Jnk1 Deficiency in Hematopoietic Cells Suppresses Macrophage Apoptosis and Increases Atherosclerosis in Low-Density Lipoprotein Receptor Null Mice

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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 2 JNK isoforms, JNK1 and JNK2, which may have different effects on cell survival and atherosclerosis.

Approach and Results—To dissect the effect of macrophage JNK1 and JNK2 on early atherosclerosis, $Ldlr^{-/-}$ mice were reconstituted with wild-type, $Jnk1^{-/-}$, and $Jnk2^{-/-}$ hematopoietic cells and fed a high cholesterol diet. $Jnk1^{-/-}$→$Ldlr^{-/-}$ mice have larger atherosclerotic lesions with more macrophages and fewer apoptotic cells than mice transplanted with wild-type 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 with $Jnk1^{-/-}$→$Ldlr^{-/-}$ and wild-type→$Ldlr^{-/-}$ 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 endoplasmic reticulum stress gradually extinguished Akt and Bad activity in wild-type cells with markedly less effects in $Jnk1^{-/-}$ macrophages, which were also more resistant to apoptosis. Consequently, anisomycin increased and JNK1 inhibitors suppressed endoplasmic reticulum stress–mediated apoptosis in macrophages. We also found that genetic and pharmacological inhibition of phosphatase and tensin homolog abolished the JNK-mediated effects on Akt activity, indicating that phosphatase and tensin homolog mediates crosstalk between these pathways.

Conclusions—Loss of *Jnk1*, but not *Jnk2*, in macrophages protects them from apoptosis, increasing cell survival, and this accelerates early atherosclerosis. (***Arterioscler Thromb Vasc Biol*. 2016;36:1122-1131. DOI: 10.1161/ATVBAHA.116.307580.)**

Key Words: apoptosis ■ atherosclerosis ■ endoplasmic reticulum stress ■ macrophages ■ MAP kinase signaling system

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 mellitus, 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.[4,5] 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 3 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,

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Nonstandard Abbreviations and Acronyms	
ER	endoplasmic reticulum
JNK	c-Jun NH_2-terminal kinases
PTEN	phosphatase and tensin homolog
WT	wild-type

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 effect 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] Although both JNK1 and JNK2 are involved in apoptotic signaling, only JNK1 is considered to be essential for apoptosis.[17] Murine embryonic fibroblasts lacking *Jnk1*, but not *Jnk2*, have reduced c-Jun phosphorylation and ultraviolet-induced cell death.[18] Loss of both *Jnk1* and *Jnk2* in murine embryonic fibroblasts 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 prosurvival phosphatidylinositol-3-kinase (PI3K/Akt).[20,21] Aikin et al[22] were the first to report cross talk between the PI3K/Akt and JNK pathways that protects islet cells from apoptosis. In addition, Sunayama et al[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 the 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 pharmacological inhibition approaches to investigate the effect of JNK1 and JNK2 on Akt signaling in mouse macrophages and atherogenesis. Our data demonstrate 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 effect 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, A). Size exclusion chromatography of serum revealed an accumulation of cholesterol in very low-density lipoprotein, low-density lipoprotein, and intermediate-density lipoproteins 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),

Table. BW, TC, and TG Levels in Male *Ldlr*$^{-/-}$ Mice Reconstituted With WT, *Jnk1*$^{-/-}$, *Jnk2*$^{-/-}$, *Jnk1*$^{+/-}$/*Jnk2*$^{-/-}$, and *Jnk1*$^{-/-}$/*Jnk2*$^{+/-}$ Hematopoietic Cells on Chow and High-Fat Diets

Type of Bone Marrow Reconstituted	Chow Diet			High-Fat Diet		
	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); *P* values	28.0±0.8; 0.21	213±13; 0.20	135±6; 0.15	29.9±0.4; 0.75	1073±84; 0.44	465±59; 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); *P* values	27.2±0.8; 0.66	213±5; 0.75	121±5; 0.12	29.3±0.7; 0.33	966±48; 0.99	345±24; 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 1-way ANOVA. BW indicates body weight; TC, total serum cholesterol; TG, triglyceride; and WT, wild-type.

erythrocytes (9.7±0.8, 9.9±1.1, and 9.7±0.9×10^6/μL), platelets (649±66, 679±73, and 613±61×10^3/μL), and white blood cells (7.8±06, 9.1±0.7, and 7.5±0.45×10^6/mL). In contrast, the extent of atherosclerotic lesions in aortic sinus of the *Jnk1*$^{-/-}$→*Ldlr*$^{-/-}$ mice was markedly increased (Figure 1A and 1B) compared with mice reconstituted with WT or *Jnk2*$^{-/-}$ marrow cells (Figure 1B; 241.6±38.1 versus 110.8±13.4 and 95.8±17.6×10^3 μm^2, respectively). Similarly, *Jnk1*$^{-/-}$→*Ldlr*$^{-/-}$ mice had significantly increased size of atherosclerotic lesions in the distal aorta compared with WT→ *Ldlr*$^{-/-}$ and *Jnk2*$^{-/-}$→ *Ldlr*$^{-/-}$ mice (Figure 1C and 1D; 0.67%±0.22% versus 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 3 groups (data not shown). The lesions predominantly consisted of macrophage-derived foam cells, and *Jnk1*$^{-/-}$→*Ldlr*$^{-/-}$ mice had significantly bigger lesion area stained with antibody to MOMA-2 versus WT→*Ldlr*$^{-/-}$ and *Jnk2*$^{-/-}$→*Ldlr*$^{-/-}$ mice (Figure 1A; 167.1±29.4 versus 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 and 1F). 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 with lesions of WT→*Ldlr*$^{-/-}$ and *Jnk2*$^{-/-}$→*Ldlr*$^{-/-}$ mice (Figure 1G). Together, the data indicate that the 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.

