Role of KCa3.1 Channels in Macrophage Polarization and Its Relevance in Atherosclerotic Plaque Instability

Rende Xu,* Chenguang Li,* Yizhe Wu, Li Shen, Jianying Ma, Juying Qian, Junbo Ge

Objective—Emerging evidence indicates that proinflammatory macrophage polarization imbalance plays a key role in atherosclerotic plaque progression and instability. The calcium-activated potassium channel KCa3.1 is critically involved in macrophage activation and function. However, the role of KCa3.1 in macrophage polarization is unknown. This study investigates the potential role of KCa3.1 in transcriptional regulation in macrophage polarization and its relationship to plaque instability.

Approach and Results—Human monocytes were differentiated into macrophages using macrophage colony-stimulating factor. Macrophages were then polarized into proinflammatory M1 cells by interferon-γ and lipopolysaccharide and into alternative M2 macrophages by interleukin-4. A model for plaque instability was induced by combined partial ligation of the left renal artery and left common carotid artery in apolipoprotein E knockout mice. Significant upregulation of KCa3.1 expression was observed during the differentiation of human monocytes into macrophages. Blocking KCa3.1 significantly reduced the expression of proinflammatory genes during macrophages polarization. Further mechanistic studies indicated that blocking KCa3.1 inhibited macrophage differentiation toward the M1 phenotype by downregulating signal transducer and activator of transcription-1 phosphorylation. In animal models, KCa3.1 blockade therapy strikingly reduced the incidence of plaque rupture and luminal thrombus in carotid arteries, decreased the expression of markers associated with M1 macrophage polarization, and enhanced the expression of M2 markers within atherosclerotic lesions.

Conclusions—These results suggest that blocking KCa3.1 suppresses plaque instability in advanced stages of atherosclerosis by inhibiting macrophage polarization toward an M1 phenotype. (Arterioscler Thromb Vasc Biol. 2017;37:226-236. DOI: 10.1161/ATVBAHA.116.308461.)

Key Words: apolipoprotein ■ atherosclerosis ■ KCa3.1 channel ■ macrophage polarization ■ plaque instability

Atherosclerotic plaque instability, which results in plaque rupture and thromboembolism, is a primary cause of acute coronary syndrome and sudden cardiac death. Emerging evidence indicates that myeloid lineage cells are the main cellular components within atherosclerotic lesions and play a critical role in the induction and progression of atherosclerotic inflammation, which contribute to plaque destabilization. Macrophages within atherosclerotic plaque respond to stimuli from the microenvironment and exhibit functional heterogeneity and plasticity. Macrophages can be broadly classified into proinflammatory (M1) or anti-inflammatory (M2) phenotypes on the basis of the cytokine environment that is created by 2 different classes of T helper cells (Th1 or Th2, respectively). Classically activated M1 macrophages, induced by Th1 cytokines such as interferon (IFN)-γ, are the most prominent macrophages at sites of inflammation and are characterized by the expression of a broad spectrum of pro-inflammatory cytokines and chemokines. M1 macrophages promote atherosclerotic lesion development and complexity. Alternatively activated M2 macrophages are primed by Th2 cytokines, such as interleukin (IL)-4, and promote tissue repair and healing. Therefore, the stability of atherosclerotic plaques is highly dependent on the balance between these macrophage polarization states. The molecules and mechanisms associated with plasticity and the polarized activation of macrophages may provide potential therapeutic targets for preventing plaque rupture.

The intermediate conductance calcium-activated potassium channel, known as KCa3.1, is part of signaling cascades that involve relatively global and prolonged calcium elevation during cellular activation, proliferation, cytokine secretion, and volume regulation in many immune cells, including T cells, B cells, microglia, and macrophages. Therefore, KCa3.1 is an attractive pharmacological target for use in immunotherapy. KCa3.1 gating is voltage independent and only requires a small increase in intracellular calcium. Intracellular calcium binds to calmodulin molecules that are constitutively associated with the channel protein and induce channel
opening, which results in $K^+$ efflux that maintains a negative membrane potential. Thus, the initial influx of $Ca^{2+}$ feeds forward, thereby, preserving the negative $V_m$ (membrane voltage) required for sustained $Ca^{2+}$ influx. The $Ca^{2+}$ influx leads to an increase in cytosolic $Ca^{2+}$ concentration, which is necessary for the translocation of nuclear factors to the nucleus and the initiation of new transcription.

Particularly, in macrophages, KCa3.1 has been shown to be involved in activation, migration, proliferation, and respiratory bursting, indicating that KCa3.1 suppression might be helpful in some macrophage-related disorders, such as asthma, multiple sclerosis, stroke, and so forth. Previous evidence has demonstrated a role for KCa3.1 in atherosclerosis. In mouse models, pharmacological inhibition of KCa3.1 by the selective blocker TRAM-34 attenuates atherosclerotic lesion formation and may provide a novel approach for the prevention and treatment of atherosclerosis. However, in advanced atherosclerotic lesions, the role of KCa3.1 in the pathogenesis of plaque instability and its underlying mechanisms are unknown. A recent study reported that IL-4 stimulation increases KCa3.1 current in microglia, which suggests that KCa3.1 may be involved in the polarization of macrophages and atherosclerotic plaque disruption. In this study, we pharmacologically modulated KCa3.1 activity and examined how this altered macrophage phenotype and determined its effect on plaque stability.

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

### Cell Culture and Differentiation
Human monocytes were isolated from peripheral blood of 12 healthy volunteers and cultured in RPMI (Roswell Park Memorial Institute) medium supplemented with 10% fetal bovine plasma. We obtained informed consent from all subjects and a license from the ethical committee. Macrophage differentiation of human monocytes was achieved by treatment with 100 ng/mL colony-stimulating factor. Human monocytes-derived macrophages were treated with 100 ng/mL IFN-γ and 10 ng/mL lipopolysaccharide (LPS) for M1 differentiation. M2 polarization was induced by incubating macrophages with 15 ng/mL IL-4.

