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Interleukin-1β promotes an atheroprotective distribution of smooth muscle cells and macrophages in late stage murine atherosclerotic lesions

Authors: Delphine Gomez[1,2,3,4], Richard A. Baylis[1,5], Brittany G. Durgin[1,2], Alexandra A. C. Newman[1,5], Gabriel F. Alencar[1,5], Sidney A. Mahan[3], Cynthia St. Hilaire[3,4], Werner Muller[6], Ari Waisman[7], Sheila E. Francis[8], Emmanuel Pinteaux[6], Gwendalyn J. Randolph[9], Hermann Gram[10], Gary K. Owens[1,2,*]

Affiliations:

[1]Robert M. Berne Cardiovascular Research Center, University of Virginia, Charlottesville, VA, USA.

[2]Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, VA, USA.

[3]Pittsburgh Heart, Lung, Blood and Vascular Medicine Institute, University of Pittsburgh; Pittsburgh, PA, USA.

[4]Division of Cardiology, University of Pittsburgh School of Medicine; Pittsburgh, PA, USA.

[5]Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA, USA.

[6]Faculty of Biology, Medicine and Health, University of Manchester, Manchester, UK.

[7]Institute for Molecular Medicine, University Medical Center of the Johannes Gutenberg University of Mainz, Mainz, Germany.

[8]Department of Infection, Immunity and Cardiovascular Disease, University of Sheffield, Sheffield, UK.

[9]Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 02114, USA.

[10]Novartis Institutes for Biomedical Research, Basel, Switzerland.

* To whom correspondence should be addressed

Dr. Gary K. Owens, PhD

University of Virginia School of Medicine

415 Lane Road, P.O. Box 801394

Room 1322 Medical Research Building 5

Charlottesville, VA 22908

phone: 434-924-2652

fax: 434-982-0055

gko@virginia.edu

Abstract

Despite decades of research, our understanding of the processes controlling the stability of late-stage atherosclerotic plaques remains poor. However, a prevailing hypothesis is that reducing inflammation may improve plaque stability. Indeed, the potent inflammatory cytokine, interleukin (IL)-1β, has been shown to be a key driver of atherosclerosis development. Importantly, initial results of the CANTOS Trial demonstrated that administration of an anti-IL-1β antibody to high-risk post-myocardial infarction (MI) patients did not improve all-cause or overall cardiovascular mortality, only modestly reduced the incidence of non-fatal myocardial infarction (MI), and was associated with a 40% increase in death by lethal infection. A subsequent CANTOS report showed a 31% reduction in CV mortality and all-cause mortality in the subset of CANTOS subjects who achieved on-treatment hsCRP levels of <2.0mg/L. However, this patient cohort represents a small fraction of all patients at risk for cardiovascular disease, and extensive further investigation is needed to better understand the mechanisms by which IL-1β inhibition impacts established lesions. Therefore, we performed intervention studies on smooth muscle cell (SMC) lineage tracing mice with advanced atherosclerosis using anti-IL-1β or IgG control antibodies. Surprisingly, we found no effect on lesion size but impaired outward remodeling resulting in a decrease in lumen diameter. In addition, there was a profound shift in the composition of the fibrous cap characterized by multiple detrimental changes including reduced collagen and SMC content but increased macrophage content, which was primarily driven by opposite effects on the proliferation of these respective cell types. By generating SMC-specific and macrophage-selective IL-1 receptor type 1 knockout mice (*Il1r1* KO) crossed with *Apoe*$^{-/-}$ mice, we found that SMC-specific *Il1r1* KO resulted in a ~60% reduction in lesion size and lesions that were nearly devoid of YFP^{+} SMC whereas macrophage-selective loss of IL-1R1 had no effect on lesion size or cell composition. This suggests that SMC are a primary cell type responding to IL-1β during atherosclerosis and that IL-1 signaling in SMC is critical for their investment and retention within the fibrous cap. In addition, we found that inhibition of IL-1β promoted an expansion of the M2 macrophage population within the fibrous cap, and that these effects may be due in part to elevated levels of IL-4. Taken together, our results show that IL-1β can promote beneficial changes in late-stage murine

atherosclerosis by promoting maintenance of a SMC/collagen-rich fibrous cap. Moreover, studies identify critical cell types and pathways that need to be considered when attempting to develop safer and more effective anti-inflammatory therapies for widespread treatment of atherosclerotic disease, including in moderate or low risk patients.

Introduction

Atherosclerosis is a chronic vascular disease characterized by the formation of plaques enriched in lipids and inflammatory cells [1]. Despite decades of research, little is known about the factors and mechanisms controlling the stability of late-stage atherosclerotic lesions [2]. However, human pathology studies have consistently shown that the cellular composition of plaques – and most importantly of the fibrous cap, a structure that separates the thrombogenic lesion contents from the blood – is a critical predictor of plaque rupture. Specifically, plaques with a higher ratio of $CD68^+$ cells relative to $ACTA2^+$ cells, presumed to be macrophages (MΦ) and smooth muscle cells (SMC), respectively, are more prone to rupture [3-5]. Indeed, it has long been assumed that SMC play a protective role in atherosclerosis by being the primary cell type responsible for formation of an extracellular matrix (ECM)-rich fibrous cap. However, the markers used for identification of the lesion cells (e.g., ACTA2 and CD68) are not specific for SMC or MΦ during atherosclerosis, which raises questions as to the origin of these of cells, and more importantly their functional roles in late-stage complications [6].

Importantly, results of recent SMC lineage tracing studies from our lab [7,8] and others [9,10] have demonstrated that SMC play a much larger role during atherosclerosis than previously appreciated, which can be either beneficial or detrimental depending on their phenotypic state. *First*, we found that >80% of SMC-derived cells within advanced lesions lack expression of the conventional SMC marker gene, ACTA2. As such, previous studies that relied on using this marker have grossly underestimated the frequency of SMC-derived cells within the lesion. *Second*, nearly 50% of SMC within advanced

atherosclerotic lesions express markers of alternative cell types including MΦ (ACTA2^{-} MYH11^{-} LGALS3^{+} F4/80^{+} CD11b^{+}), mesenchymal stem cells (ACTA2^{-} MYH11^{-} SCA1^{+} CD105^{+}), and myofibroblasts (ACTA2^{+} MYH11^{-} PDGFβR^{+}) [8,9]. *Third*, contrary to the current dogma, we found that SMC can have beneficial or detrimental roles in lesion pathogenesis depending on the nature of their phenotypic transitions. For example, Klf4-dependent transitions, including formation of SMC-derived MΦ-marker^{+} foam cells [8] exacerbate lesion pathogenesis whereas Oct4-dependent transitions [7] are necessary for the formation of a SMC-rich, protective fibrous cap. Taken together, these results highlight the importance of identifying strategies that promote beneficial and/or prevent detrimental (i.e., plaque destabilizing) changes in SMC phenotype and function.

A dominant hypothesis in the field is that excessive inflammation or failed inflammation resolution is a major contributor to plaque development as well as late-stage lesion destabilization [11,12]. Indeed, there is extensive evidence that inflammation promotes atherosclerosis development (reviewed in [13]). For example, the inflammatory cytokine interleukin-1β (IL-1β) has been shown to play a major role during atherosclerosis development using global genetic knockout strategies. Specifically, knockout of *IL-1β* [14] or its functional receptor, IL-1 receptor type 1 (*Il1r1*) [15] reduces plaque formation, whereas knockout of the endogenous IL-1 receptor 1 antagonist (*IL1ra*) increases plaque development [16]. In addition, therapeutic inhibition of IL-1β using a neutralizing antibody (mouse gevokizumab) administered throughout Western diet (WD) feeding in *Apoe*$^{-/-}$ mice showed a reduction in overall plaque burden. However, there was no reduction of brachiocephalic artery (BCA) plaque area or increase in lumen area [17]. These studies strongly implicate IL-1β as a key factor promoting plaque development. However, there are currently no preclinical studies showing direct evidence that inhibition of IL-1β after the establishment of advanced atherosclerotic lesions induces beneficial changes. Importantly, the CANTOS trial tested three doses (50, 150, and 300-mg) of an anti-IL-1β antibody, canakinumab, administered quarterly to a very high-risk cohort of post-MI patients [18,19]. The trial met its primary endpoint (a composite of nonfatal MI, nonfatal stroke, and cardiovascular death). However, this occurred only at the 150-mg dose, was driven by a reduction in recurrent nonfatal MI but no reduction in the rate of cardiovascular death or stroke, and was associated with a 40% increase in deaths due to lethal infection [20]. A subsequent CANTOS report

showed a 31% reduction in CV mortality and all-cause mortality in the subset of CANTOS subjects who achieved on-treatment hsCRP levels of <2 mg/L but no effect in subjects whose hsCRP remained above 2 mg/L[21]. However, the patient cohort tested in CANTOS represents only a small fraction of all patients at risk for cardiovascular disease (i.e. those who have survived at least one heart attack and have normalized lipids but elevated hsCRP). As such, fundamental questions must be answered before canakinumab can be expanded to lower risk patients. What are the key determinants of IL-1β antibody efficacy and safety; were the effects driven by targeting inflammation on a systemic or local level; did the therapy protect against plaque erosion, rupture, and/or another thromboembolic mechanism; what was the effect on the function of key-cell types within the lesion that regulate late-stage lesion stability? As such, there is a critical need for mechanistic studies to define how inhibition of IL-1β impacts late-stage lesion pathogenesis.