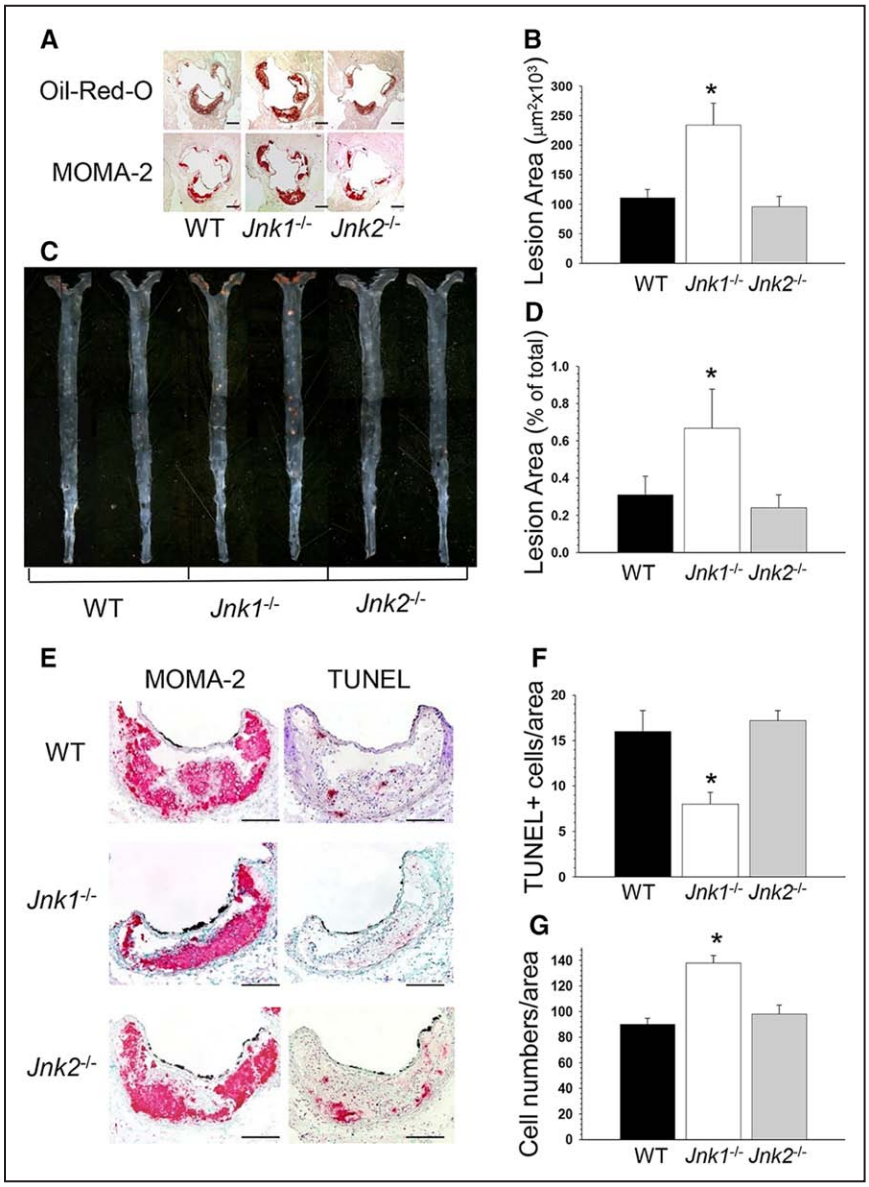

Figure 1. Loss of *Jnk1* in hematopoietic cells increases atherosclerosis. **A** and **C**, Detection of atherosclerotic lesions in the aortic sinus and aortas pinned out en face in wild-type (WT)→*Ldlr*$^{-/-}$, *Jnk1*$^{-/-}$→*Ldlr*$^{-/-}$, and *Jnk2*$^{-/-}$→*Ldlr*$^{-/-}$ mice. Serial sections of the aortic sinus were stained with Oil Red O to detect neutral lipids or with the MOMA-2 antibody followed by biotinylated goat antirat 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 bar, 200 μm; a pin size, 10 μm. **B** and **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 with control group, WT→*Ldlr*$^{-/-}$ mice, by Kruskal–Wallis 1-way ANOVA on ranks, Dunn method). **E**, Detection of macrophages by staining with anti–MOMA-2 antibodies and apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) in serial sections of the aortic sinus. Scale bar, 50 μm. **F** and **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 with the control group by 1-way ANOVA on ranks).

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 with WT cells (Figure 2A; 0.49±0.03 and 0.54±0.03 versus 1.0±0.01; $P<0.05$ by 1-way ANOVA) and JNK kinase activity (Figure I in the online-only Data Supplement). 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 effect of further genetic suppression of JNK signaling on atherosclerosis, we generated mice expressing a single allele of *Jnk1* or *Jnk2* in hematopoietic cells. Because the complete absence of both *Jnk1* and *Jnk2* causes early embryonic lethality, we intercrossed *Jnk1*$^{+/-}$/*Jnk2*$^{+/-}$ mice and collected fetal liver cells. Then, 17-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) fetal liver cells. 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, B). Macrophages isolated from mice with a single JNK allele exhibited further decrease in JNK protein content compared with *Jnk1*$^{-/-}$ and WT cells (Figure II in the online-only Data Supplement). 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 and 2F) than *Jnk1*$^{-/-}$→*Ldlr*$^{-/-}$ and WT$^{-/-}$→*Ldlr*$^{-/-}$ mice (Figure 2C, 2F, and 2G; 183% and 172% versus 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 with *Jnk1*$^{-/-}$→*Ldlr*$^{-/-}$ and WT$^{-/-}$→*Ldlr*$^{-/-}$ mice (Figure 2H and 2I; 248% and 225% versus 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

