Cross Talk Between Vascular Smooth Muscle Cells and Monocytes Through Interleukin-1β/Interleukin-18 Signaling Promotes Vein Graft Thickening

Ping Li,* Yu-lin Li,† Zhen-ya Li, Yi-na Wu, Cong-cong Zhang, Xi A, Chun-xiao Wang, Hong-tao Shi, Mi-zhou Hui, Bo Xie, Mohammed Ahmed, Jie Du

Objective—Interleukin (IL)-1β and IL-18 are key proinflammatory cytokines that play important roles in the pathophysiology of vein graft remodeling. However, the mechanism of IL-1β/IL-18 production and its role in the development of graft remodeling remain unclear.

Approach and Results—IL-1β/IL-18 were rapidly expressed in venous interposition grafts. Vascular smooth muscle cell (VSMC) death and monocyctic inflammasome activation occurred in grafted veins. Necrotic VSMCs induced the expression of IL-1β, IL-18, and other inflammasome-associated proteins in monocytes, which was partially inhibited by their antagonist, recombinant IL-1ra-Fc-IL-18bp. Activated monocytes promoted proliferation of VSMCs by activating cell growth–related signaling molecules (AKT, STAT3, ERK1/2, and mTOR [AKT/protein kinase B, signal transducer and activator of transcription 3, extracellular signal-regulated kinase 1/2, mammalian target of rapamycin]) and increasing production of platelet-derived growth factor-bb; these effects were suppressed by IL-1ra-Fc-IL-18bp. Activated monocytes also promoted migration of VSMCs, which was independent of IL-1β/IL-18 signaling. Importantly, administration of IL-1ra-Fc-IL-18bp inhibited activation of cell growth–related signaling molecules, VSMC proliferation, and vein graft thickening in vivo.

Conclusions—Our work identified an interaction among necrotic VSMCs, monocytes, and viable VSMCs through IL-1β/IL-18 signaling, which might be exploited as a therapeutic target in vein graft remodeling.

Key Words: inflammasomes ■ interleukins

Bypass surgery with vein grafts remains the treatment of choice for patients with multivessel coronary artery disease. Unfortunately, arterialized veins are associated with a high incidence of late graft failure (≤50%–60% after 10 years) because of thrombosis, graft remodeling, and advanced atherosclerosis. Moreover, there is no effective strategy to prevent this clinical problem.

Vein graft remodeling is characterized by increased vein graft medial thickening and neointima formation. The arterializations of the vein graft conduit require medial thickening. Early vein graft wall thickening is an adaptive response; however, sustained medial thickening is pathological and is a cause of late vein graft failure. Medial thickening is a complex and dynamic process, including endothelial cell dysfunction, leukocyte infiltration, vascular smooth muscle cell (VSMC) apoptosis, migration, and proliferation. VSMC apoptosis/necrosis stimulated by a sudden elevation in mechanical stress is an early event that occurs after grafting. VSMC death promotes multiple features of graft remodeling, including early loss of VSMCs, elastin fragmentation and loss, and the inflammatory response. A previous study demonstrated that inflammatory cells migrate into the vessels and a chronic inflammatory response provokes arterial thickening. Several lines of evidence have identified monocytes as a primary leukocyte involved in graft failure. Studies using monocyte chemotactic protein 1–deficient and C-C motif chemokine receptor-2–deficient mice or anti–monocyte chemotactic protein-1 gene therapy showed inhibition of vein graft thickening. The degree and persistence of monocyte infiltration have a strong direct correlation with the magnitude of media thickening.

Interleukin (IL)-1β and its related family member IL-18 are proinflammatory cytokines that cause tissue injury by
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ASC</td>
<td>apoptosis-associated speck-like protein containing a caspase recruitment domain</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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inducing inflammation, which is involved in various cardiovascular diseases, and these cytokines exhibit additive or synergistic effects in promoting inflammatory diseases. IL-1β is markedly induced in vessels affected by balloon injury or carotid artery ligation. High levels of serum IL-18 are positively correlated with carotid intima-media thickness in patients. IL-1β/IL-18 signaling initiates transcription of various inflammatory genes through the activation of NF-κB (nuclear factor kappa B) and transcription activator-1, including chemokines, proinflammatory cytokines, adhesion molecules, and colony-stimulating factors, which facilitate leukocyte infiltration. The vascular inflammatory response involves complex interactions among inflammatory cells, endothelial cells, and VSMCs. Monocytes, VSMCs, and endothelial cells express IL-1β and IL-18 receptors. p80 IL-1 type I receptor null mice exhibit attenuated intimal hyperplasia after common carotid artery ligation, whereas IL-1Ra null mice show enhanced intimal formation. The marked upregulation of IL-1β/IL-18 in injury vessels and their pleiotropic effects on most cell types involved in vascular injury and remodeling suggest that manipulation of this signaling pathway could be an effective therapy for graft failure. Therefore, it is important to define what triggers activation of IL-1β/IL-18 and how IL-1β/IL-18-mediated signaling cascades are involved in vein graft remodeling.

