Enhanced reduction in oxidative stress and altered glutathione and thioredoxin system response to unsaturated fatty acid load in familial hypercholesterolemia.

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Insulin resistance aggravates atherosclerosis by reducing vascular smooth muscle cell survival and increasing CX_3CL1/CX_3CR1 axis

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Aims Insulin resistance (IR) is a major risk factor for cardiovascular disease and atherosclerosis. Life-threatening acute events are mainly due to rupture of unstable plaques, and the role of vascular smooth muscle cells (VSMCs) in this process in IR, Type 2 diabetes mellitus, and metabolic syndrome (T2DM/MetS) has not been fully addressed. Therefore, the role of VSMC survival in the generation of unstable plaques in T2DM/MetS and the involvement of inflammatory mediators was investigated.

Methods and results Defective insulin receptor substrate 2 (IRS2)-mediated signalling produced insulin-resistant VSMCs with reduced survival, migration, and higher apoptosis than control cells. Silencing of IRS2 or inhibition of the V-akt murine thymoma-viral oncogene homologue kinase (AKT)–extracellular signal-regulated kinase (ERK)-dependent pathway in VSMCs augmented expression of the inflammatory chemokine fractalkine (CX_3CL1) and its receptor CX_3CR1, previously involved in atheroma plaque vulnerability. Interestingly, treatment of VSMCs with CX_3CL1 promoted apoptosis in the presence of other stimuli or when the AKT pathway was blocked. Analysis of a mouse model of IR–MetS and accelerated atherosclerosis, *apoE*−/−*Irs2*+/− mice, showed reduced VSMC survival, unstable plaques, and up-regulation of CX_3CL1/CX_3CR1 axis compared with *apoE*−/− mice. Human studies showed augmented soluble CX_3CL1 plasma levels and CX_3CR1 expression in monocytes from IR–MetS subjects compared with controls. A positive correlation between insulin levels, homeostatic model assessment (HOMA) index, carotid atherosclerosis, and CX_3CR1 mRNA levels was also found in all patients.

Conclusion IR increases plaque vulnerability by augmenting the CX_3CL1/CX_3CR1 axis, which is mechanistically linked to reduced VSMC survival. Thus, modulation of IRS2-dependent signalling emerges as a potential therapeutic strategy to promote VSMC survival and atheroma plaque stability and to reduce inflammatory mediators in IR–MetS.

Keywords Insulin resistance • Vascular smooth muscle cell • Unstable plaque • Inflammation • Atherosclerosis

1. Introduction

Type 2 diabetes mellitus (T2DM) and metabolic syndrome (MetS) are major risk factors for developing cardiovascular disease (CVD).[1–4] They affect 195 million people worldwide and their incidence will increase up to 500 million by 2030,[5] due to population aging and to sedentary lifestyle patterns.[1,2] Among the metabolic alterations that define MetS (abdominal obesity, glucose intolerance, hypertension, and dyslipidaemia), insulin resistance (IR) plays a pivotal role in the development of T2DM and CVD. In fact, IR is one of the linking factors between T2DM/MetS and CVD[2] and is an independent risk factor for atherosclerosis.[6,7] T2DM/MetS subjects at highest CVD risk are those

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with vulnerable atheroma plaques, which are prone-to-rupture producing life-threatening acute CVD events (thrombosis, myocardial infarction, and stroke).[3] Thus, there is an increasing recognition that quality rather than size of these plaques is determinant of patient outcomes.[8]

Stable atheromatous lesions are characterized by a thick fibrous cap consisting of vascular smooth muscle cells (VSMCs) that proliferate and migrate from the arterial wall and extracellular matrix which sits on top of the inflammatory lipid core.[9,10] VSMCs within the fibrous cap lessen the risk of plaque rupture; however, progressive accumulation of inflammatory cells and mediators beneath the cap promotes the generation of vulnerable plaques by reducing its collagen content and VSMC survival. VSMC apoptosis promotes plaque instability and has been linked to acute events like rupture, coagulation, vessel remodelling, medial atrophy, and calcification.[8,11,12] Moreover, this event is preceded by the death of neighbouring lipid-loaded macrophages coinciding with a dramatic activation of the immune system and secretion of inflammatory mediators.[11] Some studies have defined the effect of IR in these processes by characterizing the precise contribution of IR macrophages to the formation of vulnerable plaques by augmenting apoptotic mediators and impairing the clearance of apoptotic debris;[13–15] however, the role of VSMCs in IR states has been addressed to a lesser extent.[14,16] Thus, while canonical insulin signalling protects VSMCs from apoptosis[17] and impedes TNF-α-mediated activation of the inflammatory NF-κB pathway,[18] there are fewer studies describing the effect of defective insulin signalling in VSMC function and its impact on plaque vulnerability.[14]

To address this need, we have investigated the effect of IR on human aortic (ha)VSMC survival and migration by inducing defective insulin signalling by silencing a major insulin effector, insulin receptor substrate 2 (IRS2). We also hypothesized that inflammatory mediators might underlie the imbalance of VSMC. Therefore, we investigated the expression and the effect of fractalkine (Chemokine [C-X3-C motif] ligand 1, CX_3CL1) and its G-protein-coupled receptor CX_3CR1, which are main inflammatory mediators implicated in plaque vulnerability,[19,20] on VSMC survival. Moreover, VSMC survival, plaque instability, and expression of the CX_3CL1/CX_3CR1 inflammatory axis were examined *in vivo* in atheromatous plaques of *apoE*−/−*Irs2*+/− mice, a mouse model of IR and MetS with accelerated atherosclerosis. The clinical relevance of our studies was also explored in IR–MetS patients by analysing the levels of the soluble CX_3CL1 in plasma as well as CX_3CR1 mRNA and protein expression in isolated leucocytes and in atherosclerotic carotid artery plaques.

2. Methods

An expanded methods section can be found in Supplementary material online, Material.

2.1 Primary cell culture

Primary haVSMCs were obtained from a commercial supplier (Invitrogen, C-007-5C, Thermofisher Scientific, Madrid, Spain) and used until passage 5 or 6. Cell cultures were maintained on a humidified 5% CO_2 atmosphere with media containing 20% FBS/DMEM/P/S/Fungizone (Lonza, Basel, Switzerland).

2.2 Human subjects

The study was carried out in accordance with the ethical principles for medical research involving human subjects set out in the Helsinki Declaration and was approved by the ethical committee at our institution. All subjects gave written informed consent. The present investigation was a cross-sectional study of 152 unrelated individuals attending the outpatient clinic (Hospital Clínico Universitario de Valencia-INCLIVA, Valencia, Spain) over a period of 12 months. The sample was selected by opportunistic method and subjects were divided into non-IR and IR using the homeostatic model assessment (HOMA) index.[21]

2.3 Mice and diets

The animals were cared for in accordance with our institutional guidelines and approved protocols, and following the 2010/63/EU directive from the European Parliament. *apoE*−/− mice (C57BL/6J, Charles River, Barcelona, Spain) were crossed with *Irs2*+/− mice (which had been backcrossed into a C57BL/6J background for at least 10 generations).[22] Male *apoE*−/− and *apoE*−/−*Irs2*+/− mouse littermates were fed an atherogenic diet (10.8% total fat and 0.75% cholesterol; S8492-E010, ssniff Spezialdiaeten, Germany) for 2 months before analysis. Mice were sacrificed by cervical dislocation in order to obtain tissues for atheroma analysis and to harvest VSMCs.

2.4 Statistical analysis

Quantitative variables appear as means ± standard deviation and qualitative variables as percentages. Differences between mouse characteristics and cell culture studies were evaluated by Student's *t*-test and also by two- or one-way ANOVA with Fisher's *post hoc* test (GraphPad Prism 6, Inc., La Jolla, CA, USA). In human studies, differences in quantitative parameters were assessed by Student's *t*-test, qualitative variants by χ^2, and correlation studies by the Spearman correlation coefficient (SPSS 15 version, Chicago, IL, USA). Outliers identified by the Grubb's test (GraphPad) were not considered for quantification. Two-tailed probabilities (*P*) were considered to be statistically significant at a more than 95% confidence interval ($P \leq 0.05$).