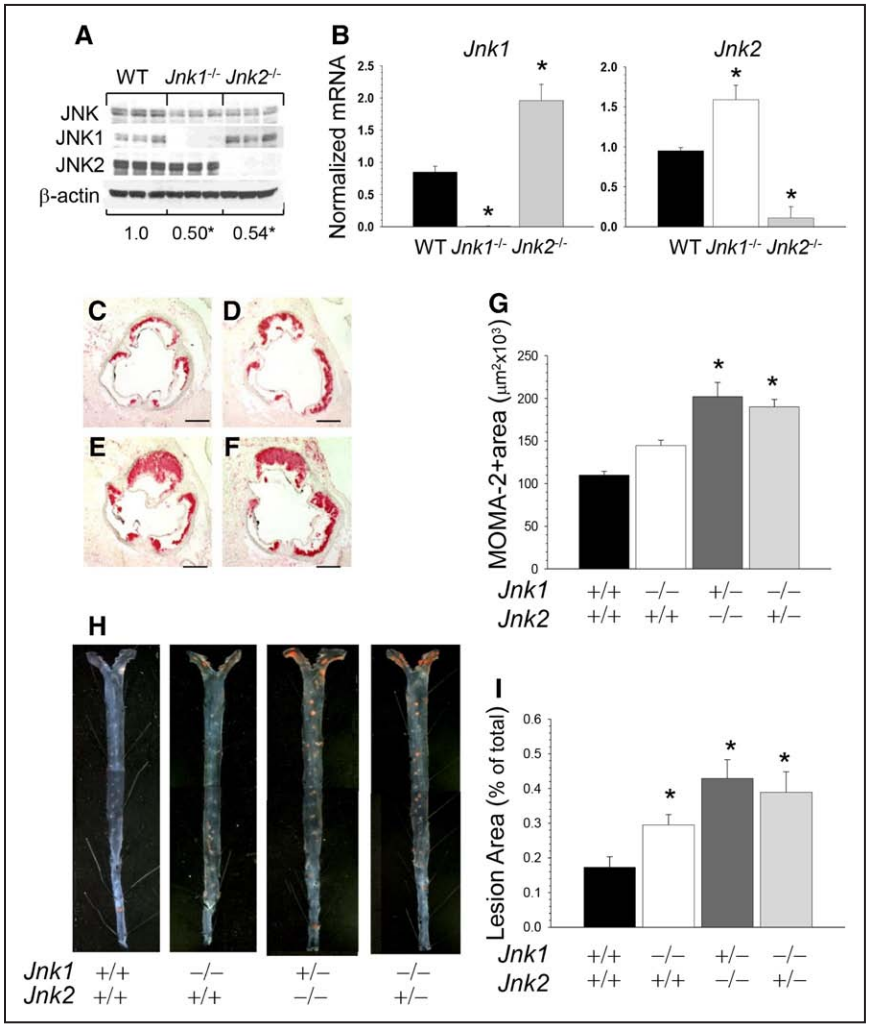

Figure 2. Genetic suppression of c-Jun NH_2-terminal kinase (JNK) signaling to a *Jnk* single allele further increases atherosclerosis. **A**, JNK protein contents in wild-type (WT), *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ macrophages (n=3 per group); proteins were isolated, and JNK protein contents were analyzed by Western blot; the ratio of JNK/β-actin is presented compared with WT cells (*$P<0.05$ by 1-way ANOVA analysis). **B**, *Jnk1* or *Jnk2* gene expression levels in peritoneal macrophages from mice reconstituted with WT (■), *Jnk1*$^{-/-}$ (□), or *Jnk2*$^{-/-}$ (■) fetal liver cells (FLC); mRNA levels were analyzed by real-time polymerase chain reaction. Graphs represent data (mean±SEM) with the same number (n=3) of mice per group (*$P<0.05$ by 1-way ANOVA). **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 bar, 50 μm. **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 1-way ANOVA, multiple comparisons vs 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, 10 μm. **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 1-way ANOVA on ranks, Dunn method, vs control group; WT→Ldlr$^{-/-}$ mice).

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 effect 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 with WT or *Jnk2*$^{-/-}$ cells (Figure 3C and 3D). 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 of *Jnk1*$^{-/-}$ macrophages (Figure 3E and 3F). 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 and 4B). 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 and 4B). Taken together, these data indicate that genetic ablation and pharmacological inhibition of JNK1, but not JNK2, eliminate the suppressive effects of JNK signaling on Akt activity.

JNK1 Deficiency Protects Macrophages From Apoptosis

JNK signaling has proapoptotic or antiapoptotic functions, depending on the 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 antiapoptotic 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 effect of anisomycin on Akt signaling in WT and *Jnk1*$^{-/-}$ macrophages treated with palmitic acid, a stress-mediated

