.9	1030±74	472±79
Jnk2$^{-/-}$ (n=13)	28.0±0.8	213±13	135±6	29.9±0.4	1073±84	465±59
p =	0.21	0.20	0.15	0.75	0.44	0.90
B.						
WT (n=10)	28.0±0.7	208±4	116±6	30.8±1.2	974±98	322±21
Jnk1$^{-/-}$ (n=10)	26.9±0.6	216±8	121±5	30.3±2.2	982±62	362±39
Jnk1$^{+/-}$/2$^{-/-}$(n=13)	27.3±0.9	218±9	118±3	29.7±1.1	955±52	334±13
Jnk1$^{-/-}$/2$^{+/-}$(n=12)	27.2±0.8	213±5	121±5	29.3±0.7	966±48	345±24
p =	0.66	0.75	0.12	0.33	0.99	0.72

Values are in mg/dl (Mean ± SEM). The number of recipient mice in each group is indicated by *n*. The differences are not statistically significant between the groups by One Way ANOVA.