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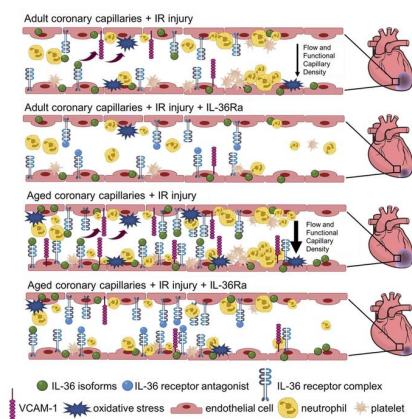
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Targeting IL-36 improves age-related coronary microcirculatory dysfunction and attenuates myocardial ischaemia-reperfusion injury in mice

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Abstract

Following myocardial infarction (MI), elderly patients have a poorer prognosis which may be linked to increased coronary microvessel susceptibility to injury. Interleukin-36 (IL-36), a newly discovered pro-inflammatory member of the IL-1 superfamily, may mediate this injury but its role in the injured heart is currently not known. We firstly demonstrated the presence of IL-36(α/β) and its receptor (IL-36R) in ischaemia-reperfusion (IR) injured mouse hearts and, interestingly, noted that expression of both increased with ageing. An intravital model for imaging the adult and aged IR injured beating heart in real-time in vivo was used to demonstrate heightened basal and injury-induced neutrophil recruitment, and poorer blood flow, in the aged coronary microcirculation when compared to adult hearts. An IL-36R antagonist (IL-36Ra) decreased neutrophil recruitment, improved blood flow and reduced infarct size in both adult and aged mice. This may be mechanistically explained by attenuated endothelial oxidative damage and VCAM-1 expression in IL-36Ra treated mice. Our findings of an enhanced age-related coronary microcirculatory dysfunction in reperfused hearts may explain the poorer outcomes in elderly patients following MI. Since targeting the IL-36/IL-36R pathway was vasculoprotective in aged hearts, it may potentially be a therapy for treating MI in the elderly.

Introduction

Treatment of ST-elevation myocardial infarction (MI) focuses on rapidly re-establishing perfusion following blockage in one or more of the epicardial coronary arteries. This can be achieved by primary percutaneous coronary interventions (PCI) using a coronary stent to open the culprit artery. Despite these interventions, a substantial proportion of patients still incur extensive muscle damage and develop heart failure post-MI (1). This is partly due to reperfusion paradoxically leading to additional tissue damage, a condition termed ischaemia-reperfusion (IR) injury. Indeed, restoration of normal epicardial blood vessel flow but with sub-optimal myocardial perfusion, can be observed in up to 50% of patients following PCI, leading to worse outcomes than in patients with full perfusion recovery (2). This suggests tissue damage likely occurs subsequent to inadequate coronary microcirculatory perfusion (3-4). Microvascular involvement is further highlighted by observations that patients may present with MI but with a normal coronary angiogram (4). Increased clinical recognition of the importance of the coronary microcirculation has meant identifying strategies to improve potential perturbations within it have gained recent attention (3-4). However, current clinical tools cannot resolve microvessels $<200\mu\text{m}$ and so little is known about the full range of cardiac microcirculatory responses to IR injury in vivo.

Age is a major risk factor for ischaemic cardiovascular disease, increasing cardiac damage caused by IR injury, independent of 'traditional' risk factors (5-7). Clinically, there is an increased incidence of heart failure, atrial fibrillation and tachycardia in patients post-MI which increases with age (7). In experimental studies, coronary blood flow is much lower post-reperfusion in senescent rats (8). Interestingly, the ability of the heart to respond to cardioprotective interventions is also lost with ageing (8-9). The term 'inflammaging'

describes the phenomenon of ageing accompanied by a chronic low-grade, sterile inflammation, that persists in the absence of an overt inflammatory stimulus (10). It is characterised by raised levels of pro-inflammatory cytokines, that contribute to gradual tissue damage as well as altered responses to acute inflammatory injuries. The drivers underlying this persistent inflammation appear to involve increased reactive oxygen species (ROS) production in the vasculature, decreased anti-oxidative ability and changes in the number, structure and function of immune cells (11). In particular, increased neutrophil presence is detected in otherwise healthy but aged tissues. Neutrophil chemotaxis is also impaired, with serious implications as adherent cells cannot exit or transmigrate out of blood vessels (12). Here, they continue to release tissue destructive proteases and ROS and cause vascular congestion as observed in aged, injured mouse lungs (13). Therefore, inflammaging may contribute to the enhanced age-related cardiovascular risk and poorer outcomes through actions on the local microcirculation. However, little is known in vivo about how age impacts the coronary microcirculation in health and whether it increases the likelihood of microvascular disturbances post-reperfusion injury.

The interleukin-1 family (IL-1F) consists of 11 known pro- and/or anti-inflammatory cytokines, some of which have been studied extensively whilst others have received less attention (14). These are frequently the first and most upstream cytokines produced in response to injury and so are considered good targets for intervention (15). Since IL-1F members critically mediate sterile inflammation, they may be key mechanistic contributors causing myocardial microcirculatory disturbances post-reperfusion (16). Indeed, the large scale canakinumab anti-inflammatory thrombosis outcomes study (CANTOS) trial provided exciting evidence that targeting this cytokine was beneficial in improving long-term outcomes post-MI in the

absence of lipid lowering (17). In the last decade, genes encoding a novel cytokine cluster, namely interleukin-36 (IL-36), with structural and functional similarities to IL-1, were discovered (18-19). IL-36, a collective name for three agonist ligands, IL-36 α , IL-36 β and IL-36 γ (previously called IL-1F6, IL-1F8, and IL-1F9), is fast emerging as a novel player regulating both innate and adaptive immune responses in a number of acute and chronic disorders. As well as amplifying IL-1 effects, IL-36 is also a potent mediator of inflammation in its own right. Indeed, its critical role in psoriasis, equalling if not surpassing that of IL-1, is well established with emerging roles in Crohn's disease, airway infections and rheumatoid arthritis recently identified (20-22).

IL-36 cytokines can be released from many sources including epithelial cells, keratinocytes, fibroblasts, macrophages, monocytes, lymphocytes and neurons, with neutrophils identified as an IL-36 source more recently (18, 24-25). They signal through the IL-36 receptor (IL-36R), a heterodimer formed of IL-1Rrp2 and an accessory co-receptor protein IL-1RacP (20). This receptor is also widely expressed at low levels in many organs and cell types including leukocytes and vascular endothelial cells. Downstream intracellular signaling leads to NF- κ B and MAP kinase activation and subsequent secretion of multiple potent pro-inflammatory mediators, including TNF α , IL-1 β , IL-6 and IL-8. The actions of the three agonists can be inhibited by a naturally occurring antagonist, namely the IL-36 receptor antagonist (IL-36Ra) (22-25). Although we know the IL-36/IL36R pathway is highly pro-inflammatory in the skin and lungs, we are still at a very early stage in our current understanding of its *in vivo* biology and no previous studies have investigated a role for this pathway in mediating inflammation in the IR injured heart. Its downstream transcription factor NF- κ B is a known mediator of coronary microvascular injury as inhibition of NF- κ B in two independent rabbit models of

myocardial IR injury reduced inflammation and the no-reflow area (26-27). Therefore, the IL-36 signalling pathway is a potential target for cardioprotective interventions.

We have previously described an intravital imaging technique that allows the microcirculation of the anaesthetised mouse beating heart to be imaged with cellular resolution in vivo (28). Using this technique we have shown that despite deceptive hyperaemic responses, increased microcirculatory flow heterogeneity, reduction in functional capillary density and a marked thromboinflammatory infiltration was present within coronary capillaries immediately post-reperfusion. The current study used this imaging model to determine whether the clinically observed age-related poorer outcomes post-MI was related to an increased susceptibility of the aged coronary microcirculation to myocardial IR injury. The presence of IL-36 and its receptor was investigated in the heart and the impact of age on their expression determined. To ascertain whether IL-36 could mediate coronary microcirculatory perturbations, the ability of IL-36 agonists to directly induce inflammation in the beating heart was imaged and the effects of treatment with the IL-36Ra determined. Mechanistic insights in to how IL36Ra may confer vasculoprotection via attenuating ROS mediated endothelial oxidative damage and vascular adhesion molecule expression is also presented.

Results

Age increased expression of IL-36R in healthy and IR injured hearts, which was also induced in endothelial cells stimulated with TNF α or IL-36 cytokines

Immunofluorescence staining of frozen heart sections demonstrated very low constitutive expression of IL-36R in adult sham hearts. This increased ($p=0.0146$) in response to IR injury in adult hearts. Basal expression of IL-36R was higher ($p<0.0001$) in aged sham hearts when compared to adult shams. This was further increased ($p=0.0003$) in aged injured hearts when compared to adult injured hearts (**Figures 1A and C**). Western blot analysis showed similar step-wise increases in the presence of the IL-36R protein. The molecular weight of IL-36R is approximately 65kDa, but it migrates to the position of approximately 85kDa in denaturing protein gels (29). Hence, two bands were observed corresponding to a 65kDa active protein due to cleavage of its signalling peptide, and an 85kDa less potent glycosylated form (**Figure 1B**). Again, adult sham hearts expressed very low levels of IL-36R. This increased in adult injured hearts which expressed the highest levels of the 85kDa protein. However, significant increases ($p<0.0001$) in IL-36R protein were expressed in aged sham hearts when compared to adult sham hearts, particularly of the more potent 65kDa protein. This expression increased ($p=0.001$) further in aged injured hearts which exhibited the highest levels of the active 65kDa IL-36R protein (**Figures 1B and D**). To further confirm at a more cellular level, the expression of IL-36R on coronary endothelial cells, hearts were collagenase digested and flow cytometrically analysed. Very low basal levels of IL-36R was noted on adult sham coronary endothelial cells, but this was significantly ($p<0.05$) increased on endothelial cells from adult IR injured hearts. Aged sham coronary endothelial cells again showed higher IL-36R than on adult sham endothelial cells, which was also significantly ($p<0.05$) increased with

injury (**Figure 1E**). Interestingly, a similar pattern of IL-36R expression was noted flow cytometrically on cardiomyocytes (**Figure 1F**). To further identify whether IL-36R was expressed specifically on endothelial cells, murine VCECs were stained for IL-36R. Constitutive expression of IL-36R on unstimulated VCECs was very low. However, stimulation of VCECs with TNF α , as well as IL-36 isoforms, significantly ($p < 0.0001$) up-regulated IL-36R expression with no differences observed between cytokine treatments (**Figures 1G-H**).

Age increased expression of IL-36 α , IL-36 β and VCAM-1 in healthy and IR injured heart

Immunofluorescence staining for IL-36 α and IL-36 β revealed a similar pattern of expression in the heart to the IL-36 receptor (**Figures 2A-C**). Expression of the endothelial surface adhesion molecule, VCAM-1, was also investigated to better understand the impact of age on a well-established inflammatory marker. VCAM-1 was expressed on the larger vasculature of the heart rather than on coronary capillaries in all 4 groups and significantly increased in a stepwise manner with injury and age (**Figures 2D-E**).