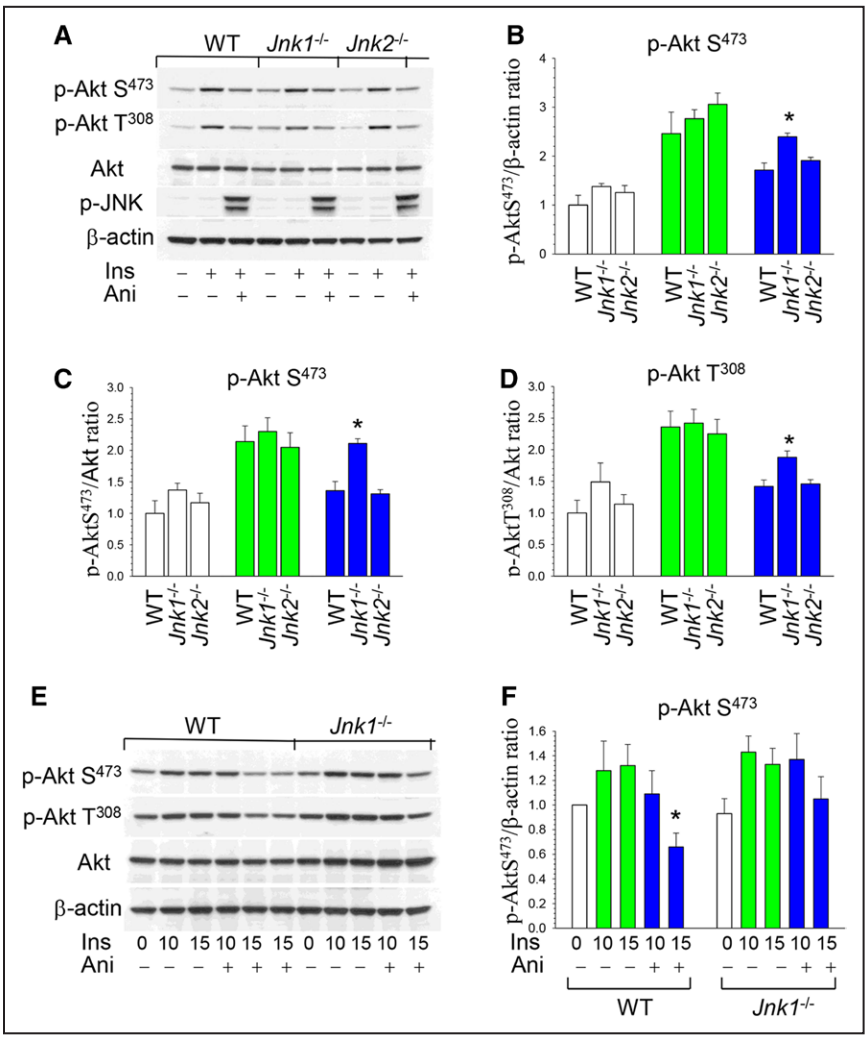

Figure 3. c-Jun NH_2-terminal kinase (JNK) signaling antagonizes p-Akt activity, and loss of JNK1 obliterated this effect. **A**, Wild-type (WT), *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ peritoneal macrophages were preincubated in serum-free media for 24 hours and then untreated or treated with insulin (100 nmol/L) alone or together with anisomycin (10 μg/mL) for 15 minutes. Macrophage proteins were extracted, resolved by electrophoresis (50 μg), and analyzed by Western blot. **B**–**D**, Ratio of p-AktS473/b-actin, p-AktS473/Akt, and p-Akt T308/Akt in untreated (white color) or treated with insulin (green color) or insulin plus anisomycin (blue color). Graphs represent data (mean±SEM) of 3 experiments (*P<0.05 by 1-way ANOVA on rank compared with control WT cells treated with insulin together with anisomycin). **E** and **F**, WT and *Jnk1*$^{-/-}$ macrophages were treated with insulin alone (green color) or together with anisomycin (blue color) for 10 and 15 minutes. Graphs represent data (mean±SEM) of 3 experiments (*P<0.05 by 1-way ANOVA on rank compared with WT cells treated with insulin).

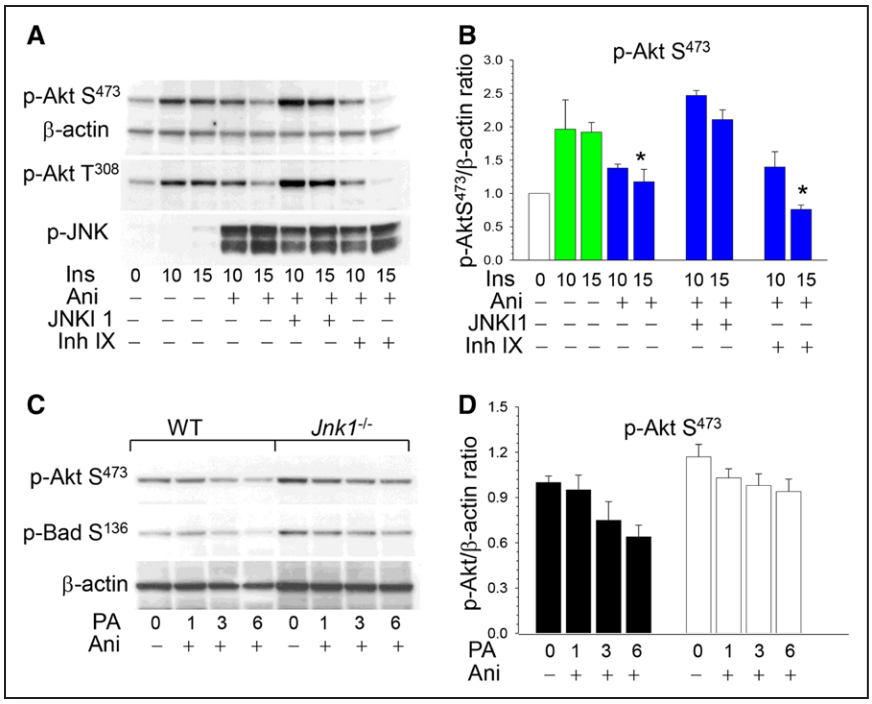

Figure 4. c-Jun NH_2-terminal kinase-1 (JNK1) inhibitor, JNKI1, preserves Akt signaling, and *Jnk1*$^{-/-}$ macrophages are more resistant to endoplasmic reticulum stress than wild-type (WT) cells. **A** and **B**, WT peritoneal macrophages were preincubated in serum-free media for 24 hours and then treated with insulin alone (green color) or together with anisomycin (Ani; blue color) without or with the specific JNK inhibitor 1, JNKI1 (3 μmol/L) or specific JNK2 and JNK3 inhibitor, inhibitor IX (50 nmol/L), for indicated time. Macrophage proteins were extracted, resolved (60 μg per well), and analyzed by Western blot with noted antibodies. Graphs represent data (mean±SEM) of experiments with 4 mice per group (*P<0.05 compared with control WT cells treated with insulin for 15 minutes by 1-way ANOVA on ranks). **C** and **D**, WT (■) and *Jnk1*$^{-/-}$ (□) peritoneal macrophages were untreated or treated with 0.5 mmol/L palmitic acid–bovine serum albumin and Ani (10 μg/mL) for the indicated time. Graphs represent data (mean±SEM) of 3 experiments.

lipotoxic factor inducing endoplasmic reticulum (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 and 4D). 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 with WT cells, *Jnk1*$^{-/-}$ macrophages were able to preserve higher levels of Akt and Bad phosphorylation, which are important protective and antiapoptotic 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 bovine serum albumin or palmitic acid. Treatment with bovine serum albumin generated only a few apoptotic TUNEL-positive (TUNEL^{+}) cells with no differences between cell types, whereas palmitic acid increased TUNEL^{+} cells 4-fold in WT and *Jnk2*$^{-/-}$ macrophages but not in *Jnk1*$^{-/-}$ cells (Figure 5A and 5B). 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 of apoptosis (Figure 5E). Importantly, when cells were loaded with human oxidized or acetylated LDL in combination with an ACAT (acetyl-coenzyme A acetyltransferase) inhibitor, *Jnk1*$^{-/-}$ macrophages generated significantly less apoptosis than WT and *Jnk2*$^{-/-}$ cells (Figure 5F). In addition, macrophages expressing a single Akt isoform (Figure III in the online-only Data Supplement), Akt1 (*Akt2*$^{-/-}$/*Akt3*$^{-/-}$), or Akt3 (*Akt1*$^{-/-}$/*Akt2*$^{-/-}$) palmitic acid–bovine serum albumin treatment induced a stepwise increase in apoptosis that was especially high in *Akt1*$^{-/-}$/*Akt2*$^{-/-}$ cells compared with WT cells. However, suppression of JNK signaling by the JNK inhibitor, SP600125, completely reversed the effect on cell survival with no differences between the groups (Figure IV in the online-only Data Supplement). 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 and 6B). Similarly, treatment with bpV(pic), a potent PTEN inhibitor, with an IC_{50} ≈10- to 100-fold lower than for other tyrosine phosphatases,[32] decreased the inhibitory effects of JNK on p-Akt (Figure 6C and 6D). Taken together, these results indicate that both genetic ablation and pharmacological 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