Despite a clear role for IL-1 signaling in atherosclerosis development, previous studies have not clearly defined the key cell types that respond to IL-1β in atherosclerosis. However, two bone marrow chimeric studies suggest that IL1 primarily acts on non-hematopoietic cell types during atherosclerosis development [22,23]. Specifically, transplantation of *Il1r1*$^{-/-}$ bone marrow into wild-type mice had no effect on lesion formation, whereas, transplantation of wild-type bone marrow into *Il1r1*$^{-/-}$ mice resulted in significantly smaller lesions, suggesting that non-hematopoietic cell types including endothelial cells (EC) and/or SMC play a key role in mediating IL-1 signaling during atherosclerosis development. Interestingly, Sui and co-workers [10] showed that SMC-selective deletion of the IκB kinase IKK-β, which is required for activation of NFκB, markedly attenuated lesion development, suggesting a critical role for inflammatory signaling in SMC. The latter results are also consistent with several *in vitro* studies identifying IL-1β as a potent regulator of SMC migration and proliferation [24-26]. However, a direct role of IL-1 signaling in SMC during atherosclerosis pathogenesis has not been studied *in vivo*.

In the current study, we tested the hypothesis that IL-1β inhibition induces beneficial changes not only during fatty streak formation, but also in the setting of established late-stage atherosclerosis. To this end, we treated SMC lineage-tracing *Apoe*$^{-/-}$ mice with a mouse monoclonal anti-IL-1β antibody between 18 and 26 or

18 and 21 weeks of WD feeding. Contrary to our expectations, late-stage IL1β antibody treatment resulted in multiple detrimental changes including i) a rapid remodeling of the fibrous cap characterized by reduced collagen and SMC content but a large increase in MΦ; and ii) no decrease in lesion size but impaired beneficial outward vessel remodeling leading to reduced lumen diameter. Equally surprising, we found that MΦ-selective KO of the *Il1r1* in *Apoe*$^{-/-}$ mice had no discernible effects, but SMC-specific *Il1r1* KO resulted in lesions highly enriched in MΦ relative to SMC with failure to form a SMC-rich fibrous cap. As such, these studies reveal an unanticipated role for IL-1β in the formation and maintenance of a protective SMC/collagen-rich fibrous cap during late-stage lesion atherosclerosis.

Results

IL-1β neutralization decreased systemic and local inflammation in advanced atherosclerotic lesions

Although several studies have previously demonstrated that global disruption of IL-1 signaling inhibits lesion development in *Apoe*$^{-/-}$ mice [14-16], they may not predict the effects of acute inhibition of IL-1β on established atherosclerotic lesions [27]. To study the impact of IL-1β inhibition on advanced lesions, we performed intervention studies in which we administered an anti-IL-1β antibody (mouse monoclonal antibody; 10mg/kg) or an isotype matched IgG control (10mg/kg) for 8 weeks to SMC lineage tracing *Apoe*$^{-/-}$ mice (*Apoe*$^{-/-}$ *Myh11* Cre ER^{T2} R26R-YFP) that had been fed a WD for 18 weeks (**Fig 1a**; **Fig S1a**). Mice were fed the same WD during the atherosclerosis lesion development (8 to 26 weeks of age) and the IL-1β antibody treatment (26 to 34 weeks of age). SMC lineage tracing *Apoe*$^{-/-}$ mice were treated with tamoxifen between 6 and 8 weeks of age, prior to initiation of WD feeding, to permanently label mature MYH11^{+} SMC and to track their fate and that of their progeny during development and progression of atherosclerosis as well as during intervention with an anti-IL1β

antibody. We have previously shown that these mice exhibit high efficiency (>95%) of SMC-specific labelling by YFP expression when treated with tamoxifen from 6-8 weeks of age [8,28]. Treatment with the IL-1β antibody potently reduced systemic inflammation including a 50% decrease in the plasma concentration of Serum Amyloid-A (SAA) (**Fig 1b**), and similar reductions in IL-1β levels in plasma and liver (**Fig 1c**). Notably, liver and plasma IL-1α concentrations were unchanged (**Fig S1b**). IL-1β neutralization did not alter body weight or plasma cholesterol and triglyceride levels (**Fig S1c-e**). To unbiasedly assess the effects of IL-1β neutralization in atherosclerotic lesions, we performed RNAseq analyses on BCA and aortic arch regions from SMC lineage tracing *Apoe*$^{-/-}$ mice after 8 weeks of treatment with the IL-1β antibody or the IgG control. Analysis of transcript expression showed that IL-1β antibody treatment induced a global reduction in inflammation within lesions as illustrated by the large number of downregulated inflammatory pathways (**Fig 1d; Fig S2**). Interestingly, there was also downregulation of cell lineage and cell cycle regulatory pathways. The most upregulated pathways in IL-1β antibody treated mice included carbohydrate and energy metabolism (**Fig 1d**). To determine whether the antibody had direct neutralizing properties within the lesion, we assessed the IL-1β antibody penetration into atherosclerotic lesions using Rag1 deficient mice which do not produce mature T cells and B cells [29]. The use of *Rag1*$^{-/-}$ mice allows for rigorous detection of the mouse monoclonal IL-1β neutralizing antibody without interference of endogenous antibodies. The IL-1β antibody was detected at higher levels than the IgG control in atherosclerotic lesions from *Rag1*$^{-/-}$ *Apoe*$^{-/-}$ mice fed 26 weeks with WD suggesting a direct effect of the neutralizing antibody within atherosclerotic lesions (**Fig S1f**). Secondly, the local inhibition of the IL-1 signaling pathway was confirmed by immunostaining showing a significant decrease in phospho-IRAK and IL-6 in SMC lineage tracing *Apoe*$^{-/-}$ mice treated with the IL-1β antibody as compared to controls (**Fig 1e**). Taken together, these results demonstrate that the IL-1β antibody treatment regimen was effective at inhibiting the IL-1 signaling pathway, resulting in a decrease in both systemic and local plaque inflammation.

Neutralization of IL-1β induced multiple unexpected changes in late-stage atherosclerotic lesions

Despite effective inhibition of inflammation, analysis of atherosclerotic plaque burden and BCA plaque morphometry showed multiple unexpected results believed to be detrimental for plaque pathogenesis. IL-1β neutralization had no impact on aortic plaque burden (**Fig 2a,b**) or BCA lesion size (**Fig 2c,d**; **Fig S3**), but completely inhibited the beneficial outward vessel remodeling that normally occurs during plaque expansion (**Fig 2c,d**; **Fig S3**). Consequently, IL-1β antibody treated mice had significantly reduced lumen area (**Fig 2d**). Even more surprising, IL-1β antibody treatment resulted in a profound reduction in YFP^+ SMC and increase in $YFP^-LGALS3^+$ MΦ within the fibrous cap area (**Fig 2e**; **Fig S4**). This change in cell composition was particularly evident in the fibrous cap area (defined as the 30μm-thick subluminal area within the lesion which is typically enriched in YFP^+ and $ACTA2^+$ cells as previously reported [8]) (**Fig 2f,g**; **Fig S4b,c**). The increase in the $LGALS3^+$ population within the fibrous cap area was driven by an increase in the number of non-SMC derived $YFP^-LGALS3^+$ MΦ (i.e., bone marrow-derived or resident MΦ) whereas the number of SMC-derived MΦ-like cells (i.e., $YFP^+LGALS3^+$) was unchanged (**Fig 2g**). However, the proportion of $YFP^+LGALS3^+$ cells normalized to the total YFP^+ SMC population was significantly increased with IL-1β antibody treatment (**Fig 2h**), suggesting that $YFP^+LGALS3^+$ cells – in contrast to YFP^+ and YFP^+ACTA2^+ populations – might be resistant to loss or clearance following IL-1β neutralization.

