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Spinal voltage-gated potassium channel Kv1.3 contributes to neuropathic pain via promotion of microglial M1 polarization and activation of the NLRP3 inflammasome

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12
13 **Keywords:** microglial activation¹, M1 polarization², neuropathic pain³, Kv1.3⁴, the NLRP3
14 inflammasome⁵, neuroinflammation⁶.

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Abstract

Studies have shown that activation of microglia is the main mechanism of neuropathic pain. Kv1.3 channel is a novel therapeutic target for treating neuroinflammatory disorders due to its crucial role in subsets of microglial cells. As such, it may be involved in the processes of neuropathic pain, however, whether Kv1.3 plays a role in neuroinflammation following peripheral nerve injury is unclear. The spared nerve injury model (SNI) was used to establish neuropathic pain. Western blot and immunofluorescence were used to examine the effect of Kv1.3 in the SNI rats. PAP-1, a Kv1.3 specific blocker was administered to alleviate neuropathic pain in the SNI rats. Neuropathic pain and allodynia occurred after SNI, the levels of M1 (CD68, iNos) and M2 (CD206, Arg-1) phenotypes were up-regulated in the spinal cord, and the protein levels of NLRP3, caspase-1 and IL-1 β were also increased. Pharmacological blocking of Kv1.3 with PAP-1 alleviated hyperpathia induced by SNI. Meanwhile, intrathecal injection of PAP-1 reduced M1 polarization and decreased NLRP3, caspase-1, and IL-1 β expressions of protein levels. Our research indicates that the Kv1.3 channel in the spinal cord contributes to neuropathic pain by promoting microglial M1 polarization and activating the NLRP3 inflammasome.

1 Introduction

Neuropathic pain is a primary subgroup of pathological pain that results from a lesion or disease of somatosensory system (Baron R et al., 2010). Hyperpathia, allodynia, and spontaneous pain are the main clinical symptoms of neuropathic pain (Jensen TS and Finnerup NB, 2014). Studies have shown many adverse effects of chronic pain on prognosis and mental health (Geneen LJ et al., 2017; Treede RD et al., 2019). Unfortunately, the mechanisms of nerve injury that induce neuropathic pain are extremely complicated, and there is a lack of preventive and therapeutic measures (Cohen S and Mao J,

38 2014;Gilron I et al., 2006) .Therefore, it is necessary to further explore the mechanisms and find a
39 novel treatment that can target neuropathic pain.

40 Kv1.3 channel is a classic voltage-gated potassium channel highly expressed in microglia. It also plays
41 a crucial role in inflammatory responses(Sarkar S et al., 2020)via promoting release of inflammatory
42 cytokines and oxidative stress(Varga Z et al., 2021),which eventually induces loss of neurons(Fordyce
43 CB et al., 2005).A recent study showed that Kv1.3 was upregulated in activated microglia(Rangaraju
44 S et al., 2015).In addition, Kv1.3 channel is considered a novel therapeutic target for treating
45 neuroinflammatory disorders due to its crucial role in subsets of T lymphocytes as well as microglial
46 cells(Tubert C et al., 2016).However, it is not clear whether Kv1.3 is involved in neuropathic pain via
47 shifting microglial phenotype and inducing inflammation.

48 Rodent models suggest that neuroinflammation contributes to neuropathic pain(Ellis A and Bennett
49 DLH, 2013) .Initial work focused on the idea that microglia can drive neuroinflammation(Muzio L et
50 al., 2021).Many research reports have demonstrated that microglia is involved in regulating various
51 types of chronic pain(Chen G et al., 2018;Tan Y-H et al., 2012),such as neuropathic pain(Pike AF et
52 al., 2022) and inflammatory pain(Decosterd I and Woolf CJ, 2000).Microglia appear to be
53 heterogeneous with two functional phenotypes:M1 phenotype and M2 phenotype, which produce
54 cytotoxic or neuroprotective effects, respectively(Song Z et al., 2017;Xiong B et al., 2020).Blocking
55 Kv1.3 channel with PAP-1 increased levels of anti-inflammatory M2 phenotype microglia and
56 inhibited M1 phenotypes in MCAO(middle cerebral artery occlusion), POCD (Postoperative cognitive
57 dysfunction), and DIO(diet-induced obesity) models(Rangaraju et al. 2015).However, the roles of M1
58 and M2 microglia in neuropathic pain are unclear.

59 The NLRP3 inflammasome was found to process damaged signals to trigger the inflammatory
60 response(Doyle TM et al., 2019).A plethora of studies have shown that the NLRP3 inflammasome

plays a central role in neuroinflammation induced by injury(Hu X et al., 2021;Kelley N et al., 2019;Liu X et al., 2021). Mechanistic connections between the NLRP3 inflammasome and Kv1.3 are becoming increasingly clear(Pike AF et al., 2022).Here, we hypothesized that Kv1.3 channel in the spinal cord can promote M1 microglial polarization and activate the NLRP3 inflammasome which results in neuropathic pain in rats. Using the SNI model and treatment with PAP-1, We first examined changes in M1 and M2 microglia in the spinal cord after SNI. Then, we investigated the role of Kv1.3 in regulating M1/M2 transformation, using PAP-1. Lastly, we examined whether Kv1.3 channel blockade exerts a neuroprotective effect through suppressing the NLRP3 inflammasome activation during SNI. These findings may provide laboratory support for translational studies in neuropathic pain of Kv1.3 inhibitors.

2 Materials and methods

2.1 Animals

A total of 142 Male SD (6–8 weeks, 200-250 g) rats were supplied from Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China. All animals were raised under controlled conditions (22–25 °C, 12-hour alternate circadian rhythm, free access to food and water, 3-4 rats per cage). All animal studies followed the protocol approved by the Animal Care and Use Committee of Tongji Hospital.

2.2 Induction of neuropathic pain

SNI models were established according to the procedures previously described(Decosterd I and Woolf CJ, 2000). The left sciatic nerve (containing the three branches: common peroneal, tibial, and sural nerve) was exposed after rat was anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally). The two branches of the sciatic nerve (the common peroneal nerve and the tibial nerve) were tightly ligated with a 5.0 silk thread (or suture) and sectioned distal to the ligation, removing 2±4 mm of the

distal nerve stump. The intact sural nerve was carefully avoided by preventing any contact or straining. Then the skin was closed. In the SHAM group, the sciatic nerve was just exposed without being ligated or sectioned.

2.3 Pain behavioral test

The mechanical paw withdrawal threshold (MPWT) of the ipsilateral hind paw was determined using Von Frey filament, which simulated mechanical allodynia as previously described. All behavioral tests took place between 8:30 a.m. and 4:30 p.m. Briefly, mice were placed in individual plastic enclosures on a metal mesh floor and given 30 minutes to acclimate. Positive responses included abrupt paw withdrawal, licking, and shaking. The MPWT was measured as previously describe(Song Z et al., 2016) and was defined as the least amount of force required to elicit a positive response (in grams). All behavioral tests were carried out by a researcher who was unaware of the study's design.

2.4 Western blot analysis

The procedure for collecting spinal tissue and preparing spinal protein samples had previously been followed(Xiong B et al., 2020).Equal amounts of spinal protein samples were separated using 10 percent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted using Millipore polyvinylidene fluoride membranes. The membranes were blocked for 1 hour at room temperature (RT) with 5% skim milk or BSA in Tris-buffered saline and Tween 20 (TBST, 0.1%), incubated with primary antibody overnight at 4 °C and then incubated for 1 hour at RT with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (1:5000 ABclonal, AS003), goat anti-rabbit secondary antibody (1:5000 ABclonal, AS014), rabbit anti-goat secondary antibody (1:5000 ABclonal, AS029). The specific primary antibodies used in this study including: GAPDH (1:1000 ABclonal, A19056), iba-1 (1:1000 abcam, ab5076), IL-1 β (1:1000 ABclonal, A1112), Arg-1 (1:1000 ABclonal, A4923), caspase-1 (1:1000 protentech, 22915-1-AP), Kv1.3 (1:400

santa, sc-398855), CD68 (1:1000 abcam, ab125212), CD206 (1:1000 abcam, ab203490), iNos (abcam, ab283655), NLRP3 (1:1000 HUABIO, ET1610-93). Chemiluminescence (Pierce ECL Western Blotting Substrate; Thermo Scientific) was used to evaluate the protein bands, and a computerized image analysis system (ChemiDoc XRS+; BIO-RAD) was used to measure them.

2.5 Immunofluorescence staining

The rats were severely sedated with an overdose of isoflurane and transcardially perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. The spinal cord was removed and preserved at 4 °C with 4% paraformaldehyde. Using a cryostat microtome, the fixed spinal cord was sectioned into 20 µm thick coronal slices (Thermo Fisher, NX50, Waltham, MA). PBS was used to wash free-floating slices at first (3 times, 8 min each). The slices were washed and incubated for 1 hour at 37 °C in blocking buffer (10% normal goat serum, 0.3% Triton X-100 in PBS). After blocking, the slices were treated with primary antibodies for 72 hours at 4 °C, including: iba-1 (1:400 abcam ab5076), CD68 (1:100 abcam, ab125212), Arg-1 (1:50 CST, 93668), iNos (1:100 abcam, ab283655), CD206 (1:200 abcam, ab203490), or NLRP3 (1:500 HUABIO, ET1610-93). And then, the sections were incubated with a Cy3-conjugated (1:200 Jackson, 711-165-152), or 488-conjugated secondary antibody (1:200 Jackson, 155707) for 2 hours at 37 °C, stained with DAPI for 10 min at RT, washed with PBS (3 times, 10 min each). Finally, a virtual microscopy slide scanning system was used to view the immunostained brain slice (Olympus, VS 120, Tokyo, Japan). Using ImageJ, images of slices containing the region of interest (ROI) were cropped and counted (National Institutes of Health, Bethesda, MD). Image J was used to quantify the cells. The average number of double-labeled cells per square millimeter in each group was used to calculate the density of double-labeled cells.