Inducible expression of IL-36R and IL-36 cytokines occurred on coronary microvasculature

Having demonstrated an increased vascular expression of IL-36R and IL-36 cytokines with IR injury and age, we further examined which component of the vascular network this increase occurred on by comparing the mean fluorescence intensity (MFI) between microvessels (capillaries) and macrovessels. Expression of IL-36R, IL-36 α , and IL-36 β significantly increased

with age and IR injury specifically on the microvasculature (**Figure 3A**). In contrast, expression on the macrovasculature remained relatively constant in all four groups (**Figure 3B**).

To further confirm that IL-36R expression was indeed present on blood vessels, heart sections were co-stained with an anti-CD31 antibody. IL-36R expression was intense within the wall of some of the larger blood vessels, particularly in the tunica adventitial layer, as well as on the coronary capillaries (**Figures 3C-D**).

Topical application of IL-36 was pro-inflammatory in adult and aged beating hearts in vivo

To determine whether the IL-36R expressed in the heart was functional in vivo, intravital microscopy was used to directly visualise the ability of topical IL-36 to elicit an inflammatory response within the healthy adult and aged beating hearts. Intravital imaging of the beating heart was successfully achieved in anaesthetised mice by attaching a 3D printed stabiliser to the left ventricle (**Figures 4A-D**). This reduced motion in the x-y plane of a small region of the ventricle sufficiently enough to permit imaging. All three IL-36 agonists were able to increase neutrophil recruitment to a similar degree in healthy adult hearts, although IL-36 γ was slightly more potent (**Supplementary Video 1**). This pro-inflammatory response was rapid and increased with time until a plateau was reached at approximately 60 minutes. In addition to neutrophil adhesion within coronary capillaries, a remarkable involvement of more medium sized blood vessels was also observed. Indeed, these vessels were not usually visible but were clearly identified when delineated with adherent neutrophils. Platelet aggregates were occasionally observed in some of these medium sized vessels, particularly in response to IL-36 β and IL-36 γ . These were mostly present in vessels where extensive clusters of adherent

neutrophils were observed (**Figures 5A-B and D-E**). A similar statistically significant pro-inflammatory response was also observed when cytokines were topically applied to the healthy aged hearts, although levels were slightly lower and the response slower than that observed in adult mice. However, unlike in adult hearts where the response plateaued, in aged hearts exposed to IL-36 α and IL-36 β , neutrophil recruitment continued to rise. Platelet aggregates were again occasionally observed but no significant differences were observed other than a decrease in their presence with IL-36 β/γ (**Figures 5A and C-E**). The number of free-flowing neutrophils generally decreased in response to all three IL-36 cytokine treatments in both adult and aged hearts (data not presented).

To ascertain whether thromboinflammatory events imaged intravitaly on the surface of the heart were also occurring in the deeper layers of the myocardium, multiphoton microscopy was used. A vibratome was used to precisely section the left ventricle wall into four 300 μ m thickness sections from the outermost layer closest to the epicardium through to the inner layer closest to the endocardium. Multiphoton z-stacks were taken from all four layers (**Figures 4E-F**). The data obtained confirmed the ability of IL-36 cytokines to mediate a pro-inflammatory response in topically treated adult and aged hearts. However, this was only noted in the outermost layer of the heart muscle exposed to the topically applied cytokine and did not extend into the depths of the muscle wall, likely explained by the inability of the cytokine to permeate during the exposure period beyond the superficial myocardial layers. Multiphoton studies also detected a high basal presence of adherent neutrophils in aged mice throughout the muscle wall (**Figure 5F**).

Age increased thromboinflammatory disturbances within healthy and IR injured coronary microcirculation in vivo, which was prevented in both age groups by IL-36R inhibition.

Intravital imaging demonstrated an increase ($p < 0.0001$) in adherent neutrophils, primarily within coronary capillaries, in response to IR injury in adult mice when compared to sham adult hearts (**Supplementary Video 2**). Basal neutrophil adhesion was also increased ($p < 0.0001$) in aged sham hearts when compared with adult shams. Neutrophil recruitment was greatest in aged IR injured hearts, which was higher ($p < 0.0001$) than adult IR injured hearts. This increase was noted at all time points post-reperfusion and continued to rise. Individual neutrophils were often difficult to demarcate and appeared as clusters in aged injured hearts. Their presence was also noted in medium sized coronary microvessels as well as in capillaries, something not observed in adult injured hearts (**Figures 6A-D**). The number of free-flowing neutrophils decreased ($p < 0.0001$ – data not presented) in all groups when compared to adult sham hearts with a concomitant and significant increase in the presence of small aggregates of platelets within coronary capillaries (**Figures 6A and E**).

Intravital imaging further demonstrated the ability of exogenous IL-36Ra treatment to reduce neutrophil adhesion in both adult ($p < 0.0001$) and aged IR injured hearts ($p < 0.0001$) when compared with their respective non-treated IR injured groups (**Figures 6A-D**). In some aged mice, neutrophil adhesion could still occasionally be observed within the medium sized coronary vessels, but this was much lower compared to non-treated groups (**Figure 6A**). There was no statistically significant impact of IL-36Ra treatment on the presence of platelet microthrombi within adult or aged IR injured hearts (**Figure 6E**).

Age increased neutrophil presence within the deeper layers of the healthy and IR injured myocardium

Minimal neutrophil presence was identified in adult sham hearts throughout the depth of the left ventricular wall when imaged ex vivo using multiphoton microscopy. However, in adult injured hearts, an increased ($p=0.035$) presence of neutrophils was observed in all four layers of the heart when compared to adult sham hearts (**Supplementary Video 3**). However, the largest neutrophil presence in response to injury occurred within the outermost 300 μ m layer. Basal neutrophil presence was also uniformly increased ($p<0.0001$) throughout all four layers of the ventricle in aged sham hearts when compared with adult sham hearts (also seen in **Figure 5F**). This was further increased ($p<0.0001$) in aged injured hearts when compared to adult injured hearts, with the greatest presence again noted within the outermost layer of the ventricle wall (**Figures 7A-C**).

IL-36R inhibition improved functional capillary density and reduced infarct size in vivo in adult and aged IR injured hearts

An extensive network of FITC-BSA perfused capillaries was observed in both adult and aged sham mice, paralleling the arrangement of muscle fibres, with cross connections along their length. Focussing up and down on the field of view showed no areas devoid of perfused capillaries. Well perfused medium-sized vessels were also visible in some fields of view. In contrast, IR injury of adult hearts was associated with multiple areas in which FITC-BSA did not perfuse. This resulted in patchy areas that appeared devoid of any microvasculature, indicating reduced functional capillary density. This was further reduced in aged IR injured

hearts. Indeed, in some fields of view, up to half of the imaged area appeared to lack perfusion. Of note, medium sized vessels were still readily visible and well perfused in both adult and aged injured hearts. The use of an IL-36RA was able to improve functional capillary density in both adult and aged hearts although some areas of no perfusion were still visible (**Figure 8A**).

Infarct size appeared slightly larger in aged mice when compared to adult mice, but this was not significant. However, a significant decrease in infarct size was observed in both adult ($p < 0.0001$) and aged IR injured hearts ($p < 0.0001$) receiving IL-36Ra treatment when compared with their respective non-treated IR injured groups. The AAR (and non-ischaemic area) was not significantly different across all four groups. (**Figures 8B-D**).

IL-36R inhibition reduced endothelial and cardiomyocyte oxidative damage and VCAM-1 expression in the IR injured adult and aged heart

To determine whether IL-36Ra treatment conferred vasculoprotection via mechanisms involving attenuation of endothelial oxidative stress and VCAM-1 expression, flow cytometry of collagenase digested adult and aged hearts and immunofluorescence on frozen adult and aged heart sections was performed. IR injury increased ROS mediated oxidative damage on both adult ($p < 0.05$) and aged ($p < 0.01$) coronary endothelial cells and adult ($p < 0.05$) and aged ($p < 0.001$) cardiomyocytes when compared to appropriate age sham heart cells as determined by flow cytometry. The damage was significantly greater in IR injured aged hearts compared to IR injured adult hearts. However, this damage was significantly ($p < 0.05$) reduced on both cell populations in all mice treated with the IL-36Ra regardless of age (**Figures 9A-B**).

Significant oxidative damage in IR injured adult ($p < 0.05$) and aged ($p < 0.001$) hearts was confirmed using immunofluorescence and was noted as punctate staining on both CD31⁺ vascular and non-vascular structures (**Figure 9C**). Moreover, this damage was noticeably greater on aged IR injured hearts. This was again reduced in all mice treated with the IL-36Ra regardless of age (**Figure 9D**). VCAM-1 was also increased in adult ($p < 0.05$) IR injured tissue, particularly around larger coronary blood vessels. In aged mice, basal VCAM-1 expression was high and did not increase further with IR injury. However, expression of this endothelial adhesion molecule decreased in both adult ($p < 0.05$) and aged ($p < 0.05$) hearts in IL-36Ra treated mice (**Figures 9C-E**).

Discussion

By 2030, it is expected that 20% of the population will be over 65 years old and cardiovascular disease is set to account for 40% of the deaths within this age group (30). Increased age is associated with a worse prognosis post-MI and may be explained by an increased microvascular susceptibility to reperfusion injury. Therefore, consideration of the effects of ageing on the post-ischaemic heart, particularly on the smallest blood vessels of the heart, is critical. This study provides original contributions on the architecture of the aged beating heart coronary microcirculation and how its response to myocardial IR injury differs from younger hearts in vivo. Ageing is associated with a chronic low-grade inflammatory cell presence in otherwise healthy hearts as well as a heightened thromboinflammatory response in the immediate aftermath of reperfusion. Importantly, what we believe to be a new mechanistic role is identified for the IL-36/IL-36R pathway. Our data demonstrates that IL-36 and its receptor were present not only in the heart but that their expression increases, particularly on coronary microvessels, with age and injury. Not only is the receptor demonstrated for the first time, as far as we are aware, to be functional in the heart but, importantly, its antagonism is shown to markedly reduce microcirculatory perturbations and consequently infarct size in adult mice. By hyperactivating innate immunity, ageing has been shown to reduce the therapeutic efficacy of both pharmacological and ischaemic pre-conditioning interventions in the heart (31-32). Indeed, pre-conditioning was unable to reduce infarct size even in middle-aged rat hearts (aged 12-13 months), demonstrating that the loss of cardioprotection manifests earlier and not only in senescence (32). Therefore, it was reassuring to observe that inhibiting IL-36R signalling remained vasculoprotective even in the highly inflamed aged injured heart.

The impact of age alone on the coronary microcirculation was not negligible. Indeed, an almost five-fold increase in neutrophil adhesion within otherwise healthy coronary capillaries was demonstrated simply as a result of the mouse age increasing from approximately 3 to 18 months. This may be linked to the observed age-related up-regulation of endothelial VCAM-1 in the heart or structural and functional changes in the neutrophils themselves (33-34). Indeed, neutrophils in aged individuals have been shown to exist in a pre-activated state whereby they constitutively secrete more neutrophil elastase and reactive oxygen species in close proximity to endothelium which can lead to vascular damage and their subsequent adhesion (35). Although age-related capillary loss or rarefaction has also been described, (36) no visible reduction in functional capillary density or increased vascular leakage was noted in non-injured aged hearts. Interestingly, a marked reduction in freely circulating neutrophils was observed, which may be linked to a possible decreased coronary blood flow in the aged heart (37). However, this would require further investigation. Furthermore, platelet aggregates were also visible in aged hearts and were sometimes occlusive as evidenced by the lack of circulating neutrophils passing through affected microvessels. Intravital microscopy therefore directly imaged the presence of a chronic low-grade inflammation in the aged heart but also eluded to a mildly pro-thrombotic state as well. These basal microcirculatory disturbances, when combined with an acute injurious insult, may create a more heightened thromboinflammatory effect in the presence of an ageing co-morbidity.

