Macrophage-Inducible C-Type Lectin/Spleen Tyrosine Kinase Signaling Pathway Contributes to Neuroinflammation After Subarachnoid Hemorrhage in Rats

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Background and Purpose—Macrophage-inducible C-type lectin (Mincle, CLEC4E) receptor is reported involved in neuroinflammation in cerebral ischemia and traumatic brain injury. This study was designed to investigate the role of Mincle and its downstream spleen tyrosine kinase (Syk) signal pathway in early brain injury after subarachnoid hemorrhage (SAH) in a rat model.

Methods—Two hundred fifteen male Sprague-Dawley rats (280–320 g) were subjected to endovascular perforation model of SAH. SAH grade, neurological score, and brain water content were measured at 24 hours after SAH. Mincle/Syk, as well as CARD9 (a member of the caspase-associated recruitment domain [CARD], involved in innate immune response), interleukin-1β, and myeloperoxidase expressions were analyzed by Western blot at 24 hours after SAH. Specific cell types that expressed Mincle were detected with double immunofluorescence staining. Mincle small interfering RNA, recombinant SAP130, and a selective Syk phosphorylation inhibitor piceatannol were used for intervention.

Results—Brain water content increased and neurological functions decreased in rats after SAH. The expression of SAP130, Mincle, Syk, and p-Syk increased at 12 hours and peaked at 24 hours after SAH. Mincle small interfering RNA reduced interleukin-1β and infiltration of myeloperoxidase positive cells, decreased brain water content, and improved neurological functions at 24 hours after SAH. Recombinant SAP130 upregulated the expression of p-Syk and CARD9 and increased the levels of interleukin-1β and myeloperoxidase, even though it did not increase brain water content nor it deteriorated neurological function at 24 hours after SAH. Syk inhibitor piceatannol reduced brain edema at 24 hours after SAH.

Conclusion—Mincle/Syk is involved in early brain injury after SAH, and they may serve as new targets for therapeutic intervention. (Stroke. 2015;46:2277-2286. DOI: 10.1161/STROKEAHA.115.010088.)

Key Words: brain injuries ♦ Mincle ♦ subarachnoid hemorrhage ♦ Syk kinase

Subarachnoid hemorrhage (SAH) is a devastating cerebrovascular disease, representing ≈5% of all strokes types, but has high rates of mortality and disability. Early brain injury was reported as the primary cause of mortality and the key target for SAH treatment. Increasing evidences suggested that innate immune response is one of the key factors in early brain injury after SAH. Therefore, it is suggested that comprehensive understanding of the activated innate immune pathways may be helpful for designing novel anti-inflammation treatments to improve the outcomes of SAH patients.

The innate immune response triggered by stroke referred to a series of reactions, including microglia activation, immune cell infiltration, and cytokine generation, which lead to blood–brain barrier disruption and neuronal dysfunction. Innate immune receptors were considered to play critical roles in the initiation of the immune response in stroke and other neurological injuries. Among the immune receptors, macrophage-inducible C-type lectin (Mincle, CLEC4E) receptor recognizes self-ligands released from necrotic cells, which leads to the production of proinflammatory cytokines, such as interleukin-1β (IL-1β) and other chemokines. Mincle belongs to C-type lectin receptor family, which was first identified as a downstream transcriptional target in peritoneal macrophages. Administration of an anti-Mincle blocking antibody suppressed neutrophil infiltration into the thymus after whole-body irradiation suggested that Mincle may be a functional sensor for damaged cells in vivo. In the central nerve system (CNS), it has been shown that Mincle and...
its downstream molecular spleen tyrosine kinase (Syk) demonstrated a pivotal role in the pathogenesis of cerebral ischemia and reperfusion. Mincle expression was elevated in the cerebrospinal fluid and brain tissue from either traumatic brain injury rodent models or patients, suggesting that Mincle signaling was involved in the pathology of traumatic brain injury. However, the specific role of Mincle signaling pathway and its downstream factors, such as CARD9 (a member of the caspase-associated recruitment domain, CARD, involved in innate immune response) and IL-1β, both are involved in innate immune responses in SAH have not yet been investigated. In this study, we examined the role of Mincle/Syk signaling pathway in early brain injury after SAH in a rat model.

