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Mincle/Syk Signaling Pathway Contributes to Neuroinflammation after Subarachnoid Hemorrhage in Rats

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Abstract

Background and Purpose—Mincle (macrophage-inducible C-type lectin, 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 Syk signal pathway in early brain injury after SAH in a rat model.

Methods—Two hundreds and fifteen (215) male Sprague-Dawley rats (280–320g) were subjected to endovascular perforation model of SAH. SAH grade, neurological score, and brain water content were measured at 24 h after SAH. Mincle/Syk as well as CARD9 (a member of the caspase-associated recruitment domain (CARD), involved in innate immune response), interleukin-1β (IL-1β) and myeloperoxidase (MPO) expressions were analyzed by western blot at 24 h after SAH. Specific cell types that expressed Mincle were detected with double immunofluorescence staining. Mincle siRNA, the endogenous ligand of Mincle receptor 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 12h and peaked at 24h after SAH. Mincle siRNA reduced IL-1β and infiltration of MPO positive cells, decreased brain water content, and improved neurological functions at 24h after SAH. The endogenous ligand of Mincle

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Disclosures

None.

receptor SAP130 up-regulated the expression of p-Syk and CARD9, and increased the levels of IL-1β and MPO, even though it did not increase brain water content nor it deteriorated neurological function at 24h after SAH. Syk inhibitor piceatannol reduced brain edema at 24h after SAH.

Conclusion—Mincle/Syk is involved in early brain injury after SAH, and they may serve as new targets for therapeutic intervention.

Keywords

Mincle; Syk; neuroinflammation; subarachnoid hemorrhage

Introduction

Subarachnoid hemorrhage (SAH) is a devastating cerebrovascular disease, representing around 5% of all strokes types[1], but has high rates of mortality and disability[2]. Early brain injury was reported as the primary cause of mortality and the key target for SAH treatment[3–5]. Increasing evidences suggested that innate immune response is one of the key factors in early brain injury after SAH[6]. 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[7, 8].

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 (BBB) disruption and neuronal dysfunction[9–12]. 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, Mincle (macrophage-inducible C-type lectin, CLEC4E) receptor recognizes self-ligands released from necrotic cells, that leads to the production of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and other chemokines[13]. Mincle belongs to C-type lectin receptor (CLR) family, which was first identified as a downstream transcriptional target in peritoneal macrophages[14]. 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[15]. In the central nerve system, it has been shown that Mincle and its downstream molecular Syk demonstrated a pivotal role in the pathogenesis of cerebral ischemia and reperfusion[16]. Mincle expression was elevated in the CSF 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[17]. 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 the present 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 experiments procedures were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

SAH Model and Experimental Protocol

Two hundred and fifteen male (280–320g) Sprague-Dawley rats (Indianapolis, IN) were used. The endovascular perforation model of SAH was performed as reported previously.[18] The details were also described in the Supplementary Text.

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 Supplementary Table.

Experiments 1: rats were divided into 6 groups (sham, and 3h, 6h, 12h, 24h, 72h after SAH, n=6 for each group). The endogenous ligand of Mincle receptor SAP130, Mincle receptor, downstream kinase Syk, and phosphorylated Syk (p-Syk) were detected by western blot. Double immunostaining of Mincle, Syk and CARD9 with calcium binding adaptor 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 (n=1) and 24h after SAH group (n=2).

Experiments 2: For outcome study, rats were randomly subjected to sham, SAH+vehicle, SAH+rSAP130, SAH+scramble siRNA, SAH+Mincle siRNA group. Vehicle or recombinant SAP130 (rSAP130, 50ng/5ul) were administrated intracerebroventricularly (ICV) at 1.5h after SAH. Macrophage-inducible C-type lectin small interfering RNA (Mincle siRNA, 500pmol/5μl) and scramble small interfering RNA (siRNA, 500pmmol/5μl) was injected ICV at 24h before SAH induction. SAH grade, neurological score and brain water content (n=6 for each group) were measured at 24h after SAH. Myeloperoxidase (MPO) were analyzed by western blot (n=6 for each group) and immunofluorescence staining to evaluate the inflammatory response at 24h after SAH in sham (n=1) and each operated group (n=2).

Experiments 3: Rats were randomly divided into sham, SAH+vehicle, SAH+rSAP130, SAH +rSAP130+scramble siRNA, SAH+rSAP130+Mincle siRNA, SAH+piceatannol, SAH +rSAP130+piceatannol group. Vehicle, Mincle siRNA, and scramble siRNA administration were described as above. Piceatannol was injected intraperitoneally at 1h after SAH. SAH grade, neurological score and brain water content were evaluated at 24h after SAH (n=6 for each group). Mincle, p-Syk, CARD9, and IL-1β expression were determined at 24h after SAH by western blots in all groups (n=6 for each group).