### Animals and Treatment
ApoE-deficient (ApoE−/−) male mice on a C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, ME) at the age of 7 weeks and were maintained in 12-hour light/dark cycles. The mice were allowed to acclimate for a week prior to the study and were fed with a high-fat diet (1.25% cholesterol and 21% fat; 42% kcal as fat) during the experiment. Unstable carotid atherosclerotic lesions were induced by combined partial ligation of the left renal artery and left internal and external carotid artery with perivascular collars. Six weeks after surgery, all mice were randomly assigned to either an interventional or a control group. Mice in the interventional group were treated with daily subcutaneous injection of TRAM-34 (120 mg/kg) for 3 weeks, whereas mice in the control group were injected with vehicle (Miglyol 812). At the end of the experiments, the animals were deeply anesthetized and euthanized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Blood was drawn from the inferior vena cava, and the carotid arteries were then harvested after in situ perfusion with heparin/saline via the left ventricle. All animal procedures were conducted in accordance with the guidelines on animal care and use of Fudan University.

### Results
#### KCa3.1 Expression Profile in Macrophages
KCa3.1 protein expression during macrophage differentiation and polarization was assessed by immunofluorescence and Western blotting (Figure 1A through 1C). Low KCa3.1 levels were present in the undifferentiated human monocytes. Compared with monocytes, significant upregulation of KCa3.1 expression was found in macrophage colony-stimulating factor–induced M0 macrophages. Macrophage polarization into the M1 and M2 subtypes is associated with inflammation and plaque stability. Using human monocyte-derived macrophages, we then investigated the expression of KCa3.1 during macrophage polarization into the M1 or M2 subtypes. M0 macrophages were stimulated with IFN-γ and LPS or with IL-4 for 48 hours to induce M1 or M2 polarization, respectively. Here, we found that IFN-γ and LPS stimulation increased KCa3.1 expression 3.2-fold, whereas IL-4 treatment increased KCa3.1 expression 2.1-fold.

Whole-cell patch-clamp experiments corroborated the protein expression data. KCa3.1 current was elicited with voltage ramps from −120 to 40 mV for 200 ms. KCa3.1 conductance was quantified from the slope of the current voltage relationship at −80 mV. Although KCa3.1 current was barely detectable in unstimulated human monocytes, the amplitude of the KCa3.1 current increased after macrophage colony-stimulating factor stimulation. In agreement with the immunofluorescence and Western blot analysis, both M1 and M2 macrophages showed significantly increased channel current amplitudes and conductance after polarization, though the increased trend was more pronounced in M1 macrophages (Figure 1D and 1E).

#### KCa3.1 Blockade Impairs Proinflammatory Genes Expression During Macrophage Polarization
To determine whether KCa3.1 channels are involved in the polarization process, macrophages were also treated with TRAM-34 during IFN-γ and LPS or IL-4 stimulation. Surface marker expression and cytokine profiles during macrophages polarization were evaluated by flow cytometry and real-time polymerase chain reaction. M1 macrophages are characterized by higher cell surface marker levels, including CD80 and CD197. M2 macrophages differed from M1 in that they expressed higher levels of CD163 and CD206. Our results showed that IFN-γ plus LPS significantly increased the expression of CD80 and CD197 with no effect on CD163 and CD206, whereas IL-4 only significantly upregulated CD163 and CD206 levels. We also revealed that coculture
Figure 1. KCa3.1 channel expression phenotypes in different macrophage subsets. A and B, Representative immunofluorescence staining of KCa3.1 channels in different macrophage subsets. The specific KCa3.1 fluorescence (green) was barely visible in undifferentiated human monocytes, though a large increase in fluorescence intensity could be observed during macrophage differentiation and polarization from human monocytes. Striking fluorescence was detected, especially in M1 macrophages (n=3 in each group). C, Immunoblots were probed for KCa3.1, and densitometry analysis showed that the relative levels of KCa3.1 protein in M1 and M2 macrophages were 3- to 4-fold and 2- to 3-fold higher, respectively, than in unpolarized M0 macrophages (n=4 in each group). Statistical significance values (P) are indicated. D, Representative KCa3.1 current for human monocytes and differentiated macrophages. These cells exhibit a voltage-independent KCa current in voltage ramps from −120 to 40 mV. The KCa current could be completely inhibited by the KCa3.1-specific blocker TRAM-34. The voltage-activated current visible above −40 mV is Kv1.3. E, The conductance of KCa3.1 channels in different macrophage subsets (n=4 in each group).
with TRAM-34 significantly reduced IFN-γ and LPS-induced CD80 and CD197 expression. In contrast, TRAM-34 treatment only slightly decreased IL-4-induced CD163 and CD206 expression, and there was no statistically significant difference (Figure 2A; Figure I in the online-only Data Supplement). Consistent with these observations, blocking KCa3.1 significantly attenuated proinflammatory cytokine and chemokine mRNA expression in M1 macrophages, including CCR7, tumor necrosis factor-α, and IL-12p35. However, M2 macrophage markers, such as CXCR4, CD206, and IL-10, were unaltered or only marginally decreased with TRAM-34 cotreatment (Figure 2B). Moreover, similar results were obtained in KCa3.1 siRNA-treated THP-1 cells, which displayed similar KCa3.1 channel expression patterns during differentiation and polarization (Figure II in the online-only Data Supplement). These in vitro data suggest that KCa3.1 channels play a more important role in the expression of proinflammatory genes during macrophage polarization, and blocking KCa3.1 channels with TRAM-34 results in a reduced M1 macrophage function. The receptors for IFN-γ (IFN-γ receptor-1), LPS (Toll-like receptor 4), and IL-4 (IL-4 receptor-α) on macrophages were also assessed. There were no differences in the expression of these receptors between the macrophages treated with or without TRAM-34, which excludes the possibility that low proinflammatory gene expression in TRAM-34-treated macrophages is because of IFN-γ receptor-1 or Toll-like receptor 4 expression defects (Figure 2B).