Indeed, this was the case in aged IR injured hearts where remarkable neutrophil adhesion was observed that surpassed that noted in adult injured hearts. Individual neutrophils were difficult to demarcate with clusters observed occupying the full width of the capillaries. Activated neutrophils are well known to become stiffer which contributes to their retention

specifically within capillaries that have a smaller diameter than their own (38). However, larger coronary blood vessels, which we assume were post-capillary venules (PCVs), were also delineated with adherent neutrophils, something not noted in adult injured hearts. Although neutrophil recruitment plateaued in the adult hearts, this was not the case in injured aged hearts where neutrophils continued to be recruited. Increased platelet presence was also noted but this was not as remarkable as the effect on neutrophils. Consequently, these thromboinflammatory occlusive events resulted in the poorest coronary microcirculatory perfusion being noted in aged injured hearts with multiple areas devoid of flow. We believe this to be the first real-time demonstration of a rapid and devastating impact of reperfusion on the smallest blood vessels of the aged heart *in vivo*. This may explain the reduced myocardial tolerance to IR injury previously demonstrated to occur as early as 12 months (middle-age) in mice (39).

IL-36 is typically one of the most upstream and up-regulated cytokines released upon tissue injury and cellular necrosis and critical in triggering subsequent synthesis and release of a multitude of inflammatory mediators (15). This study shows that IL-36R, IL-36 α and IL-36 β are constitutively expressed on vascular and non-vascular cells, albeit at very low levels, in healthy adult murine hearts. A basal expression of IL-36R is also supported by Towne and colleagues who used qPCR to demonstrate low levels in human hearts (25). Although cytokine receptor changes have not been studied extensively with age, an enhanced age-related production of cytokines such as IL-6 have previously been demonstrated (40-41). In the current study, our data demonstrated that expression of IL-36R, IL-36 α and IL-36 β increased with both age and IR injury. Expression of IL-36 agonists have been shown to increase at both the mRNA and protein level in murine kidney tissue following renal IR injury (42). Additionally,

IL-36 β mRNA expression increased in lung homogenates 24 hours after allergic lung inflammation (43). Our study assessed IL-36 after a much shorter reperfusion injury duration so the full extent of IL-36 upregulation was potentially not observed.

IL-36 and IL-36R expression was observed on the majority of coronary capillaries as evidenced by co-localisation with CD31⁺ endothelial cells. Moreover, it was only on microvessels that an age- and injury-related increase in cytokine and receptor expression was observed. This elevated age-related expression specifically on microvessels increases the likelihood of this signalling pathway exacerbating IR injury through targeting the coronary microcirculation in elderly patients with MI.

Immunofluorescence studies on VCECs confirmed expression of the IL-36R on endothelial cells. This was also recently shown on human umbilical vein and dermal lymphatic endothelial cells where it was functionally important in mediating up-regulation of ICAM-1/VCAM-1 and chemokine production in response to IL-36 stimulation (44). We further demonstrated that all three IL-36 agonists could up-regulate endothelial surface expression of the IL-36 receptor. This data provides, what we believe to be, new insights into the fundamental biology of this cytokine. The ability of some cytokines to increase expression of their receptor is not new. Indeed, Takii and colleagues showed that IL-1 could enhance gene and surface expression of its own receptor in pulmonary fibroblast cells within 2 hours (45). Autoregulation forms a positive feedback loop which drives a strengthened activation of the signalling pathway of a given cytokine. Here we show that IL-36 cytokines may also utilise this autoregulatory phenomenon to enhance their own activity.

Interestingly, intense IL-36/IL-36R staining was noted on the outer tunica adventitial layer of larger blood vessels. Inflammatory responses are generally considered to be initiated in an

'inside-out' manner through capture of circulating leukocytes by the endothelial surface. However, growing evidence supports a 'outside-in' model in which the adventitia, previously considered an inert layer that simply provides structural support, acts as an injury 'sensor' within the vessel wall and subsequently directs responses to a wide array of stimuli including ischaemia. In this model, it is proposed that resident adventitial cell such as fibroblasts, become activated and secrete inflammatory cyto/chemokines which leads to expression of endothelial surface adhesion markers such as VCAM-1 and subsequent neutrophil recruitment to the intimal layer (46). Although recent studies have extended IL-36 and IL-36R expression to include stromal cells such as fibroblasts, further studies will be required to determine whether their presence in the adventitial vascular layer is of specific significance in mediating inflammatory responses in the heart post-reperfusion injury.

A common finding in diseases where IL-36 cytokines contribute to pathology is the remarkable presence of neutrophils. Indirect evidence supporting the ability of IL-36 to recruit neutrophils has been obtained primarily from histological or flow cytometric studies in which inhibiting IL-36R signalling reduced recruitment in diseases such as psoriasis and colitis. Recent findings by Koss and colleagues pinpointed IL-36 as an early and upstream driver of acute and chronic pulmonary inflammation by promoting neutrophil recruitment and production of pro-inflammatory IL-1 family cytokines and IL-6 (47). To directly demonstrate that IL-36 could be pro-inflammatory in the heart, we topically applied agonists within the centre of the attached water-tight stabiliser ring. All isoforms were potently pro-inflammatory, something not previously shown in vivo in any organ let alone the heart. Neutrophil recruitment was observed in both coronary capillaries and PCVs but, unexpectedly, was greater in adult rather than aged hearts which could be due to reduced

neutrophil responsiveness with age (48). The inflammatory response was rapid and appeared to plateau beyond 60 minutes in adult hearts. Although it was slower and less potent in aged hearts, neutrophil recruitment did continue to rise beyond 150 minutes. It is possible that with a more prolonged imaging period, this response may have reached similar, or exceeded, maximal levels observed in adult mice. It is also possible that our observed increases in the expression of IL-36R in aged mice may be associated with a concomitant increase in circulating levels of the endogenous anti-inflammatory IL-36Ra. This would act to protect the balance of IL-36 pathway signalling in aged mice by inhibiting engagement of IL-36 cytokines with their receptor. This could also explain why the same dose of topical IL-36 was unable to elicit similar or greater inflammatory responses in aged compared to adult hearts. Indeed, this has been shown to be true for IL-1Ra where higher circulating levels are detected in elderly patients and has been suggested to play a role in the decline in the inflammatory response with age (49).

Interestingly, we also showed that topical application of IL-36 was more potent than similar doses of topical IL-1 β and TNF α at stimulating neutrophil recruitment in both adult and aged hearts (data not presented). IL-36 also increased platelet microthrombi presence although this is not known if this is driven through a direct response of IL-36 on platelets. It is possible that circulating platelets became trapped in vessels downstream of regions where substantial occlusive neutrophil adhesion occurred. Collectively, these studies provided a rationale for exploring the therapeutic potential of IL-36 signalling blockade to attenuate microcirculatory disturbances associated with injury.

The IL-36R is interesting in that it has three agonists but also two naturally occurring receptor antagonists, namely IL-36Ra and IL-38, which underpins the importance of careful

endogenous management of IL-36 pathway. Both competitively bind the IL-1Rrp2 component of the heterodimer receptor preventing recruitment of the accessory co-receptor IL-1RAcP and thus inhibiting subsequent intracellular signalling (18). We tested the vasculoprotective effects of systemically injected IL-36Ra and observed a significant reduction in neutrophil recruitment in both adult and aged injured hearts. Although no anti-platelet effect was demonstrated, IL-36Ra treatment still led to a significant decrease in infarct size in both adult and aged mice. Since one dose of IL-36Ra was administered during the ischaemic period, it is plausible that IL-36 could be targeted during PCI procedures and be therapeutically efficacious in a clinical setting.

ROS are implicated in the pathogenesis of various cardiac disorders including MI and heart failure and can promote the expression of endothelial adhesion molecules, such as ICAM-1 and VCAM-1, that are critical for neutrophil recruitment (50). Therefore, whether IL-36Ra mechanistically conferred vasculoprotection by limiting endothelial ROS damage and VCAM-1 expression was assessed in both adult and aged hearts. The anti-DNA/RNA antibody used in the study binds with high specificity and affinity to 8-hydroxy-2'-deoxyguanosine, 8-oxo-7,8-dihydroguanine and 8-oxo-7,8-dihydroguanosine. These oxidative lesions serve as excellent markers for DNA and RNA damage produced specifically by ROS. Flow cytometric and immunofluorescence studies demonstrated a significant decrease in IR injury mediated oxidative damage in the presence of the IL-36Ra. Importantly, this decrease was evident in both adult and the more damaged aged hearts. Our data supports the recent observation of reduced oxidative stress, measured using spectrophotometry of superoxide dismutase and malondialdehyde activity, in IL-36R knockout rats undergoing cardiopulmonary bypass (51). However, we further detail that the anti-oxidant effects of IL-36Ra occur specifically on both

coronary endothelial cells and cardiomyocytes. It has recently been shown that IL-36 can up-regulate VCAM-1 and ICAM-1 on dermal endothelial cells in vitro and that this can be reversed by the presence of an IL-36Ra (44). However, we demonstrate the ability of IL-36Ra to decrease VCAM-1 expression in the IR injured coronary microcirculation. Again, more importantly, this benefit was also observed in aged hearts where even basal VCAM-1 expression was high. Collectively, our data provide, what we believe to be, novel mechanistic insights into how inhibition of IL-36/IL-36R signalling attenuates oxidative stress and VCAM-1 expression in adult and aged hearts, subsequently preventing excessive neutrophil recruitment in the coronary microcirculation, which ultimately leads to decreased infarct size post-reperfusion.

Concluding Remarks

New therapies need to be designed and optimised that are effective in improving the current poor prognosis of the ageing population post-MI. We and others have recommended that this must involve specific protection of the delicate coronary microcirculation from IR injury. Although studies on age-related changes of the inflammatory and immune system have gathered pace, this study is the first, as far as we know, to explore the impact of age on the coronary microcirculation in vivo in both health and post-reperfusion injury. It is likely that the increased thromboinflammatory activation and microcirculatory perturbations that we have observed intravitaly in the aged injured heart may inhibit the therapeutic efficacy of existing and future cardiovascular drugs in the elderly. However, our finding that IL-36Ra was not only vasculoprotective, but importantly remained beneficial in the setting of an age-

related heightened inflammation in the coronary microvessels, makes it a candidate worth pursuing clinically in elderly patients undergoing PCI for MI. In support of this is the recent work by Luo and colleagues who demonstrated experimentally that deficiency of IL-36 receptor protected cardiomyocytes in the setting of cardiopulmonary bypass (51).