IL-1β is the key product of inflammasome activation. Increasing evidence has indicated that endogenous danger signals released from damaged tissue activate the inflammasome, triggering the inflammatory response. It is unknown how the inflammasome is activated and whether secreted IL-1β/IL-18 plays a role in inflammasome activation, vascular inflammation, and remodeling. IL-1ra, a natural endogenous IL-1 antagonist, competes with both IL-1α and IL-1β at the receptor level to block IL-1R1 signaling. IL-18bp naturally exhibits a higher affinity for IL-18 than the IL-18 receptor and neutralizes IL-18 biological activities by >95%. A dual domain IL-1ra-Fc-IL-18bp fusion protein was constructed by joining IL-18bp and IL-1ra cDNA to the Fc fragment of human IgG1 cDNA in an expression plasmid, in which the carboxyl-terminal sequence binds to IL-1R and the amino-terminal segment binds to IL-18. Specificity of IL-1ra-Fc-IL-18bp for IL-1β/IL-18 was validated using technetium-99 m-labeled IL-1ra-Fc-IL-18bp in vivo. Single-photon emission computed tomographic imaging analysis showed that a high accumulation of technetium-99 m-IL-18bp-Fc-IL-1ra bound to increased IL-1β/IL-18 in 12-o-tetradecanoylphorbol-13-acetate–induced inflamed ears, but invisible radioactive uptake was observed in control ears. This finding suggests that IL-1ra-Fc-IL-18bp has a strong affinity for IL-1β/IL-18 released from inflammatory tissue. In the present study, we used recombinant IL-1ra-Fc-IL-18bp to investigate (1) the mechanism of IL-1β/IL-18 production; (2) the effect of IL-1β/IL-18 on cross talk between VSMCs and monocytes; and (3) the protective role of IL-1ra-Fc-IL-18bp in vein graft thickening.

Materials and Methods

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

Results

IL-1β and IL-18 Expression Is Increased in Murine Vein Grafts

To demonstrate the expression of IL-1β and IL-18 in murine vein grafts, bypass surgery was performed in wild-type mice. Both IL-1β and IL-18 mRNA expressions were significantly increased at 1 week and sustained at 2 weeks, then decreased at 4 weeks when compared with control veins (Figure 1A and 1B). As shown in Figure 1C, a large number of IL-1β- and IL-18–positive cells were found in the graft vein at 2 weeks and only a few IL-1β– and IL-18–positive cells were found at 4 weeks. Moreover, Mac-2 (a marker of macrophages)–positive cells were significantly increased in the grafted vein at 2 weeks (Figure 1C). A nonimmune, isotype-matched immunoglobulin was used as a negative control for monoclonal antibodies.

Figure 1. Interleukin (IL)-1β and IL-18 is induced in grafted vein. A and B, RNA samples were purified from control and grafted veins after the surgery of 0, 1, 2, and 4 weeks. IL-1β and IL-18 expressions were analyzed in vein by quantitative reverse transcriptase-polymerase chain reaction. *P<0.05 compared with vein at control vein (n=6–8 per time point). C, IL-1β, IL-18, Mac-2, and IgG protein expression in grafted veins or control veins. Representative photographs are shown (bar, 50 μm).
Necrotic VSMCs Activate the Monocytic Inflammasome and IL-1β/IL-18 Secretion in an IL-1β/IL-18–Dependent Manner

The first week after grafting a vein into an artery is characterized by massive death of VSMCs predominantly and monocytic infiltration in the vein graft wall.3,9 VSMC death drives inflammation and vessel remodeling.3 Our results showed that IL-1β/IL-18 expression was highest at 1 week after vein grafting. These findings suggested that an interaction occurs between the dead VSMCs and monocytes in grafted veins. Multiple breaks in the 3′-OH terminus were present in both apoptotic and necrotic cells.20 Because DNA fragmentation is common in different kinds of cell death, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) detection in situ labels necrotic and apoptotic cells in various tissues.21,22 Therefore, we performed a TUNEL assay and found more TUNEL+ nuclei in vein grafts at 1 week when compared with control veins (Figure 2A). Costaining revealed that the TUNEL+ cells were located under the endothelium and colocalized with α-SMA+ (alpha smooth muscle actin) VSMCs (Figure 2B and 2C).

To investigate whether the inflammasome is activated in grafted veins, expression of a key structural component of the inflammasome, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), was examined and was observed in the vein 1 week after implantation (Figure 3A). To identify the cell type for expressing ASC in the grafted veins, double-immunofluorescence staining was performed using antibodies against ASC and monocytes (CD11B). Most monocytes expressed ASC in the grafted veins (Figure 3B). These results suggested that there is a link between dead VSMCs and inflammasome activation. To identify this relationship, we treated human monocytic cell line (THP1) cells with necrotic VSMC debris to mimic the in vivo phenomenon. Quantitative reverse transcriptase-polymerase chain reaction analysis demonstrated that necrotic VSMC debris significantly increased the expression of the inflammasome components ASC and NACHT, LRR, and PYD domain–containing protein 3 (Nlrp3) in monocytes (Figure 3C). Both IL-1β and IL-18 secretion were significantly increased in the supernatants of VSMC debris-treated THP1 cells (Figure 3D). Expression of these factors was not spontaneously increased in THP1 cells alone (Figure 3C and 3D). There was also a significant increase in levels of ASC, cleaved fragment of caspase-1, and the cleaved form of IL-1β in necrotic VSMC debris-treated THP1 cells (Figure 3E).

Importantly, blockade of IL-1β/IL-18 signaling with recombinant IL-1ra-Fc–IL-18bp significantly suppressed inflammasome activation (Figure 3E). Taken together, these findings suggested that necrotic VSMCs promote monocytic inflammasome activation via an IL-1β/IL-18–dependent pathway at the early stage of vein graft remodeling.