3. Results

3.1 Defective insulin signalling decreases haVSMC survival

Defective IRS2-dependent signalling produces T2DM and IR in mice and humans[22–24] thus, to investigate the effect of IR on haVSMCs survival, siRNA IRS2-gene silencing experiments were performed. haVSMCs treated with siRNA IRS2 showed decreased mRNA (*Figure 1A*, $P < 0.007$) and protein (*Figure 1B*, $P < 0.0001$) IRS2 levels compared with siRNA Control cells. Consistently, silencing of IRS2 decreased insulin-activated (phosphorylated) forms of the downstream effectors V-akt murine thymomaviral oncogene homologue kinase (AKT)1/2 and extracellular signal-regulated kinase (ERK)1/2, indicating reduced IRS2-dependent signalling (*Figure 1C*, $P < 0.03$ and $P = 0.05$).

Next, the effect of impaired insulin signalling on haVSMC survival was studied. Analysis showed a markedly decreased cell proliferation rate in siRNA IRS2-treated VSMCs compared with siRNA Control-treated cells (*Figure 2A*, $P < 0.02$). Wound-healing migration assay showed reduced migration distance and increased wound-healing area in siRNA IRS2-treated cells compared with siRNA Controls (*Figure 2B*, $P < 0.02$ and <0.03, respectively). Apoptosis analysis measured as sub-G0 cells (*Figure 2C*) demonstrated enhanced apoptosis in siRNA IRS2-VSMCs compared with VSMC Controls in basal conditions (6.7 vs. 14.3%, $P < 0.05$) as well as in cells exposed to two pro-apoptotic stimuli [UV light: 50.3 vs. 60.5%, $P < 0.01$; nitric oxide donor *S*-nitrosoglutathione (GSNO): 22.9 vs. 35.3%, $P < 0.001$]. Apoptosis analysis measured by cleaved (CL)-caspase-3 staining showed higher apoptosis percentage in siRNA IRS2-VSMCs compared with siRNA

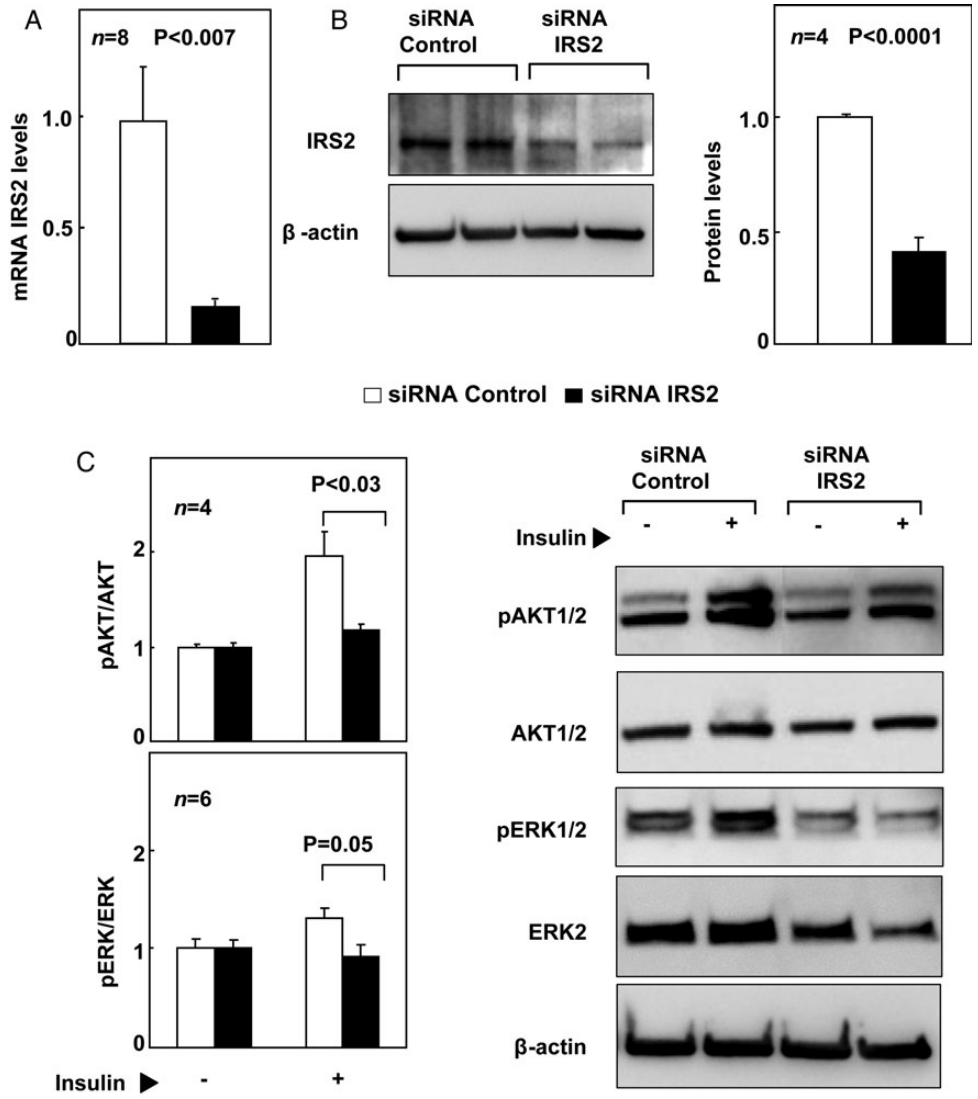

Figure 1 Effect of IRS2-gene silencing in haVSMC survival. (*A*) IRS2 mRNA levels in haVSMCs analysed by qPCR were normalized with endogenous *gapdh* mRNA levels and relativized to mRNA levels of siRNA Control VSMCs. (*B*) Western blot analysis of IRS2 protein expression in haVSMCs treated with siRNA Control and siRNA IRS2. Proteins were quantified (bar graph) by densitometry of the bands using an anti-IRS2 antibody. Protein levels were normalized to β-actin and were relativised to siRNA Control expression. (*C*) Western blot analysis of protein lysates to detect phosphorylated(activated)-AKT1/2 (pAKT1/2), AKT1/2, phosphorylated(activated)-ERK1/2, ERK2, and β-actin as a sample loading control. Phosphorylated forms were normalized to total protein levels and were relativized to the unstimulated sample. Representative blots are depicted on the right and the graphs show relative expression levels. Statistical analysis was done using Student's *t*-test.

Controls when exposed to the pro-apoptotic stimuli (UV light: 27.8 vs. 42.6%, $P < 0.05$; GSNO: 24.8 vs. 38.3%, $P < 0.05$).

Since VSMC senescence has been shown to be a relevant process in vulnerable plaques,[12,25] cellular senescence was also evaluated using senescence-associated beta-galactosidase (SABG) staining and by determining the protein expression levels of the senescence marker $p16^{ink4a}$. Senescence analysis in VSMCs (see Supplementary material online, *Figure S1*) revealed no differences in VSMC SABG-positive staining or in $p16^{ink4a}$ protein expression between siRNA Control and siRNA IRS2 cells. Thus, IRS2 signalling does not make a relevant contribution to VSMC senescence in our settings. Collectively, these findings indicate that IR induced by impaired IRS2 signalling reduces haVSMC survival pointing to a reduced capacity of IR-VSMCs to participate in the generation of stable plaques.