The reduction in SMC content was associated with an overall decrease in the $ACTA2^+$ fibrous cap thickness (**Fig 2i**). Moreover, inhibition of IL-1β also induced a significant reduction in collagen content within the fibrous cap area, a key index of plaque stability (**Fig S5b,c**). This reduction of collagen content within the fibrous cap area could be a direct consequence of the loss of SMC, described in previous studies as a major producer of collagen within the lesion [30], but could also be due to IL-1β-induced upregulation of collagenases. There was no difference in the expression of MMP3 and MMP9 (**Fig S5d,e**), two collagenases known to be regulated by IL-1β [31]. However, further studies of collagenase activity would be required to ascertain the mechanisms by which inhibition of IL-1β leads to collagen loss (i.e., decrease in production and/or increase in degradation). Interestingly, these changes

to the fibrous cap area occurred despite having no effect on necrotic core area or intraplaque hemorrhage (**Fig S5f-h**), suggesting that there has not been effective lesion regression or complete resolution of lesion inflammation. Moreover, there was no difference in the lesion calcification (**Fig S6a,b**) nor in the proportion of YFP^+ SMC expressing RUNX2, a master regulator of SMC osteogenic transitions (**Fig S6c,d**).

The main features of the IL-1β inhibition described above were also observed when mice were treated with a lower dose of the IL-1β neutralizing antibody (1mg/kg) for 8 weeks (**Fig S7a**). Treatment with the IL-1β antibody at 1mg/kg induced a significant, but to a lesser extent, deficit of the beneficial outward remodeling (**Fig S7b,c**) and modification of the cell composition within the fibrous cap area including reduced YFP^+ SMC and increased $LGALS3^+$ MΦ (**Fig S7d,e**).

Taken together the preceding results unexpectedly show that IL-1β is critical for maintaining a SMC/collagen-rich, MΦ-poor fibrous cap area.

Macrophage accumulation was driven by local proliferation rather than increased monocyte recruitment

To better understand the mechanisms responsible for the enrichment of MΦ and loss of SMC following 8 weeks of IL-1β inhibition, we investigated the cell composition after 3 weeks of treatment. *Apoe*$^{-/-}$ *Myh11* Cre ER^{T2} R26R-YFP mice were treated with the IL-1β antibody (10 mg/kg) or the IgG control between 18 and 21 weeks of WD feeding (**Fig 3a**). Remarkably, just 3 weeks of IL-1β antibody treatment resulted in profound changes to the cellular composition of the fibrous cap characterized by an increase in the proportion of $LGALS3^+$ cells and reduction in YFP^+ SMC (**Fig 3b,c**). This shift reflected a net increase in the number of $LGALS3^+$ cells within the fibrous cap of mice treated with the IL-1β antibody (**Fig 3d**). The $LGALS3^+$ population was composed predominantly of $YFP^-LGALS3^+$ cells, whereas the SMC-derived $YFP^+LGALS3^+$ population was unchanged (**Fig 3e**). Furthermore, the increase in MΦ occurred predominantly in the fibrous cap area (**Fig 3b**). These data not only support a critical role for IL-1β in maintaining the integrity of the fibrous cap but also reveal that the fibrous cap area, as a structure, is much more plastic than previously appreciated.

To determine if the increase in LGALS3+ population was driven by increased bone marrow-derived monocyte recruitment into the plaque, we quantified monocyte influx using a fluorescent latex bead trafficking assay [32,33]. In brief, Cy3-labeled beads were injected intravenously into *Apoe*$^{-/-}$ *Myh11* Cre ER^{T2} R26R-YFP mice one week prior to initiation of antibody treatment (**Fig 4a**, **Fig S8a**). Mice injected with beads received either the IL-1β neutralizing antibody or IgG control antibody for 3 weeks. Flow cytometry analysis of blood collected 24 hours after the bead injection demonstrated efficient labeling of circulating monocytes consistent with previous reports (**Fig S8b,c**)[32-34]. Importantly, there were no changes in blood cell populations after the bead and antibody treatment (**Fig S8d**). BCA plaques from IL-1β antibody and IgG control treated mice showed no differences in bead accumulation (**Fig 4b,c**). However, IL-1β antibody treated mice exhibited a marked decrease in the ratio of beads to MΦ (**Fig 4c**), suggesting that the MΦ accumulation induced by IL-1β inhibition was not due to an increase in newly recruited monocytes.

To determine if the accumulation of MΦ was driven by proliferation, we performed immunostaining for Ki67 on mice treated with 3 weeks of IL-1β antibody or IgG control (**Fig 4d**). Although the overall fraction of proliferating cells was not significantly changed (**Fig 4e**), treatment with the IL-1β antibody profoundly changed which cell types underwent proliferation in the fibrous cap. Specifically, there was marked reduction in YFP+ SMC proliferation and an increase in MΦ (YFP- LGALS3+) proliferation (**Fig 4e,f**). However, there was no change in the proliferation of YFP+LGALS3+ cells indicating that the exacerbation of LGALS3+ proliferation did not include SMC-derived MΦ-like cells (**Fig 4f**). Of note, IL-1β inhibition did not induce changes in endothelial cell proliferation (**Fig S9**). We also assessed the proportion of YFP+ and LGALS3+ cells undergoing apoptosis in these same samples. TUNEL (**Fig 4g,h**) and cleaved Caspase3 (**Fig S10**) analyses showed no differences in apoptosis of SMC or MΦ in IL-1β antibody treated mice as compared to controls. Taken together, these data show that inhibition of IL-1β profoundly impacts the cell composition within advanced atherosclerotic plaques primarily by inhibiting SMC proliferation and promoting proliferation of MΦ within lesions.

IL-1 signaling is required for SMC investment into lesions and the fibrous cap

The preceding observations demonstrate that inhibition of IL-1β in mice with advanced lesions resulted in a dramatic reduction in SMC and increase in MΦ within the fibrous cap. However, it is unclear if these changes were mediated via direct loss of IL-1 signaling in SMC, MΦ, or other cell types within the lesions. Although there is extensive evidence that IL-1β promotes atherosclerosis development [14-16] and that non-myeloid cells play a key role [22,23], the relative contribution of IL1 signaling in SMC versus MΦ in this process is unknown.

Thus, to ascertain the role of IL-1 signaling in SMC on atherosclerosis development, we generated mice with tamoxifen-inducible SMC-specific *Il1r1* KO and simultaneous SMC lineage tracing by crossing $Il1r1^{fl/fl}$ mice [35] with $Apoe^{-/-}$ *Myh11* Cre ER^{T2} R26R-YFP mice (**Fig S11a**). The resulting $Apoe^{-/-}$ *Myh11* Cre ER^{T2} R26R-YFP $Il1r1^{fl/fl}$ and littermate control $Il1r1^{WT/WT}$ mice were treated with a series of tamoxifen injections from 6 to 8 weeks of age to induce simultaneous expression of a YFP lineage tracing gene, and excision of exon 5 of the *Il1r1* gene exclusively in SMC [8,28] (designated below as $Il1r1^{SMC\ \Delta/\Delta}$ or $Il1r1^{SMC\ WT/WT}$) before being fed a WD for 18 weeks (**Fig S11b**). We observed high efficiency recombination of *Il1r1* in SMC rich tissues including aorta, carotid, and lung in tamoxifen-treated $Il1r1^{SMC\ fl/fl}$ mice (**Fig S11c**). Importantly, there was no detectable *Il1r1* recombination in $Il1r1^{SMC\ fl/fl}$ without tamoxifen, or in tamoxifen-treated $Il1r1^{SMC\ WT/WT}$ control mice. In addition, recombination of the *Il1r1* locus was associated with corresponding reductions in IL-1R1 protein expression based on immunohistochemical staining of carotid medial SMC in $Il1r1^{SMC\ \Delta/\Delta}$ mice with an anti-IL-1R1 antibody (**Fig S11d**). There were no significant differences in body weight or serum cholesterol and triglyceride levels (**Fig S11e-f**). However, remarkably, we observed a >60% reduction in BCA plaque size in $Il1r1^{SMC\ \Delta/\Delta}$ mice as compared to $Il1r1^{SMC\ WT/WT}$ mice (**Fig 5a,b**), as well as a marked reduction in collagen content (**Fig 5c,d**) but no difference in intraplaque hemorrhage (**Fig 5e,f**). Of major interest, the lesions in the $Il1r1^{SMC\ \Delta/\Delta}$ mice were almost entirely devoid of SMC (YFP^+) and $ACTA2^+$ cells, but significantly enriched in YFP^- $LGALS3^+$ cells (**Fig 5g-i**). This was particularly evident in the fibrous cap area which exhibited an >80% reduction in YFP^+ area and a 70% decrease in $ACTA2^+$ area. In addition, fibrous cap areas from $Il1r1^{SMC\ \Delta/\Delta}$ mice had a 2.8 fold increase in $LGALS3^+$ area (**Fig 5i**).