Materials and Methods
The experimental design and all experiment procedures were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

SAH Model and Experimental Protocol
Two hundred fifteen male (280–320 g) Sprague-Dawley rats (Indianapolis, IN) were used. The endovascular perforation model of SAH was performed as reported previously. The details were also described in the online-only Data Supplement. Three separate experiments were conducted. Animals were randomly divided into different groups before surgery. Detailed numbers of animals that have been used in this study are provided in the Table in the online-only Data Supplement.

Experiment 1
Rats were divided into six groups (n=6 for each group). The endogenous ligand of Mincle receptor SAP130, Mincle receptor, downstream kinase Syk, and phosphorylated Syk (p-Syk) was detected by Western blot. Double immunostaining of Mincle, Syk, and CARD9 with calcium-binding molecule 1 (Iba1), glial fibrillary acidic protein (GFAP), and neuronal nuclei (NeuN) was performed to observe Mincle expression in different cell types of the brain in sham group. The Syk phosphorylation inhibitor piceatannol was injected intraperitoneally at 1 hour after SAH.

Experiment 2
For outcome study, rats were randomly subjected to sham, SAH+vehicle, SAH+rSAP130, SAH+scramble small interfering RNA (siRNA), and SAH+Mincle siRNA group. Vehicle or recombinant SAP130 (rSAP130, 50 ng/5 μL) and scramble siRNA (siRNA, 500 pmol/5 μL) were injected intracerebroventricularly at 1.5 hours after SAH. Mincle siRNA (Mincle siRNA, 500 pmol/5 μL) and scramble siRNA (siRNA, 500 pmol/5 μL) were injected intracerebroventricularly at 24 hours before SAH induction. Exogenous SAP130 (rSAP130) was injected at 1.5 hours after SAH. The Syk phosphorylation inhibitor piceatannol was injected intraperitoneally at 1 hour after SAH.

Severity of SAH
The severity of SAH was blindly evaluated by the SAH grading system at the time of euthanasia as previously described. The basal cistern was divided into 6 segments that were scored from 0 to 3 according the amount of the subarachnoid blood clot. Rats with SAH grading c8 at 24 hours were excluded in this study.

Neurological Score
Neurological scores were assessed 1 hour before euthanasia by a blinded observer using a modified Garcia scoring system, which consisted of 6 tests covering spontaneous activity, spontaneous movements of all limbs, forelimbs outstretching, climbing ability, body perception, and response to vibrissae stimulation. The score of modified Garcia test ranged from 3 to 18.

Brain Water Content
Brains were removed at 24 hours after surgery and separated into left hemisphere, right hemisphere, cerebellum, and brain stem. Each part was weighed immediately after removal (wet weight) and after incubation in a 105°C oven for 72 hours. The following formula was used to calculate the percentage of BWC: [(wet weight−dry weight)/ wet weight]×100%.

Immunofluorescence Staining
Rats were euthanized at 24 hours after SAH for double fluorescence labeling, which was performed as previously described. Rabbit anti-Mincle (Bioss), rabbit anti-Syk (cell signaling), rabbit anti-CARD9 (Abcam), mouse anti-NeuN (EDM Millipore), goat anti-Iba-1 (Abcam), and goat anti-GFAP (Santa Cruz Biotechnology) primary antibodies were used. The expression of myeloperoxidase was detected by fluorescence labeling with rabbit antimyeloperoxidase (Santa Cruz Biotechnology). The sections were visualized using a fluorescence microscope. Microphotographs were analyzed with Image Pro Plus software (Olympus OX51, Japan). For more details, see online-only Data Supplement.