A number of anti-inflammatories, shown to be successful in experimental studies, have met with translational failure when tested in patients with MI (52). The major outcome measured in such clinical trials (and indeed experimental studies) is usually a long-term one - namely the ability to prevent post-MI remodelling, a secondary non-fatal MI or death. Whether these anti-inflammatories can also protect and keep patent the coronary microcirculation in the immediate aftermath of PCI/reperfusion has received much less interest. However, this is imperative in order to improve long-term patient outcomes. It is therefore possible that translational failure is linked to a lack of early benefit at the level of the coronary microcirculation. Since we have shown multiple microcirculatory perturbations, ultimately resulting in poor myocardial perfusion within minutes of reperfusion, it is also important that the design of an anti-inflammatory therapy involves administration immediately before interventions designed to mediate reperfusion commence (e.g. PCI). However, not all clinical trials have delivered anti-inflammatories prior to reperfusion, with some administered days later (53). Again, this may explain the lack of success of such compounds in clinical trials. Our data highlights a notable benefit to the coronary microcirculation and infarct size with early administration of IL-36Ra, which importantly is maintained in the presence of an aged co-morbidity. This indicates that early intervention with an IL-36R inhibitor is worth considering for future clinical investigations.

Methods

Myocardial ischaemia-reperfusion injury

Experiments were conducted on female C57BL/6 adult (2-4 months) or aged (18-19 months) mice in accordance with the Animals (Scientific Procedures) Act of 1986 (Project licence P552D4447). Anaesthesia was induced by an intraperitoneal administration of ketamine hydrochloride (100mg/kg) and medetomidine hydrochloride (100mg/kg), confirmed by checking the pedal reflex every 15 minutes and maintained as required via intraperitoneal administration. Mice were intubated and ventilated with medical oxygen via a MiniVent rodent ventilator (stroke volume: 220 μ l, respiratory rate: 130pm; Biochrom Ltd. Harvard Apparatus). The carotid artery was cannulated to facilitate infusion of antibodies, dyes, saline and IL-36Ra. IR injury was induced by ligating the left anterior descending (LAD) artery for 45 minutes and reperfusion allowed to proceed for 2 hours (tissue analysis), 2.5 hours (intravital observations) or 4 hours (infarct measurement). Sham surgery involved the same procedure but without LAD artery ligation. At the end of experiments, mice were euthanised by cervical dislocation and euthanasia was confirmed by ensuring cessation of the circulation by making an incision in the carotid artery.

Intravital imaging of the beating heart coronary microcirculation

Real-time intravital observations were performed as previously described (28). Briefly, a 3D printed stabilizer was permanently fixed to the left ventricle downstream of the ligation site (**Figures 4A-C**). To simultaneously image endogenous neutrophils and platelets, PE anti-mouse Ly-6G (Biolegend) and APC anti-mouse CD41 (Biolegend) were injected 5 minutes prior

to reperfusion. Intravital imaging was performed using a microscope (BX61WI, Olympus) equipped with a Nipkow spinning disk confocal head (Yokogawa CSU, Japan) and an Evolve EMCCD camera (Photometrics, USA). The first 2-minute capture was performed at 15 minutes post-reperfusion, followed by 2 minute captures every 15 minutes of the same area for 2.5 hours. In separate mice, FITC-BSA (Sigma) was injected at the end of a 2.5-hour reperfusion period to investigate overall vascular perfusion and functional capillary density.

For some studies, recombinant mouse IL-36Ra (15ug/mouse; Novus Biologicals) was injected intra-arterially at both 5 minutes pre-reperfusion and 60 minutes post-reperfusion. One of the critical factors that limits the clinical success of previously tested anti-inflammatory drugs is the time of intervention. Given the rapid development of microvascular no-reflow, the first hours, if not minutes, following reperfusion are critical. Our treatment strategy focused on introducing the antagonist during the ischaemic phase to establish an effective circulating concentration to dampen the initial reperfusion-associated inflammatory response and maintain microvascular patency. The ability of IL-36 cytokines to directly mediate thromboinflammatory events in vivo was also assessed. After placing the stabilizer on the healthy heart, 20 μ l of recombinant mouse IL-36 cytokine (α , β or γ ; 200ng/ml) or PBS was topically applied to the heart surface within the water-tight stabiliser ring for 15 minutes and a 2-minute video recorded. This was replaced with fresh cytokine / PBS for 15 minutes and the process repeated 10x for a total duration of 2.5 hours.

Data was captured, stored, and analysed using Slidebook 6 software (Intelligent Imaging Innovations, USA). Free flowing neutrophils, which passed through the coronary microcirculation without making adhesive interactions, were counted manually over the 2-minute recorded capture. To analyse adherent neutrophil and platelet presence, captured

videos were subjected to post-acquisition image repair using an in-house designed software (*Tify*) in which out-of-focus frames were removed (54). Neutrophils and platelet aggregates/microthrombi were then quantitated by placing a mask around PE-Ly6G⁺ and APC-CD41⁺ areas respectively. Integrated fluorescence density, which took into account size and fluorescence intensity, was then calculated using ImageJ (NIH).

Multiphoton imaging of heart sections

Intravital imaging captured microvascular events from the surface of the beating heart with a depth of approximately 50-60 μ m. To determine whether these events were mirrored throughout the thickness of the ventricular wall, multiphoton microscopy was performed on hearts harvested at the end of intravital experimentation. The left ventricle was sectioned into four 300 μ m sections using a tissue vibratome (Campden Instruments Limited, UK) and imaged from the epicardial through to the endocardial end using a multiphoton microscope (FVMPE-RS Olympus). Z-stacks from the four layers were rendered to form 3D stack images which were processed and displayed using ImageJ (**Figures 4E-F**). The presence of neutrophils was analysed as the sum fluorescence intensity for each section (ImageJ).

Immunohistochemistry analysis of IL-36 cytokines and IL-36R

10 μ m sections of frozen heart tissue were incubated at room temperature with either primary anti-IL-36R, anti-IL-36 α , anti-IL-36 β or IgG control antibodies (1:100 dilution; R&D Systems) and a secondary donkey anti-goat Alexa Fluor-488 antibody (1:100 dilution; Abcam). Sections were also incubated with a PE anti-mouse CD31 antibody (1:100 dilution, Biolegend),

an Alexa Fluor-647 anti-mouse CD106/VCAM-1 antibody (1:100 dilution, Biolegend) or an anti-DNA/RNA damage antibody to detect oxidative damage (1:100 dilution, Abcam). Images were captured using an EVOS FL (ThermoFisher Scientific) or multiphoton microscope (FVMPE-RS, Olympus). ImageJ was used to quantify the mean fluorescence intensity (MFI) of each image with additional analysis of MFI on regions containing only coronary capillaries or a large blood vessel.

Western blotting analysis

Total protein was extracted from harvested hearts using RIPA buffer and homogenisation with beads. Lysates were normalised using a BCA protein assay kit to 2mg/ml. Samples were run on an SDS-PAGE gel and then transferred onto a nitrocellulose membrane before being blocked for unspecific binding with 5% non-fat dried milk. The membrane was then incubated overnight at 4°C with the primary antibody for IL-36R (1:200 dilution) followed by incubation with the secondary antibody conjugated to Alexa Fluor 488 (1:1000 dilution). After washing, protein bands were visualized using a fluorescence detection system (ChemiDoc, Bio-Rad) and MFI determined.

Flow cytometric analysis of endothelial and cardiomyocyte oxidative stress

Harvested adult and aged hearts were manually minced, added to 0.1% collagenase and rotated in an incubator at 37°C for 15 minutes. The supernatant was removed, and the digestion process was repeated 2 times. The pellet was then centrifuged at 10000rpm for 10 minutes in the presence of ACK and MACS buffer to lyse red blood cells and stop enzymatic

activity respectively. The pellet was added to 20ml media and run several times through a 70µm strainer. Cells were then incubated with an anti-DNA/RNA damage antibody to detect oxidative damage (1:100 dilution, Abcam), anti-IL-36R antibody to detect IL-36R expression (R&D Systems; Alexa-647 secondary, Biolegend both at 1:100 dilution), anti-CD31 antibody to label endothelial cells (1:100 dilution, Biolegend), anti-cTnT antibody to label cardiomyocytes (1:100 dilution, Miltenyi Biotec), Zombie to detect dead cells (1:500 dilution, Biolegend) and appropriate IgG controls. Cells were then fixed using 4% formalin for 10 minutes and washed with Dulbecco's phosphate buffered saline. Acquisition of cells was performed using a CyAn™ ADP (Beckman Coulter, USA) and data analysis was performed using Summit 4.3 software (Beckman Coulter, USA). For each sample, 250000 events were captured and used in the analysis.

Endothelial cell IL-36R expression analysis in vitro

Immortalised murine vena cava endothelial cells (VCECs) were grown to confluence and stimulated for 4 hours with either experimental media (vehicle control), recombinant mouse IL-36 α , β or γ (3, 30, or 300 ng/ml; R&D systems) or recombinant mouse TNF α (3, 30, or 300 ng/ml; Boster Biological Technology). Cells were then formalin fixed and incubated overnight with a primary antibody against IL-36R (1:100 dilution), followed by incubation with a secondary antibody (1:100 dilution) and Hoechst 33342 dye (ThermoFisher). Images were captured using a multiphoton microscope and MFI determined (ImageJ).

Myocardial infarct size analysis

4 hours after reperfusion, the LAD artery was re-ligated, and 0.5% Evans blue dye (Sigma) was infused via the carotid cannula to identify the area at risk (AAR). The mouse was then sacrificed, and the harvested heart was cut into sequential slices and incubated with 2,3,5-triphenyltetrazolium chloride (TTC, Sigma). Sections were imaged using a stereomicroscope and analysis was performed using ImageJ to quantitate the infarct size (TTC negative white regions) as a percentage of the AAR (TTC positive red regions / Evans blue negative).

Statistical analysis

All statistical analysis was performed using GraphPad 7.0 software (GraphPad Software Inc., USA). Direct comparisons between two groups were performed using a Student's unpaired t-test. Multiple comparisons between three or more groups were performed by one-way ANOVA, followed by a Tukey's post-hoc test. For experiments which followed a time course, the area under the curve (AUC) was also calculated and used for subsequent analysis as a summation of the entire period. All data are presented as mean \pm SEM with statistical significance defined when $p < 0.05$.

Study Approval

Experiments were conducted on mice in accordance with the Animals (Scientific Procedures) Act of 1986 (Project licence P552D4447).

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Data Availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. Raw data supporting the findings of this study are available from the corresponding author, Dr Neena Kalia, on request.

Disclosures

The authors confirm they have no conflicts of interest to disclose.

Author Contributions

J-EA – acquired, analysed and interpreted the data, drafted the work, approved the submitted version

DPJK – analysed and interpreted the data, approved the submitted version

MR – provided material, approved the submitted version

CW – provided materials, approved the submitted version

ND – approved the submitted version

NK – obtained the funding, designed the experiments, interpreted the data, drafted the work, approved the submitted version

All authors – agreed to be personally accountable for contributions and to ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated, resolved, and the resolution documented in the literature.

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Supplemental Video Legends

Supplemental Video 1. Intravital microscopy videos of the healthy beating hearts from adult and aged mice after topical application of IL-36 isoforms. All three isoforms significantly increase neutrophil (green) adhesion in the coronary microcirculation in vivo.

Supplemental Video 2. Intravital microscopy videos of sham and IR injured beating hearts from adult and aged mice. Age significantly increases neutrophil (green) adhesion in both healthy and IR injured coronary microcirculation in vivo.