We also tested the effect of MΦ-selective *Il1r1*$^{-/-}$ on atherosclerosis development using *Apoe*$^{-/-}$ *LysM* Cre R26R-YFP *Il1r1*$^{fl/fl}$ and *Apoe*$^{-/-}$ *LysM* Cre R26R-YFP *Il1r1*$^{WT/WT}$ littermate control mice fed WD for 18 weeks (**Fig S12a,b**). Importantly, this *LysM* Cre gene targeting results in efficient gene targeting of monocytes, mature macrophages and granulocytes [36]. LysM cre driven knockout of IL1R1 induced a complete inhibition of the IL-1 signaling pathway as demonstrated by the lack of IRAK phosphorylation in LGALS3^{+}YFP^{+} cells within atherosclerotic lesions of *Il1r1*$^{MΦ\ Δ/Δ}$ (**Fig S12e**). In contrast to the profound effects of SMC-specific *Il1r1* deletion, there was no significant effect of losing IL-1R1 in LysM expressing cells on vessel size, lesion size, or the frequency of YFP^{+}, LGALS3^{+}, or ACTA2^{+} cells (**Fig S13**). Taken together, these cell-specific *Il1r1* KO studies provide compelling evidence that IL-1 signaling in SMC, rather than myeloid cells, plays a critical role during atherosclerosis development. Moreover, results suggest that the reductions in indices of plaque stability observed in our IL-1β neutralization experiments, including the marked loss of SMC and collagen, as well as increases in MΦ within the fibrous cap, are due, at least in part, to disrupted IL-1 signaling in SMC.

IL-1β neutralization increased IL-4 levels and promoted macrophage M2 polarization

To determine potential mechanisms altering the cellular composition of the fibrous cap following IL-1β antibody treatment, we performed a cytokine and chemokine array on BCA tissue extracts from mice treated with 8 weeks of either IL-1β or control antibody (**Fig 6a**). Interestingly, there was a two-fold increase in IL-4 protein in BCA lesions from the IL-1β antibody treated mice. Of interest, IL-4 has been shown to inhibit SMC [37,38] but promote resident MΦ proliferation [39,40], consistent with our *in vivo* Ki67 staining data showing profound inhibition of SMC proliferation but stimulation of MΦ proliferation (**Fig 4**). IL-4 has also been identified as a key cytokine involved in M2 polarization of MΦ *in vitro* [41,42]. To better understand the impact of IL-1β neutralization on macrophage phenotype, we performed staining for classic M1 (iNOS) and M2 (Arg-1) markers. Although there are considerable limitations to the M1/M2 paradigm *in vivo*, confocal analyses of these markers provide the spatial resolution required to study individual cells within the fibrous cap area as well as critical insights into the possible function of the accumulated macrophages. Consistent with the increased IL-4, there was a decrease in the number

of LGALS3⁺iNOS⁺ , but a large increase in Arg-1⁺ expression in macrophages within the fibrous cap area (**Fig 6b,c**). These results suggest that IL-1β neutralization induces an anti-inflammatory phenotype in the resident MΦ population within the fibrous cap that is mediated, at least in part, by increased IL-4.

Discussion

Our present study provides compelling evidence of a critical role for IL-1 signaling during late-stage atherosclerosis. However, contrary to our expectations, IL-1 signaling appeared to play multiple beneficial roles that were inhibited following either IL-1β neutralization or specific loss of *Il1r1* in SMC. Indeed, we found that IL-1 signaling in SMC was required for SMC investment into lesions and for the formation of a SMC-rich, collagen-rich fibrous cap. That is, although lesions from SMC-specific *Il1r1* KO *Apoe*$^{-/-}$ mice were smaller, they exhibited a number of changes believed to be detrimental including a failure to form a protective ACTA2⁺ fibrous cap, a reduction in collagen content, and an enrichment in macrophage content. Moreover, consistent with these findings, IL-1β antibody treatment of SMC lineage tracing *Apoe*$^{-/-}$ mice with advanced atherosclerosis, for 3 or 8 weeks, resulted in marked reductions in a number of plaque stability indices. These changes included a 40% decrease in SMC content, a >50% decrease in ACTA2⁺ coverage, a 30% reduction in collagen content, and a 50% increase in MΦ (M2) content within the fibrous cap. Taken together, these observations have several important implications for the atherosclerosis field. **First,** our results indicate an unexpected role for IL-1β in regulating fibrous cap formation in advanced lesions and suggest that excessive inhibition of inflammation could potentially have detrimental effects. Indeed, Tabas and co-workers have long emphasized the importance of improving inflammation resolution, rather than suppression, as a key therapeutic approach for attempting to stabilize lesions [11,43,44]. **Second**, the fibrous cap is far more

plastic than anticipated, a finding that if true in humans, may have important implications for atherosclerotic disease management and development of more effective therapeutic approaches. **Third**, our results highlight the need for increased emphasis on intervention rather than prevention models in preclinical studies that are attempting to identify therapeutic agents that will benefit patients with advanced atherosclerosis [27]. The importance of this concept is made particularly clear when comparing a previous prevention study that treated *Apoe*$^{-/-}$ mice from the beginning of WD feeding with an IL-1β neutralizing antibody and showed reduced plaque formation [17], with our intervention study that showed no improvement on the same measurement but revealed changes consistent with plaque destabilization. Therefore, one cannot assume that pharmacological agents that are effective at preventing fatty streak formation in young animals when treatment is initiated prior to lesion development will necessarily have beneficial effects in the setting of established atherosclerosis.

One of the most striking observations following IL-1β neutralization in advanced lesions was the loss of SMC within the fibrous cap. Our data suggest this is driven by reduced SMC proliferation, which is consistent with previous studies demonstrating that IL-1β is a potent inducer of SMC proliferation *in vitro* [25,26]. Although reduced SMC proliferation after 3 weeks of treatment may be responsible for the overall reduction in SMC number seen with 8 weeks of treatment, another likely mechanism is impaired migration and/or homing of SMC into the fibrous cap. One possible mechanism involved in the cellular redistribution would be changes in the production or sensitivity to guidance cues. Consistent with this possibility, Moore and co-workers [45,46] have identified several MΦ chemo-attractant and chemo-repulsive proteins whose production by SMC could be regulated by IL-1β.

The loss of SMC occurred in conjunction with an increase in the number of MΦ after just 3 weeks of IL-1β antibody treatment. Interestingly, the accumulation of MΦ within the lesions was restricted to $YFP^-LGALS3^+$ cells. While SMC-derived MΦ-like cells ($YFP^+LGALS3^+$) were present in the fibrous cap of both IL-1β antibody and IgG control treated mice, their numbers were unchanged by treatment. Importantly, accumulation of MΦ was driven by increased local proliferation of resident MΦ

rather than increased monocyte recruitment from the bone marrow. These observations are consistent with studies by Swirski and co-workers [47] showing that proliferation of resident MΦ, rather than increased monocyte trafficking, regulates MΦ expansion in established lesions. However, our studies are the first to show that this is a function of IL-1β and that its neutralization appears to promote M2 polarization and proliferation. These effects could be due to a direct effect of inhibiting IL-1 signaling in MΦ. However, due to the complete lack of a phenotype in the MΦ-selective *Il1r1* KO studies, it likely involves a different pathway.

Of interest, we present correlative evidence suggesting that increased expression of IL-4 may contribute to reduced proliferation of SMC while simultaneously stimulating proliferation of resident MΦ as well as their M2 polarization. Indeed, consistent with this possibility, IL-4 has previously been shown to induce these changes in cultured SMC and macrophages respectively [37,39,40]. Although M2 MΦ have been shown to play a beneficial role in atherosclerosis pathogenesis, it remains to be determined whether an influx of M2 or M2-like MΦ into the fibrous cap would be beneficial or detrimental for lesion stability. However, our results suggest that this may be detrimental due to the loss of SMC and collagen, and that IL-1β inhibition may be associated with a false sense of inflammation resolution. Specifically, the global decrease in pro-inflammatory pathways measured by RNA-seq and the enrichment in anti-inflammatory-like macrophages occurs despite failure to resolve the necrotic core, reduce the extent of intraplaque hemorrhage or calcification, or to clear plaque cholesterol crystals[48]. This false-sense of inflammation resolution may also apply to SMC that appear to rely on IL-1β stimulation to form a SMC/collagen-rich fibrous cap. Indeed, several *in vitro* studies have shown that IL-1β potently induces SMC proliferation [25], promotes matrix metalloproteinase 3 (MMP3)-dependent SMC migration [31], and markedly induces SMC collagen synthesis [30], which are all processes that would be critical for maintaining a fibrous cap. In addition, global *Il1r1* deletion was associated with reduced collagen content and loss of an $ACTA2^+$ fibrous cap in advanced atherosclerotic lesions [31]. Finally, although excessive inflammation has been clearly linked with increased risk of cardiovascular disease

[49,50], there may be an ideal level of inflammation inhibition that supports the beneficial processes induced by IL-1β while suppressing the detrimental processes (e.g., endothelial cell activation).