Intracerebroventricular Infusion

Intracerebroventricular infusion was performed as previously described[7]. Briefly, Post-anesthetized rats were placed into a stereotaxic apparatus under 2% isoflurane anesthesia during the whole procedure. Three different formats of Mincle siRNA (500pmol/5μl RNase-free water[7], Life Technologies), scramble siRNA, (500pmol/5μl, Thermo Scientific

Dharmacon) and rSAP130 Protein (50ng/5μl[17], Abnova corporation) was 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 3.2mm beneath the dural surface using a 10μl Hamilton syringe (Microliter 701; Hamilton Company, Reno, NV). In order to enhance the gene silence efficiency, three different Mincle siRNA were mixed, listed as follows: 1) 5'CACCUUAUCCUGGCUAUCAAGUCUA3'; 2) 5'GCUCACCUGGUGGUUAUCAACACAU3'; 3) 5'CCUGUUUCUUCAGUAUGCCUUGGAU3'. All the chemicals were injected by a pump at a rate of 0.5 μl/min. The syringe was kept in place for 5 min after infusion and then slowly removed. Mincle siRNA and scramble siRNA were injected at 24h before SAH induction. Exogenous SAP130 (rSAP130) was injected at 1.5h after SAH. The Syk phosphorylation inhibitor piceatannol was injected intraperitoneally at 1h after SAH.

Severity of SAH

The severity of SAH was blindly evaluated by the SAH grading system at the time of euthanasia as previous described[19]. The basal cistern was divided into 6 segments that were scored from 0–3 according the amount of the subarachnoid blood clot. Rats with SAH grading less than 8 at 24h were excluded in this study[20].

Neurological Score

Neurological scores were assessed 1h before euthanasia by a blinded observer using a modified Garcia scoring system[21], 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 24h after surgery and separated into left hemisphere, right hemisphere, cerebellum and brainstem. 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 brain water content (BWC): [(wet weight − dry weight)/wet weight]×100%[22].

Immunofluorescence staining

Rats were euthanized at 24h after SAH for double-fluorescence labeling, which was performed as previously described[22]. Rabbit anti-Mincle (Bioss), rabbit anti-Syk (cell signaling), rabbit anti-CARD9 (Abcam), mouse anti-NeuN (EMD Millipore) and Goat anti-Iba-1 (Abcam), goat anti-GFAP (Santa Cruz Biotechnology) primary antibodies were used. The expression of MPO was detected by fluorescence labeling with rabbit anti-MPO (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 Supplementary Text.

Western Blot

The perfused left brain hemispheres (perforation side) were harvested at 24h after SAH. Western blot was performed according to the protocol described previously[22]. 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-MPO (Santa Cruz Biotechnology). For more details see Supplementary Text.

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 analysis of variance (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 brainstem, particularly pronounced on the left side (Supplemental Figure I-A). At 24h post-hemorrhage, there was no significant difference of SAH grading in all SAH groups (Supplemental Figure I-B).

No rats died in sham group. No significant difference for mortality was observed among these operated groups (Supplemental Figure I-C). According to the modified SAH grading system at the time of sacrifice as previous described, 23 mild SAH rats were then excluded from this study (Supplemental Table).

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 sacrificed at 3h, 6h, 12h, 24h, and 72h after SAH. The expression level of Mincle increased, starting as early as 6h after SAH and lasting till 24h (both 12h and 24 hours were statistically significant higher than sham group, $P<0.05$), and recovered to the basal level at 72h 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 12h and peaked at 24h 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 24h after SAH (Figure 2B). Mincle was also observed expressed in neurons (Figure 2C), however not in astrocytes (Figure 2D) at 24h after SAH. There was no expression of Syk

and CARD9 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 (Supplemental Figure II-A–D).

Effect of Mincle activation by rSAP130 and Mincle/Syk signaling inhibition by Mincle siRNA and piceatannol on neurological function and brain water content at 24h after SAH

Mincle siRNA (500pmol/5μl) was injected by ICV at 24h before SAH induction, while rSAP130 (50ng/5μl) was administrated by ICV at 1.5h after SAH. Piceatannol was injected intraperitoneally at 1h after SAH. Brain water content and neurological score were measured at 24h after SAH. As shown in Figure 3A, brain water content of the left hemisphere significantly increased in both vehicle, scramble siRNA group and Mincle activation groups at 24h after SAH ($P<0.05$ vs. sham, n=6, Figure 3A). Mincle activation exhibited a potential to further increase brain water content in the left hemisphere ($P>0.05$). Consistently, there was remarkable neurobehavioral function impairment in vehicle, scramble siRNA group and Mincle activation (SAH+rSAP130) groups compared with that of sham group at 24h 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 brain water content in left hemisphere ($P<0.05$ vs. vehicle & SAP130 & scramble siRNA, n=6, Figure 3A). Consistently, Mincle siRNA was able to ameliorate neurological deficits ($P<0.05$ vs. vehicle & SAP130 & scramble siRNA, n=6, Figure 3B).

As shown in Figure 3C, the administration of rSAP130 exhibited a tendency to further increase brain water content 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 brain water content at 24h after SAH ($P<0.05$ vs. 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 ($P<0.05$ vs. SAH+rSAP130 & 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 the present study, both vehicle and Mincle activation animals showed increased expression of MPO ($P<0.05$ vs. sham group). Furthermore, Mincle activation by rSAP130 potentiated the protein expression of MPO to a level higher than the vehicle group ($P<0.05$ vs. vehicle group, Figure 4A–B). On the contrary, Mincle knockdown by specific siRNA reversed the effect of rSAP130 on MPO expressions ($P<0.05$ vs. vehicle group and scramble siRNA group, Figure 4A–4B). Similarly, immunohistochemical staining revealed elevated MPO positive cells infiltration in the cortical region after rSAP130 administration at 24h 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 pro-inflammatory effect of Mincle activation, animals were subjected to rSAP130 administration with and without Mincle siRNA

treatment at 24h 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 (Supplemental Figure III). rSAP130 administration activated Mincle/Syk pathway by increasing the expression of downstream kinase p-Syk, CARD9 and IL-1β ($P<0.05$ vs. vehicle, Figure 5B–5D). Consistently, Mincle siRNA reversed the pro-inflammatory effect of rSAP130 by down-regulating p-Syk, CARD9 and IL-1β expression ($P<0.05$ vs. 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 1h 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 ($P<0.05$ vs. 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 24h after SAH ($P<0.05$ vs. vehicle & SAH+rSAP130 group, Figure 6B–6D).