KCa3.1 Regulates Proinflammatory Genes During Macrophage Polarization Through the Signal Transducer and Activator of Transcription Pathway

Signal transducer and activator of transcription (STAT) proteins have been reported to be of critical importance for determining macrophage phenotypes in well-established signaling pathways regulating macrophage polarization. During M1 polarization, STAT-1 becomes phosphorylated and promotes M1 gene program transcription. Conversely, the exposure of macrophages to IL-4 activates the STAT-3/STAT-6 pathway and promotes the transcription of M2 phenotype-associated genes, while the activity of STAT-1 is significantly decreased. Therefore, the balance between STAT-1 and STAT-3/STAT-6 seems to dictate the fate of macrophage polarization during the immune response. In this experiment, because of the limited availability of human monocytes, we used THP-1 cells to investigate the effects of KCa3.1 blockade on STAT phosphorylation/activation. Blocking KCa3.1 with TRAM-34 significantly decreased IFN-γ and LPS-induced STAT-1 phosphorylation. Mechanistically, KCa3.1 inhibition suppressed IFN-γ and LPS-induced proinflammatory gene expression during M1 polarization by inhibiting STAT-1 activation. In contrast, TRAM-34 cotreatment did not change STAT-3/STAT-6 phosphorylation during IL-4 stimulation, which may explain why blocking KCa3.1 had few effects on M2 polarization in vitro (Figure 2C).

Blocking KCa3.1 Channels Promotes Plaque Stability and Reduces the Risk of Rupture

To determine the role of KCa3.1 channels in plaque stability, a murine model mimicking human plaque rupture was generated by combined partial ligation of the left renal artery and the left internal and external carotid arteries in 8-week-old ApoE−/− mice. TRAM-34 dissolved in Miglyol 812 was administered once daily (120 mg/kg, SC) for 3 weeks beginning at 6 weeks after surgery. High-resolution microultrasound imaging was conducted every 3 weeks to assess the progression of the carotid plaques. B-mode and pulse-wave Doppler were performed to evaluate the degree of stenosis and the hemodynamic status in the carotid artery (Figure 3A and 3B). At the beginning, no plaque was detected in the left common carotid artery. Six weeks later, atherosclerotic plaques were clearly visualized by microultrasound imaging on the left common carotid arteries. No significant changes were observed in the ultrasound-measured parameters between the 2 groups at baseline. At the end of the experiment, TRAM-34 treatment significantly lowered the ultrasound-measured plaque intimal-medial thickness (0.276±0.070 versus 0.354±0.067 mm; P=0.013), eccentric index (0.482±0.133 versus 0.623±0.192; P=0.044), and maximal systolic blood flow velocity (V_{max}; 30.2±9.4 versus 23.8±5.7 cm/s; P=0.049) compared with the vehicle-treated group (Figure 3C). Blood pressure was also measured using the tail-cuff method at each time point before microultrasound imaging. TRAM-34 treatment did not affect the hypertensive response to renal artery ligation (Table I in the online-only Data Supplement).

After the ultrasound detection at 9 weeks after surgery, the mice were euthanized, and the left common carotid arteries were isolated after pressure perfusion. Compared with the vehicle-treated group, the animals that received TRAM-34 injection showed an obviously lower incidence of fresh left common carotid arterial lumen thrombus associated with severe plaque burden (1/9 versus 6/9; P=0.016; Figure 4A). Plaque composition was assessed with special stains and immunocytochemistry coupled with quantitative image analysis (Figure 4B and 4C). TRAM-34 treatment obviously decreased the intimal surface area (149±52 versus 102±37 mm²; P=0.025) relative to the vehicle-treated mice. Although lipid deposits make plaques more prone to rupture, collagen fibers stabilize plaques. Areas of lipid deposits and collagen fibers within a plaque were defined using oil red O and adjacent Sirius-stained tissue sections, respectively, and the ratio between these 2 areas was calculated. TRAM-34-treated mice showed reduced lipid contents (15.6±4.9% versus 22.2±6.6%; P=0.014) compared with vehicle-treated control mice. No changes in the collagen contents were observed after TRAM-34 treatment (35.2±6.2% versus 36.4±8.2%; P=0.363). Consequently, TRAM-34-treated mice displayed increased collagen/lipid ratios compared with control mice (2.46±0.67 versus 1.67±0.43; P=0.005). TRAM-34 treatment increased vascular smooth muscle cells (VSMCs) content (21.9±4.8% versus 17.2±5.6%; P=0.037) and reduced macrophages (21.0±5.3% versus 31.3±8.1%; P=0.003) in carotid plaque lesions. Consequently, the VSMC/macrophage ratio (1.12±0.42 versus 0.60±0.28; P=0.003) was markedly increased in the TRAM-34-treated mice, which supports the observation that TRAM-34 treatment has beneficial effects during plaque stabilization. T cells, another major cell type within atherosclerotic plaques, have also been reported to
Figure 2. The role of KCa3.1 in M1 and M2 macrophage polarization in vitro. Macrophage colony-stimulating factor (M-CSF)-stimulated human macrophages were cotreated with vehicle or 100 nmol/L TRAM-34 during interferon (IFN)-γ and lipopolysaccharide LPS-induced M1 polarization and interleukin (IL)-4-induced M2 polarization. A, Flow cytometry analysis of cell surface markers for M1 (CD80 and CD197) and M2 (CD163 and CD206) macrophages during M1 and M2 polarization with or without TRAM-34. The flow cytometry results are expressed as MFI (n=3 in each group). B, Real-time polymerase chain reaction was performed to further evaluate the transcript expression of genes related to M1 and M2 macrophage polarization (n=5–6 in each group). C, Western blotting was conducted on cell lysates with antibodies for P-STAT1, P-STAT3, and P-STAT6; total STAT1, STAT3, and STAT6; and GAPDH. The relative densities for the phosphorylated STAT compared with total STAT are shown as histograms (n=4 in each group). Statistical significance values (P) are indicated. MFI indicates mean fluorescence intensity; and STAT, signal transducer and activator of transcription.
express KCa3.1 channels. However, TRAM-34 treatment did not significantly alter the number of CD3+ T cells (15.1±5.7% versus 17.4±5.1%; P=0.186; Figure 4D and 4E).