Monocyte-Secreted IL-1β/IL-18 Activates Cell Growth–Related Signaling

Increased vein graft thickening is characterized by the proliferation and migration of SMCs and is a main cause of late vein graft failure. In addition to local VSMCs, neointimal SMCs are derived from other sources, including adventitial fibroblasts, stem cells, or bone marrow–derived progenitors, which actively participate in the pathogenesis of vein graft thickening via differentiation into VSMCs or stimulating proliferation of local VSMCs.23–25 To clarify whether IL-1β/IL-18 mediates cross talk between VSMC and monocytes, leading to vein graft thickening, we examined signal transduction pathways in VSMCs stimulated by monocyte-secreted IL-1β/IL-18. Phosphorylated protein microarray analysis was performed on VSMCs stimulated with necrotic VSMC-activated THP1 cells (THP1+) or control untreated THP1 cells (THP1con) in the presence or absence of IL-1ra-Fc–IL-18bp. As shown in Figure 4A, MAPK (mitogen-activated protein kinase) signaling (p-ERK1/2, involved in cell mitogenesis and survival), STAT3 signaling (involved in cell differentiation and survival), and PI3K/AKT/mTOR signaling (p-AKT, p-mTOR, and p-p70S6K; involved in cell growth) were increased in THP1+–stimulated VSMCs relative to that in THP1con–stimulated VSMCs.

![Figure 2](https://example.com/figure2.png)  
**Figure 2.** Necrotic vascular smooth muscle cells are induced in grafted vein. A–C, Vascular sections were obtained from grafted vein after 1 week of implantation or normal vein. The sections were immunohistologically analyzed by TUNEL staining (A), by costaining with TUNEL and antibody against CD31 (B) or alpha smooth muscle actin (α-SMA; C). Representative photographs are shown (bar, 75 μm).
of THP1<sub>const</sub>-stimulated VSMCs. The activation of these signaling molecules in VSMCs was suppressed by IL-1ra-Fc-IL-18bp. Western blot analysis verified that activated THP1 cells induced the phosphorylation of STAT3, AKT, mTOR, and ERK1/2 in VSMCs via secretion of IL-1β and IL-18 (Figure 4B and 4C). Importantly, activation of STAT3, AKT, mTOR, and ERK1/2 was decreased significantly in the vein grafts of IL-1ra-Fc-IL-18bp–treated mice at 4 weeks when compared with that in Control-Fc–treated mice (Figure 4D and 4E). We detected the activation of cell growth signals in VSMCs in the presence of PBS, Control-Fc, or IL-1ra-Fc-IL-18bp. We found no difference in these signals in the presence of control-Fc or PBS (Figure IA and IB in the online-only Data Supplement). These data suggested that monocyte-secreted IL-1β/IL-18 may regulate the proliferation of VSMCs.

**Monocyte-Secreted IL-1β/IL-18 Promotes VSMC Proliferation**

We next investigated the effect of monocyte-secreted IL-1β/IL-18 on VSMC proliferation. Several growth factors, including platelet-derived growth factor (PDGF)-bb and vascular endothelial growth factor (VEGF), have been implicated in abnormal VSMC proliferation. Western blot analysis increased in the supernatants of VMSCs cocultured with THP1<sub>act</sub> when compared with that of VMSCs cocultured with THP1<sub>const</sub> and the elevation in PDGF-bb was abolished by IL-1ra-Fc-IL-18bp (Figure 5A). VEGF production did not differ among the 4 groups. The levels of other factors, such as basic fibroblast growth factor, nerve growth factor-β, and hepatocyte growth factor, were low and not different between the groups.
VSMCs were treated with supernatants from VSMCs cocultured with THP1<sup>act</sup> or THP1<sup>con</sup> for 0, 12, or 24 hours with or without IL-1ra-Fc-IL-18, and the proliferation of VSMCs was analyzed by BrdU (5-bromo-2-deoxyuridine) assay. Consistent with the changes in activated intracellular proliferative signaling and PDGF-bb production, activated THP1 promoted the proliferation of VSMCs in a time-dependent and IL-1β/IL-18–dependent manner (Figure 5B). To confirm this observation, we evaluated the direct effects of recombinant IL-1β or rIL-18 on VSMCs proliferation. We treated VSMCs with different concentrations of rIL-1β or rIL-18 (0.01–10 ng/mL) for 48 hours and pulsed with BrdU for last 4 hours of the incubation period. Both rIL-1β and rIL-18 induced a marked and significant proliferation of VSMCs. A significant 2.51- or 3.20-fold increase in VSMCs proliferation was observed at 1 ng/mL of rIL-1β and rIL-18, respectively (Figure 5C and 5D). To evaluate the efficiency of IL-1ra-Fc-IL-18bp on blockade of the effects of IL-1β/IL-18, the proliferation of VSMCs induced with rIL-1β or rIL-18 (1 ng/mL) in the presence of various concentrations of IL-1ra-Fc-IL-18bp (0.001–100 μg/mL) was determined. IL-1ra-Fc-IL-18bp effectively inhibited rIL-1β– and rIL-18–induced proliferation of VSMCs in a dose-dependent manner, with a 50% inhibition concentration (IC50) value of 3.762 μg/mL (for rIL-1β) or 4.012 μg/mL (for rIL-18; Figure 5E and 5F). To investigate the specificity of IL-1ra-Fc-IL-18bp, we evaluated the effects of IL-1ra-Fc-IL-18bp on proliferation of VSMCs induced by PDGF-bb. The recombinant PDGF-bb
induced a marked proliferation of VSMCs (Figure IIA in the online-only Data Supplement). The proliferation of VSMCs induced with recombinant PDGF-bb in the presence of various amount of IL-1ra-Fc-IL-18bp (0.001–100 μg/mL) was determined. The IL-1ra-Fc-IL-18bp did not inhibited recombinant PDGF-bb–induced proliferation of VSMCs (Figure IIB in the online-only Data Supplement). These results demonstrated the efficiency and specificity of IL-1ra-Fc-IL-18bp for the inhibition of IL-1β/IL-18. We also analyzed the effect of monocyte-secreted IL-1β/IL-18 on VSMC migration and found that THP1 cells promoted VSMC migration, but this effect did not involve IL-1β/IL-18 signaling (Figure 5G and 5H). These results revealed that monocyte-secreted IL-1β/IL-18 activated proliferation-related signaling pathways in VSMCs and induced VSMC proliferation.