Western Blot
The perfused left brain hemispheres (perforation side) were harvested at 24 hours after SAH. Western blot was performed according to the protocol described previously. Primary antibodies used are listed as follow: goat anti-SAP130 (Abcam Biotech Company), rabbit anti-Mincle (Bioss), rabbit anti-Syk (cell signaling), rabbit anti-phosphate-Syk (Abcam), rabbit anti-CARD9 (Abcam), rabbit anti-IL-1β (Abcam) and rabbit anti-myeloperoxidase (Santa Cruz Biotechnology). For more details, see online-only Data Supplement. Intracerebroventricular Infusion
Intracerebroventricular infusion was performed as previously described. Briefly, postanesthetized rats were placed into a stereotaxic apparatus under 2% isoflurane anesthesia during the whole procedure. Three different formats of Mincle siRNA (500 pmol/5 μL; RNase-free water, Life Technologies), scramble siRNA (500 pmol/5 μL, Thermo Scientific Dharmacon), and rSAP130 Protein (50 ng/5 μL, Abnova corporation) were delivered into the left ventricle through a burr hole at the following coordinates relative to bregma: 1.5 mm posterior, 1.0 mm lateral, and 3.2 mm beneath the dural surface using a 10-μL Hamilton syringe (Microliter 701; Hamilton Company, Reno, NV). To enhance the gene silence efficiency, 3 different Mincle siRNA were mixed, listed as follows: (1) 5′CACCUAUCCUGCUAUCAAGCUA3′; (2) 5′GCUACCGUGGGGUUAUCAACACAU3′; and (3) 5′CCUGUUUCUCAGAUAGCCUUCAU3′. All the chemicals were injected by a pump at a rate of 0.5 μL/min. The syringe was kept in place for 5 minutes after infusion and then slowly removed. Mincle siRNA and scramble siRNA were injected at 24 hours before SAH induction. Exogenous SAP130 (rSAP130) was injected at 1.5 hours after SAH. The Syk phosphorylation inhibitor piceatannol was injected intraperitoneally at 1 hour after SAH.
Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5 (GraphPad software). Data are represented as a mean±SEM. Chi-square test was used to analyze the mortality of all the groups. All other data were analyzed by one-way ANOVA followed by Tukey post hoc test. *P value of <0.05 was considered statistically significant.

Results

Mortality and Exclusion

There was no significant difference of physiological variables (body temperature, blood gases, and body weight) among different experimental groups (data not shown). For SAH animals, filament puncture induced widespread subarachnoid bleeding around the Willis Circle and along the ventral brain stem, particularly pronounced on the left side (Figure IA in the online-only Data Supplement). At 24-hour posthemorrhage, there was no significant difference of SAH grading in all SAH groups (Figure IB in the online-only Data Supplement).

No rats died in sham group. No significant difference for mortality was observed among these operated groups (Figure IC in the online-only Data Supplement). According to the modified SAH grading system at the time of euthanasia as previously described, 23 mild SAH rats were then excluded from this study (Table in the online-only Data Supplement).

Temporal Patterns of Mincle, SAP130, Syk, and p-Syk Expression in the Left Hemisphere After SAH Induction

Western blot was performed to determine the protein expression of Mincle, the endogenous ligand of Mincle receptor SAP130, Syk, and p-Syk in sham group and in animals euthanized at 3, 6, 12, 24, and 72 hours after SAH. The expression level of Mincle increased, starting as early as 6 hours after SAH and peaked at 24 hours after SAH (A). SAP130 (B), Syk (C), and p-Syk (D) expressions significantly increased at both 12 and 24 hours after SAH. The expression of Mincle and its downstream factors recovered to the basal level at 72 hours after SAH. Relative densities of each protein have been normalized against the sham group. n=6 for each group. *P<0.05 vs sham.
hours after SAH and lasting till 24 hours (both 12 and 24 hours were statistically significant higher than sham group, \( P<0.05 \)), and recovered to the basal level at 72 hours after SAH (Figure 1A). A similar tendency was observed in the expression of SAP130, the endogenous ligand of Mincle receptor, and the downstream kinase Syk and p-Syk. All of them started to increase at 12 hours and peaked at 24 hours after SAH (Figure 1B–1D).

**Mincle, Syk, and CARD9 Distribution in Cells in the Left Hemisphere After SAH Induction**

Double immunostaining of Mincle, Syk, and CARD9 with Iba1, NeuN, and GFAP indicated that Mincle was expressed in microglias (Figure 2A) and Mincle expression was increased at 24 hours after SAH (Figure 2B). Mincle was also observed expressed in neurons (Figure 2C), however, not in astrocytes (Figure 2D) at 24 hours after SAH. There was no expression of Syk and CARD9 in sham animals. Both of them were colocalized only with Iba1 and NeuN positive cells, but not GFAP positive cells in animals at 24 hours after SAH (Figure IIA–IID in the online-only Data Supplement).