Supplemental Video 3. Multiphoton microscopy videos of sham and IR injured heart sections from the outermost to the innermost layer from adult and aged mice. Age increases neutrophil (green) presence within the deeper layers of both healthy and IR injured myocardium in all layers of the heart.

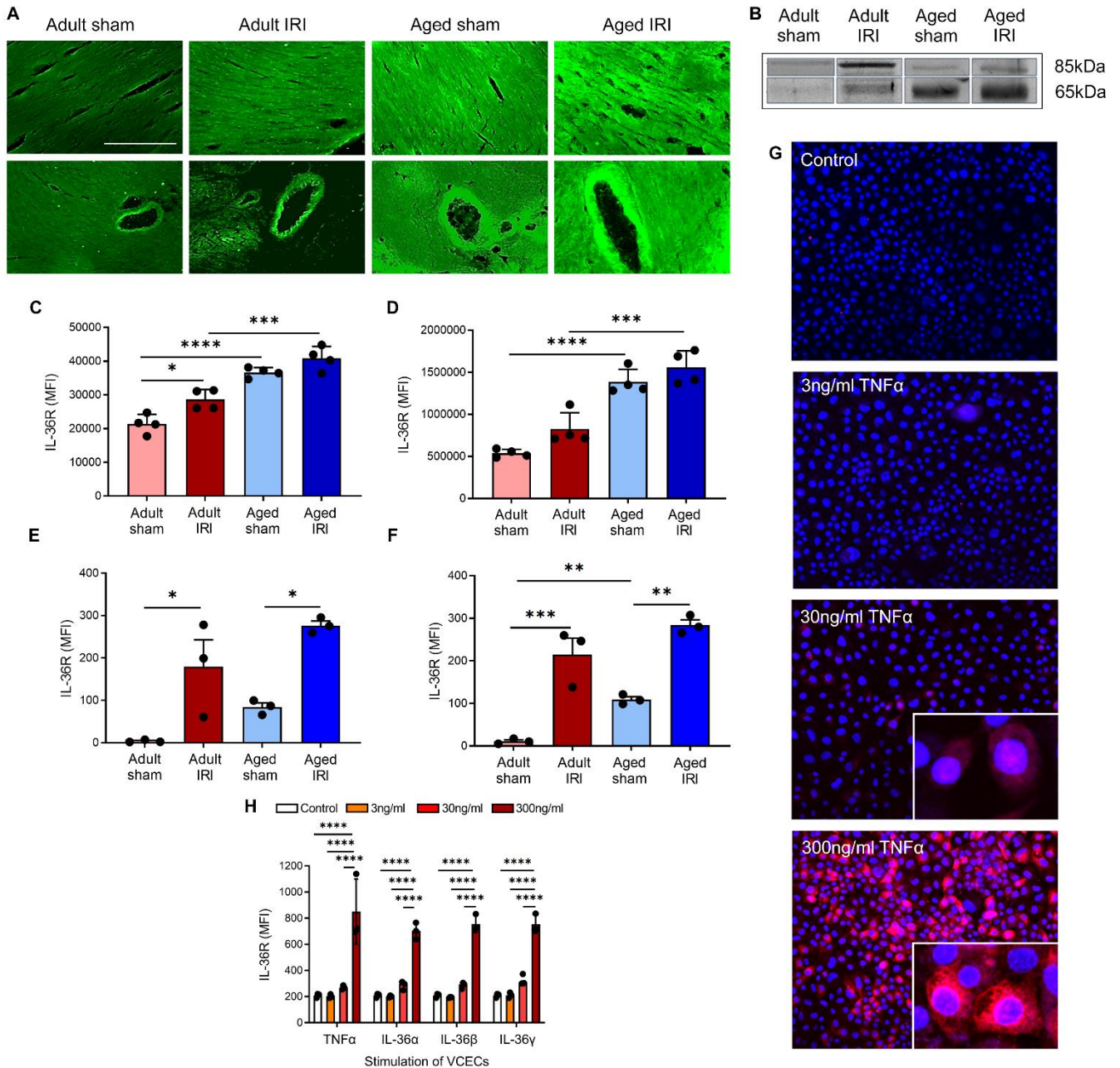


Figure 1. Age increases expression of IL-36R in healthy and IR injured hearts, which can also be induced in endothelial cells with TNF α or IL-36 cytokine stimulation. Hearts were assessed for IL-36R using immunostaining, western blot analysis and flow cytometry. Representative images of IL-36R (green) staining of **(A)** frozen heart sections and **(B)** western blots. The molecular weight of IL-36R is about 65kDa, but it migrates to the position of about 85kDa in denaturing protein gels. Hence, two bands were observed corresponding to 65kDa, the more active protein due to cleavage of its signalling peptide, and 85kDa, the less potent glycosylated form. Quantitative analysis of the **(C)** immunofluorescent images (n=4/group) and **(D)** western blots (n=4/group). Hearts were also collagenase digested and analysed flow cytometrically for IL-36R expression. Flow cytometry analysis confirmed that IR injury and age induced a significant increase in IL-36R on **(E)** coronary endothelial cells and **(F)** cardiomyocytes (n=3/group). **(G-H)** Murine vena cava endothelial cells (VCECs) were cultured

and stimulated for 4 hours with experimental media (control), an IL-36 cytokine (α , β or γ) or TNF α . **(G)** Representative images of IL-36R (red) expression on stimulated non-permeabilised cells (Hoechst 33342 stained nuclei in blue). **(H)** Quantitative analysis of IL-36R expression on VCECs following stimulation (n=3/group). Scale bar indicates 200 μ m. *p<0.05, *p=0.0146 **(C)**, **p<0.01, ***p=0.0003 **(C)**, ***p<0.001, ***p=0.001 **(D)**, ****p<0.0001 as determined using a one-way ANOVA followed by a Tukey's post-hoc test.

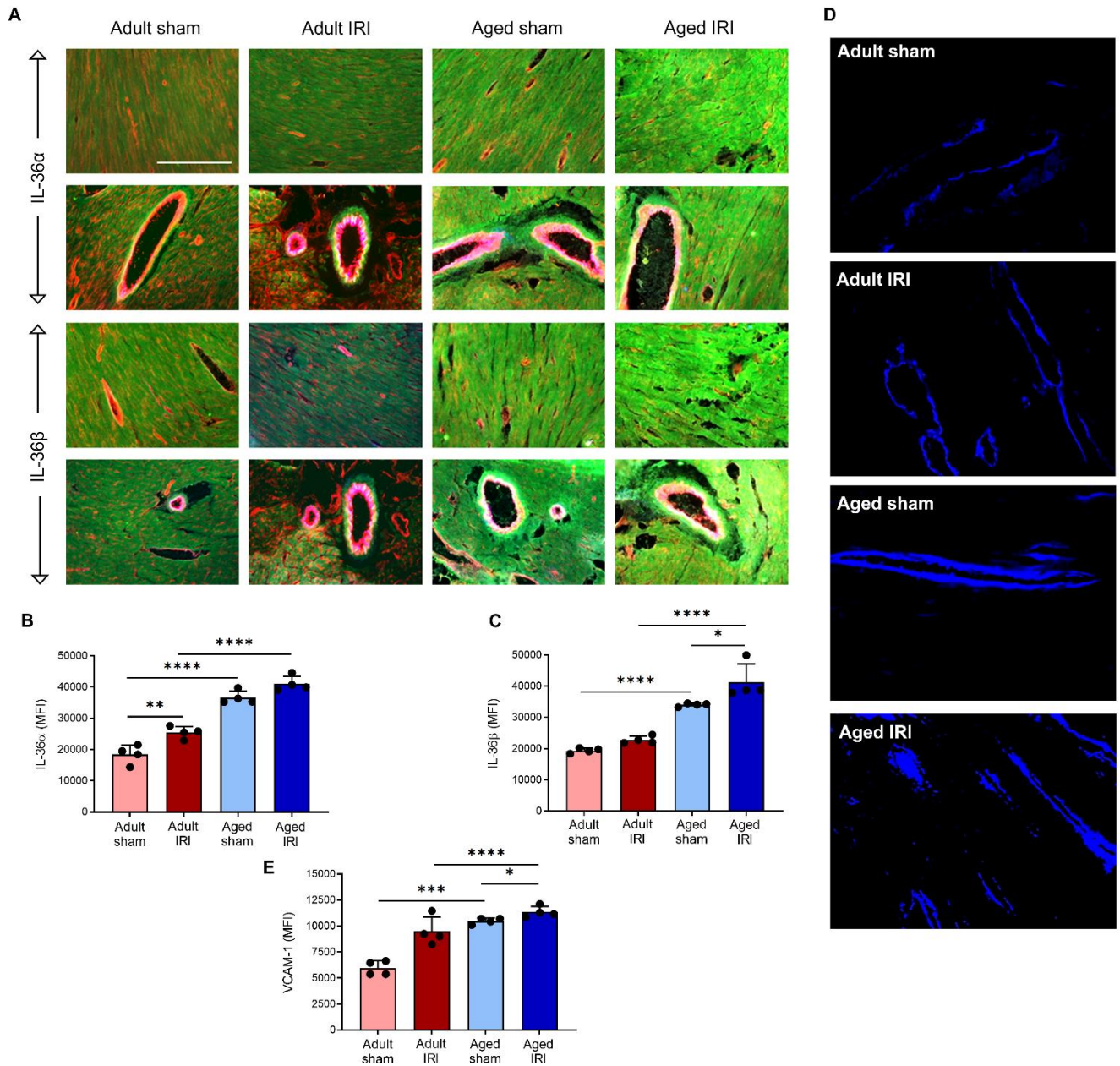


Figure 2. Age increases expression of IL-36 α , IL-36 β and VCAM-1 in healthy and IR injured hearts. Hearts from adult and aged, sham and IR injured mice were immunostained with an anti-IL-36 α/β , anti-CD31 and anti-VCAM-1 antibody. **(A)** Representative images of IL-36 α (green; top two rows) and IL-36 β (green; bottom two rows) staining of frozen heart sections co-stained with CD31 (red) and VCAM-1 (blue). The upper row of each cytokine panel shows coronary microvessels with the lower row selected to demonstrate staining of a large coronary blood vessel. Quantitative analysis of the immunofluorescent images for **(B)** IL-36 α and **(C)** IL-36 β expression. **(D)** Representative images of VCAM-1 (blue) staining of frozen heart sections. **(E)** Quantitative analysis of immunofluorescent images of VCAM-1 expression. Scale bar indicates 200 μ m. n=4/group. *p=0.0193 **(C)**, *p=0.0308 **(E)**, **p=0.0054, ***p=0.0002, ****p<0.0001 as determined using a one-way ANOVA followed by a Tukey's post-hoc test.