**IL-1ra-Fc-IL-18bp Treatment Does Not Affect Organ Function**

To determine whether IL-1ra-Fc-IL-18bp treatment (5 mg/kg, IV injection daily for 8 weeks) results in organ toxicity, we examined the hearts, livers, lungs, kidneys, and spleens of IL-1ra-Fc-IL-18bp–treated and control mice that underwent subsequent vein grafting surgery. Evaluation of histological sections of these organs did not show detectable pathological
Abnormalities on hematoxylin and eosin staining (Figure IIIA in the online-only Data Supplement). Hematoxylin and eosin-stained sections showed normal muscle morphology in the heart; normal hepatic lobular architecture in the liver; normal alveoli in the lung; normal glomeruli, proximal, and distal tubules, and interstitium in the kidney; and normal follicles and vascular sinusoïds in the spleen in both groups. We next assessed organ-associated toxicity by measuring the organ function of control or IL-1ra-Fc-IL-18bp–treated mice. Liver function tests (aspartate transaminase, alanine transaminase, total bilirubin, and direct bilirubin levels) and renal function tests (blood urea nitrogen and creatinine levels) were similar between IL-1ra-Fc-IL-18bp–treated and control groups (Table I in the online-only Data Supplement).

Administration of IL-1ra-Fc-IL-18bp Inhibits Vein Graft Thickening

To study the effect of inhibition of IL-1β/IL-18 on vein graft thickening in vivo, mice were intravenously injected with IL-1ra-Fc-IL-18bp daily, beginning 1 day before vein graft surgery. First, we evaluated cell survival and inflammasome activation at 1 week after vein grafting in mice that received IL-1ra-Fc-IL-18bp or control-Fc treatment. TUNEL+ cells were seen in the vein grafts of both IL-1ra-Fc-IL-18bp and control-Fc groups, with no significant difference between the groups (Figure 6A). The numbers of ASC-positive cells were significantly decreased in the vein grafts of IL-1ra-Fc-IL-18bp–treated mice when compared with those of control mice (Figure 6B). These results suggested that inhibition of IL-1β/IL-18 has no effect on cell death but inhibits inflammasome activation in grafted veins, consistent with the in vitro data. Then, we evaluated the effect of IL-1ra-Fc-IL-18bp on vein graft remodeling. Vein grafts collected at 4 and 8 weeks were examined for morphological changes. Hematoxylin and eosin staining and Elastica van Gieson staining indicated that the graft wall was markedly thinner in the vein grafts of IL-1ra-Fc-IL-18bp–treated mice than in control-treated mice (Figure 6C and 6D). Vein grafts from IL-1ra-Fc-IL-18bp–treated mice had a significantly larger lumen area and thinner wall when compared with those in control-treated mice (Figure 6E and 6F). To test whether the Fc fragment itself has any effect on vascular remodeling, we compared the vein graft thickening in mice that received PBS or control-Fc administration. After 4 and 8 weeks of vein graft implantation, hematoxylin and eosin staining and Elastica van Gieson staining indicated that the thickness of the graft wall (lumen area and thinner walls) was not different in the vein grafts of PBS or control-Fc–treated mice (Figure IVA–IVD in the online-only Data Supplement). To test the effect of IL-1ra-Fc-IL-18bp on VSMC proliferation in vivo, proliferating cell nuclear antigen (PCNA)-positive cells were evaluated in grafted veins of control and IL-1ra-Fc-IL-18bp–treated mice 4 weeks after vein graft implantation. The numbers of PCNA-positive cells were significantly decreased in the vein grafts of IL-1ra-Fc-IL-18bp–treated mice compared with those in control mice (Figure 6G).

Discussion

The major findings of this study are (1) IL-1β, IL-18, and ASC were significantly expressed in grafted veins and necrotic VSMCs stimulated inflammasome activation in monocytes and release of IL-1β/IL-18, which was partially IL-1β/IL-18 signaling dependent; (2) cross talk between viable VSMCs and activated monocytes via IL-1β/IL-18 signaling augmented cellular proliferation as revealed by activation of cell growth–related signaling and elevation of PDGF-bb production; and (3) IL-1ra-Fc-IL-18bp treatment suppressed the activation of cell growth–related signaling, VSMC proliferation, and vein graft thickening in a murine vein graft model. To the best of our knowledge, the present study indicates for the first time that IL-1β/IL-18 signaling plays an important role in the mutual cross talk between VSMCs and monocytes, including monocyctic inflammasome activation and VSMC proliferation, during the progression of vein graft thickening, and that pharmacological inhibition of IL-1β/IL-18 has protective effects against vein graft thickening. These findings clarify the molecular events that occur during initial vein injury and remodeling after vein graft implantation and identify IL-1β/IL-18 and the inflammasome as novel therapeutic targets for vein graft remodeling. Increasing evidence has indicated the importance of inflammation in the pathophysiology of vascular injury and remodeling, and interventions targeted against inflammatory mediators substantially reduce neointimal hyperplasia in grafted veins. In particular, blockade of IL-1β/IL-18 reduces vascular injury and intimal formation, suggesting that IL-1β/IL-18 is a key mediator of the pathophysiology of vein injury/remodeling after implantation. Our results demonstrated that IL-1β/IL-18 expression was increased in vein grafts, and that blockade of IL-1β/IL-18 signaling suppressed vein thickening.