As reported, KCa3.1 channels play an important role in the regulation of inflammatory cytokine expression. To investigate this further, proinflammatory cytokine plasma levels were assessed (Figure 4F). TRAM-34-treated mice exhibited a significant reduction in plasma proinflammatory cytokine levels, including IL-2, IL-6, tumor necrosis factor-α, and monocyte chemoattractant protein, and increased the plasma levels of the anti-inflammatory cytokine IL-10. TRAM-34 treatment did not affect plasma IL-1β and macrophage inflammatory protein-1α levels. We also analyzed the plasma lipid profiles in these mice. Lipids were highly elevated in ApoE−/− mice fed a high-fat diet. However, there were no significant differences in glucose, triglycerides, and total cholesterol between the vehicle- and TRAM-34-treated groups (Table I in the online-only Data Supplement).

Figure 3. Microultrasound imaging evaluation of carotid artery plaque. A, Representative microultrasound long-axis views of the left common carotid arteries in a mice model of unstable atherosclerotic lesions treated with vehicle or TRAM-34. B, Representative view of carotid blood flow obtained by pulse-wave Doppler assessment. C, Quantification of ultrasound-derived plaque intima-media thickness (IMT), eccentric index, and maximal systolic velocity of blood flow in the vehicle- and TRAM-34-treated group (n=9 in each group). Statistical significance values (P) are indicated.
Figure 4. Histopathologic analysis of carotid lesions in vehicle- and TRAM-34-treated animal models. A, Representative gross anatomy of the left common carotid arterial segments at 9 weeks after surgery. B, Representative carotid artery cross-sections from vehicle- and TRAM-34-treated mice were stained with hematoxylin and eosin (H&E), Sirius red, or oil red O. C, Quantification of the intimal (Continued)
TRAM-34 Treatment Suppresses M1 and Promotes M2 Macrophage Polarization Within Atherosclerotic Lesions

Recent advances indicate that the relative abundance of macrophage phenotypes, rather than the absolute number, is more relevant to atherosclerotic plaque phenotype and lesion progression. To determine whether the enhanced plaque stability in TRAM-34-treated animals correlated with a decreased M1:M2 macrophage ratio, we performed double immunofluorescence experiments in carotid cross-sections to analyze macrophage polarization in atherosclerotic lesions. These studies showed that Mac3-positive macrophages from carotid lesions in TRAM-34-treated mice exhibited decreased expression for M1 markers, including fatty acid–binding protein-4 and CD36, and increased expression for M2 markers, including arginase-1 and peroxisome proliferator-activated receptor γ coactivator-1 (Figure 5A, 5B, and 5D). Moreover, the expression of KCa3.1 on Mac3-positive macrophages within the plaque was markedly suppressed in TRAM-34-treated mice, which also indicated the effectiveness of the treatment (Figure 5C and 5D).

Discussion

The calcium-activated K+ channel KCa3.1 plays an important role in several macrophage functions, including respiratory burst, migration, and macrophage-mediated pathogen killing in vitro and in vivo. Here, we extend these findings and present the first evidence suggesting that KCa3.1 channels also play a key role in regulating proinflammatory gene expression during macrophage polarization. Based on these observations, the KCa3.1 blockers likely reduce inflammation and stabilize atherosclerotic plaques. Here, we provide proof-of-concept evidence that blocking KCa3.1 with TRAM-34 reduces atherosclerotic lesion burden and enhances plaque stability with a concomitant decrease in proinflammatory markers and an increase in anti-inflammatory markers in macrophage population within atherosclerotic lesions. Altogether, our results highlight a critical role for KCa3.1 channels in determining...
proinflammatory genes expression in macrophage polarization and atherosclerotic plaque instability.

Monocytes and their descendant macrophages are consistently found in atherosclerotic lesions and contribute to plaque growth and activity. During the early stages of atherosclerosis, circulating monocytes are involved in the initiation and progression of the disease by infiltrating the vessel wall and becoming lesional macrophages.\(^{21,22}\) KCa3.1 expression is high in macrophages that infiltrate atherosclerotic plaques but not in monocytes. Therefore, treating ApoE−/− mice with TRAM-34 resulted in reduced atherosclerosis, which was associated with decreased recruitment of monocytes into the vascular wall.\(^{16}\) Our results also confirmed that KCa3.1 expression was significantly upregulated during the differentiation of human monocytes into macrophages. However, during the later stages of plaque development, local proliferation accounted for ≈90% of macrophage accumulation in established disease.\(^{23}\) Moreover, the results from studies on human atherosclerotic plaques have demonstrated that the relative proportion of macrophage subsets within plaques is a better predictor of plaque compositional phenotype and related stability than the total number of macrophages. Changing the composition of resident macrophages during advanced atherosclerosis stages would affect plaque instability; therefore, the identification of agents that are responsible for M1 or M2 polarization is of great importance.