2.6 Experimental designs and drugs treatment

This study was designed as demonstrated in Figure 1A and Figure 2A. First, we examined the protein levels of Kv1.3 in the spinal cord. Next, we further tested the activation of microglia and the NLRP3 inflammasomes in the spinal cord. Then, PAP-1 (5 µg, sigma) or Vehicle was intrathecally(i.t.) administered from day 7 to day 12 after surgery to explore whether it alleviated hyperpathia in the SNI rats. we designed three drug administration methods to explore the therapeutic effect of PAP-1 in neuropathic pain, which include a single dose (2.5 µg/100g), multiple doses (2.5 µg/100g,5 days), and a prophylactic dose (2.5 µg/100g, 5 days). A pre-experiment was used to determine the PAP-1 dosage, such as (5 µg/kg, 25 µg/kg, 50 µg/kg, i.t.)(which is showed as supporting information in the supplemental material, Figure1).

2.7 Statistical analysis

All results are shown as mean ±SEM. For analyses, when comparing two groups, an unpaired Student's *t*-test was used, for multiple groups, one-way ANOVA followed by the Bonferroni post hoc test was utilized. Two-way ANOVA followed by Bonferroni post hoc test was used to analyze the MPWT. Pearson coefficients were applied to statistically express pertinence. GraphPad Prism 7.0 was used for statistical analysis, and $P < 0.05$ was considered statistically significant in this study.

3 Results

3.1 SNI caused mechanical allodynia and increase of Kv1.3 in the spinal cord

To evaluate the development of mechanical allodynia after SNI, the MPWT was tested on day 0, day 1, day 3, day 7, and day 14 (fig1A). The results obtained are consistent with our previous results of similar studies that the MPWT was distinctly decreased in the SNI rats compared to the SHAM group (fig1B). Next, we examined the protein levels of Kv1.3 channel in the spinal cord by western blot. The results showed that the expression of Kv1.3 was increased on day 1 and continued up until day 14 after

SNI (fig1C). Then, immunofluorescence was used to detect the co-localization of Kv1.3 and microglia (iba-1). Kv1.3 was extensively co-localized with iba-1 in the spinal cord, and the immunoreactivity was increased after SNI (fig1D). These results indicate that the protein expression of Kv1.3 was increased and co-localized with microglia in the spinal cord of the SNI rats.

3.2 PAP-1 reversed mechanical allodynia caused by neuropathic pain

PAP-1 is the selective inhibitor of Kv1.3. We then used PAP-1 to further study the role of Kv1.3 in neuropathic pain. At the same time, the MPWT was performed after using PAP-1(fig2A). Drug dose and Tmax (peak time of drug) were obtained by pre-experiment (which is showed as supporting information in the supplemental material). The results suggest that a single administration of PAP-1 after SNI could effectively increase the MPWT and continued for 6 hours (fig2B). In addition, continuous use of PAP-1 7 days after establishing the model can also improve mechanical allodynia of the SNI rats (fig2C). However, prophylactic use of PAP-1 had no effect on the MPWT of the SNI rats (fig2D). Next, the expression of Kv1.3 was examined by western blot. The result showed that administration of PAP-1 clearly decreased the level of PAP-1 in the spinal cord of the SNI rats. As a result, we concluded that PAP-1 can decrease the level of Kv1.3 protein and increase the MPWT in the SNI rats.

3.3 Microglia were activated in the spinal cord of the SNI rats

Kv1.3 channel is highly expressed in microglia and is a key therapeutic target for inflammatory diseases. Next, microglia expression in the spinal cord was examined by western blot and immunofluorescence. The results showed that the expression of iba-1 protein was remarkably increased and continued up to day 14 in the SNI rats (fig3A). Besides, compared to the SHAM group, immunostaining of iba1 was enhanced and microglial bodies were enlarged with retraction of the protuberances on day 3 and continued until day 14 in the spinal cord of the SNI rats (Fig3B). Then, we

quantitatively analyzed the mean fluorescent intensity of iba-1(fig3C) and the number of iba-1⁺ cells (fig3D) in the spinal cord. The results demonstrated that the mean fluorescent intensity and the number of iba-1⁺ cells were significantly increased after SNI, compared with the SHAM group. These results indicate that microglia are activated in the spinal cord after SNI.

3.4 Microglia were activated and mainly expressed as the M1 phenotype in the spinal cord after SNI.

The changes in microglia, M1 versus M2, after SNI were examined by western blot with CD68 and iNos identified as markers of the M1 phenotype, and CD206, and Arg-1 as markers of the M2 phenotype. The results showed that compared with the SHAM group, the level of CD68 increased in a time-dependent manner on days 1, 3, 7, and 14 (fig4A), while the expression of iNos significantly increased in the SNI rats (fig4B). In contrast, both Arg-1 and CD206 increased early after SNI and then gradually decreased to baseline by day 14 (fig4C). At the same time, we examined the colocalization of iba-1 with CD68, iNos, CD206 and Arg-1(fig4E). The results revealed that the ratios of CD68⁺ and iNos⁺ cells in microglia clearly increased in a time-dependent manner in the spinal cord of the SNI rats which are consistent with western blot results (fig4F,4G). The ratio of CD206⁺ cells in the microglia increased on days 1, 3, and 7, but decreased on day 14 (fig 4H). Although, the ratio of Arg-1⁺ cells increased on day 1 and day 3, it reduced to baseline at day 7 and day 14 (fig4I). These findings indicate that microglia are activated and mainly expressed as the M1 phenotype in the spinal cord after SNI.

3.5 Neuroinflammation activated the NLRP3 inflammasome in the spinal cord after SNI

Many researchers suggest that the NLRP3 inflammasome is activated by neuroinflammation. We next examined the protein levels of NLRP3, caspase-1(p20), and IL-1 β (p17) in the spinal cord. The results showed that the expressions of NLRP3, caspase-1, and IL-1 β increased in the spinal cord in a time-dependent manner after SNI (fig5A,5B,5C). Then, we analyzed the co-location of NLRP3 and iba-1 in

the spinal cord. Double immunofluorescence staining of iba-1 and NLRP3 was markedly increased after SNI (fig5D), and the mean fluorescent intensity was upregulated (fig5E). These results imply that neuroinflammation activates the NLRP3 inflammasome after SNI which is associated with neuropathic pain.

3.6 PAP-1 reversed the polarization of microglia in the spinal cord of the SNI rats

Next, PAP-1 was used to further investigate the role of Kv1.3 in neuroinflammation after SNI. The results showed that the expression of iba-1 decreased in the spinal cord of the SNI rats after administration of PAP-1(fig6A). Meanwhile, the levels of CD68 and iNos (markers of the M1 phenotype) were significantly downregulated after using PAP-1, but the levels of CD206 and Arg-1 (markers of the M2 phenotype) were upregulated (fig6B,6C,6D,6E). Similarly, the results of immunofluorescence were consistent with those of western blot (fig6F). The level of iba-1+ cells decreased particularly those of CD68+/iba-1+ and iNos+/iba-1+ cells, while the levels of CD206+/iba-1+ and Arg-1+/iba-1+ cells increased in the spinal cord of the SNI rats after using PAP-1(fig6G,6H,6I,6J,6K). These results suggest that PAP-1 reverses the polarization of microglia induced by upregulation of Kv1.3 channel in the spinal cord of the SNI rats.

3.7 PAP-1 reversed activation of the NLRP3 inflammasome in the spinal cord of the SNI rats

Blockade of Kv1.3 with PAP-1 reversed the polarization of M1 microglia in the SNI rats. Subsequently, we examined the expression of the NLRP3 inflammasome by western blot and immunofluorescence. The results showed that the expressions of NLRP3, caspase-1, and IL-1 β decreased remarkably after administration of PAP-1 in the SNI rats (fig7A,7B,7C). The mean fluorescent intensity of NLRP3/iba-1 was also reduced in the SNI rats after using PAP-1 (fig7D, 7E). These results denote that PAP-1 effectively reverses activation of the NLRP3 inflammasome in the SNI rats.

4 Discussion

220 Neuropathic pain is a clinically intractable disease that seriously affects patients' quality of life. In the
221 present study, we used PAP-1 as a tool to examine the role of Kv1.3 in microglia in neuropathic pain.
222 Our results showed that PAP-1 attenuated SNI-induced microglial polarization, neuroinflammation,
223 and allodynia. We found that PAP-1 reduced SNI-induced Kv1.3 upregulation and NLRP3 production,
224 reverted the ratio of M1/M2 reactive microglia in the spinal cord, and relieved mechanical allodynia
225 after peripheral injury. These results demonstrate that Kv1.3 contributes to neuropathic pain via
226 promotion of microglial M1 polarization and activation of the NLRP3 inflammasome.