Sterile inflammation is triggered by endogenous danger signals released from damaged tissue and is mediated by inflammasome activation. Mechanical stress-induced VSMC death is one of the earliest cellular events that occur in vein grafts, which evokes an inflammatory response. We found that the VSMCs within grafted veins became apoptotic within the first week of implantation. However, multiple cytokine array results revealed that apoptotic VSMCs did not release IL-1β. The apoptotic cells that are not efficiently cleared by phagocytosis by local defenses (VSMCs) or bone marrow mobilization (neutrophils and monocytes) undergo necrosis and release danger signals. It was reported that IL-6 and monocyte chemotactic protein-1 were released from viable VSMCs stimulated by necrotic VSMCs, with no significant production of IL-1β and IL-18, suggesting that necrosis does not activate the inflammasome in necrotic VSMCs and necrotic VSMCs do not activate the inflammasome in viable VSMCs. Thus, we hypothesized that the effect of necrotic VSMCs could paracrine stimulate inflammasome activation in monocytes. We provided evidence that an inflammasome protein (ASC) expressed by infiltrating monocytes in grafted veins and necrotic VSMCs induced inflammasome activation in monocytes (increased ASC, Nlrp3, and cleaved caspase-1 expression) and secretion of IL-1β (including cleaved-IL-1β) and IL-18. Moreover, we demonstrated that blockade of the IL-1β/IL-18 signaling pathway by IL-1ra-Fc-IL-18bp suppressed necrotic VSMC-induced
inflammasome activation, including decreased expression of ASC, cleaved caspase-1, and cleaved IL-1β. These findings highlight that the monocytic inflammasome is activated by necrotic VSMCs, leading to the release of IL-1β and IL-18, and this process is at least partially dependent on the IL-1β/IL-18 signaling pathway. Necrosis is characterized by the disruption of the plasma membrane, organelle breakdown, and release of endogenous damage-associated molecular patterns. Damage-associated molecular patterns, such as extracellular ATP, S100 proteins, double-stranded DNA, and high mobility group protein-1, are known to trigger inflammasome activation.33,34 Necrotic cell debris contains broken membrane pieces, damaged organelles, and double-stranded DNA fragments. For example, membrane vesicles from necrotic endothelial cells contain active oxidized phospholipids, which induce monocyte activation.35 Damaged mitochondria from necrotic cells activate monocytes.36 Genomic double-stranded DNA from dying cells induces inflammation in immune or nonimmune cells.37–39 Thus, multiple damage-associated molecular patterns from the necrotic cell
Synergy between mechanical stress and inflammation results in the proliferation and migration of VSMCs, which eventually causes medial thickening. Our and other studies indicated that interactions between tissue cells (VSMCs and fibroblasts) and infiltrating leukocytes (monocytes/macrophages) promote the pathological process in cardiovascular disease.40-43 In the present study, we elucidated the role of IL-1β/IL-18 in cross talk between VSMCs and monocytes and the molecular basis by which IL-1β/IL-18 induces proliferation and migration of VSMCs. Proliferative signals, such as AKT, mTOR/p70-S6K, ERK1/2, and STAT3 in VSMCs, were stimulated by necrotic cell–activated THP1 cells. IL-1β and IL-18 use the same downstream signaling molecules and activate NF-kB translocation. IL-1/IL-18 receptors also induce activation of other signaling cascades, such as the PI3K/AKT, MAPK, and STAT3 pathways in other cells.44,45 Our results showed that suppression of IL-1/IL-18 signaling by IL-1ra-Fc-IL-18bp significantly inhibited the activation of AKT, mTOR, ERK1/2, and STAT3, as well as VSMC proliferation in vitro and in vivo, which indicated that activated monocytes induced VSMC proliferation through IL-1β/IL-18 signaling.

Moreover, activated monocytes indeed induced PDGF-bb expression via IL-1β/IL-18. PDGF-bb is one of the most potent mitogens and chemoattractants for VSMCs. 46 In response to PDGF-bb stimulation, SMCs from the saphenous vein exhibit enhanced proliferation when compared with the internal mammary artery, contributing to venous graft failure.47 Several studies have demonstrated that inhibition of PDGF-bb suppresses pathological vascular remodeling. Ferns et al48 discovered that administration of a PDGF-neutralizing antibody inhibited balloon catheter injury-induced neointimal VSMC accumulation in the carotid artery. PDGF-β receptor antibodies inhibit intimal hyperplasia induced by balloon catheterization.49 Nanoparticle-mediated delivery of imatinib mesylate, a PDGF receptor tyrosine kinase inhibitor, significantly inhibits neointima formation and VSMC proliferation in rabbit vein grafts.50 Suramin, a growth factor antagonist, inhibits neointima hyperplasia through inhibition of PDGF receptor activation in murine vein grafts.51 Therefore, PDGF-bb might be a mediator of activated monoocyte-induced VSMC proliferation, which leads to vein graft thickening.

The activity of IL-1 and IL-18 signaling is tightly regulated by IL-1ra and IL-18bp, respectively.16,17 The balance of free IL-18/IL-18bp and IL-1α/IL-1ra affects the severity of inflammatory diseases.52,53 Transgenic mice overexpressing human IL-18bp produce high levels of bioactive circulating IL-18bp, which protects against inflammatory stimuli.54 The absence of IL-1ra allows unopposed action of IL-1, resulting in life-threatening systemic inflammation.55 We demonstrated for the first time that the administration of IL-1ra-Fc-IL-18bp inhibited vein graft thickening after implantation, thus highlighting the protective effect of IL-1ra-Fc-IL-18 against vein graft remodeling. Moreover, the administration of IL-1ra and IL-18bp has an excellent safety profile. In the present study, no pathological abnormalities or organ-associated toxicity was observed in multiple organs of mice that received IL-1ra-Fc-IL-18bp treatment for 8 weeks. It should be noted that the current mouse vein graft models hold disadvantages and bear little resemblance to the human scenario. After vein grafting in this murine model, most of the cells die and are repopulated by recipient cells from the circulation and surrounding tissues.23,56 Therefore, this murine model is not same as human vein grafts; however, in the present study, we used this model to address the important role of IL-1/IL-18, inflammation, and neointima formation, which are the main events of human venous bypass graft remodeling.