As mentioned earlier, the KCa3.1 channel is pivotal in regulating multiple macrophages functions. Moreover, recent studies have indicated that expression of KCCN4, the gene that encodes the KCa3.1 channel, is upregulated in IL-4-treated human macrophages.\(^{17,24}\) However, the expression pattern of KCa3.1 channels and their role in modulating the balance of macrophage phenotypes are still not well defined. Using the whole-cell patch clamp recording method and Western blot assays, we demonstrated increased KCa3.1 levels in IFN-γ and LPS-treated macrophages, as well as in IL-4-treated macrophages, though the increase was significantly larger in the IFN-γ and LPS-induced M1 macrophages. These results support the potential importance for KCa3.1 channels during macrophage polarization. In the present study, we showed that specific gene silencing or blocking KCa3.1 dramatically inhibited the expression of proinflammatory markers and cytokines during macrophages polarization in vitro.

In agreement with our in vitro studies, in vivo data showed a reduced number of M1 proinflammatory macrophages in advanced plaques found in TRAM-34-treated mice. It is interesting that blocking KCa3.1 significantly increased the expression of M2 macrophage markers in atherosclerotic lesions, which is different from the in vitro results. Indeed, depending on environmental signals, macrophages can display a spectrum of activation states in vivo. A continuum of pro- and anti-inflammatory macrophages, with extreme polarization phenotypes M1 and M2 macrophages, can be found in atherosclerotic lesions.\(^1\) The local microenvironment present in the atherosclerotic lesion, which predominantly determines the polarization of the plaque macrophages, is more complex than typically described by the M1/M2 paradigm.\(^{25}\) Therefore, the local cytokine milieu can determine macrophage polarization and plasticity. In our study, TRAM-34 treatment promoted anti-inflammatory and inhibited proinflammatory cytokine expression, which may explain the shift in the macrophage spectrum toward an M2 phenotype in vivo. Consequently, increased collagen/lipid and smooth muscle cell/macrophage ratios were observed in TRAM-34-treated mice, which are suggestive of more stable and less complex lesions.\(^{26}\) Notably, blood pressure, plasma cholesterol, and triglycerides profiles in the vehicle-treated mice versus the TRAM-34-treated mice were not significantly different.

In addition to macrophages, KCa3.1 channels have been demonstrated to express on VSMCs, T cells, and endothelial cells, all of which are also involved in the pathogenesis of atherosclerosis.\(^{11,16}\) It has been reported that long-term blocking KCa3.1 therapy with TRAM-34 strikingly inhibited VSMCs proliferation and T cells infiltration and reduced atherosclerosis in ApoE−/− mouse.\(^{16}\) In our experiments, however, TRAM-34 therapy was administered for 3 weeks and did not change the proportion of T cells and even increased the VSMC content. In the early stages of atherosclerosis, most of the T cells present in atherosclerotic plaques are naive and central memory T cells that express high numbers of KCa3.1 channels; however, in more advanced stages, they convert to effector memory T cells and contribute to the destabilization of atherosclerotic lesions.\(^{16,27}\) Different from naive and central memory T cells, Kv1.3 is the functionally dominating K⁺ channel in effector memory T cells.\(^{27}\) Therefore, in vivo therapy with TRAM-34 during the advance stages of atherosclerosis did not decrease CD3⁺ T cell in lesions. Similarly, there is also a phenotypic switching of VSMCs during the progression of atherosclerosis.\(^{28}\) Increased VSMCs proliferation is observed during early atherogenesis and promotes plaque formation. In contrast, VSMCs derived from advanced atherosclerotic plaques undergo reduced proliferation, and VSMC apoptosis seems to be a critical event in plaque rupture. It has been suggested that VSMCs apoptosis is potentially induced by macrophages via death ligand/death receptor interactions and tumor necrosis factor-α secretion.\(^{28,29}\) In our study, the mechanism underlying the increased VSMCs content in TRAM-34-treated mice is possibly an indirect effect on macrophages that induce VSMCs apoptosis in the lesion. However, we cannot completely exclude that it is not a direct effect of TRAM-34 treatment on VSMCs in advanced lesions. In endothelial cells, the blocking KCa3.1 channels acts as a double-edge sword. On the one hand, KCa3.1 inhibition can cause endothelial dysfunction and, thereby, contribute to vascular diseases;\(^{30}\) in contrast, KCa3.1 channels are involved in endothelial cell proliferation and angiogenesis, which contribute to the progression of advanced atherosclerotic plaques.\(^{31}\) The inhibition of angiogenesis in advanced plaques may contribute to TRAM-34’s therapeutic effect. Additionally, given the role of TRAM-34 treatment in systemic immunosuppression,\(^{11}\) further studies are needed to clarify the potential role of KCa3.1 in systemic inflammation that can contribute to plaque instability.

Regarding the signaling pathways by which KCa3.1 regulates macrophage polarization, recent studies have determined that there is a balance between the STAT-1 and STAT-3/STAT-6 pathways and macrophage polarization. Exposing
macrophages to M1 phenotype inducers, such as IFN-γ and LPS, leads to the phosphorylation of STAT-1, which promotes the transcription of the M1 gene program. In contrast, IL-4 stimulation causes the phosphorylation of STAT-6 and the transcription of M2 phenotype–associated genes. The KCa3.1 potassium channel has been demonstrated to impact the function of macrophages by regulating intracellular Ca2+ homeostasis. Furthermore, a recent study indicated that intracellular Ca2+ overloading triggers greater macrophage production of inflammatory mediators and promotes classically activated macrophage M1 polarization. In the present study, we found that decreasing STAT-1 phosphorylation underscores the role of blocking KCa3.1 in inhibiting proinflammatory genes expression during macrophage polarization and attenuating plaque instability. However, further study is needed to understand the mechanisms that underlie the interplay between KCa3.1 and JAK/STAT1 signaling in detail.