Discussion

We have obtained the following new observations: firstly, SAH enhanced the expression of Mincle, SAP130 (an endogenous ligand of Mincle receptor), Mincle downstream factors Syk, and phosphorylated Syk at 12h-24h in the ipsilateral hemisphere (puncture side) of rats. The expression of Mincle and downstream factors recovered to basal level at 72h after SAH. Secondly, Mincle expressed mostly in microglia but also in neurons but not in astrocytes. SAH enhanced the expression of Mincle in microglia. Thirdly, activation of Mincle by rSAP130 had a tendency to increase brain water content ($p>0.05$) but did not affect the neurological functions, while knockdown Mincle by Mincle siRNA and Syk inhibition by piceatannol reduced brain water content and improved neurological function. Fourthly, SAH increased IL-1β and MPO levels, and Mincle activation by rSAP130 potentiated the effect of SAH on IL-1β and MPO, while Mincle knockdown by siRNA reduced the effect of SAH. Fifthly, 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 24h after SAH.

Early brain injury represents most neurological injuries within 72hr after SAH, including global ischemia, cortical spreading depolarization, neuroinflammation, and resulted in BBB disruption and brain edema[23]. The innate immune system is a major contributor to acute inflammation induced by microbial infection or tissue damage[24]. Increasing evidences have demonstrated a role of innate immune response, may be triggered by pattern recognition receptors, after CNS injury including stroke and TBI[25]. Several publications have demonstrated the potential roles of Toll-like receptor (TLR) and NOD-like receptor (NLR) complexes mediated inflammatory responses in the pathogenesis of early brain injury

following SAH[7, 26]. The current study may expand the pattern recognition receptors by the identification of Mincle, one of the C-type lectin receptors (CLR), as a contributor to the innate immune response after SAH.

Mincle (macrophage-inducible C-type lectin), also called CLEC4E was originally recognized as a downstream target of NF-IL6 (C/EBP) in murine peritoneal macrophages[14]. Mincle has been identified as an essential receptor for TDM (cord factor) of M. tuberculosis, the pathogenic fungi Candida albicans, and the function of Mincle has been suggest playing an important role in the immune response to mycobacteria and fungi[27]. Yamasaki *et al* demonstrated that Mincle, when activated by endogenous SAP130 which is a component of small nuclear ribonucleoprotein released from necrotic cells, could up-regulate pro-inflammatory mediators and enhance neutrophils infiltration into damaged tissue. Though 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[28]. 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 2–22h after reperfusion[16]. Mincle and endogenous SAP130 were found elevated in the CSF and injured brain tissue in TBI patients and rodents[17]. Consistent with those abovementioned observations, the present study demonstrated that Mincle, endogenous SAP130, and Mincle downstream kinase Syk/p-Syk increased after SAH. In the present study, Mincle was found to co-localize 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[16]. 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 that Iba1 expression increased after CNS injuries[29]. In addition, it was reported that Mincle[30], as well as P2X7R and inflammasome[31], also expressed in neurons. We have had similar observations that Mincle co-localized 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 MPO. It was reported that IL-1β activation was able to induce MMP-9 expression via JNK pathway after SAH[32]. MMP-9 then degraded extracellular matrix proteins, such as type IV collagen of cerebral microvessels, leading to the blood-brain barrier (BBB) disruption and neuronal dysfunction. The results from present study also demonstrated the increased brain water content and MPO 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 pro-inflammatory cytokines TNFα[17]. Similar results were obtained in this present study that rSAP130 administration significantly enhanced the expression of p-Syk, CARD9 (Caspase recruitment domain-containing protein 9) and pro-inflammatory 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 MPO 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[33], and IL-1β are established as contributors for neuroinflammation in multiple CNS injuries including SAH[7]. 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, has been reported by others that piceatannol prevented tissue injury in the retina[34], local intestine and remote lung[35] as well as brain[16] after ischemia-reperfusion.