In conclusion, our study revealed a novel mechanism involving cross talk between VSMCs and monocytes that induced inflammasome activation and amplification of VSMC proliferation via IL-1β/IL-18 signaling, which function as critical inductor molecules for vein graft remodeling. Therefore, the IL-1β/IL-18 pathway may constitute a novel therapeutic target to interrupt/attenuate the inflammatory process and vascular remodeling associated with vein grafting.

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Disclosures
None.

References
The sustained vein graft thickening remains the main cause of late vein graft failure. Inflammation has a strong direct correlation with the magnitude of media thickening. However, it is unknown how the inflammatory process leads to vein graft thickening. This study finds an upregulation of interleukin (IL)-1β and IL-18 in monocyte that infiltrate into the vein graft vessel wall that is mediated by necrotic vascular smooth muscle cells (VSMCs), via inflammasome activation. Furthermore, we show that the cross talk between necrotic VSMCs and monocytes augments VSMC proliferation and consequently vein graft wall thickening. Treatment with a fusion protein IL-1ra-Fc-IL-18bp strongly inhibit inflammasome activation, activation of cell growth signaling, VSMC proliferation, and vein graft thickening in vivo in a mouse model. Our work identified an interaction between necrotic VSMCs and monocytes and viable VSMCs through IL-1β/IL-18 signaling might be exploited for therapeutic target in vein graft remodeling.

**Significance**

The sustained vein graft thickening remain is the main cause of late vein graft failure. Inflammation has a strong direct correlation with the magnitude of media thickening. However, it is unknown that how the inflammatory process leads to vein graft thickening. This study finds an upregulation of interleukin (IL)-1β and IL-18 in monocyte that infiltrate into the vein graft vessel wall that is mediated by necrotic vascular smooth muscle cells (VSMCs), via inflammasome activation. Furthermore, we show that the cross talk between necrotic VSMCs and monocytes augments VSMC proliferation and consequently vein graft wall thickening. Treatment with a fusion protein IL-1ra-Fc-IL-18bp strongly inhibit inflammasome activation, activation of cell growth signaling, VSMC proliferation, and vein graft thickening in vivo in a mouse model. Our work identified an interaction between necrotic VSMCs and monocytes and viable VSMCs through IL-1β/IL-18 signaling might be exploited for therapeutic target in vein graft remodeling.
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Supplemental Materials

Supplemental Figure I. Effect of PBS, control-FC or IL-1ra-Fc-IL-18bp on monocyte induced cell-growth intracellular signaling in VSMCs

A-B, VSMCs were stimulated necrotic VSMCs activated THP1 (THP1act) in the presence of PBS, control-FC, IL-1ra-Fc-IL-18bp. The intracellular signaling was detected with western-blot. *p<0.05 compared with THP1act+PBC.

Supplemental Figure II. The effect of IL-1ra-Fc-IL-18bp on rPDGF-bb-induced VSMCs proliferation

A, VSMCs were treated with rPDGF-bb (10ng/ml) or control for 48 hours and pulsed with BrdU for last 4 hours of the incubation period, then performed BrdU assay. *p<0.05 compared with no rPDGF-bb treatment. B, Proliferation of VSMCs induced with rPDGF-bb (10ng/ml) in presence of...
various amounts of IL-1ra-Fc-IL-18bp (0.001-100 μg/ml) was determined. Data are from 3 independent experiments.

Supplemental Figure III. IL-1ra-Fc-IL-18bp treatment fails to reveal any detectable pathologic abnormalities

A, H&E staining of paraffin-embedded sections of the heart, liver, lung, kidney and spleen from untreated and IL-1ra-Fc-IL-18bp-treated groups of mice. There were no observable histopathologic differences in these tissues. (Bar=50μm). Representative photographs are shown (n=6 to 8 mice per group).

Supplemental Table I. Plasma biochemical measurement of control or IL-1ra-Fc-IL-18bp treated mice after vein graft surgery

<table>
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<tr>
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<th>Control-Fc</th>
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</table>

Values are mean±SEM. n=5.

Alanine transaminase (ALT); Aspartate transaminase (AST); Total bilirubin (TBIL); Direct bilirubin (DBIL); Creatinine (Cr) Blood urea nitrogen (BUN).
Supplemental Figure IV. Effect of PBS or control-FC on vein graft thickening

A-B. Section from PBS and control-FC treated mice on weeks 4 and 8 of surgery were stained with H&E. Pictures of ×100 (Bar=200 μm) magnification were presented. Staining for Elastica van Gieson were presented (B, Bar=50 μm). C-D, The lumen area and wall thickness were measured and calculated. (n=5 mice per group).
Materials and Methods

**Vein graft surgery**

We used 10-to 12-week-old C57BL/6 male mice. Mice were maintained in specific pathogen-free conditions and given free access to food and water. The experiment was approved by Animal Care and Use Committee of Capital Medical University. A venous interposition was placed in the carotid artery as described previously. In brief, the caval veins from a donor mouse was grafted between 2 ends of a carotid artery by “sleeving”. In the recipient, the right carotid artery was dissected and cut in the middle. A polyethylene cuff was placed at both ends of the carotid artery. At the ends, the carotid artery was everted around the cuff and ligated. The grafts was sleeved over the cuffs and secured with 8.0 silk sutures. Pulsations and turbulent blood flow within the graft confirmed successful engraftment. At the end points of control and treatment groups, the vein grafts were harvested after 5 minutes in vivo perfusion with ice-cold PBS followed by 4% paraformaldehyde perfusion-fixation to reserve the best of circumstances of grafted vein. For the IL-ra-Fc-IL-18bp treatment, mice were intravenously injected with IL-1ra-Fc-IL-18bp (5mg/kg, gift from Dr. Mizhou Hui) every day, beginning 1day prior to vein graft surgery. Control mice were injected with human IgG1-Fc (5mg/kg, every day) or PBS.