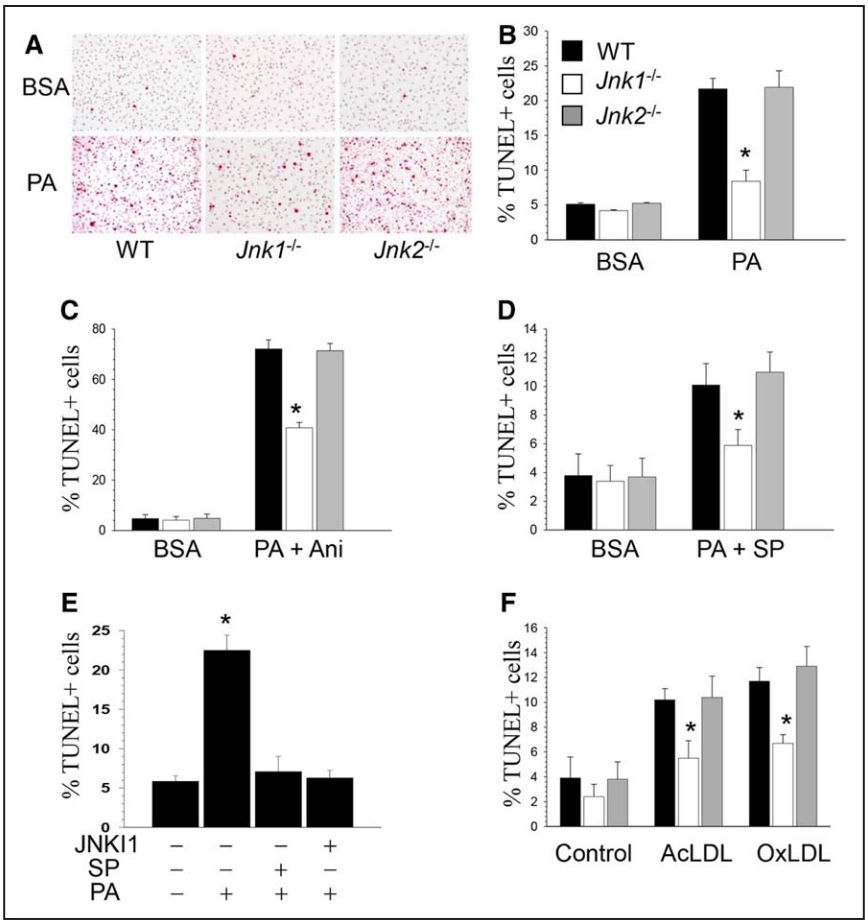

Figure 5. *Jnk1*$^{-/-}$ macrophages are protected from apoptosis and anisomycin (Ani) increases, whereas JNK inhibition suppresses endoplasmic reticulum–mediated apoptosis. **A**, Detection of apoptosis in wild-type (WT), *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ macrophages treated with bovine serum albumin (BSA; control) and 0.5 mmol/L palmitic acid (PA)–BSA for 24 hours by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (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 1-way ANOVA on rank compared with WT cells treated with PA-BSA). **C** and **D**, Percent of TUNEL^{+} WT, *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ macrophages treated with BSA or PA-BSA together with Ani (10 mg/mL) or the JNK inhibitor, JNKI1 (3 μmol/L), for 24 hours (*P<0.05 by 1-way ANOVA on rank compared with WT cells treated with PA+Ani or PA+SP600125 [SP]). **E**, Percent of TUNEL^{+} cells in WT macrophages untreated or treated with 0.5 mmol/L PA-BSA alone or together with JNK inhibitors, SP (100 nmol/L) or JNKI1 (3 μmol/L) for 24 hours (*P<0.05 by 1-way ANOVA on rank compared with untreated WT cells). **F**, Percent of TUNEL^{+} in WT, *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ macrophages untreated (control) or treated with human acetylated low-density lipoprotein (AcLDL; 100 μg/mL) in the presence of the ACAT (acetyl-coenzyme A acetyltransferase) inhibitor CP-113,818 (2 μg/mL) or human oxidized LDL (OxLDL; 100 μg/mL) for 48 hours (*P<0.05 compared with control WT cells treated with AcLDL by 1-way ANOVA on rank).

JNK isoforms have produced varying degrees of effect in different models, perhaps because of interactions between isoforms and redundancies.[8] In fact, a recent report using *Jnk1* and *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 effect 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 with 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 with *Jnk1*$^{-/-}$→*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. On the basis of 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 overexpressed 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 affect macrophage viability and atherosclerosis. In fact, Ricci et al[16] were the first to report the involvement of JNK2 in atherosclerosis showing that *Jnk2*$^{-/-}$/*apoE*$^{-/-}$ mice developed less atherosclerosis compared with 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