**Effect of Mincle Activation by rSAP130 and Mincle/Syk Signaling Inhibition by Mincle siRNA and Piceatannol on Neurological Function and BWC at 24 hours After SAH**

Mincle siRNA (500 pmol/5 \( \mu L \)) was injected intracerebroventricularly at 24 hours before SAH induction, and rSAP130 (50 ng/5 \( \mu L \)) was administrated intracerebroventricularly at 1.5 hours after SAH. Piceatannol was injected intraperitoneally at 1 hour after SAH. BWC and neurological score were measured at 24 hours after SAH. As shown in Figure 3A, BWC of the left hemisphere increased significantly in vehicle, rSAP130, and scramble siRNA groups at 24 hours after SAH (\( P<0.05 \) versus sham, n=6, Figure 3A). Mincle activation exhibited a potential to further increase BWC in the left hemisphere (\( P>0.05 \)). Consistently, there was remarkable neurobehavioral function impairment in vehicle, scramble siRNA, and Mincle activation (SAH+rSAP130) groups compared with that of sham group at 24 hours after SAH (\( P<0.05 \), n=6, Figure 3B). However, the difference between vehicle and Mincle activation groups was not significant (\( P>0.05 \)). On the contrary, Mincle siRNA treatment significantly reduced BWC in left hemisphere (\( P<0.05 \) versus vehicle, rSAP130, and scramble siRNA, n=6, Figure 3A). Consistently, Mincle siRNA was able to ameliorate neurological deficits (\( P<0.05 \) versus vehicle, rSAP130, and scramble siRNA, n=6, Figure 3B).

As shown in Figure 3C, the administration of rSAP130 exhibited a tendency to further increase BWC in the left hemisphere, however, not significantly (\( P>0.05 \)). Mincle knockdown by specific siRNA and Syk inhibition by piceatannol reversed the effect of rSAP130 on BWC at 24 hours after SAH (\( P<0.05 \) versus rSAP130+scramble siRNA group, n=6, Figure 3C). Consistently, Mincle siRNA and piceatannol were able to ameliorate neurological deficits by Mincle activation compared with that of SAH+rSAP130 group (\( P<0.05 \) versus SAH+rSAP130 and rSAP130+scramble siRNA group, n=6, Figure 3D).

**Effect of Mincle Activation by rSAP130 and Mincle Knockdown by Mincle siRNA on Inflammatory Response After SAH**

SAP130 activated Mincle signaling in macrophages, as well as in cultured neurons.\(^{17}\) In this study, both vehicle and Mincle activation animals showed increased expression of myeloperoxidase (\( P<0.05 \) versus sham group). Furthermore, Mincle activation by rSAP130 potentiated the protein expression of myeloperoxidase to a level higher than the vehicle group (\( P<0.05 \) versus vehicle group, Figure 4A and 4B). On the contrary, Mincle knockdown by specific siRNA reversed the effect of rSAP130 on myeloperoxidase expressions (\( P<0.05 \) versus vehicle group and scramble siRNA group, Figure 4A and 4B).
Similarly, immunohistochemical staining revealed elevated myeloperoxidase positive cells infiltration in the cortical region after rSAP130 administration at 24 hours after SAH, which was also decreased by Mincle siRNA (Figure 4C).

Mincle siRNA Inhibited Mincle/Syk Signaling and IL-1β Production Induced by rSAP130 Administration After SAH

To explore the signaling pathway involved in proinflammatory effect of Mincle activation, animals were subjected to rSAP130 administration with and without Mincle siRNA treatment at 24 hours before SAH induction. As shown in Figure 5A, rSAP130 administration did not have any direct effect on the expression level of Mincle. However, Mincle siRNA was able to significantly knockdown Mincle expression (Figure III in the online-only Data Supplement). rSAP130 administration activated Mincle/Syk pathway by increasing the expression of downstream kinase p-Syk, CARD9, and IL-1β \( (P<0.05 \text{ versus vehicle, Figure 5B--5D}) \). Consistently, Mincle siRNA reversed the proinflammatory effect of rSAP130 by downregulating p-Syk, CARD9, and IL-1β expression \( (P<0.05 \text{ versus SAH+rSAP130, Figure 5B--5D}) \).