As previously mentioned, several reports suggest that blocking KCa3.1 has beneficial effects on chronic neurodegenerative disorders, ulcerative colitis, obliterative airway disease, neuroinflammation associated with ischemia/reperfusion stroke, and resistance to oxidative stress. These pathological processes all involve the activation of an acute or chronic inflammatory response. Therefore, the potential of blocking KCa3.1 to change the balance in M1 and M2 macrophages and promote alternative, anti-inflammatory macrophage phenotypes presents another possible mechanism that contributes to its protective effects in these diverse pathological conditions.

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Disclosures

None.

References


**Highlights**

- Our results highlight a critical role for KCa3.1 channels in determining macrophage phenotype and atherosclerotic plaque instability.
- Blocking KCa3.1 reduced the expression of proinflammatory genes during macrophage polarization.
- In an animal model, blocking KCa3.1 with TRAM-34 reduced atherosclerotic lesion burden and enhanced plaque stability with a concomitant decrease in M1 polarization and an increase in M2 polarization in macrophage population within atherosclerotic lesions.
Role of KCa3.1 Channels in Macrophage Polarization and Its Relevance in Atherosclerotic Plaque Instability

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**Supplementary Table I.** Blood pressure and metabolic characteristics of mice in vehicle- and TRAM-34-treated groups at the end of the experiments.

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**M1 polarization**

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**M2 polarization**

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Fluorescence intensity
**Supplementary Figure I.** Representative results of flow cytometry analysis are shown as histograms. Dashed lines indicate stimulated and/or treated macrophages.

**Supplementary Figure II.** Expression pattern and function of KCa3.1 channels in THP-1 monocytes and their derived THP-1 macrophages. A and B, Patch-clamp and immunoblots were probed for KCa3.1 expression in different macrophage subsets. C, Real-time polymerase chain reaction was performed to evaluate transcript expression of genes related to M1 and M2 macrophage polarization. *P<0.05 vs. vehicle or control group

**Supplementary Figure III.** Animal model of unstable atherosclerotic lesions induced by combined partial ligation of the left renal artery and left internal and external carotid artery with
perivascular collars in ApoE<sup>−/−</sup> mice. Partial ligation of the left renal artery (A) and left internal and external carotid artery (C). B, Representative kidney images at 9 weeks after surgery.

Supplementary Figure IV. Plasma cytokine analysis. A, Cytokine array detecting a panel of 7 cytokines. B and C, Representative results in vehicle- and TRAM-34-treated animals.
Materials and Methods

Human monocyte isolation and macrophage differentiation

Human venous blood (100ml) was collected from 12 healthy volunteers after obtaining informed written consent. We obtained a license from the ethical committee of Zhongshan Hospital. Peripheral blood mononuclear cells (PBMCs) were isolated from heparin-treated blood by density gradient centrifugation with Ficoll-Paque (GE Healthcare). CD14⁺ monocytes were purified from PBMCs by positive selection using the MACS system (Miltenyi Biotec). PBMCs were resuspended at 10⁷ cells/ml in MACS buffer and incubated with anti-CD14 magnetic beads at 6°C for 15 min. The cell suspension was applied to a separation column, with the CD14⁺ cells being retained in the column. Separated CD14⁺ populations had greater than 95% purity as demonstrated by flow cytometry. CD14⁺ cells were resuspended at 3 x 10⁵ cells/ml in Roswell Park Memorial Institute medium (RPMI 1640, Gibco) culture medium supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (complete medium) and pipetted into tissue-culture-treated 24-well plates. Cells were allowed to adhere for 2 h at 37°C after which the non-adherent cells were removed by washing with the medium. Adherent monocytes were differentiated to M0 macrophages by culturing for 7 days in complete medium supplemented with 100 ng/ml of macrophage colony stimulating factor (M-CSF, PeproTech). Following macrophage differentiation, the macrophages were polarized to M1 macrophages by incubation for 48 h with 100 ng/ml of interferon (IFN)-γ (PeproTech) and 10 pg/ml of lipopolysaccharide (LPS) (Sigma), or to M2 macrophages by incubation for 48 h with 15 ng/ml of interleukin 4 (IL-4, PeproTech). On the day of the treatment, 100 nmol/l TRAM-34 or vehicle (DMSO) was added to replicate cell polarization.

THP-1 cell culture

The THP-1 human acute monocytic leukaemia cell line was purchased from ATCC (Manassas, VA) and cultured at 3 x 10⁵ cells/ml in complete medium. Macrophage differentiation of THP-1 cells was induced by treatment for 48 h with 100 nM of phorbol 12-myristate13-acetate (PMA, Sigma) followed by a 24 h incubation in RPMI 1640 medium. THP-1-derived macrophages were polarized by incubation for 48 h with 100 ng/ml of IFN-γ and 10 pg/ml of LPS (M1 polarization) or 15 ng/ml of IL-4 (M2 polarization).

KCa3.1 silencing
M0 macrophages were transfected with either siRNA targeting KCa3.1 or negative control siRNA using the Hiperfect transfection reagent (QIAGEN) according to the manufacturer’s instructions. The sequence of the KCa3.1-targeting siRNA was 5'-GCACCUUUCAGACACAUU-3' (GenePharma). After transfection, the cells were treated with IFN-γ and LPS or IL-4 for 48 h.