A critical question is to determine to what extent, if any, our IL-1β antibody intervention studies in mice translate to patients, including those in large anti-inflammatory atherosclerosis clinical trials, and, most importantly, the Novartis-sponsored CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcomes Study) Phase III Clinical Trial that was recently completed [20]. This trial enrolled over 10,000 patients who were treated with canakinumab at three different dosages (50, 150, and 300mg) or placebo and tracked over a median period of 3.7 years. Importantly, CANTOS enrolled a unique patient population that is at very high risk for future cardiovascular events (>4.50 events per 100 person-years). Specifically, each patient had already survived at least one MI and had elevated biomarkers of inflammation (hsCRP≥2mg/L) despite standard of care therapy including high-intensity statin therapy in most cases [19]. Of major significance for the cardiovascular field, CANTOS met its primary endpoint of reducing a composite of nonfatal MI, nonfatal stroke, and cardiovascular death[20,21] thus providing the most direct evidence to date validating the inflammation hypothesis of atherosclerosis in man, and achieving a reduction in cardiovascular risk via a therapy independent of lipid lowering (see editorials by us[51] and others[52,53]). However, it is critical to appreciate that: 1) CANTOS met its primary endpoint only at the intermediate of three dosages tested; 2) there was no benefit on all cause or cardiovascular mortality; and 3) there was a 40% increase in death due to lethal infection[20]. Importantly, a subsequent CANTOS report showed a 31% reduction in CV mortality and all-cause mortality in the subset of CANTOS subjects who achieved on-treatment hsCRP levels of <2.0mg/L versus no effect in subjects whose hsSRP >2mg/L[21]. These latter results are quite exciting in that they indicate that Canakinumab may be an appropriate therapy, at least for this subset of very high risk post-MI normolipidimic patients, but would seem to be at complete odds with our findings that IL-1β antibody treatment of *Apoe*$^{-/-}$ mice with advanced lesions resulted in rapid loss of SMC and collagen within the fibrous cap (Figures 2-3).

However, there are major limitations in directly comparing the results of our mouse intervention studies with the outcome of the CANTOS Trial. **First**, there are inherent differences between humans and mice that confound direct translation of our results to clinical trials. These include differences in disease time-course (i.e., weeks in mice versus decades in humans), fundamental differences in the immune system, a lack of genetic diversity in mice, and much higher lipid levels in *Apoe*$^{-/-}$ mice which is necessary for lesion formation [54]. In addition, >80% of CANTOS subjects had a percutaneous coronary intervention or coronary bypass; >90% were taking antithrombotic, lipid-lowering, and/or anti-ischemic agents; and >79% were on inhibitors of the renin-angiotensin system. **Second**, it is possible that we are modeling fundamentally different mechanisms than those operative in CANTOS patients including effects of a prior MI that was not a component of our experimental design. **Third**, there are several studies reporting improved glycemic control and insulin sensitivity with inhibition of IL-1 signaling [10,55,56]. Therefore, patients treated for 3-5 years with canakinumab are likely to have long-term benefits on metabolic parameters as well as a reduction in microvascular inflammation and dysfunction, which are unlikely to be evident in our relatively short murine intervention studies. **Fourth**, the IL-1β antibody dosing regimens are very different between our mouse (weekly) and the CANTOS human (quarterly) studies due at least in part to the half-life of Canakinumab being over twice as long (27 days) as the mouse IL-1β antibody (12 days). However, we feel this is unlikely to be the key driver of the surprising effects given that we observed very similar effects with a ten-fold lower dose (1mg/kg) of the IL-1β antibody in our mouse studies, suggesting that our results are not a function of an overly aggressive dosing strategy and/or due to excessive suppression of IL1β. That being said, the results of CANTOS clearly show that the clinical benefit of canakinumab in reducing all-cause and cardiovascular mortality is highly dependent on the response of an individual patient to a given dosage, and more specifically to the degree of hsCRP lowering. The implication is that there may be a very narrow window where canakinumab is both effective and safe for a given patient especially given the marked increase in lethal infections among patients treated with canakinumab. **Fifth**, our mice are severely hypercholesterolemic whereas the CANTOS patients had relatively well managed cholesterol levels

(e.g., LDL cholesterol <83mg/dL). As such, our model in hypercholesterolemic mice most closely emulates cholesterol-driven plaque rupture events and our indices of lesion stability are derived from histological features of thin-capped fibroatheromas (plaques most vulnerable to rupture). However, it is possible that the CANTOS patients are more at risk for plaque erosion, rather than rupture, events. Indeed, CANTOS patients had been treated aggressively with lipid lowering agents (e.g., high-intensity statin treatment) in addition to canakinumab and had relatively well-controlled cholesterol levels. As such the current thought is that these patients have reduced plaque lipid content and are at greater risk for plaque erosion events [57], which our model poorly replicates. Unfortunately, the CANTOS trial did not differentiate non-ST elevation MI (more associated with plaque erosion) versus ST-elevation MI (more associated with plaque rupture).

Despite these limitations, our results provide novel insights as to how global suppression of IL1β impacts the composition of advanced lesions, may help explain why canakinumab treatment did not benefit the majority of patients treated, and/or may help to identify patients who might be harmed by this treatment. Indeed, our results remind us that inflammatory processes evolved to protect the host and that great care should be applied when targeting these pathways. A major challenge moving forward will be to determine to what extent canakinumab or other broadly acting anti-inflammatory agents should be extended to lower-risk patients. Specifically, it will be critical to determine strategies to identify subsets of patients that will exhibit the most benefit from these therapies, and perhaps more importantly to determine if there are patients that may be more susceptible to the adverse effects. In support of this hypothesis, two clinical studies are consistent with our findings and suggest that inhibition of IL-1 signaling may unexpectedly increase the risk of some cardiovascular events. **First**, the MRC-ILA Heart Study was a Phase IIb, double-blinded, randomized, placebo-controlled study of Anakinra, a recombinant IL-1 receptor antagonist (IL-1ra), administered for two weeks to patients following a non-ST elevation MI [58]. Although not powered to measure long-term outcomes, the study showed that Anakinra treatment increased major adverse cardiovascular events, including MI, at one year by greater than four-fold. **Second**, studies by the IL-1 Genetics Consortium [59] investigated two

common variants located upstream of the *IL-1ra* locus known to increase its expression. This retrospective study of 746,171 patients showed that increased levels of IL-1ra are correlated with reduced hs-CRP and IL-6, consistent with reduced systemic inflammation, but with an allele-dependent increase in coronary artery disease. As such, we believe the greatest value of our late-stage atherosclerosis intervention studies in mice is to generate hypotheses, which might be tested in man to better understand the mechanistic and physiological aspects of IL-1β inhibition. In addition, armed with a greater understanding of the basic vascular biology, the field can identify alternative (or combinatorial) anti-inflammatory therapeutic targets that are both effective and safe for treating a broader and lower risk cohort of human subjects.

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Author contributions

D.G. and G.K.O. originally conceived of and designed the experiments. D.G. performed experiments, analyzed data, performed statistical analysis and wrote the manuscript. R.B. designed and performed experiments, analyzed data, and contributed to the manuscript. B.G.D. performed collagen staining and analysis, DAB staining. A.A.C.N. performed and analyzed necrotic core and Ter119 data. G.F.A. analyzed the RNAseq dataset. S.A.M. performed immunofluorescent staining and analyzed data. C.S.H. performed Von Kossa staining and Immunofluorescent staining. A.W., W.M., S.E.F. and E.P. provided IL-1R1$^{fl/fl}$ mice. G.J.R. helped for the monocyte trafficking assay. All co-authors read the manuscript.

Competing interests

The authors declare no competing interests.

Data and materials availability

Further details regarding methods, data analyses, and materials can be found on the Owens laboratory website at https://www.cvrc.virginia.edu/Owens/protocols.html. The RNA-seq data is available at the NCBI Gene Ontology Omnibus (GEO) database under GEO accession number GSE111535.

Figure Legends

Figure 1: **IL-1β antibody treatment resulted in systemic and local downregulation of the IL-1 signaling and pro-inflammatory pathways.**

a – Schematic of intervention studies during which *Apoe*$^{-/-}$ *Myh11* ER^{T2} Cre YFP mice fed a WD for 18 weeks were injected weekly with the IL-1β antibody or an isotype-matched IgG control antibody at a concentration of 10mg/kg for 8 weeks. Assessment of systemic inflammation by quantification of plasma Serum Amyloid A (**b**) and IL-1β in plasma and liver (**b**) (n=12 per group). Data were analyzed with a non-parametric Mann-Whitney *U*-test (**b and c**) and presented as the mean ± SEM. **d** – Results of RNA-seq analyses on tissue extracts from the BCA of IL-1β antibody and IgG control treated mice (n=4 per group). Pathway-enrichment charts display pathways that were downregulated or upregulated in IL-1β antibody treated mice as compared to IgG control treated mice. The red lines indicate results are significant with an adjusted *P* value of $P_{adj} \leq 0.05$. Enrichment is shown as the $-\log_{10}$ of P_{adj} values. Underlined pathways are related to immunity and inflammation. **e** – Pie charts show functional annotation analyses of pathways downregulated (upper pie charts) or upregulated (bottom pie chart) in BCA extracts of IL-1β antibody treated mice as compared to control as determined by RNAseq analysis. **f** – Representative images of phospho-IRAK and IL-6 immunostaining in BCA sections of IgG control and IL-1β antibody treated mice. Scale bar, 100 µm. **g** – Quantification of phospho-IRAK and IL6 staining by integrated optical densitometry analysis. Data were analyzed by an unpaired t-test and presented as the mean ± SEM.