227 Kv1.3 was first described in human T-cells, where it regulates T-cell activation(Panyi G, 2005). Other
228 immune cells include macrophages and B cells, which can express Kv1.3 under specific
229 conditions(Beeton C et al., 2006) . It is notable that a systems pharmacology-based study identified
230 functional roles for Kv1.3 in pro-inflammatory microglial activation(Di Lucente J et al., 2018).
231 Increase of microglial Kv1.3 expression has been observed in both human and rodent models of
232 Alzheimer's disease (AD)(Rangaraju S et al., 2015) and Parkinson's disease (PD)(Tubert C et al.,
233 2016) , suggesting that Kv1.3 may be an effective strategy for reducing neuroinflammation in the
234 context of a variety of central nervous system diseases. Kv1.3 inhibitors have also been shown to
235 ameliorate disease severity and neurologic deficit in rodent models of ischemic stroke(Ma D-C et al.,
236 2020). Similarly, PAP-1 treatment reduced neuroinflammation, decreased cerebral amyloid load,
237 enhanced hippocampal neuronal plasticity, and improved behavioral deficits in mouse models of
238 AD(Du Y et al., 2021). Peimine, one of the main components in Fritillaria which has a long history of
239 use as an anti-inflammatory and pain-relieving herb in ancient Chinese medical practice, was found to
240 be able to inhibit Kv1.3 channels(Xu J et al., 2016). Similar to non-steroidal anti-inflammatory drugs
241 (NSAIDs) it could effectively suppress the Kv1.3 currents in T-lymphocytes and thus exerted
242 immunosuppressive effects to more rapidly reduce headache after vaccination(Kazama I and Senzaki
243 M, 2021). But it is not clear whether the neuropathic pain and allodynia induced by SNI are attributable

to the effects of Kv1.3 of microglia. In our study, SNI induced an on-going upregulation of Kv1.3 in the spinal cord and PAP-1 effectively increased the MPWT of the SNI rats. An interesting finding is that preventive treatment with PAP-1 before establishing SNI model could not prevent the development of SNI. Possibly, since the small molecule Kv1.3 blocker PAP-1 is lipophilic(Lam J and Wulff H, 2011) and the t1/2 of PAP-1 in rodents is short (3.8 hours) (Beeton C et al., 2006). The pharmaceutical concentration of preventive treatment is unamenable to play an instant immunosuppressive role in alleviating mechanical allodynia after surgery. The decrease of the MPWT at 6 hours after a single dose seems also to confirm this. However, continuous administration of PAP-1 for 5 days after surgery remarkably reversed the mechanical allodynia and maintained this effect in the SNI rats, indicating that blocking Kv1.3 could prevent neuropathic pain through a blocking process such as neuroinflammation. As alluded to earlier, Kv1.3 plays an important role in immune cell activation in nervous system diseases through the high expression of pro-inflammatory microglia(Felipe A, 2018). In the present study, we observed an imbalanced microglial polarization of M1 and M2 in the spinal cord of the SNI model. Alterations in M1/M2 polarization are associated with excessive inflammatory activation(Orihuela R et al., 2016) and may play an important role in the development and progression of SNI. Indeed, we found that during the development of SNI, reactive microglia were mainly expressed as M1 microglia, with very few M2 microglia. It is pertinent that lipopolysaccharide(LPS)-induced microglial activation, which transforms into classical (“M1”) phenotype, has been shown to require the participation of microglial Kv1.3(Di Lucente J et al., 2018).In mice genetically devoid of Kv1.3, LPS failed to activate microglia, or produce neuroinflammation(Nicolazzo JA et al., 2022). Progranulin could inhibit LPS-induced macrophage M1 polarization via NF-κB and MAPK signaling pathways(Liu L et al., 2020). But whether Kv1.3 channel is associated with the reshaping of microglial phenotype in neuropathic pain is still unclear. Our findings showed that the expression of M1 microglia was reduced, and the expression of M2 was increased when mechanical allodynia was relieved in the SNI rats after intrathecal injection of PAP-1. These results suggest that Kv1.3 contributes to

269 neuropathic pain via the promotion of microglial M1 polarization. Activation of Kv1.3 channel in
270 cytomembrane induces K^+ efflux, while the low intracellular potassium concentration has a
271 fundamental role in activating the NLRP3 inflammasome(He Y et al., 2016). It has been acknowledged
272 that the NLRP3 inflammasome activation mediates inflammatory responses after nerve injury(Chen R
273 et al., 2021). Thus, it seems plausible to infer that Kv1.3 channel may play a role in the NLRP3
274 inflammasome activation in microglia after nerve injury, however, there is a paucity of studies in this
275 area. Given that the cleavage of caspase-1 and IL-1 β are the hallmarks of inflammasome activation and
276 IL-1 β is a well-known pain-inducing molecule(Starobova H et al., 2020), the effect of Kv1.3 on them
277 was investigated in the present study. Our data provides the first evidence about the association of
278 Kv1.3 with the NLRP3 inflammasome activation in microglia after SNI. Therefore, we concluded that
279 blockage of cellular K^+ efflux with PAP-1 may inhibit the decrease of intracellular potassium
280 concentration, thus compromising the NLRP3 inflammasome activation after SNI in the current study.
281 But we noticed that there was an expression peak of IL-1 β on the first day after SNI. This discrepancy
282 of IL-1 β might be due to microglia and neurons responding at different times after damage(de Rivero
283 Vaccari JP et al., 2009).

284 It must be admitted that there are some limitations in our study. Firstly, iba1, CD68, iNos, Arg-1, and
285 CD206 were used to isolate microglia and identify microglial phenotypes in the present study.
286 Although they have been used as molecular markers of activated microglia, they are not specific to
287 microglia. These markers are also expressed by the central nervous system monocyte-derived
288 macrophages(Crain JM et al., 2013). Thus, additional experiments such as morphology analysis are
289 warranted to illustrate the effects of Kv1.3 blockade on microglial activation. Secondly, the initial
290 concept of M1 or M2 phenotype is set in macrophages for experimental examination of inflammation.
291 Although it has utility in describing microglial heterogeneity on inflammatory responses(Ransohoff
292 RM, 2016). To translate the paradigm to microglia, efforts towards characterization would include

assessing the morphological phenotype, discriminating between microglia (resident macrophages) and macrophages (infiltrating macrophages), metabolism, and functional features of the cells. Thirdly, it has become clear that any efforts to classify microglia in distinct activation or polarized states cannot rely on the standard M1/M2 polarization paradigm, but rather will require more complex phenotypes, especially *in vivo*. Lastly, in the present study, the relationship among Kv1.3, the NLRP3 inflammasome activation and M1 polarization was indirectly demonstrated by the transformation of biomarkers. Future studies are still warranted to examine the interrelationship of Kv1.3 effects on activation of the NLRP3 inflammasome and M1 polarization, which will provide the real evidence.

5 Conclusion

In conclusion, PAP-1, the Kv1.3 blocker, significantly attenuated mechanical allodynia of SNI. The present study provided evidence that Kv1.3 contributes to neuropathic pain via the promotion of microglial M1 polarization and activation of the NLRP3 inflammasome (fig8). Pharmacological inhibition of Kv1.3 may thus, be a promising strategy in the treatment of neuropathic pain and PAP-1 may provide effective therapy for the management of pain in the clinic.

6 List of abbreviations

MCAO :60 min of ischemia followed by reperfusion; POCD: Postoperative Cognitive Dysfunction; DIO: mimicking metabolic syndrome; SNI: spared nerve injury; MPWT: The mechanical paw withdrawal threshold. PAP-1: 5-(4-Phenoxybutoxy)psoralen

7 Declarations

7.1 Ethics approval and consent to participate

313 All experiments were approved by the Experimental Animal Care and Use Committee of Tongji
314 Medical College, Huazhong University of Science and Technology, and were in agreement with the
315 National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

316 **7.2 Consent for publication**

317 Not applicable.

318 **7.3 Availability of data and materials**

319 The data and materials supporting the conclusions of this study are available from the corresponding
320 author on reasonable request.

321 **7.4 Competing interests**

322 The authors declare that they have no competing interests.

323 **7.5 Funding**

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326 **7.6 Authors' contributions**

327 Xiaoman Yuan conceived the research, carried out the model building, performed the Western blot,
328 coordinated the lab work and drafted the manuscript. Siyi Han and Anne Manyande performed the
329 statistical analysis and drafted the manuscript. Feng Gao and Jie Wang took care of the MPWT and
330 drafted the manuscript. Wen Zhang, Anne Manyande and Xue-Bi Tian participated in its design and
331 coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

332 **7.7 Acknowledgements**

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Figure legend

Figure 1. Expression and cellular localization of Kv1.3 in the spinal cord of the SHAM and SNI group. (A) Experimental designs and animal groups. (B) The mechanical paw withdraw threshold was measured by Von Frey, and the MPWT was decreased at day 1 and continued until day 14 in the SNI rats, (n = 8). (C) Compared with the SHAM group, the expression level of Kv1.3 was increased in the spinal cord of the SNI rats at day 1 and continued up to day 14, (n = 6). (D) Double immunostaining of Kv1.3 and iba-1 in the spinal cord. The immunoreactivity of iba-1 was increased with Kv1.3 in the SNI rats, (person= Pearson Correlation Coefficient). All data are presented as mean \pm SEM. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$, compared with the SHAM group. D: day.

Figure 2. Effects of Kv1.3 blocker PAP-1 on the MPWT in the SNI rats. (A) Experimental designs and animal groups. (B) MPWT was evaluated hours after the PAP-1 injection, (n = 4). (C) PAP-1 effectively reversed the decrease of the MPWT in the SNI rats, (n = 8). (D) Prophylactic administration of PAP-1 had no effect on SNI-induced decrease of the MPWT, (n = 8). (E) PAP-1 dramatically downregulated the protein level of Kv1.3 in the spinal cord, (n = 6). All data are presented as mean \pm SEM. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$, compared with the SNI group, ##### $P < 0.0001$, compared with the SNI+saline group. D: day. Red arrow: time point of administration.

Figure 3. Activation of microglia in the spinal cord of the SNI rats. (A) The protein level of iba-1 was increased in the spinal cord of the SNI rats and continued until day 14, (n = 6). (B) Immunostaining of iba-1 was enhanced on day 3 and continued to day 14 in the spinal cord of the SNI rats. (C) Quantification of the mean fluorescent intensity of iba-1 positive cells in the spinal cord. (D) Quantification of the number of iba-1 positive cells per square millimeter in the spinal cord, (n = 4). All data are presented as mean \pm SEM. $**P < 0.01$, $****P < 0.0001$, compared with the SHAM group. D: day.

Figure 4. Microglia were activated and mainly expressed as the M1 phenotype in the spinal cord of the SNI rats. (A-B) The protein levels of CD68 and iNos which are the markers of M1 phenotype microglia were increased on day 1 and continued until day 14 in the spinal dorsal of the SNI rats. (C-D) The expression levels of CD206 and Arg-1 were increased on day 1 and decreased to baseline on day 14 in the spinal cord of the SNI rats, (n=6). (E) Double immunofluorescence staining of iba-1(red) and CD68 or iNos (green) for M1 phenotype, CD206 or Arg-1 (green) for M2 phenotype. (F) Quantification of the ratio of CD68 in the iba-1 positive cells of the spinal cord. (G) Quantification of the ratio of iNos in the iba-1 positive cells of the spinal cord. (H) Quantification of the ratio of CD206 in the iba-1 positive cells of the spinal cord. (I) Quantification of the ratio of Arg-1 in the iba-1 positive cells of the spinal cord, (n=4). All data are presented as mean \pm SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, compared with the SHAM group. D: day.