**Flow cytometry**

Flow cytometry analysis was performed to confirm M1 and M2 induction and was later used to evaluate the effect of TRAM-34 on macrophage phenotype. Macrophages were treated as described above and then stained with fluorochrome-conjugated mAbs for surface markers to delineate the macrophages M1 and M2 phenotypes. The following antibodies were used for flow cytometry: (1) phycoerythrin (PE)-conjugated anti-CCR7 antibodies, (2) PE-conjugated anti-CD80 antibodies, (3) PE-conjugated anti-CD163 antibodies and (4) PE-conjugated anti-CD206 antibodies (eBioscience, cat. no. 12197941, 12080941, 12163941 and 12206941). The IgG1 and IgG2a isotype antibodies (eBioscience, cat. no. 12471441 and 12432141) were used as the negative control. Conjugated monoclonal antibodies specific for the extracellular markers were added to 100 μl of cell suspension and incubated for 30 min on ice. The cells were washed twice with 3 ml of 0.5% phosphate buffer (PBS)/bovine serum albumin (BSA) and fixed in fixation buffer (PBS with 2% formaldehyde) at 4°C. All of the samples were analyzed by flow cytometry (FACSCanto, BD Biosciences).

**Quantitative real time RT-PCR**

Total RNA was isolated from treated and untreated M1 and M2 macrophages with the RNeasy Mini Kit (Qiagen) and was processed directly to cDNA using RevertAid™ reverse transcriptase (MBI Fermentas). Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed with FastStart Universal SYBR Green Master (Invitrogen) on an ABI Prism 7900 Sequence Detection System (Thermo Scientific). Gene expression was analyzed using the comparative ΔΔCt method with target gene mRNA levels normalized to GAPDH. The primers used for the specific genes are given below.

**CCR7**—sense: GGTTGGTGCTCTCTTGATT; antisense: GCTTTAAAGTTCCGCACGTCCCTT.

**TNF-α**—sense: CAGGGGCCACCACGCTCTTCTC; antisense: CTTGGGGCAGGGGCTCTTGAC.

**IL-12p35**—sense: CCACTCCAGACCAAGGATG; antisense: GACGGCCTCAGCGGTT.

**CXCR4**—sense: GCCTTATCTGCCTGTATTG; antisense: CGAAGAAAGCCAGGATGAGA.
CD206—sense: CGTTTACCAAATGGCTTCGT; antisense: CCTTGCTCTTGAGTTCTCAGCT.
IL-10—sense: ACCTGCCTAACATGCTTCGAG; antisense: CTGGGTCTTGGTTCTCAGCT.
TLR-4—sense: AGCCCTGCGTGGAGGTGGTT; antisense: GAAGGGGAGGTTGTCGGGGA.
IFNGR-1—sense: GTATTGTCGCTTCTGGCTCCTT; antisense: AAGGCTAGCCGAGGCAAAC.
IL-4Rα—sense: TCCGCACTTCTACGTGTGAG; antisense: AGACCACAGTTCCAGCCAGT.
GAPDH—sense: GCACCGTCAAGGCTGAGAAC; antisense: TGGTGAAGACGCCAGTGGA.

**Western Blot**

Protein lysates from human monocytes/THP-1 cells before or after M-CSF/PMA stimulation and from treated and untreated M1 and M2 macrophages were resolved on 12.5% polyacrylamide gels and then transferred onto nitrocellulose membranes. The membranes were incubated with antibodies overnight at 4°C. The antibodies used targeted KCa3.1 (Abcam, cat. no. ab83740), P-701-STAT1 (Cell Signaling, cat. no. 7649), STAT1 (Cell Signaling, cat. no. 9172), P-705-STAT3 (Cell Signaling, cat. no. 9145), STAT3 (Cell Signaling, cat. no. 4904), STAT 6 (Cell Signaling, cat. no.5397), P-641-STAT6 (Cell Signaling, cat. no. 9361) and GAPDH (Santa Cruz, cat. no. sc-365062). After washing, the membranes were exposed to the appropriate horseradish peroxidase secondary antibody for 1 h at room temperature and developed by chemiluminescence (Thermo). Prestained molecular weight marker proteins (Gibco) were used to calculate the molecular weights of the proteins. Densitometry analysis of the bands was conducted using the Image J 2x software.

**Patch-clamp electrophysiology**

KCa3.1 channel expression was studied in monocytes and THP-1 cells before or after stimulation in the whole-cell mode with the patch-clamp technique with an EPC-10 HEKA amplifier. Data were acquired and analyzed using Pulse/Pulsefit (v. 8.11) (HEKA) and Igor Pro (v. 6.1). All experiments were conducted at room temperature. KCa3.1 currents were elicited with voltage ramps from −120 to 40 mV of 200 ms duration applied every 10 seconds. The pipette solution contained (in mmol/l): 145 K+ aspartate, 2 MgCl2, 10 HEPES, 10 K2EGTA, and 8.5 CaCl2 (1 μmol/l free Ca2+), pH 7.2, 290 mOsm. To reduce chloride “leak” currents, we used a Na+ aspartate external solution containing (in mmol/l): 160 Na+ aspartate, 4.5 KCl, 2 CaCl2, 1 MgCl2, and 5 HEPES, pH 7.4, 300 mOsm. Whole-cell KCa3.1 conductance was calculated from the slope of the current voltage relationship at −80 mV.
Immunofluorescence labelling and confocal microscopy

THP-1 monocytes were seeded at $1 \times 10^5$ cells/well in 24-well plates containing a coverslip and were differentiated as described above. Undifferentiated monocytes were attached on coverslips by drying a PBS drop containing $1 \times 10^5$ cells. For labeling, the cells were fixed for 10 min with 4% paraformaldehyde in cold PBS, washed three times with 2% PBS/BSA and incubated overnight at 4°C with primary antibodies against KCa3.1 (Abcam, cat. no. ab83740). Next, the cells were washed three times with 2% PBS–BSA and then incubated for 1 h with secondary antibody conjugated to a fluorophore. The cells were then were stained with DAPI for nuclei. Images were collected with a confocal microscope.