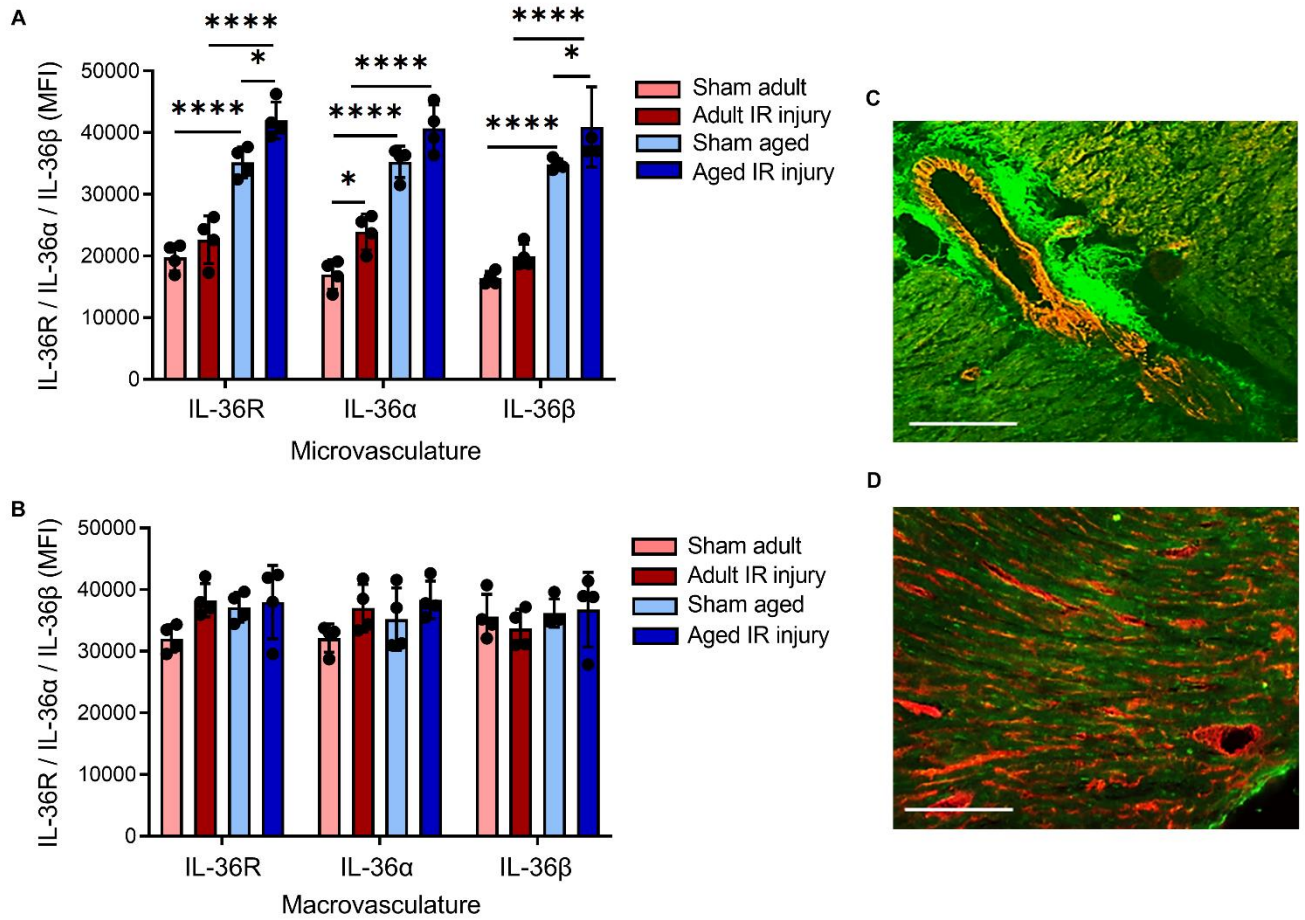


Figure 3. Changes in the expression of IL-36 cytokines and its receptor occurs on the coronary microvasculature and not on the large blood vessels. Quantitative analysis of IL-36R, IL-36α, and IL-36β expression using immunofluorescence is shown for the (A) microvasculature and (B) macrovasculature of the adult and aged, sham and IR injured heart. $n=4/\text{group}$. $*p=0.0191$ (IL-36R), $*p=0.0182$ (IL-36α), $*p=0.0321$ (IL-36β), $****p<0.0001$ as determined using a one-way ANOVA followed by a Tukey's post-hoc test. To further determine whether IL-36R (green) expression was vascular in nature, heart sections were co-stained with an anti-CD31 antibody (red) and imaged using a multiphoton microscope. Representative image of (C) coronary macrovasculature and (D) microvasculature for an adult IR injured mouse.

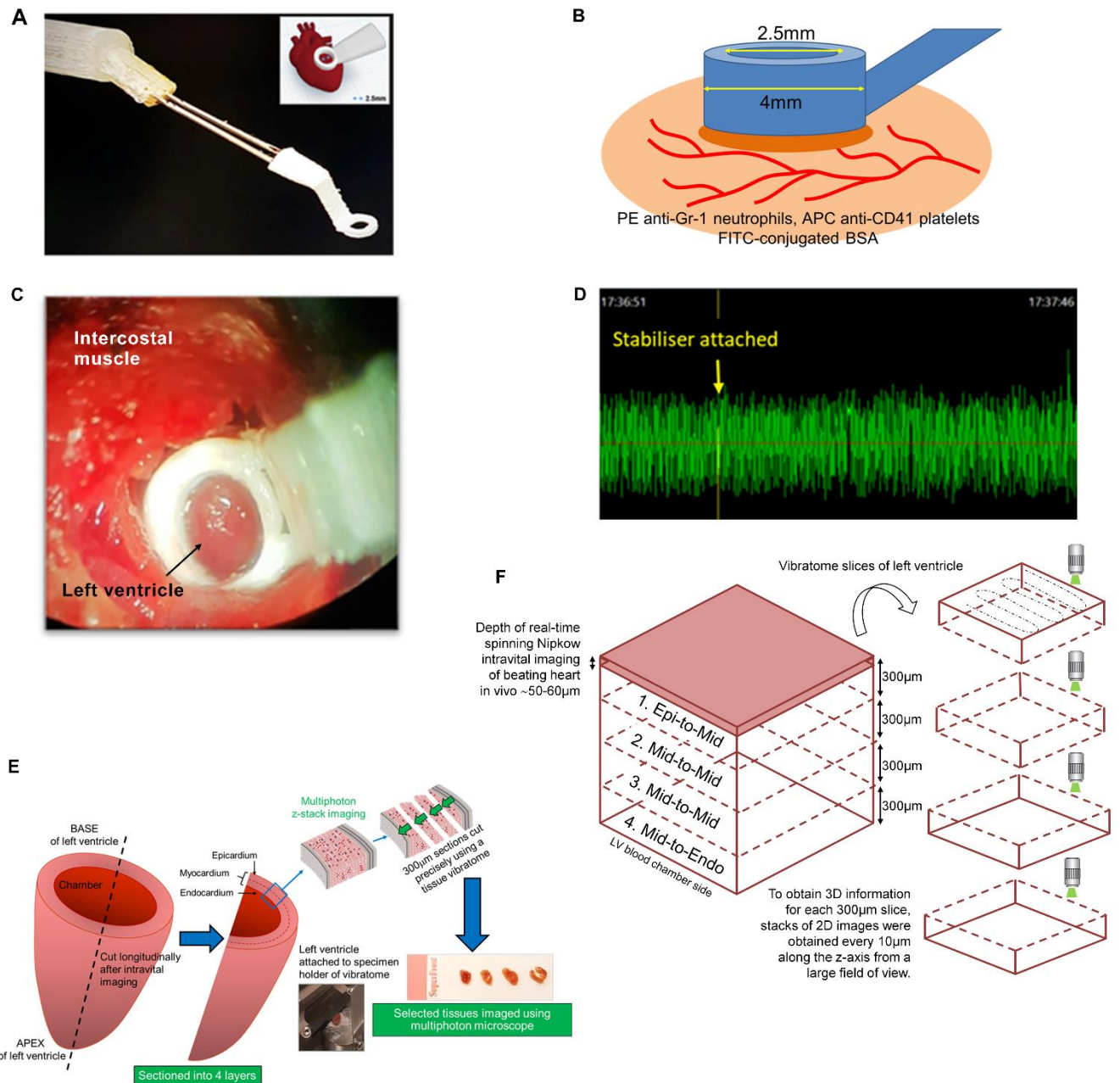


Figure 4. Intravital imaging of the mouse beating heart microcirculation *in vivo* and multiphoton imaging of the heart *ex vivo*. (A-C) An in-house designed 3D printed stabiliser is lowered on to the beating left ventricle allowing confocal intravital imaging in its centre. Only a small surface of the beating heart has its motion reduced sufficiently enough to permit imaging. (D) No BP/HR changes were detected using this approach as determined by photoplethysmography in both adult and aged sham and IR injured mice. The graph presented shows BP, which remains constant even after the stabiliser is attached. (E) To ascertain whether any thromboinflammatory and vasculoprotective events imaged intravitaly on the surface of the heart were also occurring in the deeper layers of the myocardium, multiphoton microscopy was used. The heart was cut in half longitudinally from the base to apex to expose the inner endocardial layer lining the left ventricle chamber. It was then placed on a specimen holder and attached to a tissue vibratome to precisely section the left ventricle wall into 4 x 300µm thickness sections from the outermost layer closest to the epicardium,

through to the inner layer closest to the endocardium. **(F)** Multiphoton z-stacks were taken from all 4 layers, namely the (i) outermost layer closest to the epicardium – epi-to-mid (ii) outer myocardial layer – mid-to-mid (iii) inner myocardial layer – mid-to-mid and the (iv) innermost layer closest to the endocardium – mid-to-endo, avoiding the last section if it had ‘missing’ myocardium due sectioning through the actual ventricle chamber. Images from each layer were then rendered to form 3D stack images.