Syk Inhibition by Piceatannol Reduced IL-1β Production Induced by Mincle Activation After SAH

To further determine the role of Syk in Mincle signaling pathway, piceatannol, a selective Syk phosphorylation inhibitor, was injected intraperitoneally at 1 hour after SAH. Both Syk inhibition and Mincle activation had no effect on Mincle expression (Figure 6A). The increased expression of p-Syk, CARD9, and IL-1β after SAH was further enhanced by rSAP130 treatment \( (P<0.05 \text{ versus vehicle, Figure 6B--6D}) \). However, Piceatannol was able to reverse the effect of
Mincle activation by reducing p-Syk, CARD9, and IL-1β expression at 24 hours after SAH (P<0.05 versus vehicle and SAH+rSAP130 group, Figure 6B—6D).

Discussion

We have obtained the following new observations: first, SAH enhanced the expression of Mincle, SAP130 (an endogenous ligand of Mincle receptor). Mincle downstream factors Syk and phosphorylated Syk at 12–24 hours in the ipsilateral hemisphere (puncture side) of rats. The expression of Mincle and downstream factors recovered to basal level at 72 hours after SAH. Second, Mincle expressed mostly in microglia but also in neurons but not in astrocytes. SAH enhanced the expression of Mincle in microglia. Third, activation of Mincle by rSAP130 had a tendency to increase BWC (P>0.05) but did not affect the neurological functions, whereas knockdown Mincle by siRNA and Syk inhibition by piceatannol reduced BWC and improved neurological function. Fourth, SAH increased IL-1β and myeloperoxidase levels, and Mincle activation by rSAP130 potentiated the effect of SAH on IL-1β and myeloperoxidase, whereas Mincle knockdown by siRNA reduced the effect of SAH. Fifth, Mincle activation by rSAP130 did not change the protein expression of Mincle but enhanced the expression of p-Syk and CARD9, and Mincle knockdown by siRNA reduced the protein levels of Mincle, p-Syk, and CARD9. Finally, Syk inhibitor piceatannol did not affect the protein expression of Mincle but decreased the p-Syk, CARD9, and IL-1β at 24 hours after SAH.

Early brain injury represents most neurological injuries within 72 hours after SAH, including global ischemia, cortical spreading depolarization, neuroinflammation, and resulted in blood–brain barrier disruption and brain edema. The innate immune system is a major contributor to acute inflammation induced by microbial infection or tissue damage. Increasing evidences have demonstrated a role of innate immune response, may be triggered by pattern recognition receptors, after CNS injury, including stroke and traumatic brain injury. Several publications have demonstrated the potential roles of Toll-like receptor and nucleotide-binding oligomerization domain–like receptor complexes mediated inflammatory responses in the pathogenesis of early brain injury after SAH. The current study may expand the pattern recognition receptors by the identification of Mincle, one of the C-type lectin receptors, as a contributor to the innate immune response after SAH.