Figure 5. The NLRP3 inflammasome in the spinal cord was activated after SNI. (A-C) The expression levels of NLRP3, caspase-1, and IL-1 β proteins were increased remarkably in the spinal cord of the SNI rats, (n=6). (D) Double immunofluorescence staining of iba-1 (red) and NLRP3 (green) in the

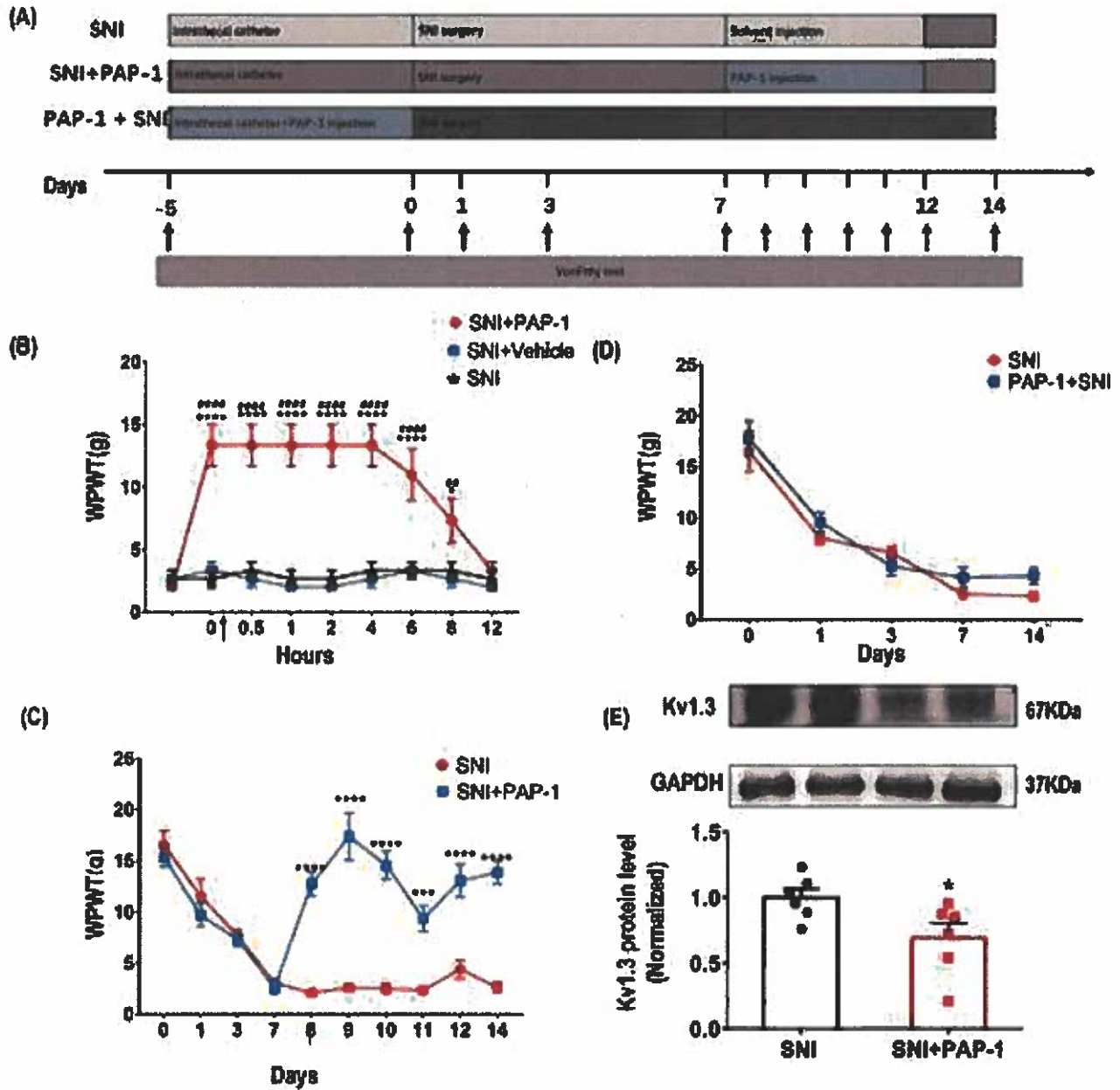
spinal cord. (E) Quantification of the mean fluorescent intensity of the iba-1 positive cells in the spinal cord, (n=4). All data are presented as mean \pm SEM. * P < 0.05, ** P < 0.01, **** P < 0.0001, compared with SHAM group.

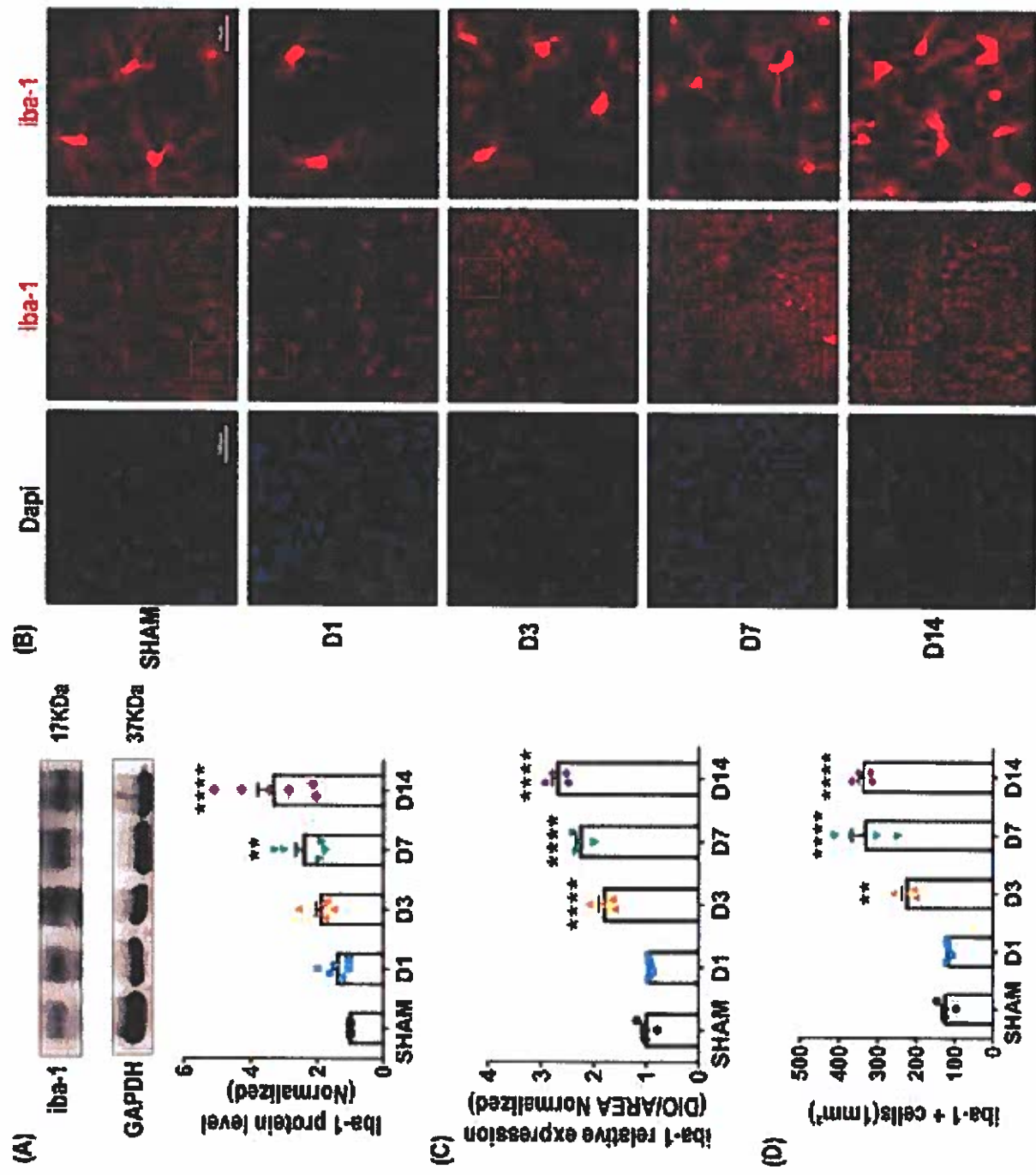
Figure 6. Effects of PAP-1 on microglial polarization in the spinal cord. (A) The expression levels of iba-1 in the spinal cord were observably reduced after administration of PAP-1 in the SNI rats, (n = 6). (B-C) The expression levels of CD68 and iNos in the spinal cord were observably reduced after administration of PAP-1 in the SNI rats, (n = 6). (D-E) The protein levels of CD206 and Arg-1 were increased after using PAP-1 in the SNI rats, (n=6). (F) Double immunofluorescence staining of iba-1 (red) and CD68 or iNos (green) for M1 phenotype, CD206 or Arg-1 (green) for M2 phenotype.(G) Quantification of the iba-1 positive cells in the spinal cord.(H) Quantification of the ratio of CD68 in the iba-1 positive cells in the spinal cord. (I) Quantification of the ratio of iNos in the iba-1 positive cells in the spinal cord. (J) Quantification of the ratio of CD206 in the iba-1 positive cells in the spinal cord. (K) Quantification of the ratio of Arg-1 in the iba-1 positive cells in the spinal cord, (n=4). All data are presented as mean \pm SEM. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, compared with the SNI group.