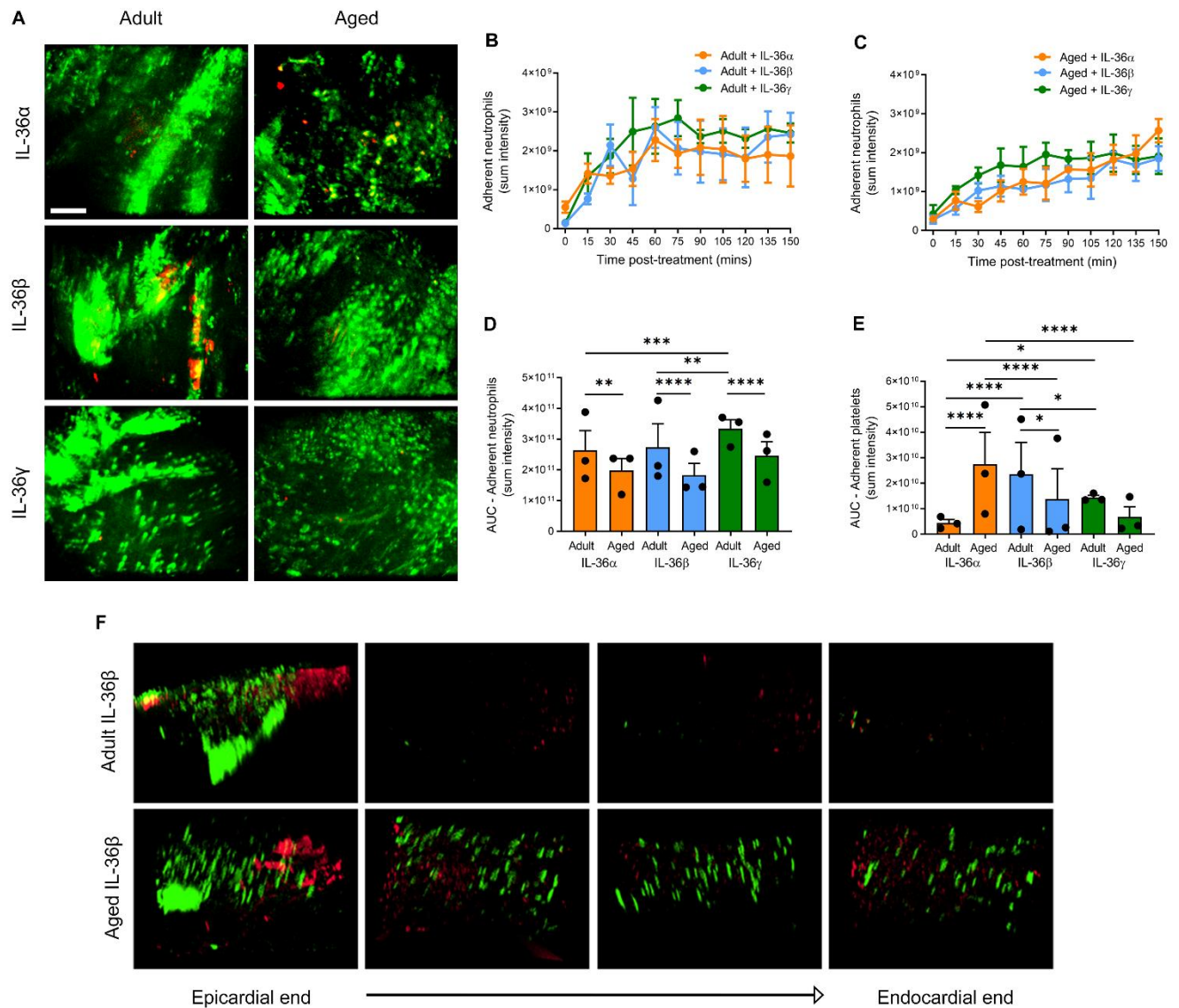


Figure 5. Topically applied IL-36 is pro-inflammatory in the adult and aged beating heart in vivo. IL-36 α , IL-36 β or IL-36 γ (200ng/ml) was topically applied to the healthy adult and aged beating heart left ventricle. **(A)** Representative intravital images of the beating heart showing adherent neutrophils (green) and platelets (red) in the coronary microcirculation at 120 minutes post-application. Quantitative analysis of the intravital data for adherent neutrophils for the **(B)** adult and **(C)** aged groups, and the area under the curve (AUC) for **(D)** adherent neutrophils and **(E)** platelets over a time course of 150 minutes. **(F)** Representative multiphoton images of neutrophils (green) and platelets (red) in all 4 layers of the left ventricle exposed to IL-36 β in adult (top row) and aged (lower row) mice. Images are taken from the outermost layer closest to the epicardium, outer myocardial layer, inner myocardial layer and the innermost layer closest to the endocardium. Scale bar indicates 100 μ m. n=3/group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 as determined using a one-way ANOVA followed by a Tukey's post-hoc test.

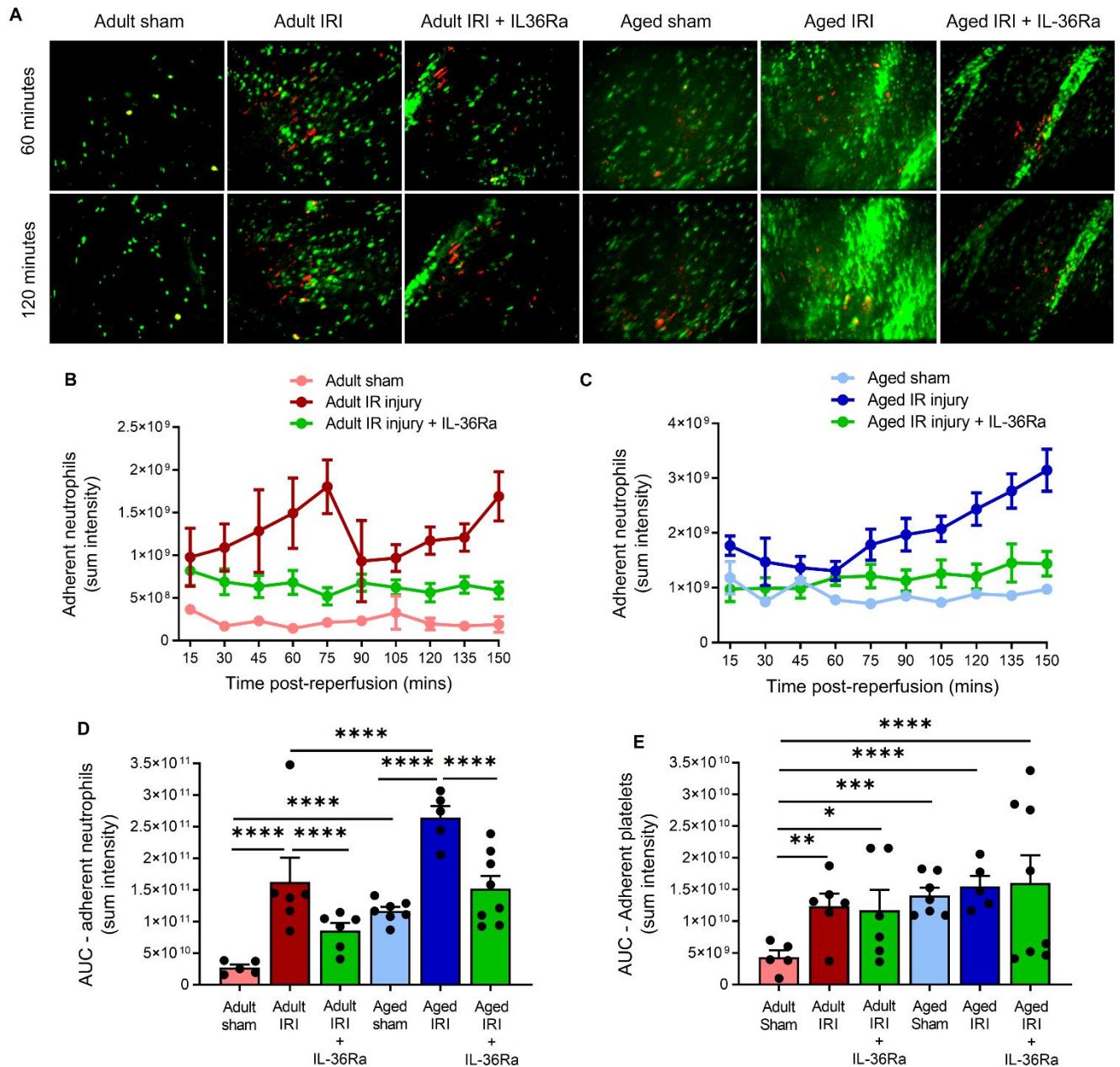


Figure 6. Age increases thromboinflammatory disturbances within the healthy and IR injured beating heart coronary microcirculation in vivo, which can be prevented in both age groups by IL-36R inhibition. An IL-36 receptor antagonist (IL-36Ra; 15 μ g/mouse) was injected intra-arterially at 5 mins pre-reperfusion and 60 mins post-reperfusion in adult and aged mice. **(A)** Representative intravital images of the beating heart showing adherent neutrophils (green) and platelets (red) in the coronary microcirculation at 60- and 120-mins in sham hearts or 60- and 120-mins post-reperfusion in injured hearts. Adherent neutrophils (green) and platelet microthrombi (red) are primarily within coronary capillaries in injured hearts. Intensely fluorescent green areas in aged IR injured hearts correspond to medium sized blood vessels that have become delineated by the presence of a significant number of adherent neutrophils. Quantitative analysis of the intravital data for adherent neutrophils in **(B)** adult and **(C)** aged IR injured hearts and the area under the curve (AUC) for **(D)** adherent neutrophils

and **(E)** platelets over a time course of 150 minutes. Scale bar indicates 100 μ m. Adult sham - n=5/group; Adult IRI - n=6/group; Adult IRI + IL-36Ra – n= 5/group ; Aged sham - n=7/group; Aged IRI - n=5/group; Aged IRI + IL-36Ra – n=8/group. *p=0.0339, **p=0.0014, ***p=0.0003, ****p<0.0001 as determined using a one-way ANOVA followed by a Tukey's post-hoc test.

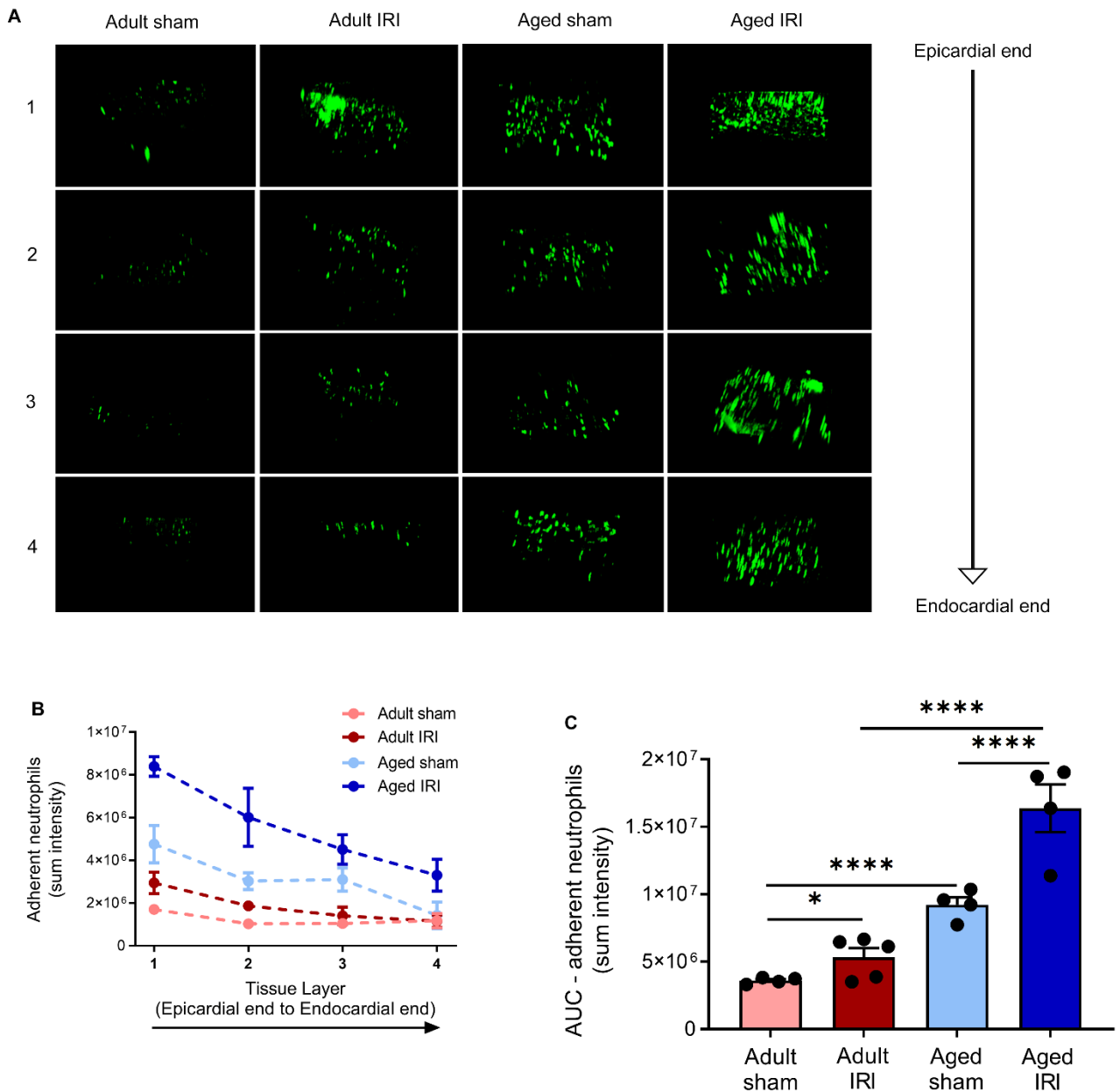


Figure 7. Age increases neutrophil presence within the deeper layers of the healthy and IR injured myocardium. (A) The left ventricle was vibratome sectioned into four 300 μ m sections and imaged using a multiphoton microscope. Representative z-stack multiphoton images of neutrophils (green) in the 4 layers of the left ventricle taken from the outermost layer closest to the epicardium (1), outer myocardial layer (2), inner myocardial layer (3) and the innermost layer closest to the endocardium (4). Quantitative analysis of the multiphoton data at various depths for (B) adherent neutrophils and corresponding (C) AUC for adherent neutrophils. Adult sham - n=4/group; Adult IRI - n=5/group; Aged sham - n=4/group; Aged IRI - n=4/group. *p=0.0325, ****p<0.0001 as determined using a one-way ANOVA followed by a Tukey's post-hoc test.