There are a few limitations in this study. Firstly, the physiological role of rSAP130 and Mincle siRNA were 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 the present study. Second, in a previous study of ischemic stroke animal model that Mincle was shown expressed in neurons and promoted neuronal apoptosis[30]. 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[21]. Third, Mincle was reported to form a heterodimer with another CLR or TLRs, which may amplify signaling, expand ligand specificity, or confer multiple functions[36]. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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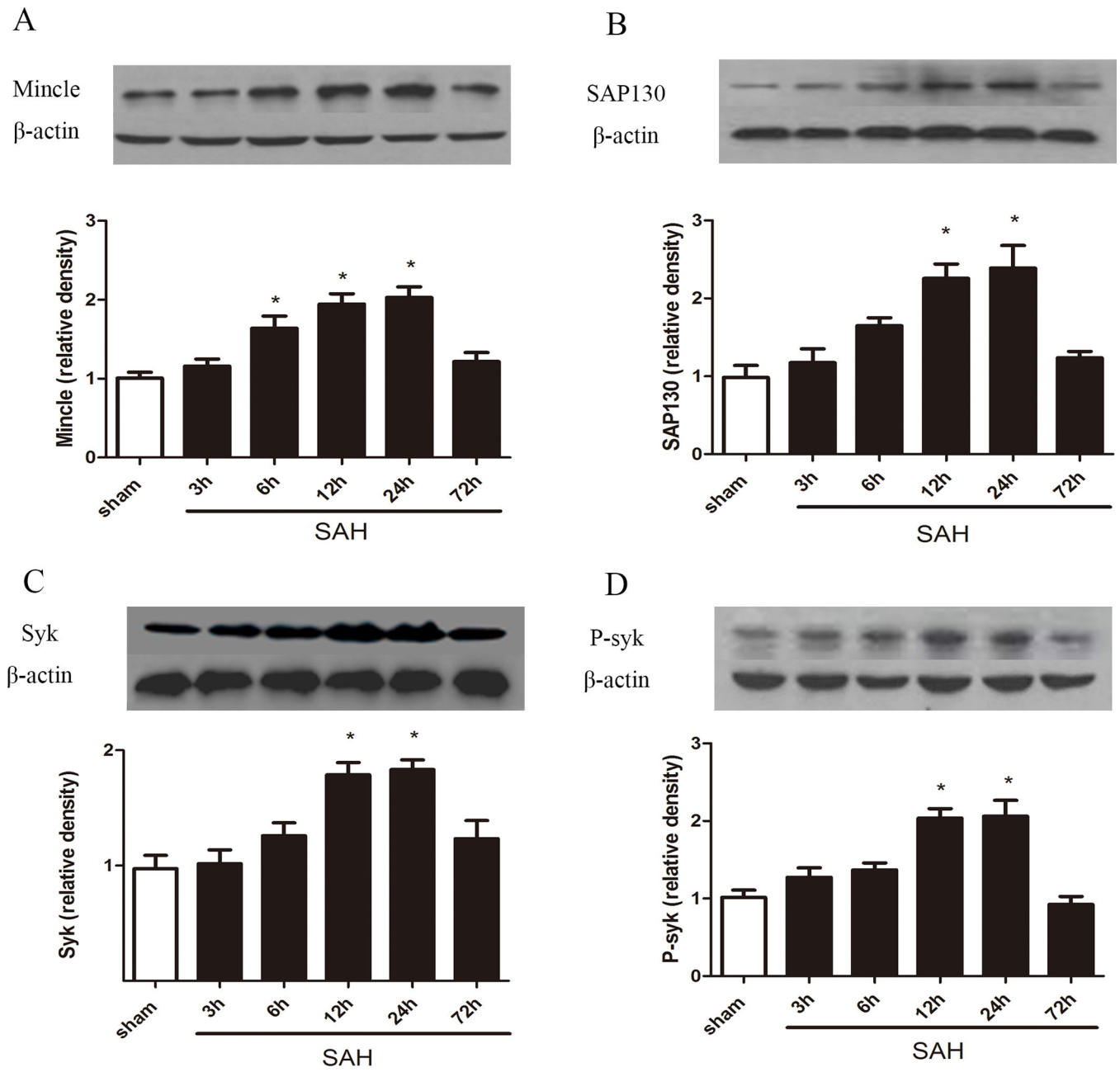

Figure 1. Time course of Mincle, SAP130, Syk and p-Syk expression in the left hemisphere after SAH

Quantitative analysis of western blot shows that there was an increased expression level of Mincle, started as early as 6h after SAH and peaked at 24h after SAH (A). SAP130 (B), Syk (C) and p-Syk (D) expressions significantly increased at both 12h and 24h after SAH. The expression of Mincle and its downstream factors recovered to the basal level at 72h after SAH. Relative densities of each protein have been normalized against the sham group. n=6 for each group. * $P<0.05$ vs. Sham.