3.2 Decreased VSMC survival mediated by defective IRS2 signalling is linked to augmented expression of the inflammatory CX_3CL1/CX_3CR1 axis

To analyse whether defective insulin signalling in VSMCs is linked to changes in the inflammatory CX_3CL1/CX_3CR1 axis, which has been related to plaque progression and vulnerability,[19,20,26] analyses of mRNA and protein expression in siRNA IRS2 and siRNA Control

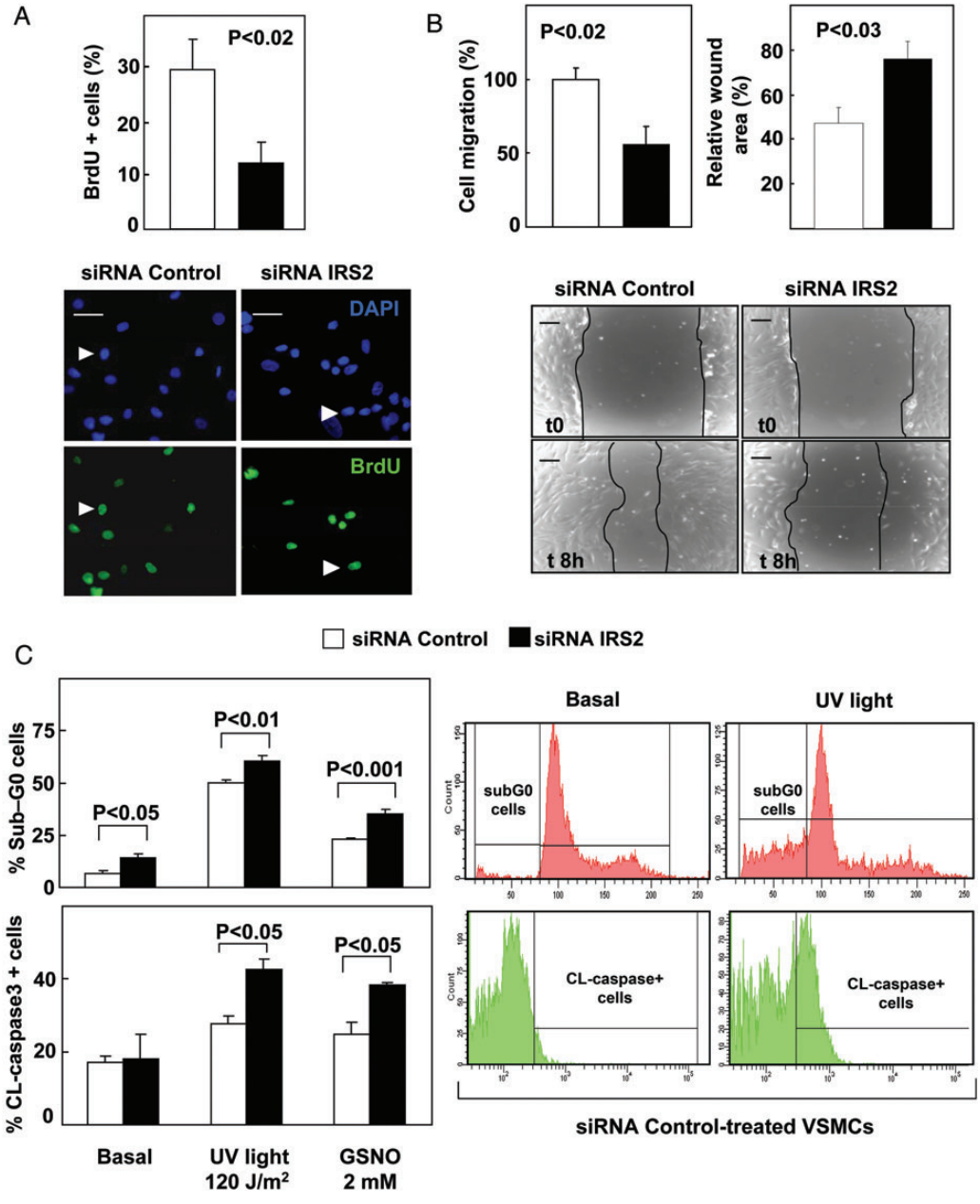

Figure 2 Impaired IRS2-mediated signalling reduces survival of haVSMCs *in vitro*. (*A*) Percentage of proliferating VSMCs analysed by BrdU incorporation and detected by immunofluorescence. Representative images are shown. White arrows point to nucleus of cells. Scale bar: 20 μm. (*B*) Wound-healing migration assay expressed as relative migration distance (left panel) and as a percentage relative wound area (right panel) at the 8-h time point (t8 h) relative to the wound area at the starting point (t0 h) of siRNA IRS2 and siRNA Control-treated VSMC monolayers. Representative images of migration at different time points are shown. Scale bar: 200 μm. (*C*) Apoptosis analysis of siRNA VSMCs, non-treated (basal), irradiated with UV light (120 J/m^2), or incubated with GSNO (2 mM) determined by flow cytometry as sub-G0 cell population or as a percentage of CL-caspase-3-immunoreactive cells relative to the total cell count. Representative flow cytometry plots for both apoptotic analysis approaches are shown for non-treated (basal) and UV light-treated siRNA Control VSMCs. Results are the average of at least three independent experiments, each in triplicate. Statistical analysis was performed using the Student's *t*-test (*A* and *B*) and by two-way ANOVAs (*C*).

VSMCs were performed. Compared with vehicle-treated cells, TNF-α treatment significantly increased the mRNA expression of the CX_3CL1/CX_3CR1 axis in siRNA IRS2-treated VSMCs (*Figure 3A*, $P < 0.01$ and <0.001, respectively). Comparison between TNF-α-stimulated siRNA IRS2-VSMCs and siRNA Control cells (*Figure 3A*) demonstrated higher CX_3CL1/CX_3CR1 mRNA levels ($P < 0.05$ and 0.01, respectively) in siRNA IRS2-VSMCs. Compared with vehicle-treated cells, co-stimulation with TNF-α/IFN-γ enhanced CX_3CL1 protein expression in both siRNA IRS2 and siRNA Control VSMCs (*Figure 3B*; in siRNA Control: $P < 0.001$ and in siRNA IRS2: $P < 0.0001$). CX_3CR1 protein expression after stimulation with TNF-α/IFN-γ augmented only in siRNA IRS2-treated VSMCs (*Figure 3C*, $P < 0.05$). Comparison between siRNA IRS2 and siRNA Control VSMCs showed significantly increased levels of CX_3CL1 protein (*Figure 3B*, $P < 0.05$) and a modest, but not significant, increase