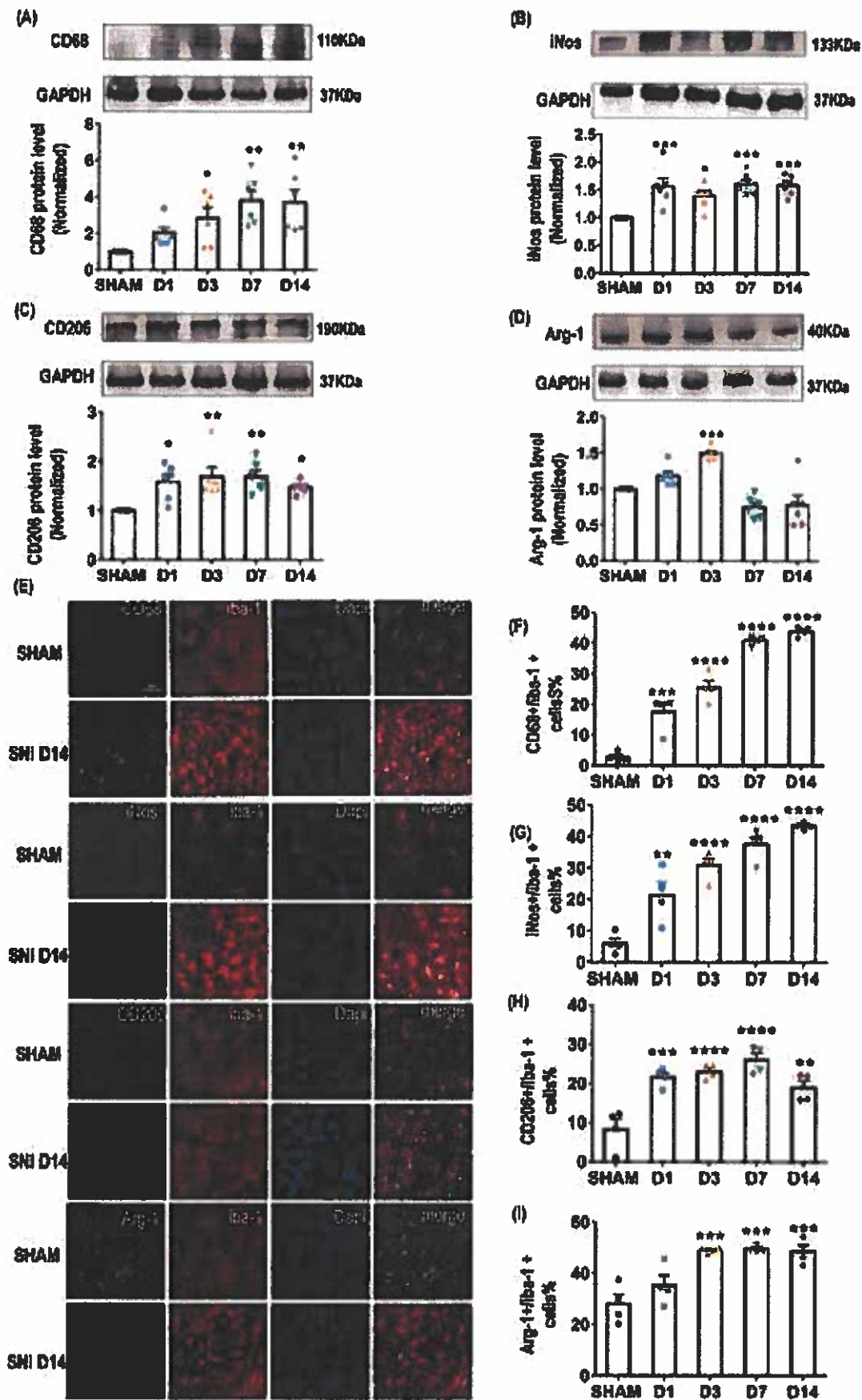
Figure 7. The effects of PAP-1 on the NLRP3 inflammasome in the spinal cord of the SNI rats. (A-C) The protein levels of NLRP3, caspase-1, and IL-1 β were clearly decreased in the spinal cord of the SNI rats. (n=6). (D) Double immunofluorescence staining of iba-1 (red) and NLRP3 (green) in the spinal cord. (E) Quantification of the mean fluorescent intensity of iba-1 positive cells in the spinal cord, (n=4). All data are presented as mean \pm SEM. * P < 0.05, ** P < 0.01, *** P < 0.001, compared with the SNI group.

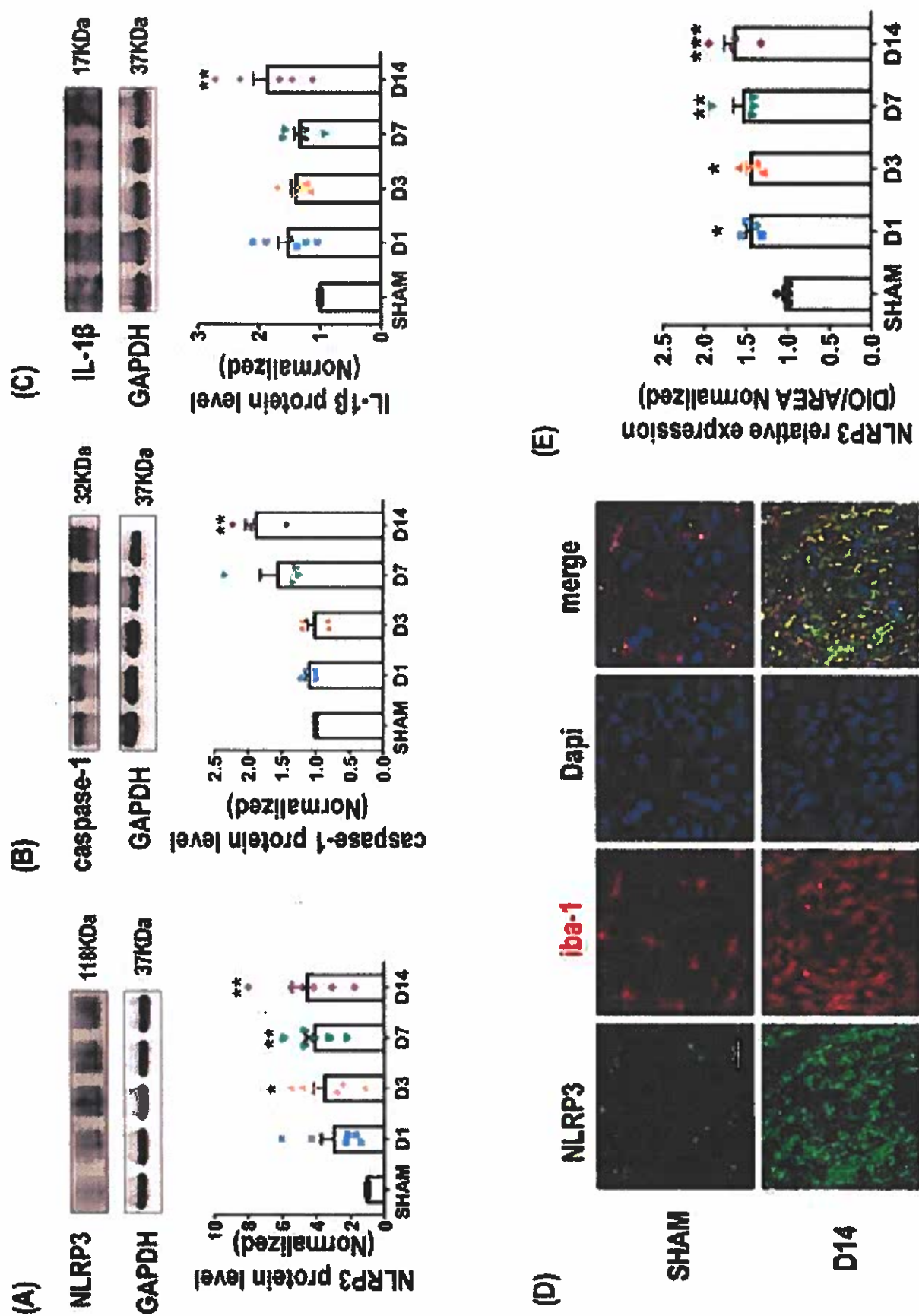
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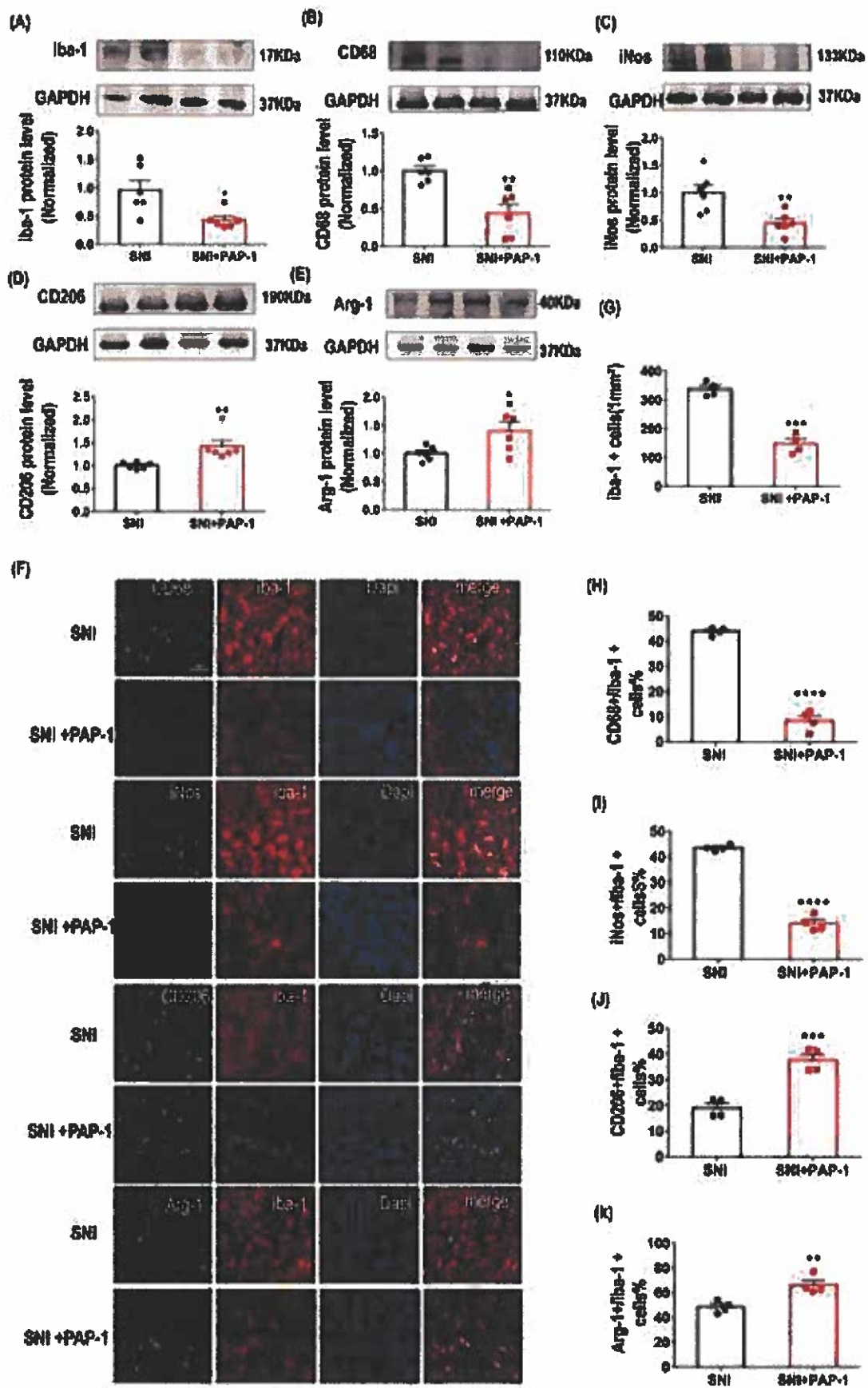
525 **Figure 8.** Schematic diagram. During the development of SNI, Kv1.3 was upregulated and induced
526 microglial M1 polarization, which secreted inflammatory cytokines. Meanwhile, increased K⁺ efflux
527 led to the activation of the NLRP3 inflammasome which presented as the subsequent upregulation of
528 NLRP3, caspase-1, and IL-1 β .