**Cell culture and stimulation**

Freshly discarded human saphenous veins were obtained from the patients undergoing coronary artery bypass surgery with ethical approval from ethical committee of Anzhen Hospital affiliated Capital Medical University. Primary human VSMCs were grown from human saphenous vein segments and cultured in collagen-coated culture dishes as previously reported. VSMCs were grown in DMEM (high glucose; Hyclone, Cramlington, UK) supplemented with 10% fetal bovine serum 10% fetal bovine serum (FCS) (Hyclone), penicillin (100 U/ml, Invitrogen, Illkirch, France) and streptomycin (100 mg/ml, Invitrogen) at 37°C in the presence of 5% CO2. Only early passages of VMSCs (passage 3-5) were used in our experiments. Human monocytic leukemia cell line (THP1) cells were cultured in RPMI-1640 medium (Hyclone), 10% FCS, penicillin and streptomycin. Necrotic VMSCs were induced by 500μM H2O2 stimulation for 8 hours, which based on the previous study that 500μM H2O2 treatment induced cell necrosis not apoptosis. After treatment of VSMCs with H2O2, the residual H2O2 was decomposed by the 15 mins incubation with 30μg/ml catalase based on a previous study, then replaced with the new fresh medium. Cellular homogenate was first centrifuged at 300 x g to remove unbroken
cells. The supernatant was next centrifuged at 15,000 × g. The pellets were resuspended and immediately used for stimulating THP1 cells.

**Quantitation of H$_2$O$_2$**

The presence of H$_2$O$_2$ in the resuspended necrotic VSMCs was determined by quantitative measurement with an Amplex Red H$_2$O$_2$ assay kit (Invitrogen). Necrotic VSMCs supernatant (50 μL) was added to a 96-well microplate containing 100 μmol/L Amplex Red and 0.2 U/mL horseradish peroxidase. The plate was incubated for 30 mins while being protected from exposure to light. Absorbance was measured spectrophotometrically at 560 nm. No H$_2$O$_2$ residues are detected in the necrotic VSMCs debris.

**Inflammasome activation**

For experiments, THP-1 cells were cultured at 1 × 10$^6$ cell/ml and then stimulated with necrotic VSMCs debris for 12 and 24 hours. IL-1ra-Fc-IL-18bp (10μg/ml) was added into THP-1 culture media prior to stimulation of necrotic VSMCs debris. The control THP1 cells were incubated under the same conditions without the addition of necrotic VSMCs debris or IL-1ra-Fc-IL-18bp. THP-1 cells were harvested for further qRT-PCR and western blot.

**VSMCs proliferation**

Human VSMCs were seeded at 1 × 10$^6$ cells/ml and growth arrested by culture in 0% FCS for 48 hours. VSMCs were incubated with activated THP1 cells (pretreatment with necrotic VSMCs for 24 hours) or control THP1 cells at the ratio (1:1) for 1 hour. VSMCs lysates were harvested for phosphorylated protein microarray analysis and western-blot to detect the intracellular signaling. The supernatant of co-cultured VSMCs-THP1 cells was collected for detection of growth factors and for further experiment. IL-1ra-Fc-IL18bp (10μg/ml) was also added to VMSCs media prior to the addition of THP1 cells to check the effect of IL-1β/IL-18 on THP-1 proliferation. Human VSMC were seed at 3,000 cells/well in 96-well plates and then stimulated with the 600 μl conditioned media from VSMCs cocultured with THP1$_{act}$ or THP1$_{con}$ for 12 hours and 24 hours. 10uM BrdU were added to culture media and VSMC BrdU uptake was determined by using the Cell Proliferation ELISA, BrdU assay (Millipore Corporation, Temecula, CA ) as manufacturer’s protocol. BrdU incorporation into DNA is measured by photometric analysis using a microplate reader (Bio-Rad; Hercules, CA) and is quantified by optical density (λ). Results represent experiments done in three separate donors performed in triplicate.
**VSMCs migration**

VSMCs migration was performed using a microchemotaxis chamber containing apolycarbonate filter (Transwell) with 8 \( \mu \)m pores. \( 1 \times 10^4 \) VSMCs were added to the upper chamber and the lower chamber was filled with 600 \( \mu \)L of conditioned media from VSMCs cocultured with THP1_{act} or THP1_{con} for 24 hours with or without IL-1ra-Fc-IL-18bp (10\( \mu \)g/ml). After 5 h, VSMCs on the upper side of membrane were removed and inserts fixed in ice-cold paraformaldehyde for 20 min. VSMCs were stained with DAPI and viewed on Nikon Eclipse TE2000-S microscope (Nikon, Japan). The number of VSMCs that migrated to the lower surface of the filter was counted in 10 fields from triplicate experiments.

**Western-blot analysis**

Total protein was isolated from cells or tissues using lysis buffer solution supplemented with a protease/phosphotase inhibitor cocktail (1%) and PMSF (Sigma, St. Louis, MO). Lysis extracts were sonicated and after centrifugation, supernatants were collected and protein content measured (BCA assay, Pierce). Proteins were denatured by boiling (95°C, 5min), separated by SDS-PAGE and transferred to nitrocellulose membranes. For THP-1 lysates, the membranes were immunoblotted with primary anti-caspase-1, anti-IL-1β and anti-ASC/TMS1 antibodies (Abcam, Cambridge, MA). For VSMCs, the membranes were immunoblotted with primary anti-p-AKT, anti-p-STAT3, anti-p-mTOR and anti-p-ERK1/2 antibodies (Cell Signaling Technology, Danvers, MA). Proteins were then detected with appropriate secondary rabbit polyclonal antibody (Sigma Aldrich) conjugated to horseradish peroxidase followed by enhanced chemiluminescence (ECL; Fisher, Illkirch, France). After stripping, the membranes were reprobed with primary anti-total-AKT antibody, anti-total-STAT3, anti-total-mTOR, anti-total-ERK1/2 antibodies (Cell Signaling Technology). Actin served as loading control for all western-blot.