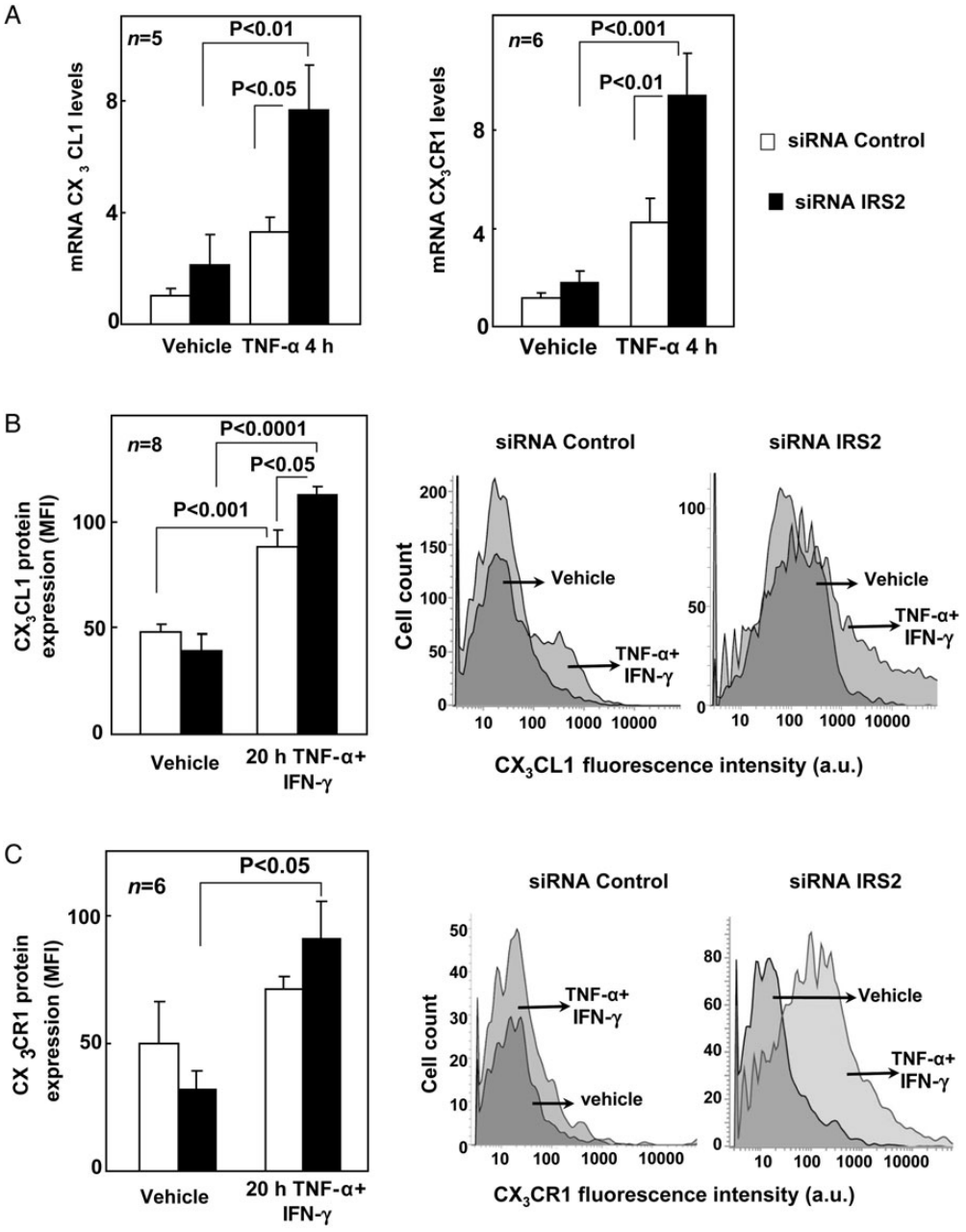

Figure 3 Impaired IRS2 signalling increases the expression of CX_3CL1/ CX_3CR1 in haVSMCs. (*A*) mRNA expression levels of CX_3CL1 and CX_3CR1 were analysed in siRNA Control and siRNA IRS2-treated VSMCs stimulated with TNF-α or vehicle for 4 h. mRNA levels, normalised with endogenous control *gapdh* mRNA levels, were relativised to vehicle-treated siRNA Control mRNA levels. (*B*) CX_3CL1 and (*C*) CX_3CR1 cell surface protein expression analysis of siRNA Control and siRNA IRS2-VSMCs stimulated with TNF-α/IFN-γ or vehicle for 20 h measured by flow cytometry (MFI, median fluorescence intensity). Representative plots from the flow cytometry are shown on the right. Statistical analysis was performed by two-way ANOVAs.

of the receptor, CX_3CR1, in siRNA IRS2-treated cells compared with siRNA Controls. Thus, defective IRS2-dependent signalling in VSMCs up-regulates the CX_3CL1/CX_3CR1 axis.

As IRS2 silencing significantly reduced the activation of the downstream effector kinases AKT/ERK, we examined a possible connection between the CX_3CL1/CX_3CR1 inflammatory axis and these kinases. Inhibition of either AKT or ERK produced a significant increase in both CX_3CL1 and CX_3CR1 mRNA levels in TNF-α-stimulated haVSMCs compared with vehicle-treated cells (*Figure 4A*, AKT inhibition: $P < 0.03$ and <0.02, respectively; ERK inhibition: $P = 0.05$ and $P < 0.005$, respectively). The effectiveness of the inhibitors is shown in Supplementary material online, *Figure S2*. These results indicate that impaired IRS2/AKT/ERK-dependent signalling produces increased CX_3CL1/CX_3CR1 expression.

Because impaired IRS2-mediated signalling reduced haVSMC survival and increased expression of CX_3CL1, we explored the effect of soluble CX_3CL1 on haVSMC apoptosis. The treatment of haVSMCs with CX_3CL1 combined with either AKT inhibition (*Figure 4B*) or UV light

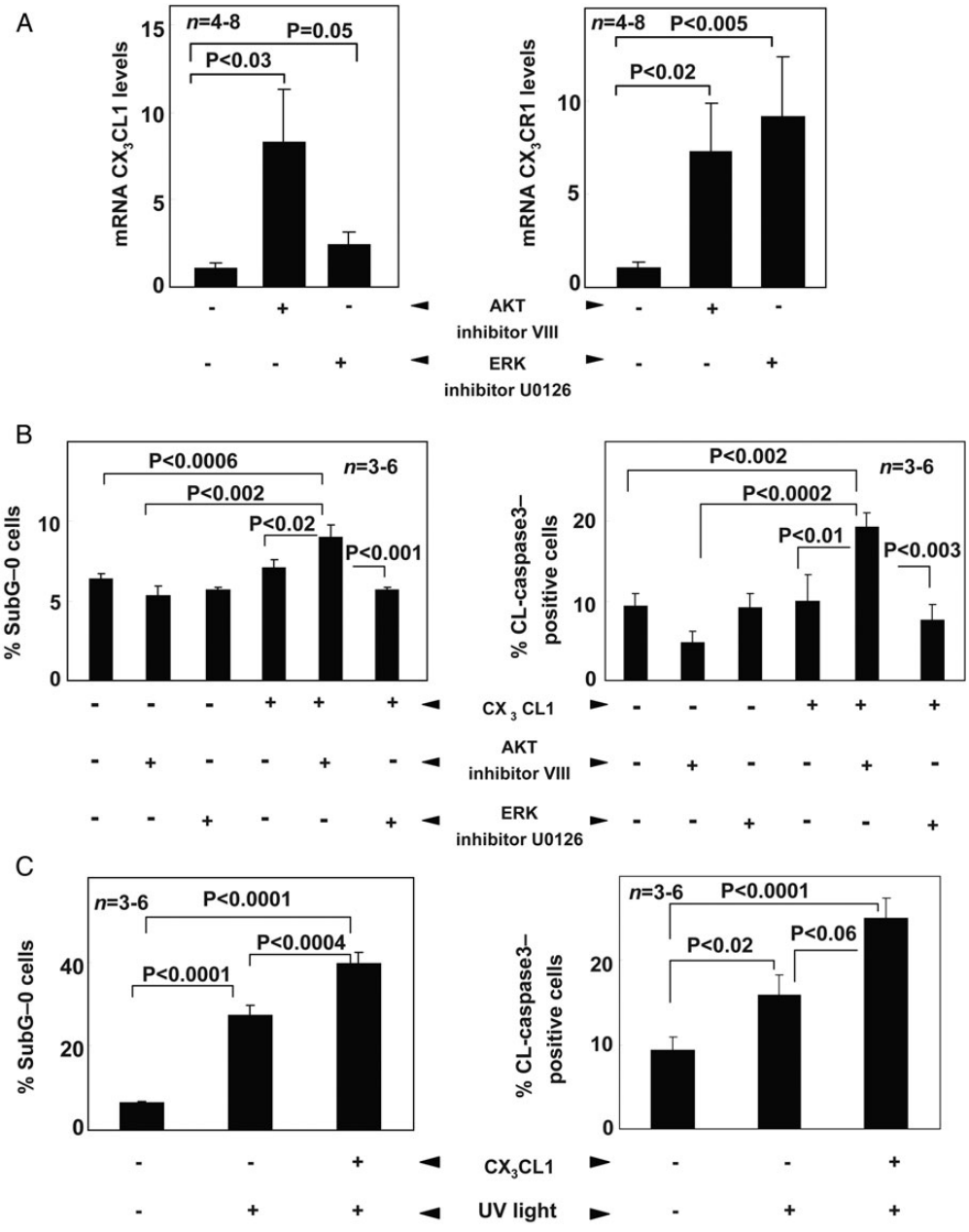

Figure 4 Effect of AKT–ERK inhibition on CX_3CL1/CX_3CR1 expression and CX_3CL1 effect on haVSMC survival. (*A*) mRNA expression levels of CX_3CL1 and CX_3CR1 were analysed in haVSMCs stimulated with TNF-α or vehicle (4 h) after specific inhibition of AKT and ERK kinases (using AKT inhibitor VIII and U0126 inhibitors). mRNA levels, normalised with endogenous control *gapdh* mRNA levels, were relativised to vehicle-treated cells. (*B*) Apoptosis in untreated haVSMCs (basal), or in haVSMCs treated with an AKT inhibitor, with an ERK inhibitor, with CX3CL1 alone, or treated with CX3CL1 combined with an AKT or ERK inhibitors. Apoptosis was measured as a percentage of sub-G0 cells (left graph) and as a percentage of CL-caspase-3-positive cells (right graph). (*C*) Apoptosis analysis of haVSMCs untreated, treated with UV light (60 J/m^2) only and irradiated with UV light and incubated with CX_3CL1 (30 ng/mL) 48 h. Flow cytometry was performed as in *B*. Results are the average of at least three independent experiments each in triplicate. Statistical analysis was performed by one-way ANOVA with Fisher's *post hoc* test.