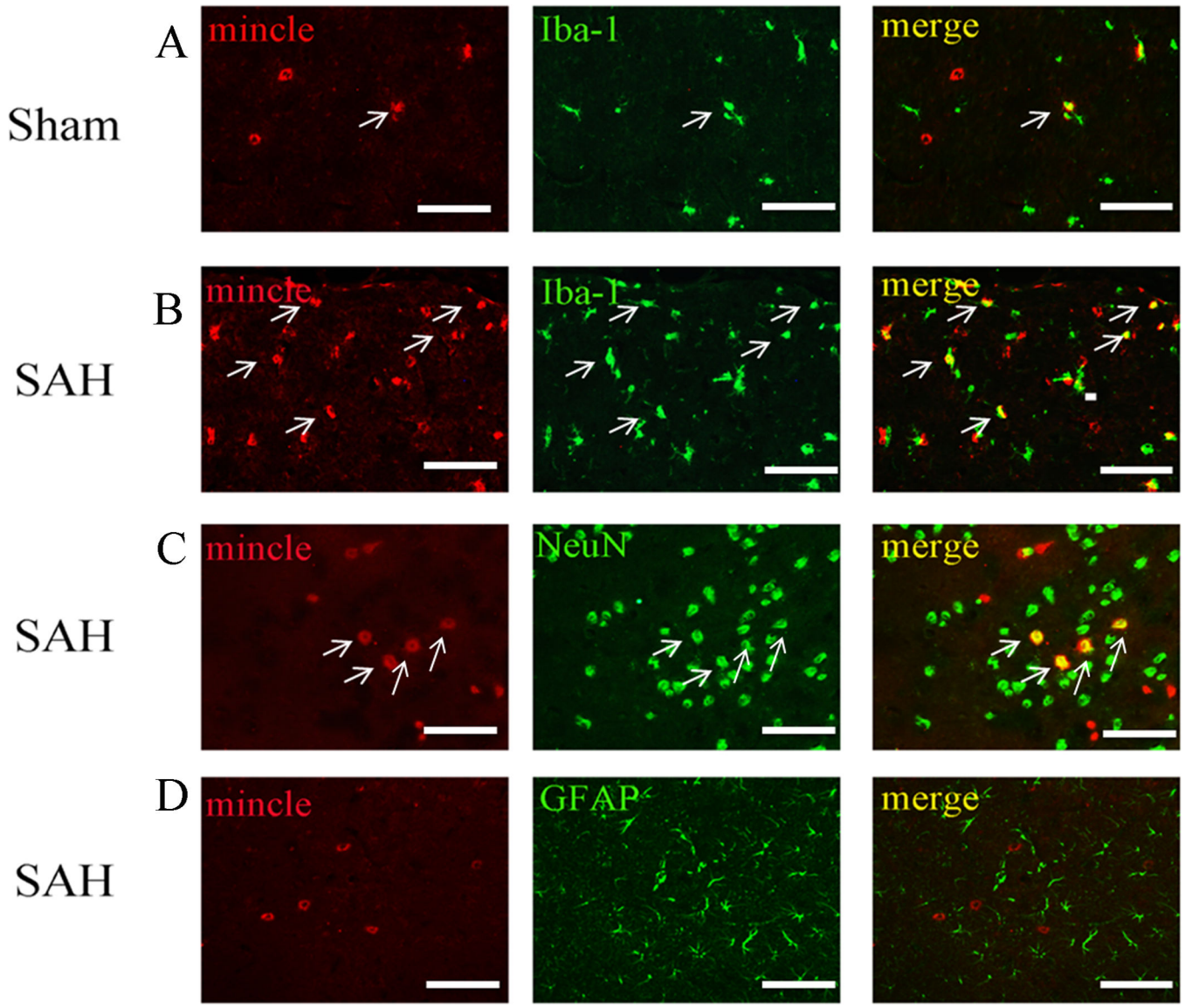

Figure 2. Co-localization of Mincle with cell type markers in the left hemisphere after SAH

Mincle co-localized with Iba1 in sham animals (A) and in animals at 24h after SAH (B). More Iba1 positive cells are observed after SAH when compared with Sham. There was some positive co-localization of Mincle and NeuN cells(C), but not with GFAP positive cells (D) at 24h after SAH. Scale bar=100μm.