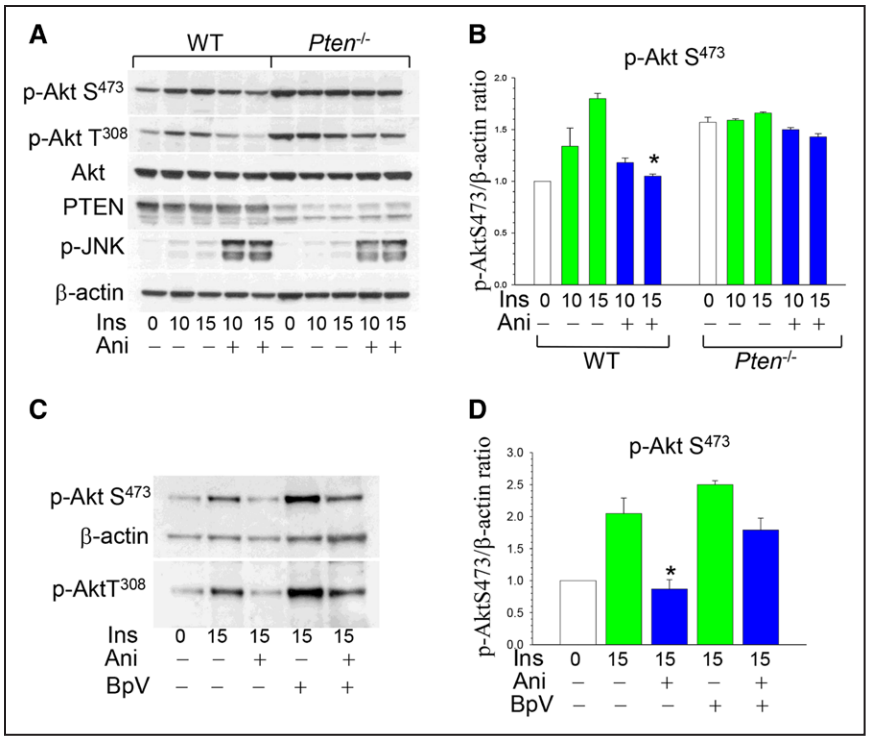

Figure 6. Genetic and pharmacological inhibition of phosphatase and tensin homolog (PTEN) eradicates anisomycin-mediated suppression of p-Akt in macrophages. **A** and **B**, Akt signaling in wild-type (WT) and *Pten*$^{-/-}$ macrophages treated with insulin and anisomycin. Cells were preincubated with serum-free media for 16 hours and 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 3 experiments (*P<0.05 between untreated and treated cells by 1-way ANOVA). **C** and **D**, PTEN inhibitor bpV(pig) preserves p-Akt signaling in WT peritoneal macrophages treated with anisomycin. Cells were preincubated in serum-free media for 24 hours and treated with insulin alone (green color) or with anisomycin (blue color) with or without bpV(pig) (0.1 μmol/L) for 15 minutes. Graphs represent data (mean±SEM) of 2 experiments (*P<0.05 by 1-way ANOVA on rank compared with cells treated with insulin).

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 effect 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. Because of 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 the 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 used 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 caused by atherosclerosis.[43] In fact, this would be quite reminiscent of the role of certain ER stress responses that are also related to macrophage death.[2] For example, C/EBP homologous protein 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*$^{-/-}$→*Ldlr*$^{-/-}$ mice had a dramatic decrease in apoptosis and increased numbers of macrophages in their atherosclerotic lesions compared with lesions of WT→*Ldlr*$^{-/-}$ and *Jnk2*$^{-/-}$→*Ldlr*$^{-/-}$ mice. These results suggested that *Jnk1* deficiency changes the balance between survival and proapoptotic 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 (3–15 minutes) 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 and apoptosis of immature thymocytes[49] and plays a role in control of $CD8^+$ T-cell expansion in vivo, whereas 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 prosurvival 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 and 4D). Compared with 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 mellitus 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 use 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 pharmacological 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.

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Disclosures

None.

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Highlights

- c-Jun NH_2-terminal kinase-1 (JNK1) signaling antagonizes prosurvival Akt activity in mouse macrophages.
- *Jnk1* null macrophages were less affected by the stress factors and more protected from apoptosis than wild-type 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 with mice reconstituted with wild-type or *Jnk*1 null bone marrow.

Arteriosclerosis, Thrombosis, and Vascular Biology

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Jnk1 Deficiency in Hematopoietic Cells Suppresses Macrophage Apoptosis and Increases Atherosclerosis in Low-Density Lipoprotein Receptor Null Mice

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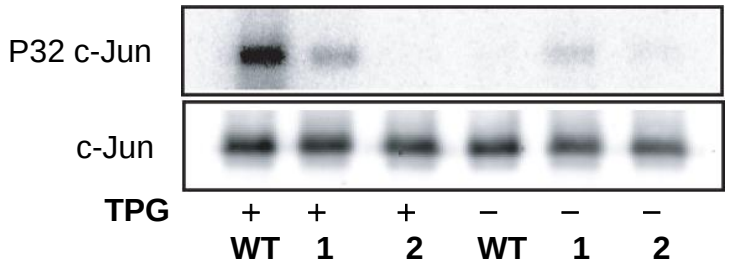

Figure SI. **c-Jun kinase assay in WT, *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ macrophages treated with thapsigargin.**
Peritoneal macrophages from WT, *Jnk1*$^{-/-}$(1) and *Jnk2*$^{-/-}$(2) mice were isolated and two days later, untreated or treated with 1mM thapsigargin (TPG) for 6 hours. Then proteins were extracted, resolved (50μg/well) and analyzed by Western blot.

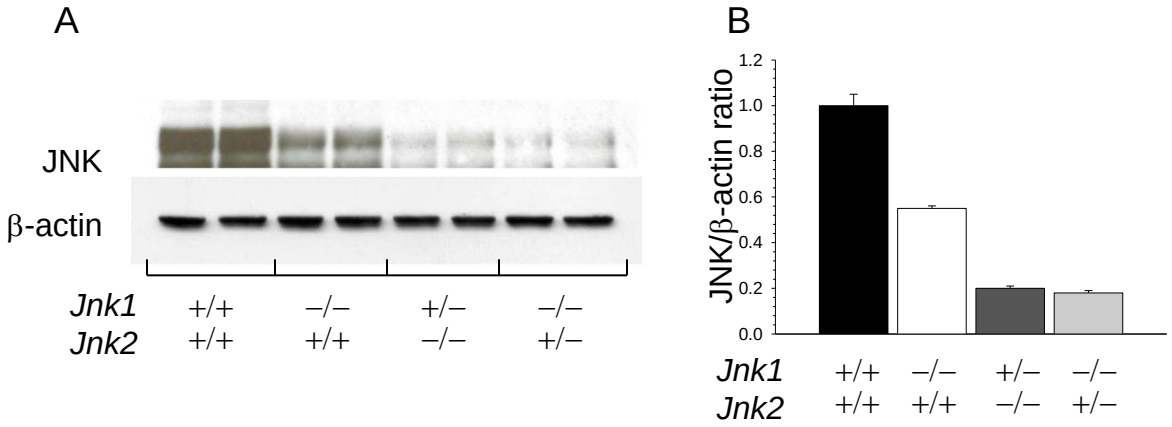

Figure SII. **Dose-dependent suppression of JNK protein contents in wild type, *Jnk1*$^{-/-}$ and single *Jnk* allele macrophages.**
A,**B**. JNK protein contents in WT, *Jnk1*$^{-/-}$, *Jnk1*$^{+/-}$/*Jnk2*$^{-/-}$ and *Jnk1*$^{-/-}$/*Jnk2*$^{+/-}$ macrophages and the ratio of JNK/β-actin is presented compared to WT cells. Macrophage proteins were extracted, resolved (40μg/well) and analyzed by Western blot.