(*Figure 4C*) significantly increased apoptosis of VSMCs. No changes were observed on apoptosis after AKT inhibition alone. Apoptosis was not affected by ERK inhibition either in the absence or presence of CX_3CL1 (*Figure 4B*). VSMC incubation with CX_3CL1 after UV light treatment augmented apoptosis in a dose-dependent fashion, starting at 1 ng/mL CX_3CL1 dose and reaching a maximum response at 30 ng/mL dose, but after inhibition of AKT, apoptosis was only enhanced at the highest dose (30 ng/mL) of CX_3CL1 (see Supplementary material online, *Figure S3A* and *B*). Apoptosis induced by CX_3CL1 addition of 30 ng/mL dose in combination with UV light was optimum at 48 h after the stimulus was applied (see Supplementary material online, *Figure S3C*), while the treatment of haVSMCs with different doses of soluble CX_3CL1 alone (see Supplementary material online, *Figure S3D*) did not affect apoptosis. Collectively, these findings indicate that CX_3CL1 might enhance the susceptibility of VSMC to succumb to apoptosis.

3.3 IR reduces VSMC survival *in vivo* in a mouse model of IR and MetS *apoE−/−Irs2+/−* mice

Next, VSMC proliferation and apoptosis *in vivo* were studied in atheroma lesions of an IR–MetS mouse model which develops increased atherosclerosis, *apoE−/−Irs2+/−* mice[22] and were compared with *apoE−/−* controls both fed with an atherogenic diet for 8 weeks. Analysis of proliferative rate, in aortic root cross-sections, demonstrated an overall reduced number of Ki67-immunoreactive cells as well as reduced double Ki67+/SM-α-actin+ proliferating VSMCs in *apoE−/−Irs2+/−* mouse lesions compared with *apoE−/−* controls (*Figure 5A*, $P < 0.05$). *In vivo* apoptosis analysis showed augmented of both total CL-caspase-3 immunostaining and double CL-caspase 3+/SM-α-actin immunostained VSMCs in *apoE−/−Irs2+/−* mice compared with *apoE−/−* mice (*Figure 5B*, $P < 0.02$ and <0.04, respectively). Interestingly, analysis of the CL-caspase-3-positive area showed that medial VSMC invasion and disruption of the elastic lamins, an event associated with vulnerable plaques, was also increased in *apoE−/−Irs2+/−* mice (*Figure 5B*—right panel, $P < 0.03$). Therefore, VSMCs in the lesions of *apoE−/−Irs2+/−* mice exhibit reduced survival compared with *apoE−/−* controls. Consistent with these results, mouse aortic VSMCs derived from *apoE−/−Irs2+/−* mice also exhibited impaired survival *in vitro* compared with *apoE−/−* VSMCs (see Supplementary material online, *Figure S5*). Thus, disruption of IRS2 signalling *in vivo* reduces survival of VSMCs within the atheroma plaques of IR–MetS mice.

3.4 IR increases plaque instability *in vivo* in *apoE−/−Irs2+/−* mice and is mechanistically linked to increased expression of the inflammatory CX3CL1/CX_3CR1 pathway

To evaluate the effect of the reduced VSMC survival on plaque vulnerability in IR–MetS, characteristics of plaque vulnerability in mouse lesions were investigated. Analysis of atheromas demonstrated significantly augmented necrotic core area, a reduced fibrous cap thickness, and an increase in elastic lamin breaks in *apoE−/−Irs2+/−* mice compared with *apoE−/−* controls (*Figure 6A–C*, $P < 0.05$, <0.004, and <0.04, respectively). No differences were found in collagen or Matrix metallopeptidase-9 (MMP-9) content (see Supplementary material online, *Figure S2A* and *D*). A tendency towards a lower VSMC content and an increased percentage of macrophages was observed, but the differences were not significant (see Supplementary material online *Figure 5B* and *C*).

Given the results described above, and taking into account that progressive inflammation is a key component in making plaques more unstable plaque, we also explored the expression of CX_3CL1/CX_3CR1 axis . Expression analysis demonstrated that the enhanced plaque vulnerability in *apoE−/−Irs2+/−* mice was accompanied by augmented expression of CX_3CL1 ($P < 0.002$) and CX_3CR1 ($P < 0.004$) mRNA levels in the aortic tissue of *apoE−/−Irs2+/−* mice compared with controls (*Figure 6D*). Double immunofluorescence of CX_3CL1/SM-α-actin or CX_3CR1/SM-α-actin to identify VSMCs expressing the inflammatory axis was also performed. Analysis of the immunofluorescence showed an overall enhancement in CX_3CL1 expression in the lesion and in SM-α-actin-positive fibrous cap areas of *apoE−/−Irs2+/−* mice compared with *apoE−/−* mice (*Figure 6E*). The number of double-positive CX_3CR1/SM-α-actin cells was higher in the lesions and in the fibrous caps of *apoE−/−Irs2+/−* mice compared with *apoE−/−* controls (*Figure 6F*). As previously reported, CX_3CL1 expression was abundant in the necrotic core areas and positive staining was also observed in VSMCs in the media.[26] CX_3CR1 was expressed in VSMCs and also in other cell types. These results provide *in vivo* experimental data linking impaired IR-VSMC survival with increased plaque vulnerability and up-regulation of the CX_3CL1/CX_3CR1 inflammatory pathway in atheromatous plaques from IR *apoE−/−Irs2+/−* mice.

3.5 Expression of CX_3CL1/CX_3CR1 axis is enhanced in IR and MetS patients and positively correlates with atherosclerosis development

We have previously shown that IR–MetS patients exhibit reduced IRS2–AKT–ERK-dependent signalling.[22] Given that IR induced by IRS2 silencing, provoked features of plaque vulnerability in haVSMCs, we investigated the relationship between MetS, IR, and atherosclerosis progression in human subjects. The demographic and clinical characteristics of patients are summarized in Supplementary material online, *Table S1*. No significant differences in gender and age distribution were found. Compared with non-IR control individuals, IR–MetS subjects had higher body mass index, waist circumference, diastolic and systolic blood pressure, and HOMA index score, as well as increased plasma levels of fasting glucose, insulin, total cholesterol, apolipoprotein B, triglycerides, and high-sensitivity C-reactive protein, and lower plasmatic levels of high-density lipoprotein cholesterol. IR–MetS subjects had a higher frequency of T2DM and took hypoglycaemic oral medication more frequently than non-IR patients.

As expected, examination of human carotid artery by ultrasound revealed an increased common carotid artery intima-media thickness (CC-IMT) and a higher percentage of atheroma plaques in IR–MetS patients compared with non-IR subjects (*Figure 7A*: 0.685 vs. 0.574, $P < 0.00001$ and 32 vs. 16%, $P < 0.04$, respectively). Moreover, consistent with previous studies linking IR to atherosclerosis,[27,28] CC-IMT positively correlated with insulin levels ($P < 0.002$) and HOMA index scores ($P < 0.0001$) in our cohort of patients (*Figure 7B*).

Since IRS2 silencing in haVSMCs was mechanistically linked to increased CX_3CL1/CX_3CR1 expression, we wondered whether IR–MetS subjects might exhibit changes in these inflammatory mediators. Expression studies showed higher CX_3CR1 mRNA levels in white blood mononuclear cells (WBMCs) from IR–MetS patients compared with non-IR controls (*Figure 7C*, $P < 0.0006$). Analysis in circulating lymphocytes and monocytes demonstrated enhanced cell surface CX_3CR1 protein expression in CD14+ monocytes (*Figure 7D*, $P < 0.02$) and a non-significant moderate increase in CD3+ lymphocytes from IR–MetS subjects compared with controls. Moreover, CX_3CR1 mRNA levels in WBMCs positively correlated with plasmatic insulin levels, the HOMA index, as well as with CC-IMT (*Figure 7E*, $P < 0.0001$, <0.0001, and <0.02) in all patients, indicating that CX_3CR1 levels might predict the severity of IR and the likelihood of developing atherosclerosis. Plasma analysis of CX_3CL1 levels also demonstrated an increase in IR–MetS patients compared with control subjects (*Figure 7F*, $P < 0.04$).