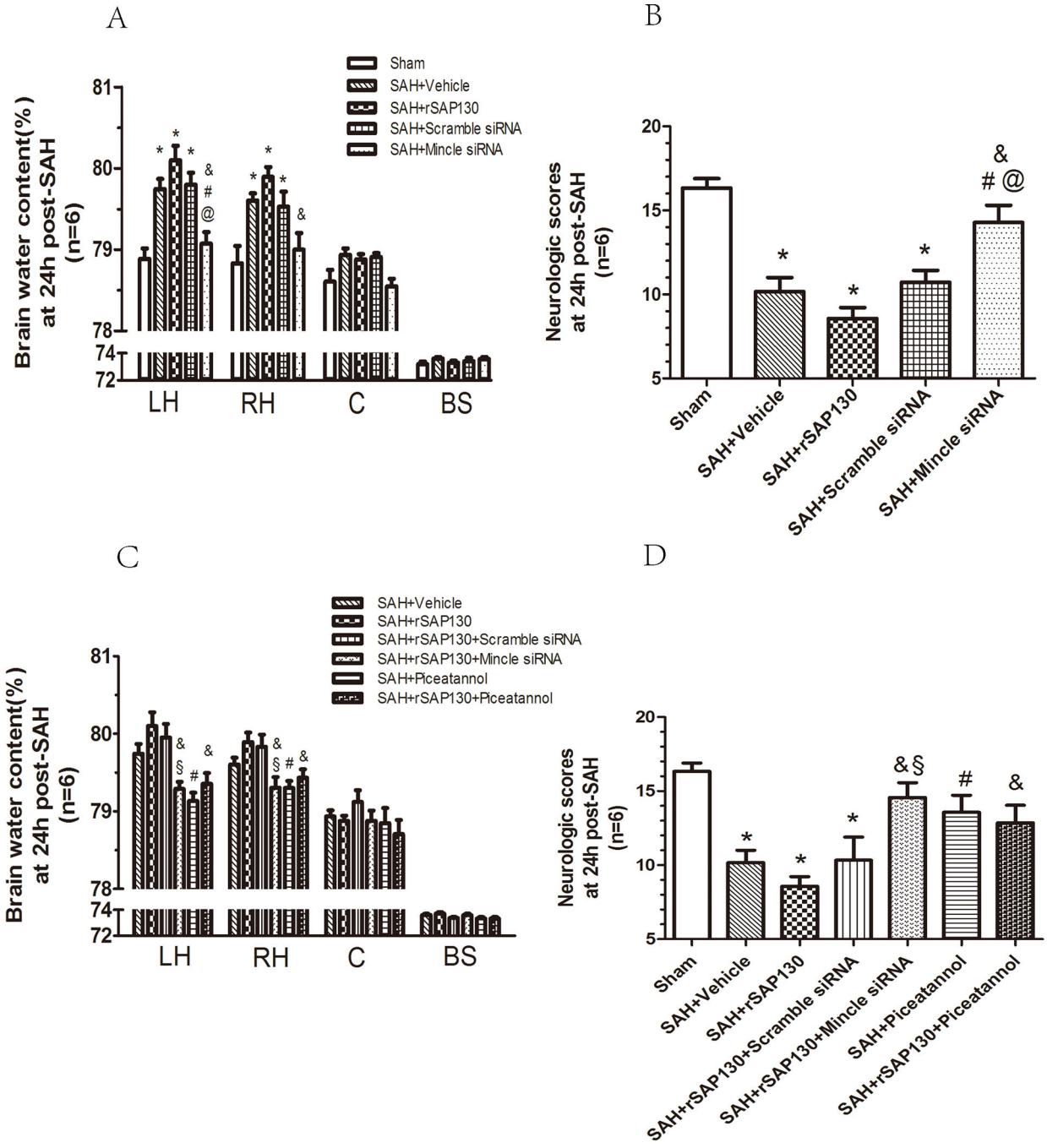

Figure 3. Effect of Mincle activation by rSAP130 and Mincle/Syk signaling inhibition by Mincle siRNA and piceatannol on neurological function and brain water content at 24h after SAH

SAH increased brain water content and decreased neurological scores (A,B). rSAP130 administration showed a tendency to increased brain water content (A) and decreased neurological function (B) but the results are not statistical significant. Knockdown of Mincle significantly reduced brain water content (A) and ameliorated neurological deficits (B) at 24h after SAH. Mincle siRNA and Syk inhibition by piceatannol reversed the effect of rSAP130 on brain water content (C) and ameliorated neurological deficits (D) at 24h after SAH. Scramble siRNA failed to affect either brain water content or neurological function.

n=6 for each group. * P<0.05 vs. Sham; # P<0.05 vs. SAH+vehicle; @ P<0.05 vs. SAH +scramble siRNA; & P<0.05 vs. SAH+rSAP130; § P<0.05 vs. SAH+rSAP130+scramble siRNA.