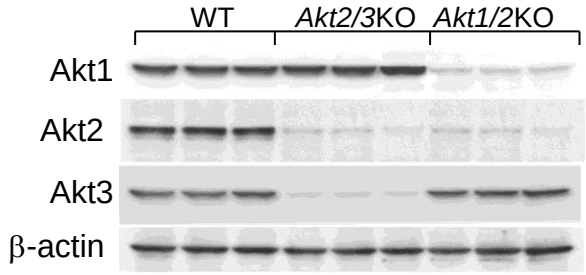

Figure SIII**. Akt isoform expression in *Akt2/3* and *Akt1/2* knockout macrophages** Peritoneal macrophages were isolated from mice reconstituted with WT, *Akt2/3KO* and *Akt1/2KO* FLC (n = 3/group). Proteins were extracted from macrophages, resolved (50μg/well) and analyzed by Western blot using an Akt isoform sampler kit.

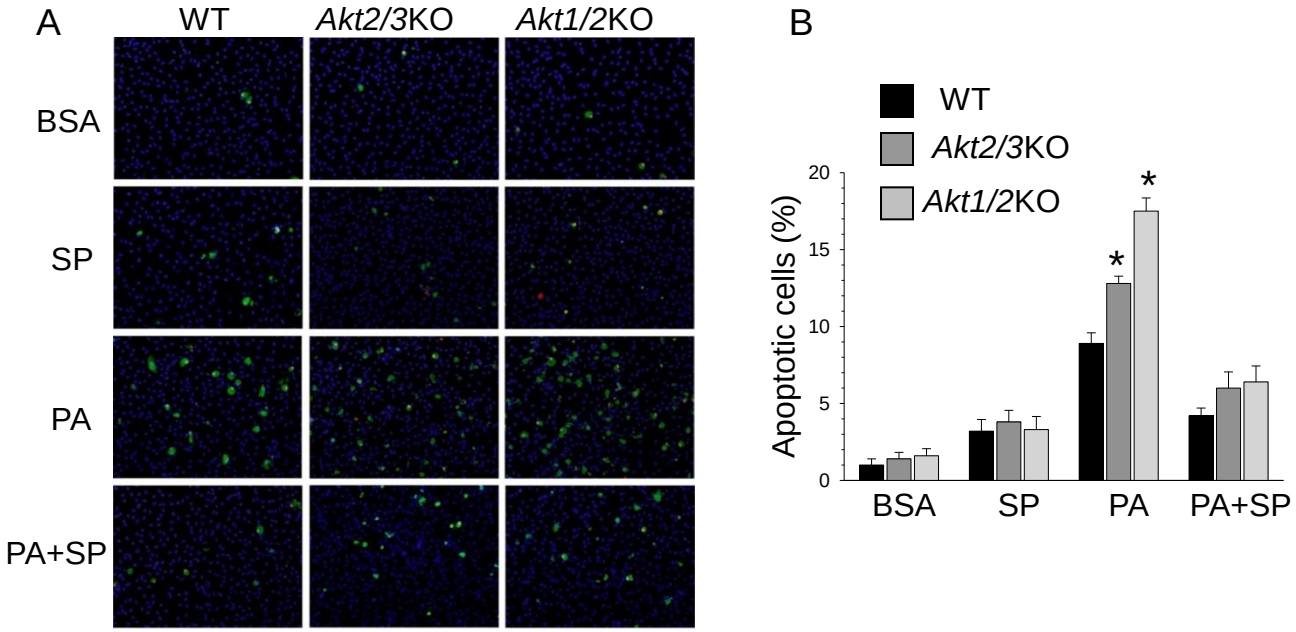

Figure SIV. **Macrophages with a single Akt1 or Akt3 isoform are more sensitive to ER stress and apoptosis but the JNK inhibition eliminates the increase in apoptosis**.
A. WT, *Akt2/3KO* and *Akt1/2KO* macrophages were treated with BSA, the JNK inhibitors, SP600125 (SP, 100nM), 0.5mM PA-BSA (PA) and mixture of PA plus SP for 24 hours. Apoptotic cells were detected by the Alexa Flour 488 Annexin V/Dead cell apoptosis kit. Note the Annexin revealed apoptotic cells (green), propidium iodide detected dead cells (red) and DAPI stained nuclei (blue).
B Percent of apoptotic cells in WT, *Akt2/3KO* and *Akt1/2KO* macrophages treated with BSA, SP, PA or PA plus SP (*$p<0.05$ by One Way Analysis of Variance on Rank compared to WT cells treated with PA).

Material and Methods

Animal Procedures: Mice deficient for *Jnk1* and *Jnk2* were on the C57BL/6 background (10th backcross), recipient *Ldlr*$^{-/-}$ (on C57BL/6 background) and C57BL/6 mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Mice were maintained in microisolator cages on a rodent chow diet containing 4.5% fat (PMI 5010, St. Louis, MO) or a Western type diet containing 21% milk fat and 0.15% cholesterol Teklad, Madison, WI). Animal care and experimental procedures were performed according to the regulations of Vanderbilt University's Animal Care Committee.

Bone Marrow Transplantation and Serum Lipid Analyses: Recipient mice were lethally irradiated (9Gy) from a cesium gamma source and transplanted with 5x10^6 bone marrow cells as described [1]. FLC were collected as described [2]. The serum total cholesterol and triglyceride levels were determined on 24-hour fasting samples using enzymatic assays (Roche) as described [3]. Fast performance liquid chromatography (FPLC) was performed on an HPLC system model 600 (Waters, Milford, MA) using a Superose 6 column (Pharmacia, Piscataway, NJ).

Analysis of Aortic Lesions: Aortas were flushed through the left ventricle and the entire aorta was dissected for en face analysis as described [4]. Cryosections of the proximal aorta (n=15/sinus) were analyzed using an Imaging system KS 300 (Kontron Electronik GmbH.) [2].

I*mmunocytochemistry***:** Serial 5-micron cryosections of the proximal aorta were fixed with acetone and stained with the appropriate antibodies including rat antibody to macrophages, MOMA-2 or CD68 (Accurate Chemical & Scientific Corp., Westbury, NY), rat anti-mouse T-cells, CD90.2 (Thy 1.2 from BioLegend), rat anti-CD3 antibody (17A2 from BD Biosciences), and mouse antibody to smooth muscle cells (1A4 from Sigma). A non-immune rat and mouse sera were used as a negative control. Double staining of macrophages with MOMA-2 and cell nuclei with DAPI with analysis of nucleus numbers in macrophage area as previously described [5].