These results indicate that IR–MetS subjects with more severe atherosclerosis exhibit up-regulation of the inflammatory CX_3CL1/CX_3CR1 axis, which is consistent with previous studies describing enhanced cell surface expression of CX_3CR1 in monocytes from coronary artery diseased (CAD) patients.[19] Moreover, these data are also consistent with our IR mouse model results described above.

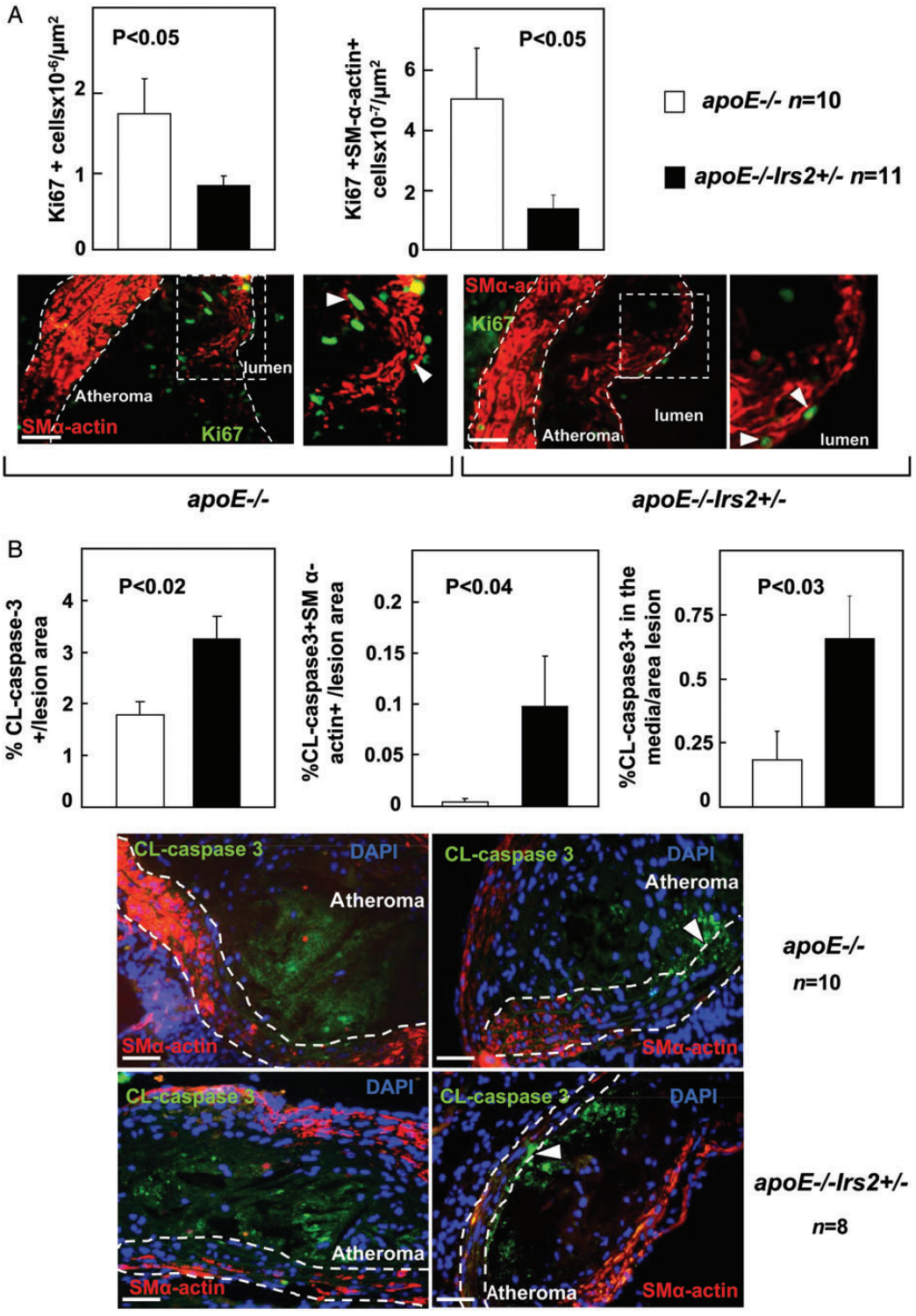

Figure 5 VSMC survival is reduced in IR–MetS *apoE*−/−*Irs2*+/− mice. Proliferation rate (*A*) and apoptosis (*B*) in atheromatous lesions of aortic root cross-sections of *apoE*−/− and *apoE*−/−*Irs2*+/− mice. (*A*) Total proliferating cells and proliferating VSMCs were detected by Ki67+ and double Ki67+/SM-α-actin+ staining, respectively. (*B*) Apoptosis analysis (as percentage) was measured in the whole lesion as the CL-caspase-3+ area (left panel), in VSMCs by double CL-caspase-3+/SM-α-actin+ staining (middle panel), and in medial VSMCs as the CL-caspase3+ area within the media (right panel). Scale bar: 50 µm. The white arrows point to double Ki67+/SM-α-actin+ staining and to CL-caspase-3+ staining in the media. Statistical analysis was performed by the Student's *t*-test.