Animal models

ApoE-deficient (ApoE−/−) male mice on a C57BL/6 background (n=18) were obtained from the Jackson Laboratory (Bar Harbor, ME) at an age of 7 weeks and were maintained in 12 h light/dark cycles. The mice were allowed to acclimate for a week prior to the study and were fed a high-fat diet (1.25% cholesterol and 21% fat; 42% kcal as fat) during the experiment. Unstable carotid atherosclerotic lesions were induced by combined partial ligation of the left renal artery and left internal and external carotid artery with perivascular collars (Supplementary Figure III). Mice were anesthetized with 100 mg/kg pentobarbital sodium and maintained at 37°C on a heating pad. The left renal artery and left internal and external carotid artery were bluntly dissected. Stenosis was initiated by placement of a sterilized polytetrafluoroethylene tube (ID 0.20 mm x OD 0.36 mm x length 0.5 mm) (Braintree Scientific, SUBL 140) around the arteries. Six weeks after surgery, all of the mice were randomly assigned to the interventional or control group. Mice in the interventional group were treated with a daily s.c. injection of TRAM-34 (120 mg/kg) for 3 weeks, while mice in the control group were injected with the vehicle (Miglyol 812).

Blood pressure measurement and micro-ultrasound imaging

Blood pressure and ultrasound imaging parameters for the left common carotid artery were evaluated at baseline, as well as at 6 and 9 weeks after surgery. Blood pressure was measured using the tail-cuff method under conscious state as protocol (NIBP system, Kent Scientific). Ultrasound imaging parameters were measured with use of the Vevo770 system (Visualsonics). Imaging involved a 55-MHz scan head, with a 4.5-mm focus and an axial resolution of 30 µm. Mice were anesthetized with 2% isoflurane and body temperature was maintained with a heating
The mouse neck hair of mice was defoliated and ultrasound transmission gel was applied liberally. First, on B-mode, a long axis view was used to visualize the plaque length in the carotid artery and to measure the maximal and minimal intima-medial thickness (IMT). Then, the scan head was adjusted to less than 60° to the left common carotid artery. The sample volume was placed in the lumen of the left common carotid artery to record the maximal flow velocity ($V_{max}$) proximal to the stenotic lesion by pulse-wave Doppler mode. The images were analyzed by two independent researchers in a blinded manner. The eccentric index (EI) was calculated by the following formula: (maximal IMT – minimal IMT)/maximal IMT. An EI of 1.0 indicates a completely eccentric plaque distribution.

**Tissue collections and analysis**

After finishing the ultrasound detection at 9 weeks after surgery, the mice were anesthetized and harvested at 17 weeks of age. Blood was drawn from the inferior vena cava and collected in heparinized tubes. Plasma was obtained by centrifugation (3000 rpm) at 4°C for 15 min and stored at -80°C. Subsequently, the mice were perfused with 4°C heparin/saline through the left ventricle under physical pressure. After perfusion, the carotid arteries were harvested and embedded in paraffin or in an optical cutting temperature (OCT) compound (Tissue-Tek). A cytokine/chemokine array kit (QAM-CUST Kit, Ray Biotech) was used to detect a panel of 7 plasma cytokines and chemokines, which included IL-1β, IL-2, IL-6, IL-10, TNF-α, MIP-1α and MCP-1. The reaction conditions and data analysis were performed according to the manufacturer’s recommended protocol (Supplementary Figure IV). The plasma concentrations of glucose, triglyceride and total cholesterol were measured on a Hitachi 7180 autoanalyzer (Hitachi).

**Histopathology and Immunofluorescence**

Serial 4 µm cryosections were cut for paraffin-embedding and 5 µm for OCT-embedding at 50 µm intervals along the carotid artery specimens, starting 200 µm downstream from the origin of the internal and external carotid artery. Sections were stained with Harris hematoxylin and eosin (Sigma) and Masons Trichrome (Sigma). OCT imbedded frozen tissue was serially sectioned and stained with Oil Red O and Mayer’s Hematoxylin (Sigma). Images were collected and the lesion area was quantified using Image J 2x software as described previously. Briefly, the intimal
surface area was determined on H&E stained sections, lipid content by measuring the area of oil red O staining, and collagen area by measuring the area of the Sirius red staining.

For immunofluorescence analysis, paraffin embedded serial sections were immunostained using primary antibodies against Mac3 (BD Transductions, cat. no. 553322), α-SMA (Santa Cruz, cat no. sc-53142), CD3 (GeneTex, cat. no. GTX16669), KCa3.1 (Abcam, cat. no. ab83740), FABP4 (Cell Signaling, cat. no. 3544), CD36 (Santa Cruz, cat. no. sc-9154), Arg1 (Santa Cruz, cat. no. sc-20150) and Pgc1 (Santa Cruz, cat. no. sc-67286). The serial sections were stained with pre-immune IgG in place of primary antibodies, to control for non-specific staining. Appropriate secondary antibodies (BD transductions) conjugated to a fluorophore were used for detection. Immunofluorescent staining intensities were quantified using Image J 2x software. To analyze the different plaque parameters, sections equally spaced over the entire length of the artery at 100 μm intervals were used for analyses; five sections were used per animal for each type of analysis. The analysis was performed by two independent observers and the sections were randomly mixed prior to analysis.

Statistical analysis

The results were analyzed and presented as the mean±SD for the continuous variables and as percentages for the categorical data. Continuous variables were analyzed with a 1-tailed t test for unpaired observations. The categorical data were analyzed with the χ² test. Statistical significance was defined when P values <0.05.

Reference