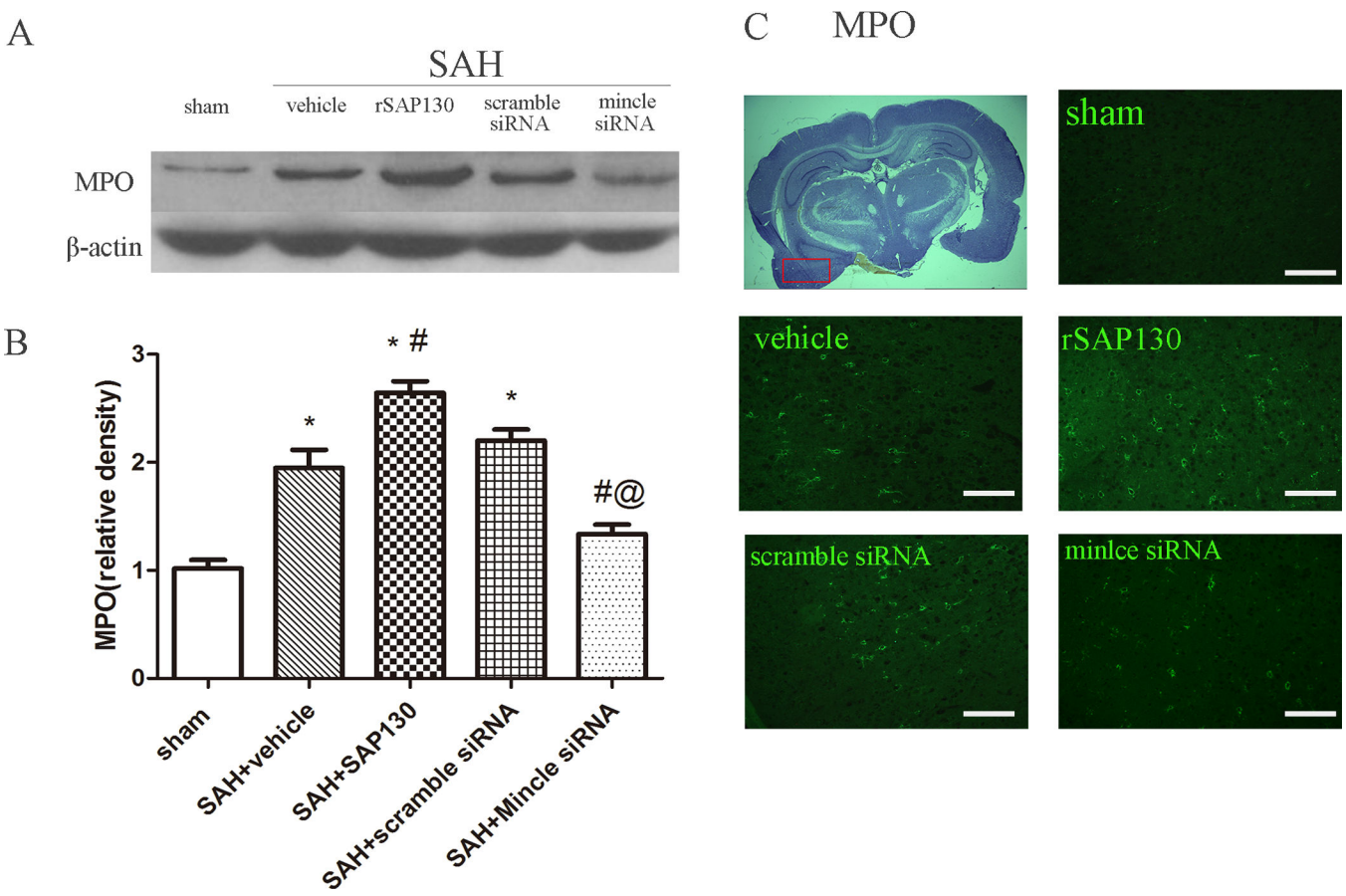

Figure 4. Effect of Mincle activation and Mincle knockdown on inflammatory response after SAH

Representative western blot bands are shown for MPO (A). Relative densities of each protein have been normalized against the sham group. The expression of MPO (B) was increased by rSAP130 administration, which were prevented by Mincle siRNA. Scramble siRNA did not show an effect. Representative photograph of immunofluorescence staining (C) for MPO showed that the MPO-positive cells were increased in the rSAP130 group and decreased in Mincle siRNA group at 24 h after SAH. Scale bars: 50μm. n=6 for each group.* P<0.05 vs. Sham; # P<0.05 vs. SAH+vehicle; @ P<0.05 vs. SAH+ scramble siRNA.