Figure 2: IL-1β inhibition induced multiple detrimental changes in the pathogenesis of late stage atherosclerotic lesions including loss of SMC-rich fibrous cap and inhibition of beneficial outward remodeling.

a – Sudan IV staining of aortic plaque burden. **b** – Quantification of Sudan IV positive area (n=6). **c** - Representative images of Movat stained BCA sections. **d** – Vessel, lesion and lumen area of BCA cross-sections from 18 week WD fed, 26 week WD fed IgG control treated, and 26 week WD fed IL-1β antibody treated *Apoe*$^{-/-}$ *Myh11* ER^{T2} Cre YFP mice (n=10 per group). **e** – Representative pictures showing modifications in the distribution of YFP^{+}, LGALS3^{+}, and ACTA2^{+} cells in the fibrous cap area of mice treated with the IL-1β antibody and IgG control. **f** – Quantification of YFP^{+} and YFP^{+}ACTA2^{+} cells normalized to the total number of cells within the fibrous cap area. **g** – Quantification of YFP^{-}LGALS3^{+} and YFP^{+}LGALS3^{+} cells normalized to the total number of cells within the fibrous cap area. **h** – Proportion of YFP^{+}LGALS3^{+} normalized to the SMC YFP^{+} population within the fibrous cap. **i** – Fibrous cap thickness (µm) as determined by the average thickness of ACTA2+ cells from the endothelial layer.

Data were analyzed by an unpaired *t*-test (**b**, **d**, **f-i**) and presented as the mean ± SEM. Scale bar, 100 μm (**c, e**).

Figure 3: Three-week IL-1β antibody treatment resulted in an increased number of macrophages within the fibrous cap.

a – Schematic of short-term treatment of *Apoe* $^{-/-}$ *Myh11* ER ^{T2} Cre YFP fed a WD for 18 weeks with weekly injections with IL-1β or IgG control antibodies at a concentration of 10 mg/kg for 3 weeks (n=8). **b** – Representative images of the modified distribution of YFP $^{+}$, LGALS3 $^{+}$, and ACTA2 $^{+}$ cells following 3 weeks of treatment with the IL-1β antibody. Scale bar, 100 μm. **c** – Proportion of YFP $^{+}$, LGALS3 $^{+}$ and ACTA2 $^{+}$ cells normalized to the total number of cells within the fibrous cap area. **d** – Number of cells normalized to the area of the fibrous cap (μm ^{2}). **e** – Quantification of YFP $^{-}$LGALS3 $^{+}$ cells versus YFP $^{+}$LGALS3 $^{-}$ cells within the fibrous cap. Data were analyzed by unpaired *t*-test (**c-e**) and presented as the mean ± SEM.

Figure 4: IL-1β inhibition for three-weeks resulted in increased proliferation of local macrophages but no change in monocyte trafficking or apoptosis.

a – Schematic representing the experimental design of bead uptake assays performed in *Apoe* $^{-/-}$ *Myh11* ER ^{T2} Cre YFP mice treated with IL-1β antibody or IgG control. (**B**) Representative images of bead uptake within the BCA lesions stained for LGALS3 $^{+}$ and visualizing Cy3-labeled beads. (**C**) Quantification of bead uptake with the total number of beads per section (left) and the number of beads normalized to the number of LGALS3 $^{+}$ cells (n=5). (**D**) Assessment of cell proliferation and representative images of staining for YFP, ACTA2, LGALS3 and Ki67 in BCA sections of IL-1β antibody treated and control mice. (**E**) Quantification of Ki67 $^{+}$ cells and Ki67 $^{+}$ subpopulations (Ki67 $^{+}$YFP $^{+}$ and Ki67 $^{+}$LGALS3 $^{+}$; n=6). Data were analyzed using an unpaired *t*-test (**E**) and a non-parametric Mann-Whitney *U*-test (**C**) and presented as average ± SEM. Scale bar, 100 μm (**B** & **D**).

Figure 5: **IL-1 signaling within SMC was required for SMC investment into the lesion and the fibrous cap.**

a – Movat staining of BCA sections from *Il1r1* $^{SMC\ WT/WT}$ and *Il1r1* $^{SMC\ \Delta/\Delta}$ littermates fed a WD for 18 weeks. **b** – Morphometric analysis of the vessel, lesion, and lumen area of *Il1r1* $^{SMC\ WT/WT}$ and *Il1r1* $^{SMC\ \Delta/\Delta}$ BCA cross-sections (n = 12 per group). **c** – Representative images of Picrosirius Red staining (polarized light). **d** – Collagen content quantification expressed as a percentage of lesion area (n = 8 per group). **e** – Representative images of Ter119 staining used to detect intraplaque hemorrhage. **f** – Distribution of intraplaque hemorrhage positive and negative lesions in *Il1r1* $^{SMC\ WT/WT}$ and *Il1r1* $^{SMC\ \Delta/\Delta}$ mice. **g** – Immunofluorescent staining of BCA sections from *Il1r1* $^{SMC\ WT/WT}$ and *Il1r1* $^{SMC\ \Delta/\Delta}$ mice for YFP,

ACTA2 and LGALS3. A high magnification image of the fibrous cap is delineated by a dashed line. **h-i** – Pixel quantification of YFP^+, $ACTA2^+$, and $LGALS3^+$ areas normalized to the lesion area (**h**) and the 30μm fibrous cap area (**i**) (n = 12 per group). Data were analyzed with an unpaired *t*-test (**b,d**), an exact Fisher test (**f**), or a non-parametric Mann-Whitney *U*-test (**h,i**) and presented as the mean ± SEM. Scale bar, 100 μm (**a,c,e,g**).

Figure 6: IL-1β inhibition was associated with polarization of fibrous cap macrophages to an M2 phenotype. a – A cytokine and chemokine array was performed on protein extracts from BCA of mice treated with IL-1β antibody for 8 weeks as compared to control (n=5 per group). Protein abundance of monocyte and macrophage colony stimulating factors (CSF2 and CSF3), monocyte chemoattractant CCL2, pro-inflammatory cytokine IL-6 and anti-inflammatory cytokines IL-10 and IL-4 are shown. **b** – Quantification of the number of $LGALS3^+iNOS^+$ and $LGALS3^+Arg\text{-}1^+$ cells normalized to DAPI or the total number of $LGALS3^+$ cells in BCA lesions of mice treated for 3 weeks with the IL-1β antibody or IgG control (n=6 per group). **c** – Representative images of immunostaining for ACTA2, LGALS3, M1 marker iNOS (upper panels) or M2 marker Arg-1 (lower panels). Scale bar, 100 μm. Data were analyzed using an unpaired *t*-test (**a,b**) or non-parametric Mann-Whitney *U*-test (**b**) and presented as the mean ± SEM.