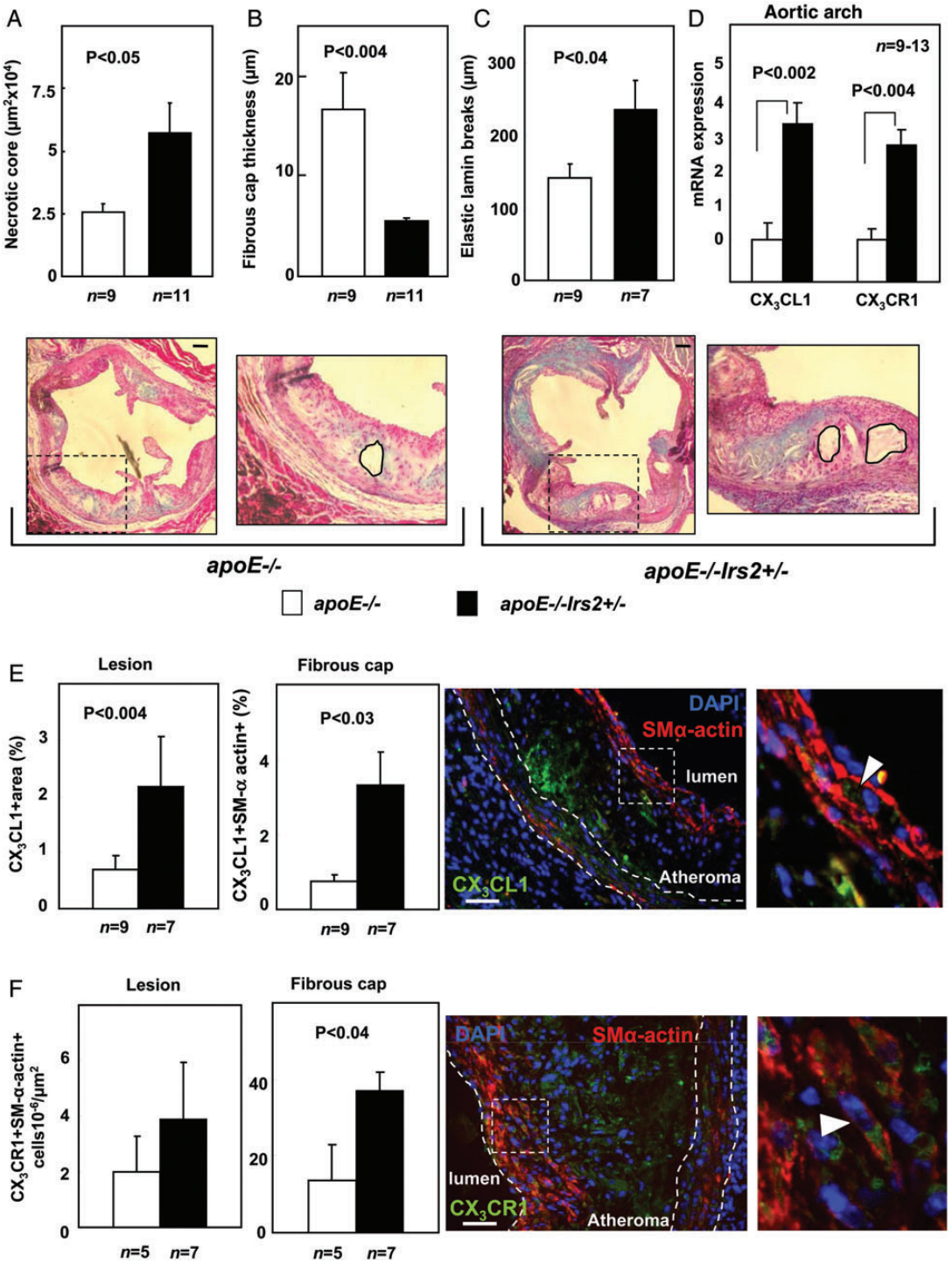

Figure 6 IR induces features of plaque vulnerability and increases the CX_3CL1/CX_3CR1 inflammatory axis in atheromas of *apoE*−/−*Irs2*+/− mice fed an atherogenic diet. Quantification of necrotic core area (*A*) and fibrous cap thickness (*B*) in Masson's trichrome-stained mouse lesions. (*C*) Length of medial elastic lamin breaks. Solid lines delineate necrotic core limits. Scale bar: 100 μm. (*D*) CX_3CR1 and CX_3CL1 mRNA levels in aortic tissue from *apoE*−/− and *apoE*−/−*Irs2*+/− mice. mRNA levels were normalized by the endogenous *cyclophilin* mRNA levels and relativized to *apoE*−/− mouse mRNA levels. (*E*) Quantitative analysis of CX_3CL1-positive staining within the lesion (left panel) and in fibrous cap VSMCs (right panel) determined by double immunofluorescence CX_3CL1+/SM-α-actin+ to identify VSMCs expressing CX_3CL1. (*F*) Quantitative analysis of CX_3CR1-positive staining in lesional VSMCs (left panel) and in fibrous cap VSMCs (right panel) determined by double immunofluorescence CX_3CR1+/SM-α-actin+ to identify VSMCs expressing CX_3CR1. Representative images from the immunofluorescence analysis are shown. The white arrows point to double-positive CX_3CL1/SM-α-actin and CX_3CR1/SM-α-actin. Statistical analysis was performed using the Student's *t*-test. Scale bar in *F* and *E*: 50 μm.

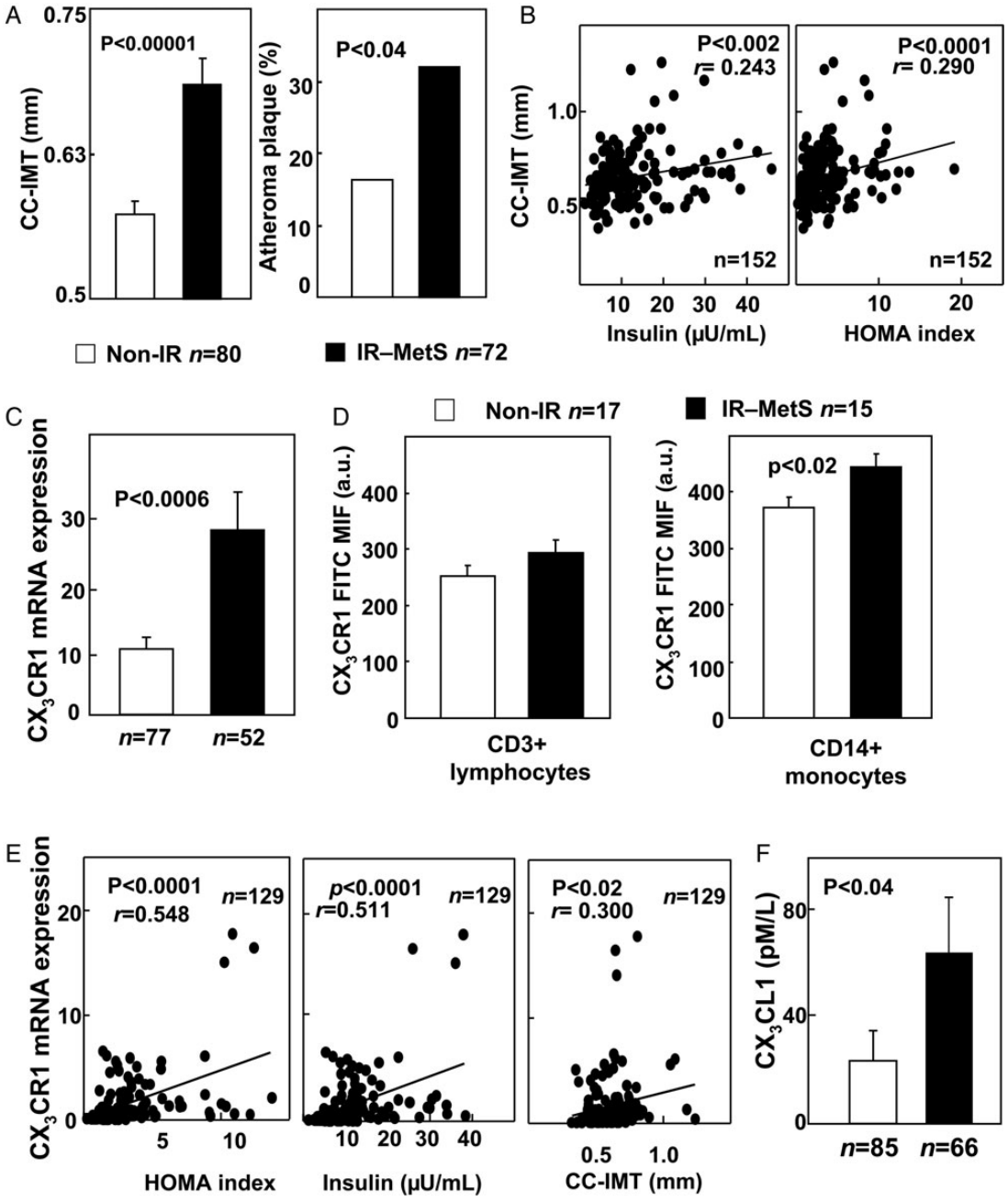

Figure 7 Atherosclerosis burden analysis and CX_3CL1/CX_3CR1 inflammatory axis expression in IR–MetS and non-IR control subjects. (*A*) The CC-IMT (left panel) and the presence of atherosclerotic plaques depicted as the frequency of plaques (percentage) (right panel) analysed by ultrasound in IR–MetS and non-IR control subjects. (*B*) Correlation studies between CC-IMT and insulin and HOMA index in all patients. (*C*) CX_3CR1 mRNA expression levels in WBMCs in IR–MetS patients compared with non-IR controls. mRNA levels were normalised with *gapdh* mRNA levels and relativised to non-IR mRNA levels. (*D*) CX_3CR1 expression in circulating lymphocytes and monocytes identified with anti-human CD3 and anti-human CD14 antibodies, respectively, in IR–MetS and non-IR subjects. (*E*) Correlation studies of CX_3CR1 mRNA levels in WBMCs with the HOMA index, insulin, and with the CC-IMT in all patients. (*F*) Plasma circulating soluble CX_3CL1 levels in both groups of patients. Data are shown as mean ± SEM (*A* left panel, *C*, *D*, and *F*), as a percentage (*A* right panel), and as individual data points for correlation studies (*B* and *E*). Statistical analysis was done using the Student's *t*-test (*A* left panel, *C*, *D*, and *F*), χ^2 test (*A* right panel), and the Spearman correlation coefficient (*B* and *E*).