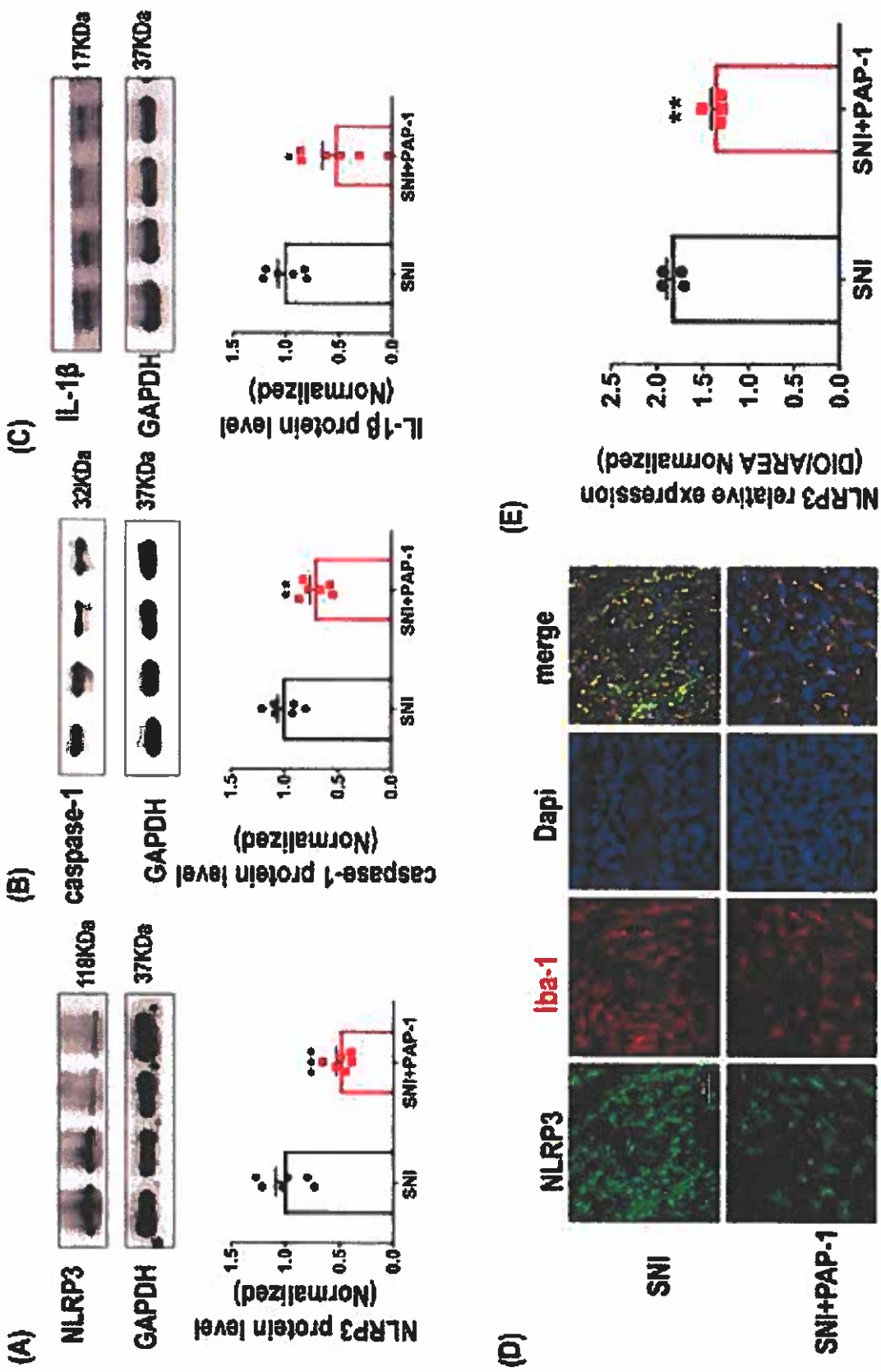


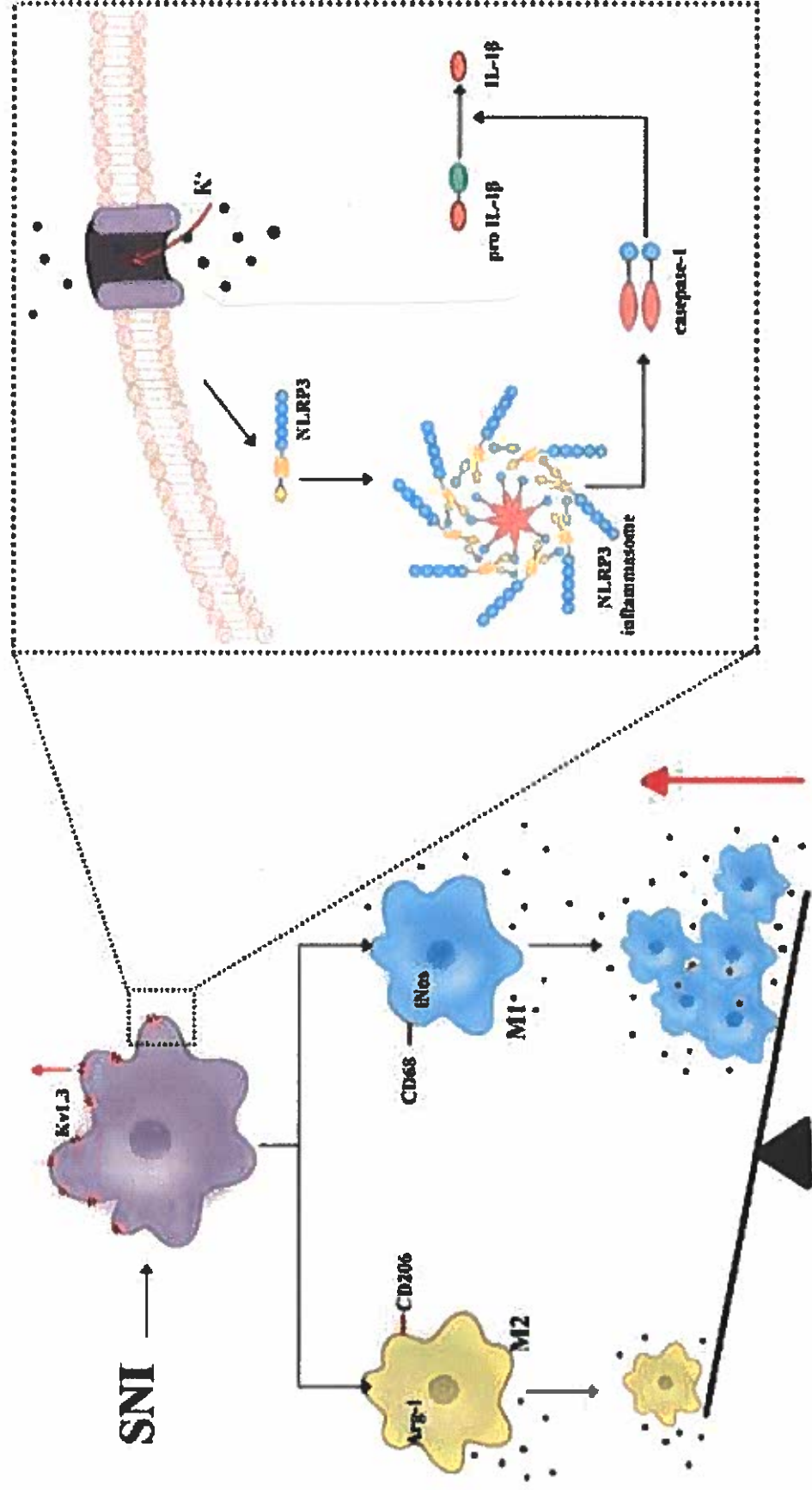


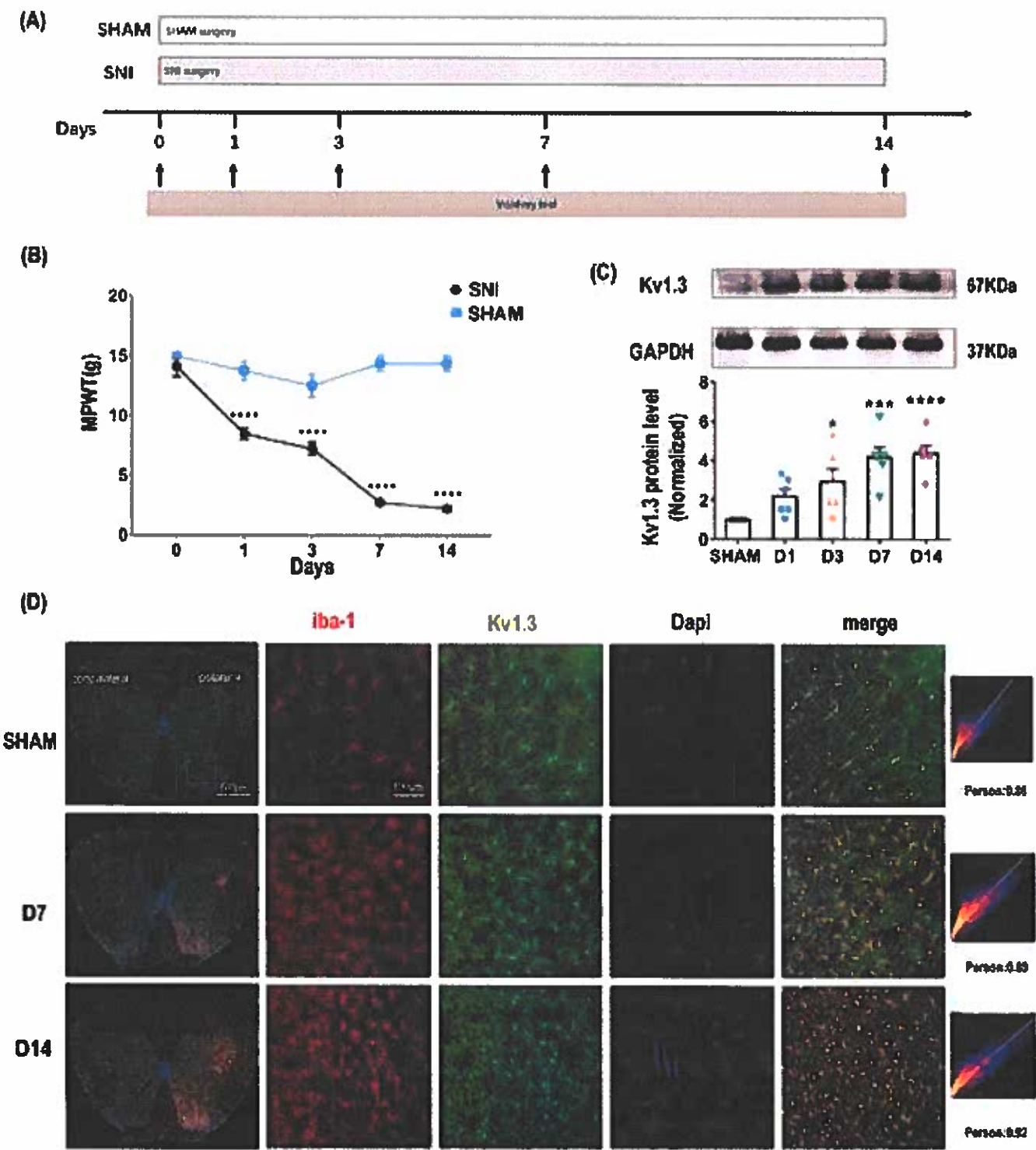


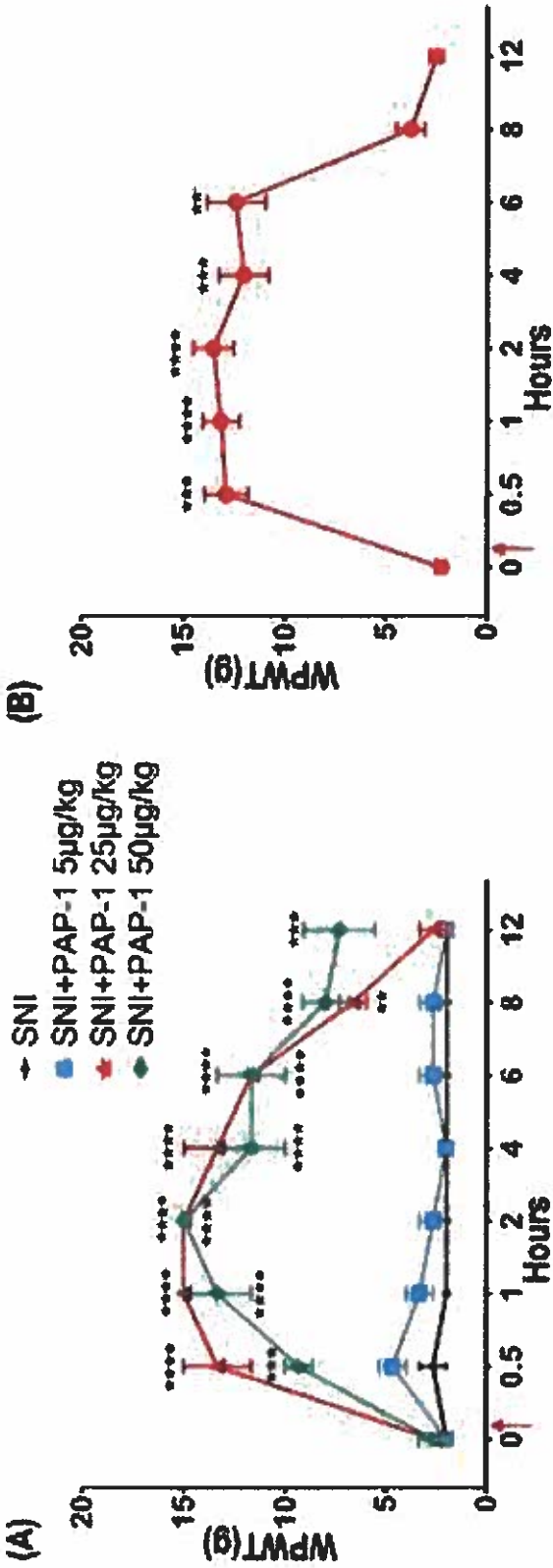












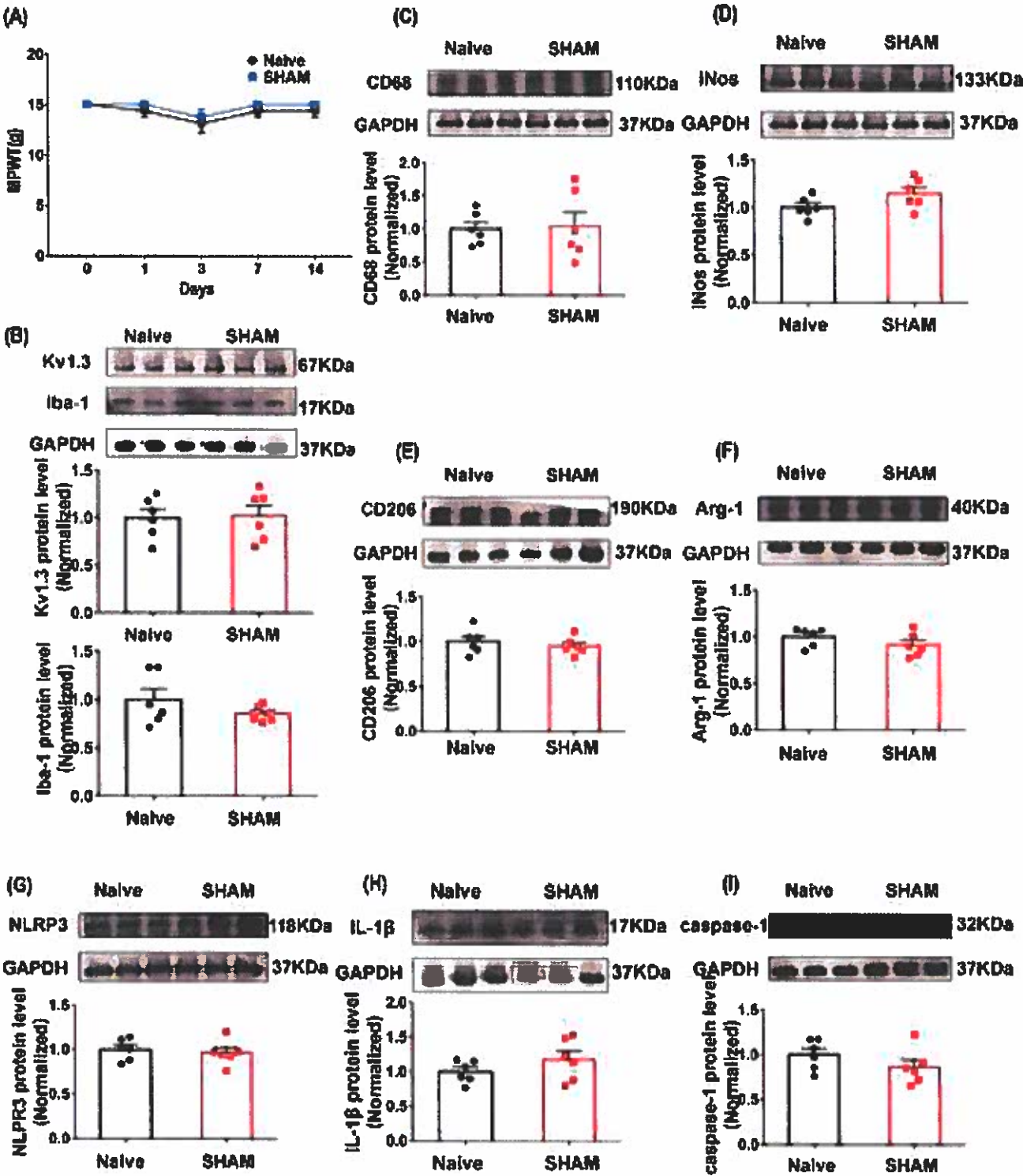


Figure 1. Effects of Kv1.3 blocker PAP-1 on MPWT in the SNI rats. (A) Effects of different doses of PAP-1 on SNI-induced decrease of MPWT, (n = 3). All data are presented as mean \pm SEM. $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, compared with the SNI group, $###P < 0.001$, $####P < 0.0001$, compared with the SNI group. (B) MPWT was evaluated at hours after PAP-1 injection (n = 8). The peak reached immediately after administration and lasted for 6 hours. All data are presented as mean \pm SEM. $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, compare with baseline. Red arrow: time point of administration.