Peritoneal macrophage isolation and treatment. Thioglycollate-elicited peritoneal macrophages were isolated as described from WT, *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ mice reconstituted with wild type, bone marrow. Then macrophages were treated with bovine pancreatic insulin alone or with anisomycin (both from Sigma-Aldrich), palmitic acid complexed to BSA prepared as described [6], the JNK inhibitors, JNKI1, IX, and SP600125, and the PTEN inhibitor, bpV(pic) (all from EMD Millipore); AcLDL and oxLDL (from Intracel, Inc) and ACAT inhibitor, CP-113,818 (Sigma).

RNA Isolation and real-time PCR. Total RNA was isolated from peritoneal macrophages using the Trizol reagent (Life Technologies, Inc.) and purified by RNA Easy kit (Qiagen, Valencia, CA). Relative quantitation of the target mRNA was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, ABI) and normalized with 18S ribosomal RNA as an endogenous control.

Apoptosis assessment. Peritoneal macrophages were cultured in Laboratory-Tek chamber slides (Nalge Nunc International). Cryosections of 5-micron were obtained from the proximal aorta. Cultured cells and sections were fixed in 2% paraformaldehyde, and apoptotic cells were detected by the Alexa Flour 488

Annexin V/Dead cell apoptosis kit (Life Technologies) and by the TUNEL (TdT-mediated dUTP nick end labeling) technique using the in situ cell death detection kit (Roche Applied Science). TUNEL-positive (TUNEL+) cells were counted in 6 different sections from each aorta.

Macrophage-specific Pten knockout mice. Using the *Cre-loxP* recombination system, we generated mice with *Pten* knockout in myeloid cells. Mice with *loxP* sites flanking exon 5 of the *Pten* gene (from the Jackson laboratory, stock number 006440) were crossed with a mouse *Cre* line under the control of the murine M lysozyme promoter [7] on the C57BL/6 background (nine backcross into C57BL6) similarly as described [5]. These mice were viable and fertile with no notable differences in body weight or plasma lipid levels when compared with control C57BL6 mice.

Western blotting. Cells were lysed on ice with a lysis buffer (Cell Signaling Technology, Danvers, MA) containing a protease (Sigma) and phosphatase (Pierce) inhibitor cocktail. Protein concentrations were determined with the DC Protein assay kit (Bio-Rad Laboratories). Lysates (20 or 100 µg/*lane*) were resolved by NuPAGE Bis-Tris elecrophoresis (Invitrogen) and transferred onto polyvinylidene difluoride nitrocellulose membranes (Amersham Bioscience). Blots were probed with rabbit antibodies to Akt, p-Akt S^{473}, p-Akt T^{308}, p-Bad S^{136}, p-JNK and PTEN (all from Cell signaling), β-actin antibody (Abcam, Inc. Cambridge, MA) and goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Sigma). Proteins were visualized with ECL western blotting detection reagents (GE healthcare) on X-ray films. Protein levels were quantified by densitometry normalizing to β-actin.

Statistical Analyses: Data are provided as means ± SEM. Multiple groups were compared by One-way ANOVA or non-parametric Kruskal-Wallis test using a SPSS Statistics Premium 22 (IBM, Armonk, NY:IBM Corp). These tests initially measure normality to choose parametric or nonparametric analysis (the details are presented in each figure legend). A difference was considered to be statistically significant at a *P*-value less than 0.05.

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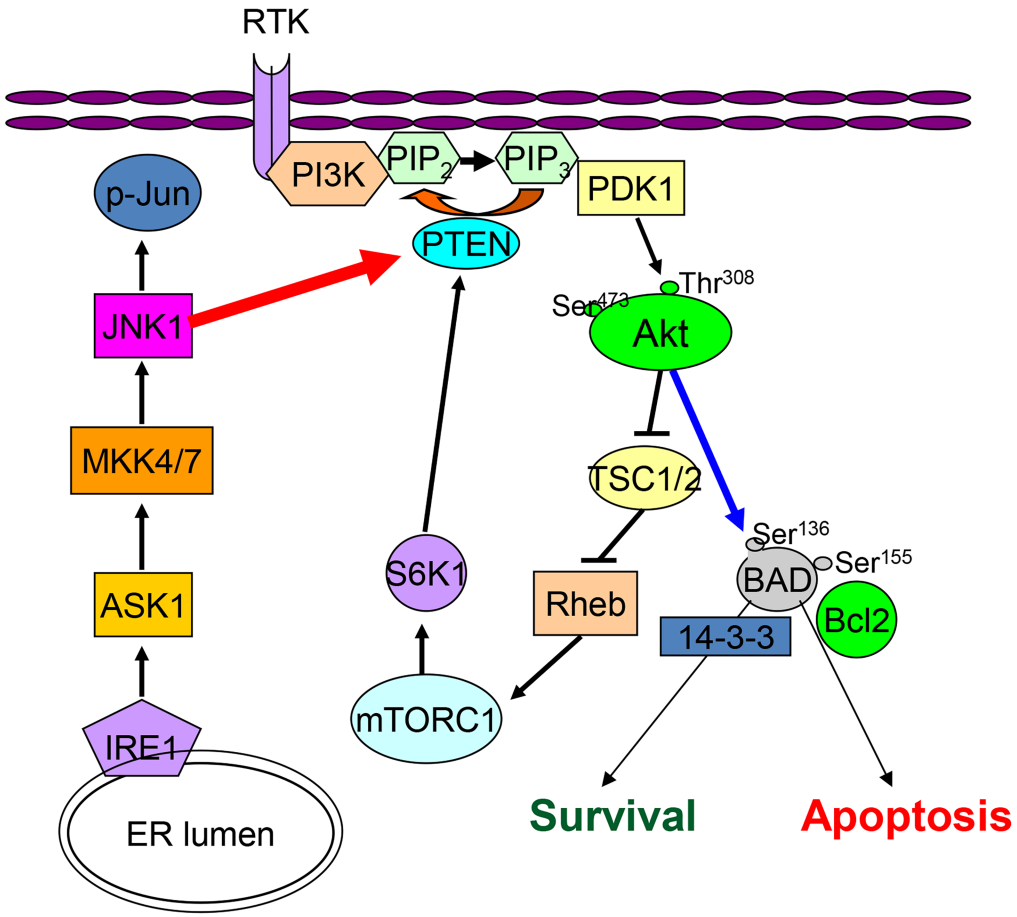
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mTORC1
Ser473
Thr308
Akt
TSC1/2
Rheb
Ser136
Ser155
BAD
Bcl2
14-3-3
Survival
Apoptosis