**Cytokines measurements**

The supernatants were analyzed in the following ELISAs: IL-1β and IL-18 (R&D Systems, Minneapolis, MN). The assays were performed following the manufacturer’s specifications, and absolute cytokine levels were calculated based on comparison to assay performance in the presence of known quantities of recombinant cytokine standards. The growth factors were measured by mouse Procarta Immunoassay Kit according to the manufacturer’s protocol (Affymetrix, USA). For the protein quantification assays, Bio-PlexTM 200 System (Bio-Rad, USA) was used to analyze the concentration of each cytokine in each sample.
**Phosphorylated protein microarray assay**

Intracellular signaling screening was performed with PathScan® Intracellular Antibody Array kit purchased from Cell Signaling Technology as manufacturer’s protocol. Briefly, smooth muscle cell lysates were prepared with 1X Cell Lysis Buffer and diluted to 0.2 – 1.0 mg/ml in Array Diluent Buffer. After affixing the multi-well gasket to the glass slide, 75 μl diluted lysate was added to each well covered with sealing tape, followed by 2-hour incubation at room temperature on an orbital shaker. After serial washing, 75 μl 1X Detection Antibody Cocktail was added to each well, followed by 1-hour incubation at room temperature on an orbital shaker. After serial washing, 75 μl 1X DyLight 680®-linked Streptavidin was added to each well, followed by 30-minute incubation at room temperature on an orbital shaker. After serial washing, fluorescent image of air-dry slide was captured by LICOR-Odessey fluorescent digital imaging system. Spot intensities were quantified with LICOR commercially available array image analysis software.

**Quantitative real-time PCR**

Total RNA was extracted by the Trizol reagent method (Invitrogen). Aliquots of 2μg of total RNA were used for first-strand cDNA synthesis with M-MLV reverse transcriptase (Promega, Madison, USA). Quantitative real-time PCR (qPCR) was performed with an iCycler IQ system (Bio-Rad, USA) as previous describle. Relative mRNA levels of IL-1β and IL-18 were calculated after normalization to GAPDH; relative mRNA levels of NLRP3 and ASC were calculated after normalization to β-actin. The primer sequences were described:

**IL-1β:** 5′-CTTCCCCAGGGCATGTTAAG-3′, 5′-CTTCCCCAGGGCATGTTAAG-3′;

**IL-18:** 5′-GCCATGTCAGAAGACTCTTGCGTC-3′; 5′-GTACAGTGAAGTGCCGAGCAGAAG-3′;

**GAPDH:** 5′-GGTTGTCTCTGCGACTTCA-3′; 5′-GGTGCGCTCCAGGGTTTCTTACTC-3′;

**ASC:** 5′-AACCCAAGCAAGATGCGGAAG-3′; 5′-TTAGGGCTGGAGGAGCAAG-3′

**NLRP3:** 5′-ATGCCAGGAAGACAGCATGTG-3′; 5′-TCATCGAAGCCGTCCTGAGT-3′

**β-actin:** 5′-CTGGGACGACATGGGAGAAA-3′; 5′-AAGGAAGGCTGGAGAAGTGC-3′

**Histology and immunohistochemistry**

Mice were euthanized with an overdose of sodium pentobarbital and excised grafted vein, heart, kidney, lung, liver and spleen were embedded in paraffin and sectioned at 5μm thick. The sections of heart, kidney, lung, liver and spleen were stained with hematoxylin-eosin (H&E) staining. The vessel sections were performed with H&E and elastica van gieson staining. Images were captured under a Nikon.
Eclipse TE2000-S microscope (Nikon, Japan). Vein graft wall thickening (defined as the thickness between lumen and adventitia), and lumen diameter area was measured in H&E -stained sections was analyzed using Image Pro Plus 3.0 (Nikon). For immunohistochemical analysis, the paraffin-embedded vessel sections were incubated with primary antibodies against ASC, IL-1β and IL-18 (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with secondary antibodies. The reaction was developed using the DAB substrate and the sections were then counterstained with hematoxylin. For immunofluorescence, frozen vessel sections were labeled with primary antibody against mouse ASC and CD11B (Abcam), and then incubated with FITC-and TRITC–conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Images were captured by the use of a Nikon Eclipse TE2000-S microscope (Nikon, Japan).

**TUNEL Staining**

The frozen vessel sections from control or grafted vein was analyzed by the terminal deoxynucleotidyl-transferase–mediated dUTP nick-end labeling (TUNEL) assay according to the manufacturer’s protocol (Promega, Madison, USA). Then the sections was performed the immunofluorescence assay against CD31 (Abcam) or α-SMA (sigma) antibody.

**Biochemical assays**

Blood was collected by cardiac puncture. ALT and AST activities in plasma samples were measured by commercially available kits. Plasma samples were assayed for Cr and BUN in milligram per deciliter by means of an autoanalyser (RA 1000; Technicon Instruments, NY, USA).

**Statistical Analysis**

All data are presented as means ± SEM. The normality of distribution was checked by the Kolmogorov-Smirnov test prior to 1-way ANOVA statistical tests. All of variables were normally distributed. Statistical analysis involved 1-way ANOVA followed by Bonferroni test for selected pairs with use of GraphPad Prism 4.01 (GraphPad Software Inc., San Diego, CA). P<0.05 was considered statistically significant.

**Reference**