Figure 1

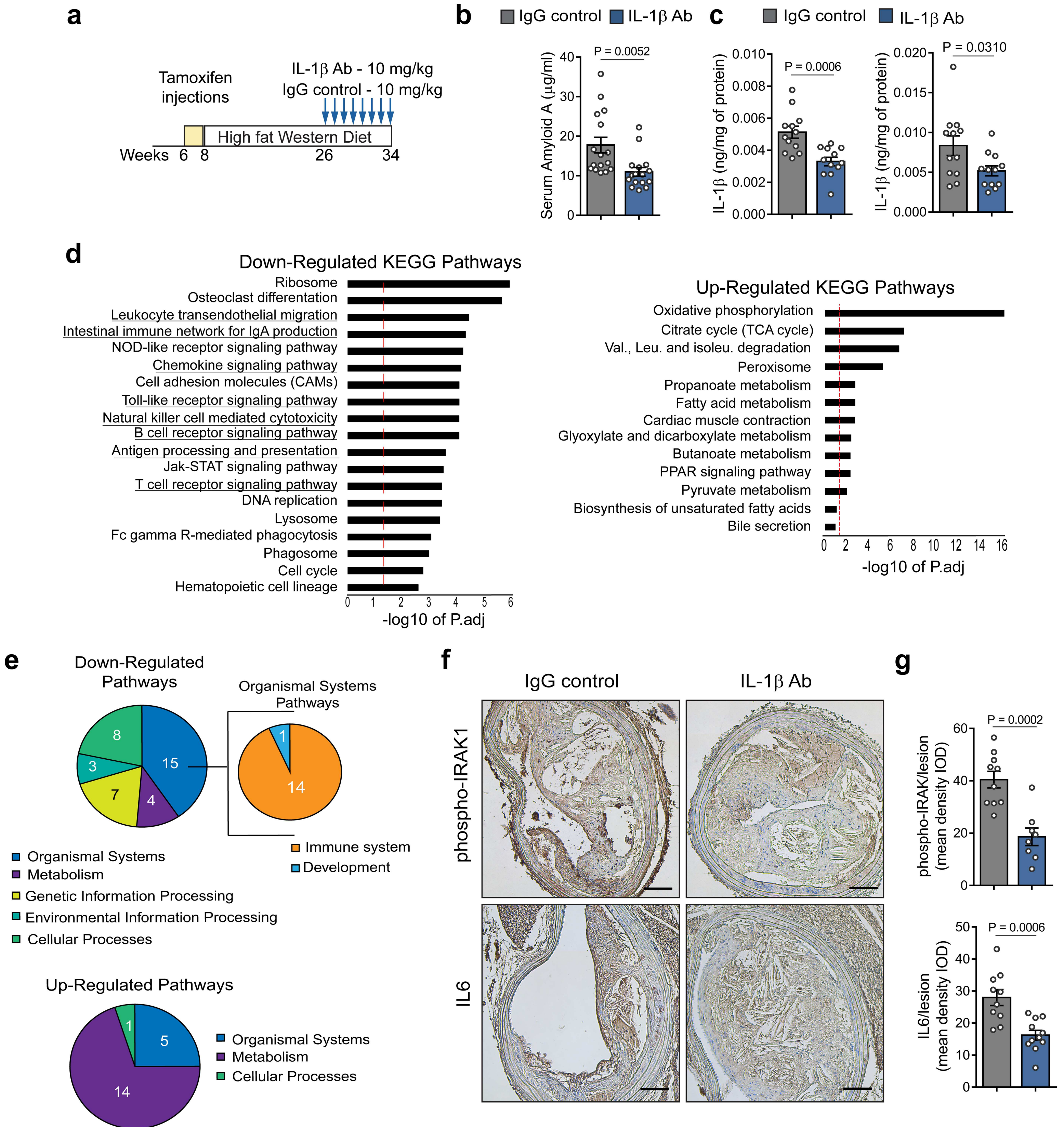
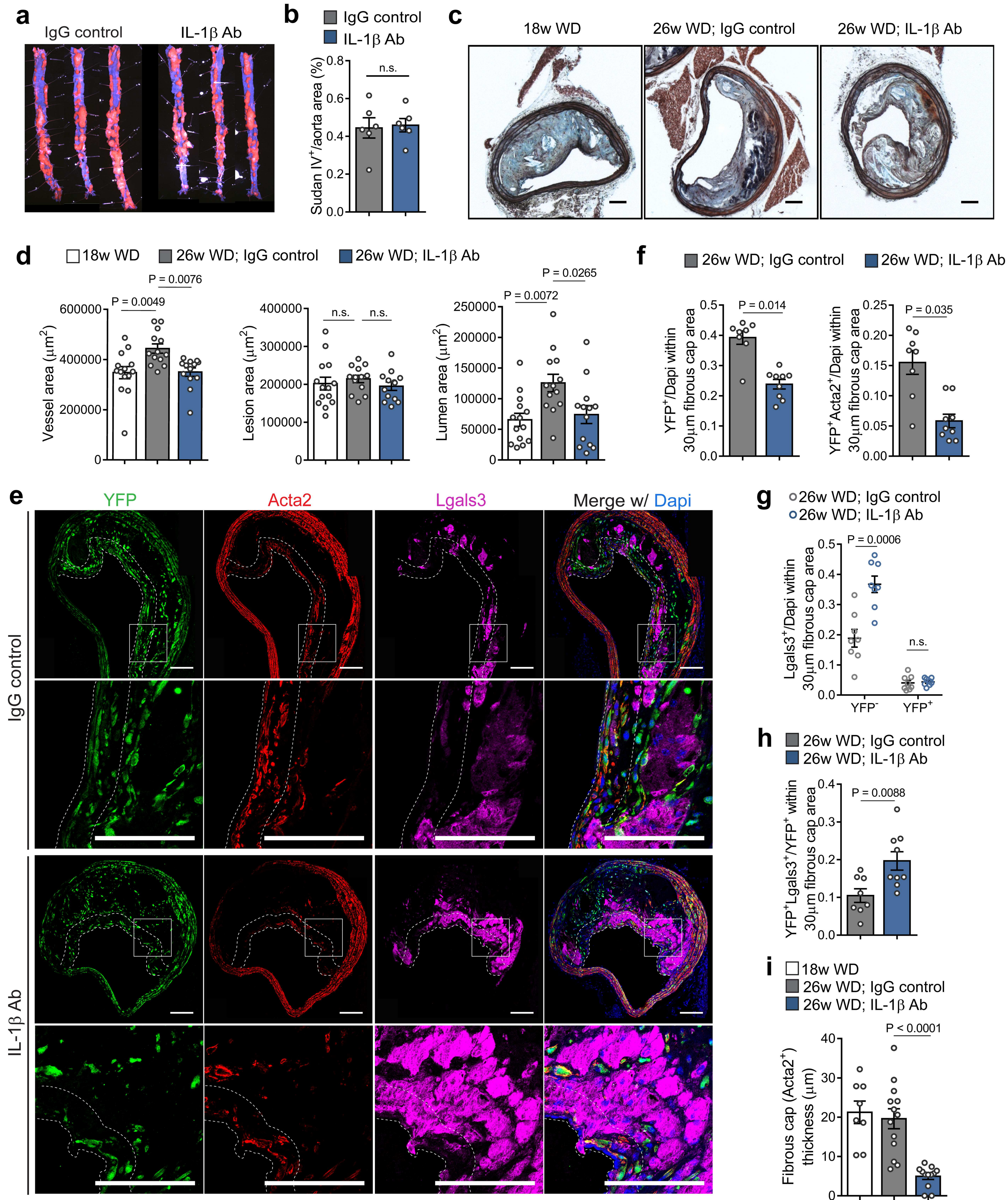


Figure 2



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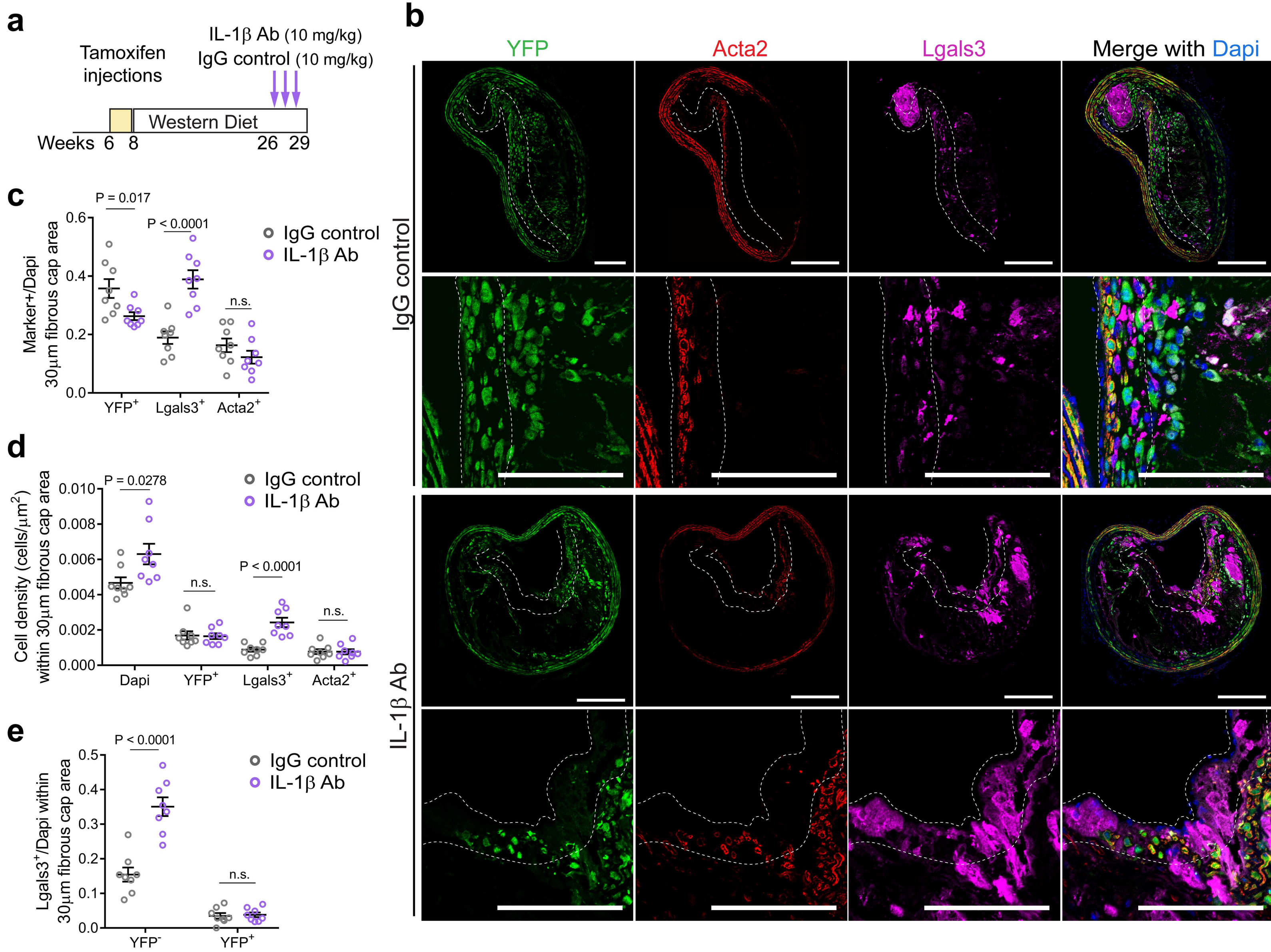