Mincle, also called CLEC4E, was originally recognized as a downstream target of NF-IL6 (C/EBP) in murine peritoneal macrophages. Mincle has been identified as an essential receptor for TDM (trehalose-6,6-dimycolate; cord factor) of Mycobacterium tuberculosis, the pathogenic fungi Candida albicans, and the function of Mincle has been suggested playing an important role in the immune response to mycobacteria and fungi. Yamasaki et al demonstrated that Mincle, when activated by endogenous SAP130 which is a component of small nuclear ribonucleoprotein released from necrotic cells, could upregulate proinflammatory mediators and enhance neutrophils infiltration into damaged tissue. Although Mincle is at a low expression level in the steady-state condition, it was strongly upregulated after exposure to various stimuli, such as injury and stress. There were a few studies have focused on the role of Mincle in neuronal disorders. In a cerebral ischemia mouse model, the expression of Mincle and endogenous SAP130 were increased at 2–22 hours after reperfusion. Mincle and endogenous SAP130 were found elevated in the cerebrospinal fluid and injured brain tissue in traumatic brain injury patients and rodents. Consistent with those abovementioned observations, this study demonstrated that Mincle, endogenous SAP130, and Mincle downstream kinase Syk/p-Syk increased after SAH. In this study, Mincle was found to colocalize mostly with Iba1 or expressed mostly in microglia. This observation is different from the previous report that Mincle localized in CD11b (macrophage) positive cells but not Iba1 in a mouse cerebral ischemia model. Even though we did not study macrophage, and Iba1 expressed in macrophages and microglia, the immunohistochemistry staining in our study showed clearly that microglia expressed Mincle especially after SAH. This observation is consistent with most studies...
Figure 5. The effects of macrophage-inducible C-type lectin (Mincle) small interfering RNA (siRNA) on the expression of Mincle, phosphorylated Syk (p-Syk), caspase-associated recruitment domain 9 (CARD9), and interleukin-1β (IL-1β) in the presence of rSAP130 administration after subarachnoid hemorrhage (SAH). rSAP130 did not affect the expression of Mincle but potentiated the expression of p-Syk, CARD9, and IL-1β. Mincle siRNA decreased the protein expression of Mincle (A), p-Syk (B), CARD9 (C), and IL-1β (D) at 24 hours after SAH. Scramble siRNA failed to affect the expression of Mincle, p-Syk, CARD9, and IL-1β. Relative densities of each protein have been normalized against the sham group. n=6 for each group. *P<0.05 vs sham; #P<0.05 vs SAH+vehicle; &P<0.05 vs SAH+rSAP130; and §P<0.05 vs SAH+rSAP130+scramble siRNA.

that Iba1 expression increased after CNS injuries. In addition, it was reported that Mincle, as well as P2X7R and inflammasome, also expressed in neurons. We have had similar observations that Mincle colocalized with NeuN, but only in some neurons but not in most of the neurons (Figure 2). Further studies are needed to identify what types of neurons express Mincle. The overall observations in this study indicated that Mincle may serve as a pattern recognition receptor that increased its expression after SAH and silencing Mincle via Mincle siRNA decreased its innate immune reaction, such as the expression of IL-1β and myeloperoxidase. It was reported that IL-1β activation was able to induce matrix metalloproteinase-9 expression via c-Jun N-terminal kinase pathway after SAH. Matrix metalloproteinase-9 then degraded extracellular matrix proteins, such as type IV collagen of cerebral microvessels, leading to the blood–brain barrier disruption and neuronal dysfunction. The results from this study also demonstrated the increased BWC and myeloperoxidase positive cells infiltration following Mincle activation, which initially induced neuroinflammation.

It seems neuroinflammation is one of the results after Mincle and Syk activation after CNS injuries. Indeed in a previous study, when activated by SAP130, Mincle triggers intracellular signaling via activation of Syk, leading to enhanced production of proinflammatory cytokines
Similar results were obtained in this study that rSAP130 administration significantly enhanced the expression of p-Syk, CARD9, and proinflammatory cytokines IL-1β, however without increasing Mincle expression. The most likely reason is that Mincle might have different self- and non–self-ligands other than SAP130. Thus, it was not fully combined with endogenous SAP130 after SAH and the exogenous rSAP130 treatment could enhance the expression of p-Syk, CARD9, and IL-1β without further increasing Mincle expression. Furthermore, based on the pathophysiological response of Mincle activation, there was no feedback response of Mincle expression to its activation. The expression of Mincle did not change when exhibiting its function. Since the research of SAP130 and its receptor Mincle is limited to date, it is less known of molecular pathway which induces Mincle expression after SAH and the remaining pathophysiological effects of Mincle. CARD9 signaling plays an essential role in the innate immune response and inflammation, and IL-1β are established as contributors for neuroinflammation in multiple CNS injuries, including SAH. Mincle knockdown by specific siRNA and Syk inhibition by piceatannol reduced substantially the expression of CARD9 and IL-1β. Similar results of piceatannol, a selective Syk inhibitor that suppresses phosphorylation of Syk, have been reported by others that piceatannol prevented tissue injury in the retina, local intestine, and remote lung, as well as brain, after ischemia–reperfusion.
There are a few limitations in this study. First, the physiological role of rSAP130 and Mincle siRNA was not evaluated in normal rats. We searched but did not find any studies that reported an effect of either rSAP130 or Mincle siRNA on the blood pressure, blood glucose, or cerebral blood flow, factors may affect the results from this study. Second, in a previous study of ischemic stroke animal model that Mincle was shown expressed in neurons and promoted neuronal apoptosis. We did not study the potential apoptotic effect of Mincle and its downstream pathways but rather focused on neuroinflammation. One of the reasons is that as shown in Figure 2 that only a few but not most neurons showed Mincle expression after SAH. Further studies are required to establish the type of neurons that express Mincle and if Mincle contributes to neuronal apoptosis after SAH. It has been reported that neuronal apoptosis occurred and contribute to the poor functional outcomes after SAH. Third, Mincle was reported to form a heterodimer with another C-type lectin receptor or toll-like receptors, which may amplify signaling, expand ligand specificity, or confer multiple functions. Therefore, the connections between Mincle and other innate immune receptors in neuroinflammation after SAH require further studies in the future.