4. Discussion

T2DM/MetS morbidity and mortality rates are highly attributed to cardiovascular acute events precipitated by the rupture of vulnerable plaques.[2,14,29] In the present study, we demonstrated that IR produced by impaired IRS2 signalling induces a cell phenotype in haVSMCs, which is characteristic of unstable plaques consisting of reduced proliferation and migration and increased VSMC apoptosis. In addition, IRS2–AKT–ERK-impaired signalling in haVSMCs was accompanied by augmented expression of CX_3CL1, and its receptor, CX_3CR1, a chemokine system previously linked to increased vulnerable atheromatous plaques in mice and to enhanced CAD in humans.[19,26,30–32]

Interestingly, CX_3CL1 also induced apoptosis in haVSMCs when combined with pro-apoptotic stimuli or when the AKT pathway was blocked, thus suggesting a connection between the inflammatory pathway and increased susceptibility to apoptosis. We also obtained relevant experimental evidence *in vivo* linking plaque vulnerability, dysfunctional VSMCs, and increased CX_3CL1/CX_3CR1 axis expression in a mouse model of IR and atherosclerosis, *apoE−/−Irs2+/−* mice. Atheromatous plaques in IR–MetS *apoE−/−Irs2+/−* mice exhibited *in vivo* reduced VSMC proliferation, augmented apoptosis, unstable plaque features, and increased CX_3CL1/CX_3CR1 expression in atheroma VSMCs compared with control *apoE−/−* mouse lesions. IR–MetS patients with significantly enhanced atherosclerosis also exhibited higher CX_3CR1 mRNA levels in WBMCs, CX_3CR1 monocyte cell surface expression, and CX_3CL1 circulating plasma levels, suggesting that a global systemic activation of this inflammatory pathway is associated with IR and atherosclerosis. Taking all of these data together, we conclude that IR produced by impaired IRS2-mediated signalling reduces VSMC survival and activates the CX_3CL1/CX_3CR1 axis *in vitro* and *in vivo* in a mouse model of IR and atherosclerosis which also exhibits plaque vulnerability features.

IRS2-dependent signalling is impaired in a variety of cells and tissues exhibiting IR.[16,22,33–36] Notably, impaired IRS2–AKT–ERK-mediated signalling in IR–MetS patients is associated with an increased CVD risk.[22] In agreement with previous studies,[27,28,37] we also observed correlation between IR and CC-IMT and a higher frequency of atheromatous plaques in IR–MetS patients. Progression of atherosclerosis is characterized by the generation of unstable plaques due, among other reasons, to reduced cell survival within theses plaques.[8,11,12] Thus, the reduced haVSMC survival induced by IR observed in our *in vitro* experiments could be a potential mechanism, contributing to the progression of plaques to instability in IR. In support of this hypothesis, *in vivo* analysis of atheromatous plaques from *apoE−/−Irs2+/−* mice exhibited both increased VSMC death and enhanced vulnerable plaques, indicating that IR-mediated VSMC death contributes to *in vivo* plaque instability. These results are compatible with a previously reported mechanism which describes increased apoptosis in IR macrophages whose ability to clear apoptotic debris is impaired and thus accelerates plaque progression.[14,36] In further support of a pro-apoptotic role of IR, activation of insulin receptor signalling,[17] overexpression of the IGF1 signalling[38] and activation of the downstream effector of the insulin receptor, Akt,[39] protected VSMCs from apoptosis. All these data demonstrate a global pro-apoptotic role of IR in macrophages and VSMCs present in advanced atheromatous plaques.

Diminished VSMC proliferation would rather appear as a protective mechanism by avoiding progression of atherosclerosis from early fatty streak lesions to mid-stage VSMC-rich fibrous cap atheromas. However, proliferating VSMCs overlying the inflammatory core might attenuate the risk of plaque rupture in advanced plaques. Thus, the reduced proliferative rate observed *in vitro* in IR-haVSMC and *in vivo* in vulnerable atheromatous lesions found in *apoE−/−Irs2+/−* mice indicate that IR might limit VSMC fibrous cap formation. Previous studies have reported diminished VSMC proliferation and migration after AKT1 signalling disruption[40] or after pharmacological inhibition of the Mitogen-activated protein kinase (MAPK)/ERK1/2 pathway,[41] consistent with this, we showed that IRS2-impaired signalling decreased the activation of AKT1/2 and ERK1/2 survival pathways and VSMC proliferation. Our results demonstrate that besides the previously known role of IRS2 in the survival, replication, and maintenance of pancreatic β-cells,[23,42] IRS2/AKT/ERK-mediated signalling might also play an important function in the maintenance of VSMCs in atheromatous plaques. Collectively, these findings suggest that IR induced by a defective IRS2–AKT–ERK-mediated signalling could promote plaque instability by reducing VSMC survival in atheroma plaques.

One of the main features of vulnerable plaques is a massive accumulation of inflammatory cells and mediators beneath the fibrous cap.[8] In humans, CX_3CR1 gene polymorphisms that result in decreased CX_3CR1 expression have been linked to a reduced risk of CAD,[43] while CX_3CR1 up-regulation has been associated with increased CAD.[19] Consistent with this, deletion of either CX_3CL1[31] or CX_3CR1[26] in mice decreases plaque size and vulnerability,[26,31] while advanced vulnerable plaques exhibit augmented CX_3CL1 levels.[20,32] In our study, expression of the CX_3CL1/CX_3CR1 pathway was enhanced in: (i) *in vitro* IR-VSMCs with reduced survival and (ii) *in vivo* in lesion VSMCs and in the atheromas of IR–MetS *apoE−/−Irs2+/−* mice, which also exhibited increased VSMC death and plaque instability. These results suggest that the activation of this inflammatory axis in VSMCs could be at least one of the mechanisms contributing to progression of atherosclerosis in IR states and are consistent with previous reports showing abundant CX_3CL1 and CX_3CR1 levels in highly unstable murine and human atheromatous plaques.[19,20,32,44] In agreement with a role of this inflammatory axis in the progression of atherosclerosis, IR–MetS subjects with increased atherosclerosis exhibited augmented soluble CX_3CL1 plasma levels, and there was a positive correlation between CC-IMT and CX_3CR1 levels in WBMCs indicating the clinical relevance of our findings. Concurring with previous studies showing up-regulation of CX_3CR1 in monocytes from CAD patients,[19] we also found increased cell surface expression of CX_3CR1 in monocytes from IR–MetS patients. On the basis of these findings, we propose a connection between atherosclerosis and the global up-regulation of this inflammatory axis in IR. In addition, soluble CX_3CL1 is able to induce apoptosis in haVSMCs *in vitro*, in the presence of other pro-apoptotic stimuli, suggesting that VSMC apoptosis mediated by the chemokine might be one of the mechanisms playing a role in atheromatous plaque vulnerability. However, to specifically address the contribution of the CX_3CL1/CX_3CR1 axis in VSMCs in plaque vulnerability, additional studies involving modulation of the axis in VSMC-specific deficient mice must be performed.

Taken together, our observations suggest a global activation of the CX_3CL1/CX_3CR1 axis in IR–MetS states, which might be mechanistically linked to defective IRS2–AKT–ERK-mediated signalling and reduced VSMC survival. This scenario would be aggravated by: (i) a defective dead cell removal by impaired IR macrophages[14,16] and (ii) a diminished IR-VSMC proliferative capacity unable to perform its essential reparative proliferative process in order to form the thick fibrous cap and thus avoid plaque rupture. These processes would therefore promote necrotic core formation, plaque instability, and increased risk of acute CVD events.

In summary, we conclude that IR reduces VSMC survival and promotes atheroma plaque vulnerability which is partly due to augmented CX_3CL1/CX_3CR1 inflammatory pathway expression within the plaque. Notably, *in vivo* enhanced soluble CX_3CL1 levels in IR–MetS patients and the correlation between CX_3CR1 expression and carotid atherosclerosis suggest that this inflammatory pathway might be useful in future diagnostic tools destined to predict atheroma progression in IR–MetS states. Therefore, we suggest that IR–MetS patients might benefit from strategies based on the stimulation of VSMC survival and reduction in CX_3CL1/CX_3CR1 inflammatory axis to promote plaque stability by modulating IRS2-dependent signalling.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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