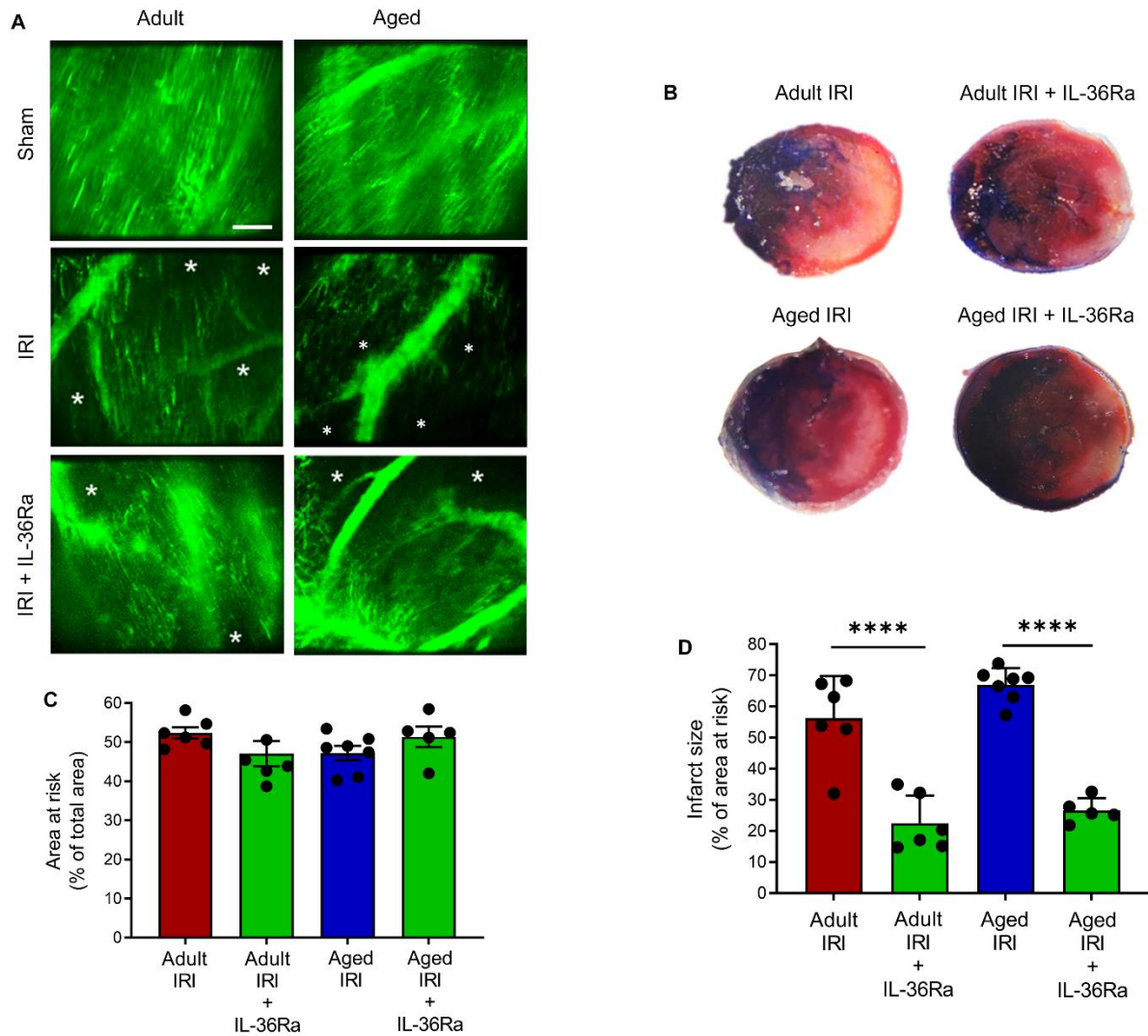


Figure 8. IL-36R inhibition improves functional capillary density and reduces infarct size in vivo in the IR injured adult and aged heart. An IL-36 receptor antagonist (IL-36Ra; 15 μ g/mouse) was injected intra-arterially at 5 mins pre-reperfusion and 60 mins post-reperfusion in adult and aged mice. **(A)** Representative intravital images of FITC-BSA (green) perfused coronary microvessels at 150 mins in sham hearts or 150 mins post-reperfusion in injured hearts. *Areas not perfused with FITC-BSA. Scale bar indicates 100 μ m. **(B)** Representative images of the TTC stained infarct size in all 4 groups. **(C)** Quantitative analysis of the area at risk in all 4 group. **(D)** Quantitative analysis of infarct size in all 4 groups. n=6/group (n=5 for Aged IRI + IL-36Ra group). ****p<0.0001 as determined using a one-way ANOVA followed by a Tukey's post-hoc test.

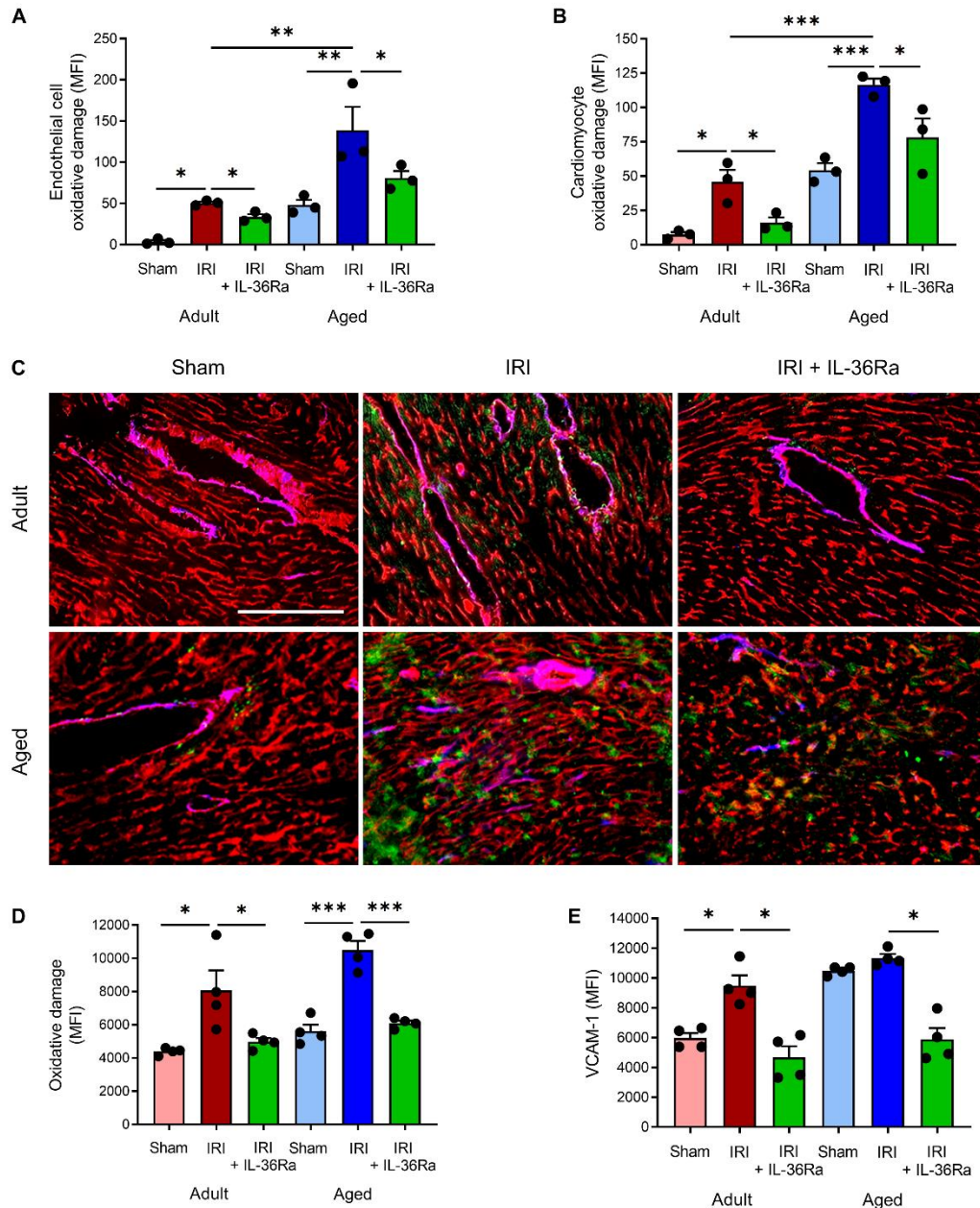


Figure 9. IL-36R inhibition reduces endothelial and cardiomyocyte oxidative damage and VCAM-1 expression in the IR injured adult and aged hearts. Hearts from adult and aged sham, IR injured, and IR injured + IL-36Ra mice were either collagenase digested and analysed flow cytometrically or sectioned and analysed using immunofluorescence for oxidative damage and/or VCAM-1 expression. Flow cytometry analysis demonstrated that IR injury increased oxidative damage of both **(A)** adult and aged coronary endothelial cells and **(B)** adult and aged cardiomyocytes when compared to appropriate age sham cells. This was reduced in both cell populations in adult and aged mice treated with the IL-36Ra. $n=3/\text{group}$. **(C)** Representative immunofluorescence images of CD31 (red) co-stained with DNA/RNA oxidative damage (green) and VCAM-1 (blue) in sham, IR injured and IL-36Ra treated adult and aged mice. Quantitative analysis of the immunofluorescent images for myocardial **(D)**

oxidative damage and **(E)** VCAM-1 expression. Scale bar indicates 200 μ m. n=4/group. *p<0.05, **p<0.01, ***p<0.001 as determined using a one-way ANOVA followed by a Tukey's post-hoc test.