In conclusion, this observation for the first time demonstrated that Mincle/Syk signaling pathway played an important role in the innate immune response and neuroinflammation after SAH. Mincle/Syk may have potentials to serve as future novel treatment targets for control neuroinflammation after SAH or other CNS disorders.

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Disclosures
None.

References


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SUPPLEMENTAL MATERIAL
Supplemental methods
Animal Model
Briefly, rats were anesthetized using 3% isoflurane in 70%/30% medical-air/oxygen. A 4-0 sharpened monofilament nylon suture was gently inserted rostrally into the left internal carotid artery from the external carotid artery stump to the bifurcation of the anterior and middle cerebral arteries. Suture was further advanced for an extra 5mm to cause the perforation of the artery. After withdrawing the suture immediately, the external carotid artery was ligated. Sham-operated rats were subjected to all surgical procedures except suture puncture.

Immunofluorescence staining
Brain sections were incubated with a certain mixture of primary antibodies over night at 4°C, followed by a mixture of appropriate secondary antibodies in the dark for 1 hour at room temperature. For negative controls, the primary antibodies were omitted and the rest staining procedures were performed.

Western Blot
Equal amounts of protein (50µg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes. Membranes were incubated with the respective primary antibodies. Immunoblots were processed with appropriate secondary antibodies (Santa Cruz Biotechnology) for 2 hours at room temperature. Bands were visualized after incubating the membranes with the ECL Plus chemiluminescence reagent kit (Amersham Bioscience, Arlington Heights, IL). The signal density was quantified using Image J (National Institutes of Health, Bethesda, MD). Results were presented as relative density ratio, normalized to the average value of the sham group.
**Supplemental Table:**

**Table Numbers of animals that has been used in each group**

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<td>6</td>
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<tr>
<td>SAH+rSAP130+piceatannol</td>
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<td>6</td>
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<tr>
<td><strong>Total</strong></td>
<td></td>
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<td>215*</td>
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* Seven animals were excluded from the study because of the intubation related complication.
Supplemental Figure  I  Representative Photograph of SAH model, SAH grading and mortality among each group. (A) Representative photographs of sham and SAH model. Subarachnoid hemorrhage was pronounced at the left side. (B) There was no significant difference of SAH grading among all the experimental groups at 24 hours after SAH. (C) The mortality for each group are listed as follow: SAH group 21.4% (12/56), SAH+vehicle group 15.8% (3/19), SAH+rSAP130 group 21.1% (4/19), SAH+scramble siRNA group 15.78% (3/19), SAH+Mincle siRNA 12.5 % (2/16); SAH+rSAP130+scramble siRNA group 18.8% (3/16), SAH+rSAP130+Mincle siRNA group 17.6% (3/17), SAH+piceatannol group 14.3% (2/14), SAH+rSAP130+piceatannol group 16.7% (3/18). No statistically significant difference was observed among all the operated groups.
Supplemental Figure II  The expression of Syk and CARD9 in sham animals and in animals at 24h after SAH. There was no expression of Syk (A) and CARD9 (C) in sham animals. Both of them were co-localized only with Iba1 and NeuN positive cells, but not GFAP positive cells in animals at 24h after SAH (B and D). Scale bar=100 µm.
Supplemental Figure III The efficiency of Mincle siRNA. Quantitative analysis of western blot demonstrated that Mincle siRNA knocked down the expression of Mincle by around 50%. # P<0.05 vs. vehicle. @ P<0.05 vs. Scramble siRNA.