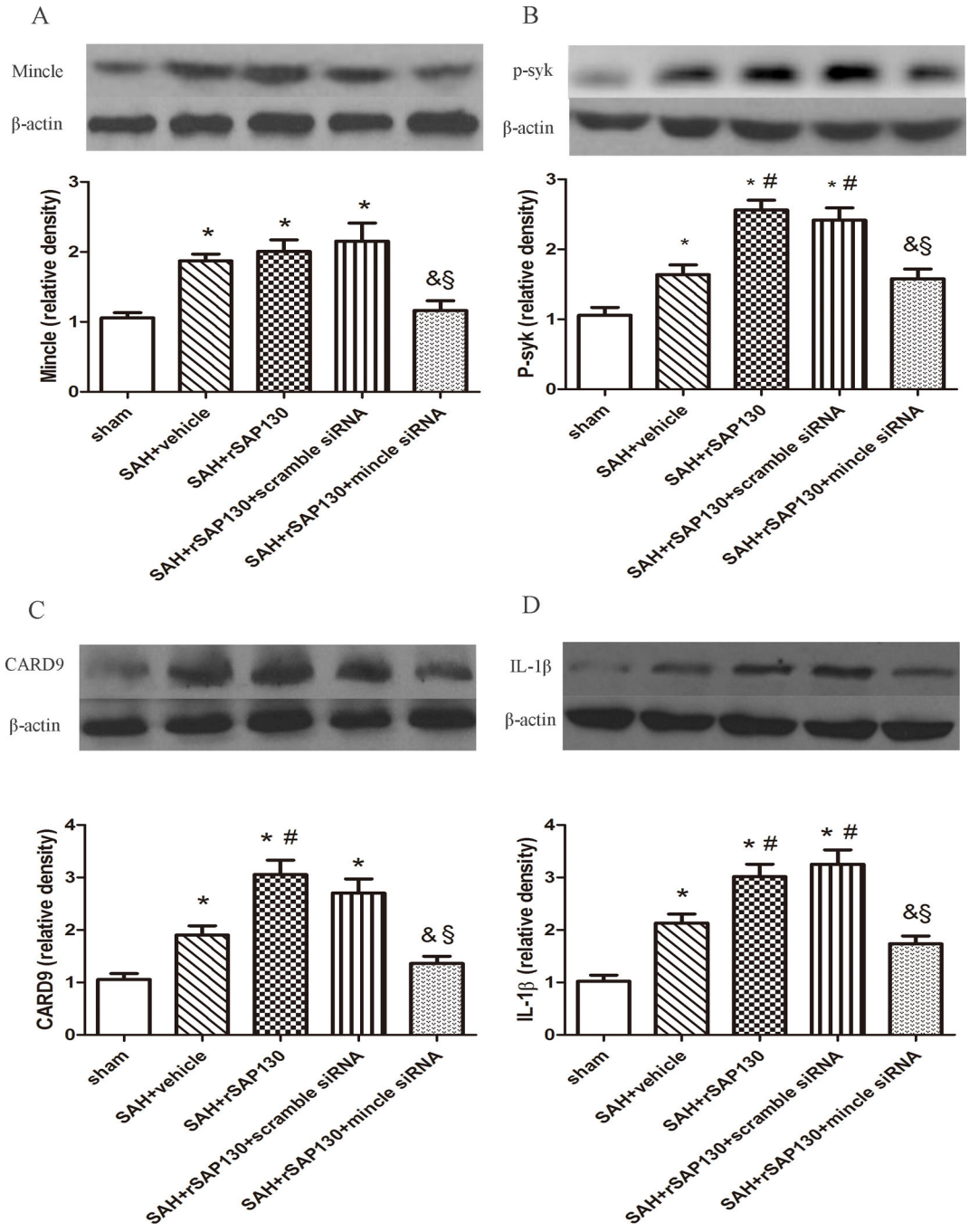

Figure 5. The effects of Mincle siRNA on the expression of Mincle, p-Syk, CARD9 and IL-1β in the presence of rSAP130 administration after SAH

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 24h 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. § P<0.05 vs. SAH+rSAP130+scramble siRNA.

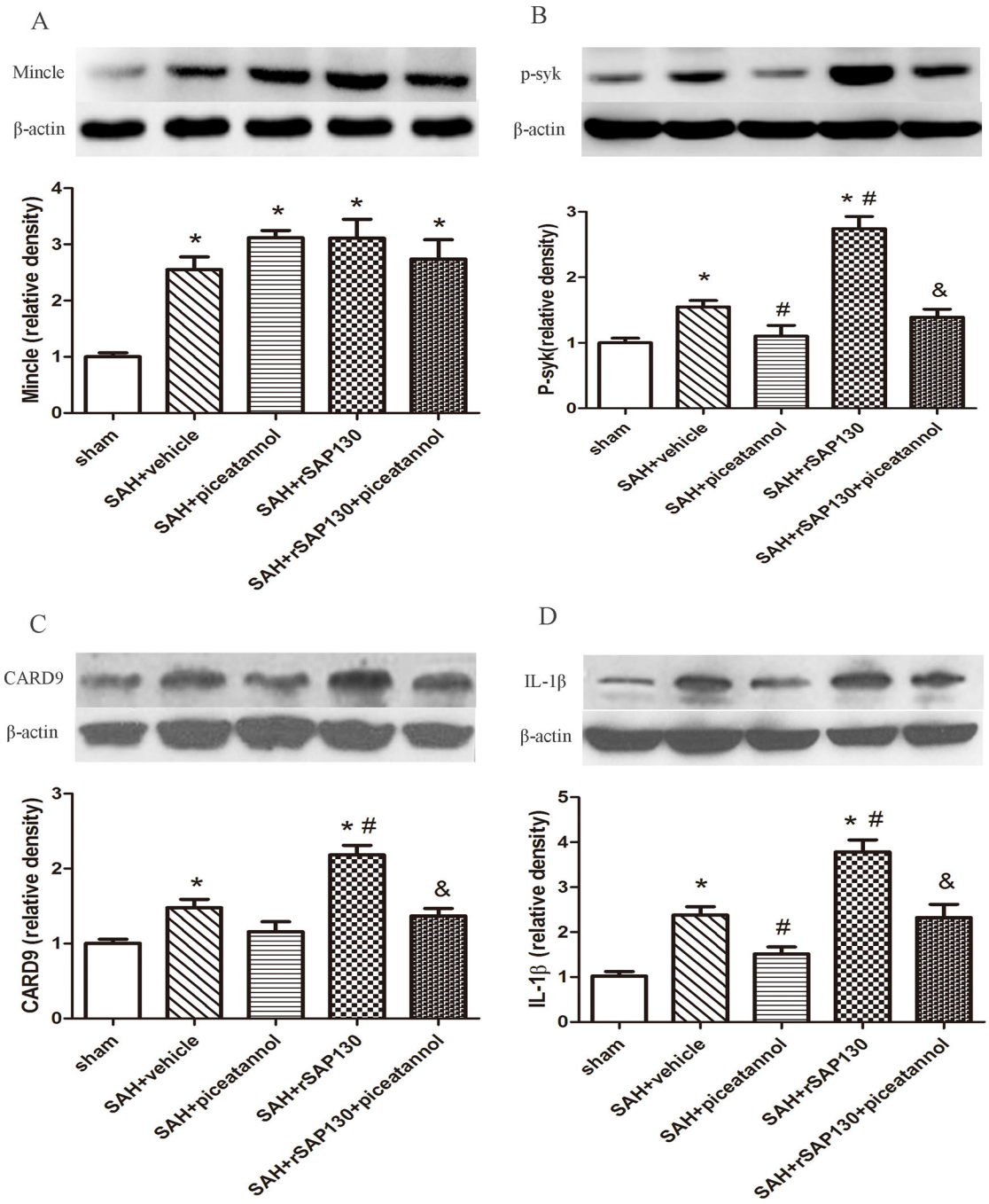

Figure 6. Syk inhibition by Piceatannol on the expression of Mincle, p-Syk, CARD9 and IL-1β in the presence of rSAP130 administration after SAH

Piceatannol did not affect the protein expression of Mincle (A), but decreased p-Syk (B), CARD9 (C) and IL-1β (D) expression at 24h after SAH. Piceatannol showed similar effects in the presence of rSAP130 after SAH. Relative densities of each protein have been normalized against the sham group. 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.