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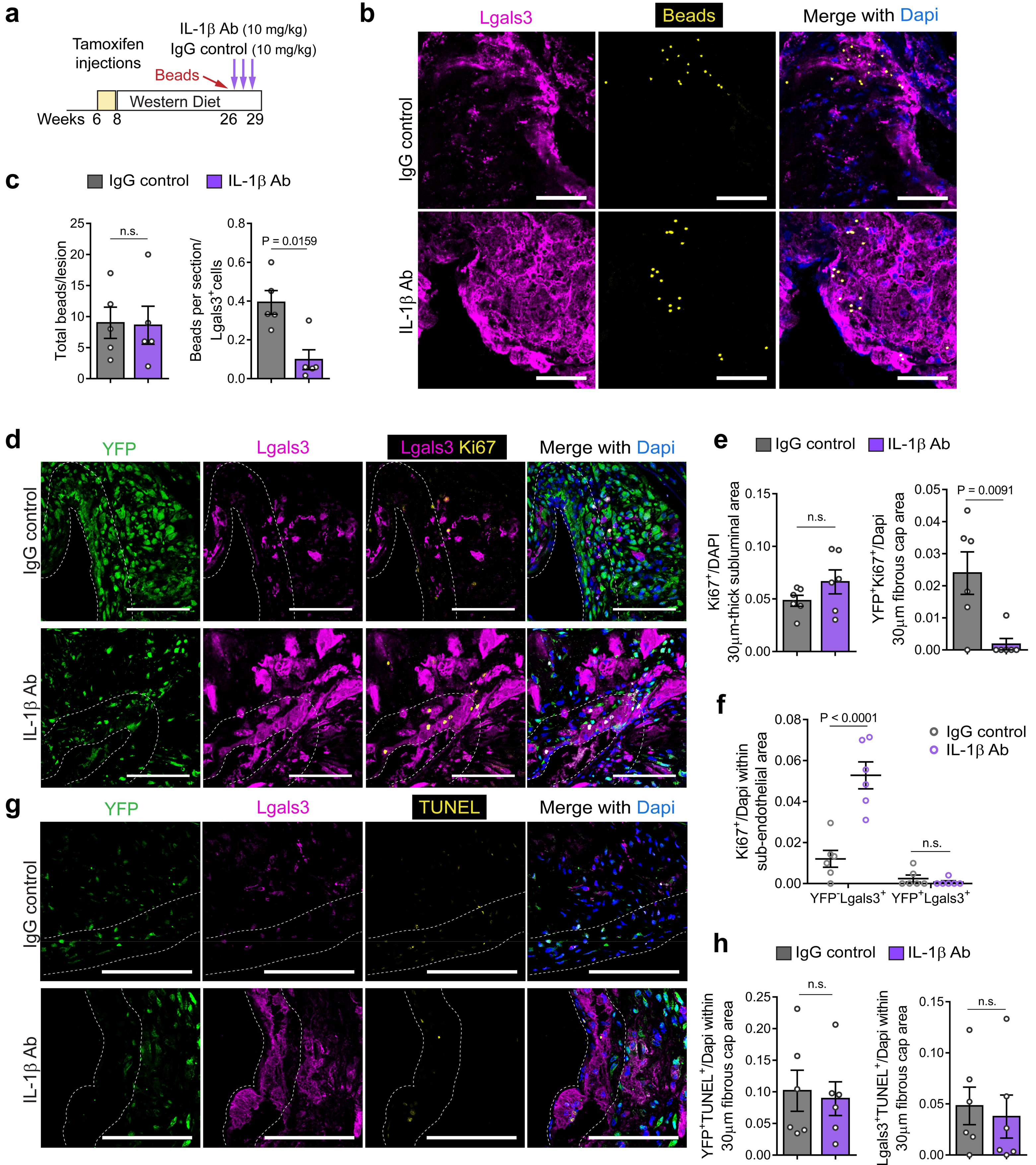

Figure 5

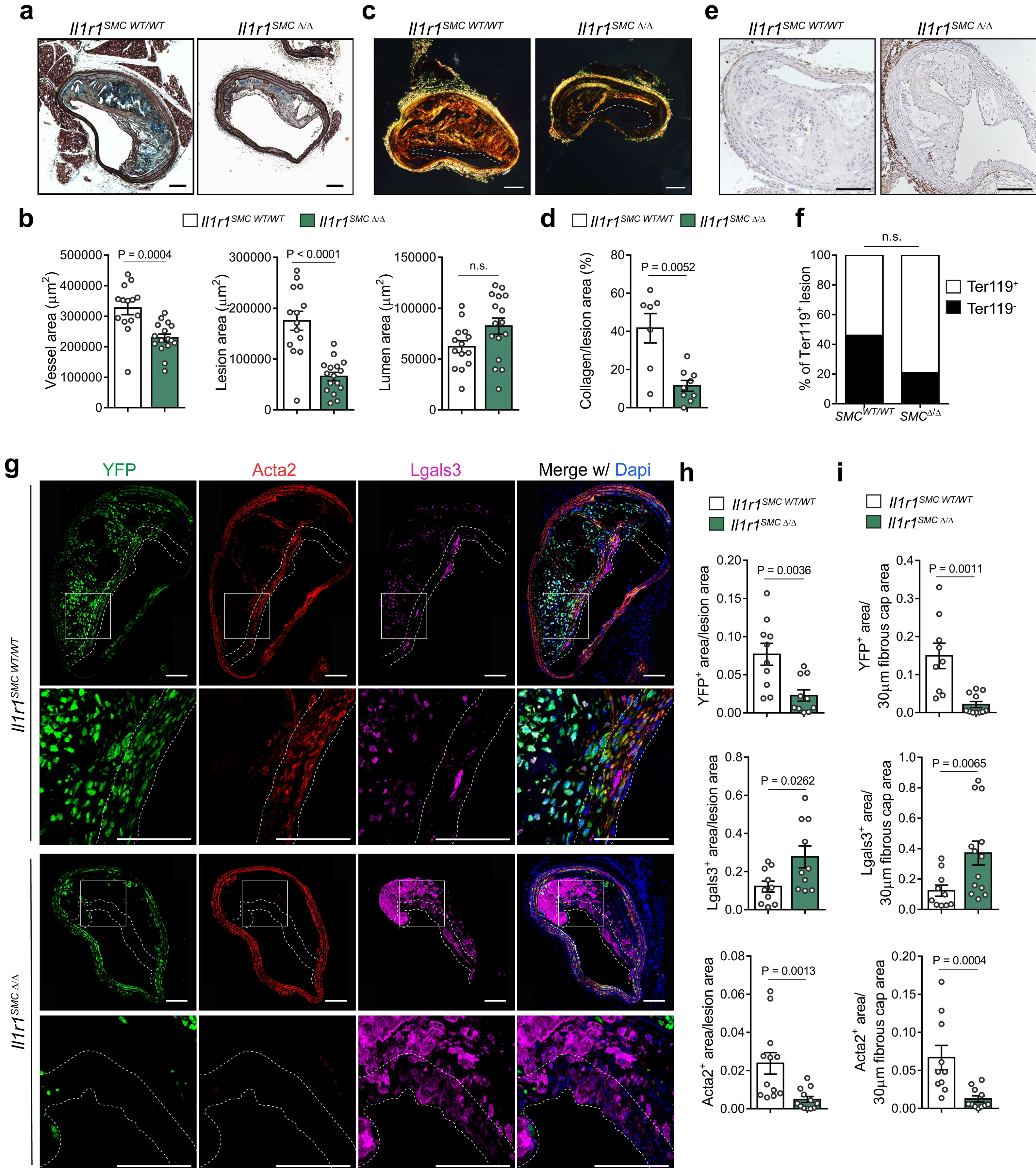

Figure 6