Figure 2. MPWT and relevant protein expressions in the spinal cord of the Naive and Sham rats. (A) The mechanical paw withdraw threshold was measured by Von Frey, and the MPWT was decreased at day 1 and continued until day 14 in the Naive and Sham rats, (n = 8). (B) The protein levels of Kv1.3 and Iba-1 had no statistical difference between the sham and naive groups, (n=6). (C-D) There was no difference of the expression levels of CD68 and iNos had no between the sham and naive groups, (n=6). (E-F) The expression levels of CD206 and Arg-1 had no statistical difference between the sham and naive groups, (n=6). (G-I) There was no significant difference of the protein levels of NLRP3, caspase-1, and IL-1 β between the sham and Naive group, (n=6).

Fig1 Kv1.3(sham D1 D3 D7 D14)

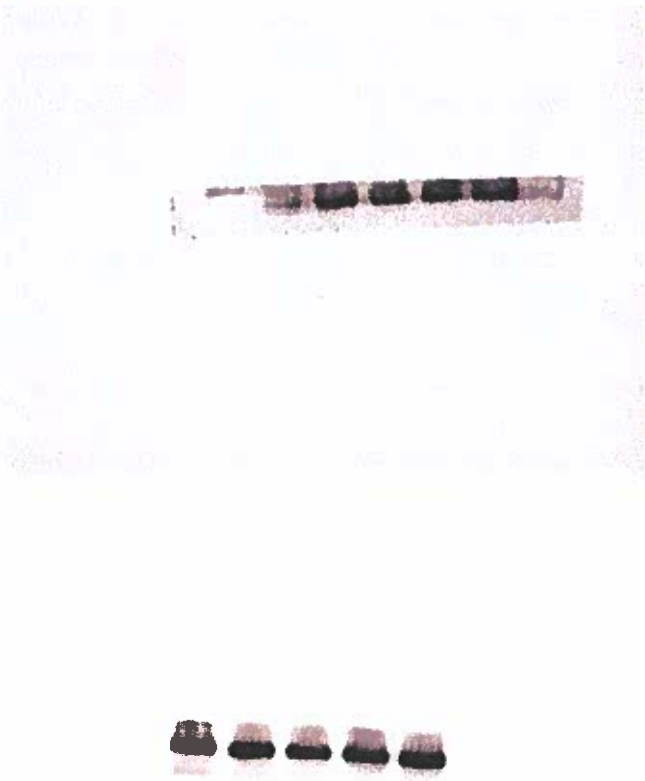


Fig2 Kv1.3 (sham d1 d3 d7 d14 d14 PAP-1 PAP-1)





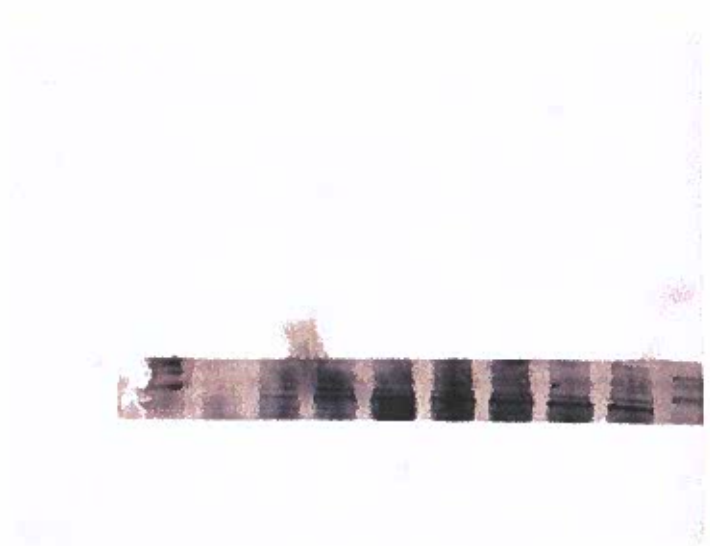
Fig3 Iba-1(sham D1 D3 D7 D14)



17KD



Fig4
CD68(sham D1 D3 D7 D14 D14 PAP-1 PAP-1)

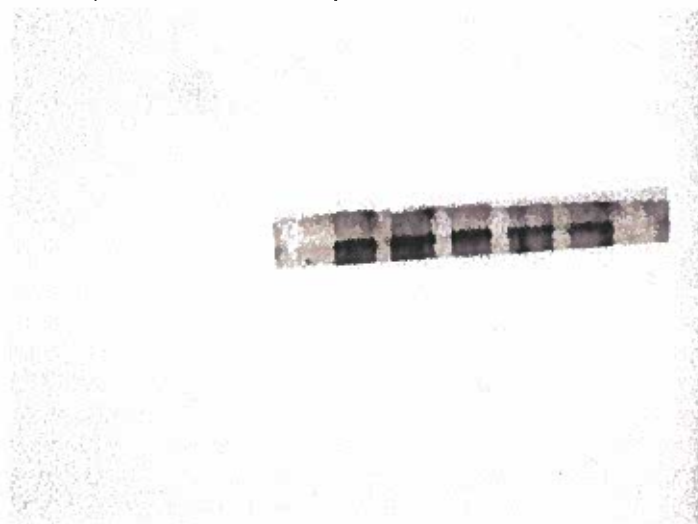


iNos(sham D1 D3 D7 D14 D14 PAP-1 PAP-1)





CD206(sham D1 D3 D7 D14)



Arg-1(sham D1 D3 D7 D14)

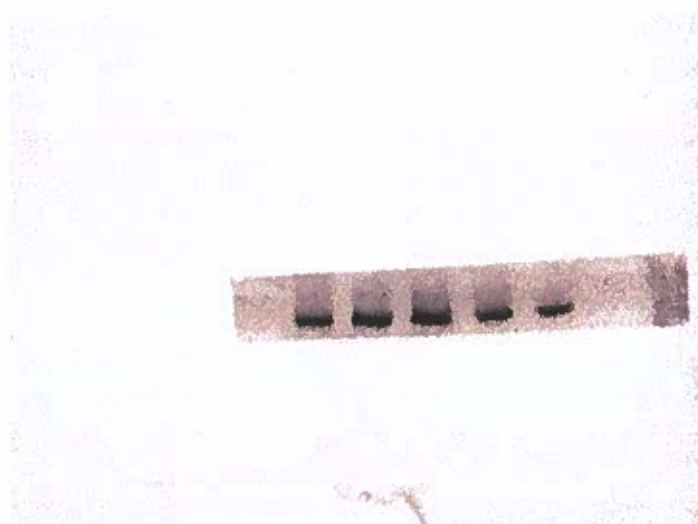


Fig5

NLRP3(sham D1 D3 D7 D14 D14 PAP-1 PAP-1)





Capase-1(sham D1 D3 D7 D14)



IL-1 β (sham D1 D3 D7 D14 D14)

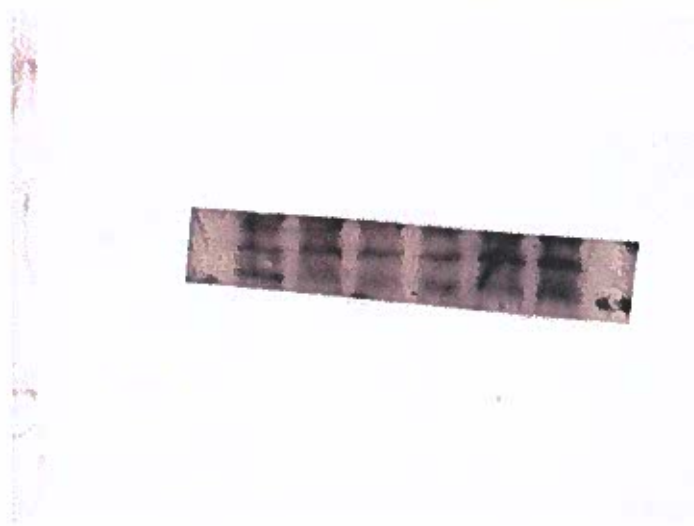


Fig6

Iba-1((D14 D14 D14 PAP-1 PAP-1 PAP-1))

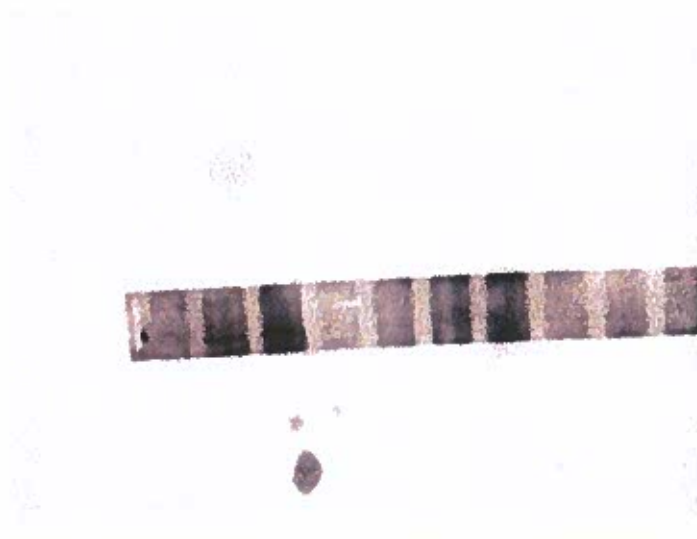




CD68(D14 D14 PAP-1 PAP-1 D14 D14 PAP-1 PAP-1)



Inos(D14 D14 PAP-1 PAP-1 D14 D14 PAP-1 PAP-1)



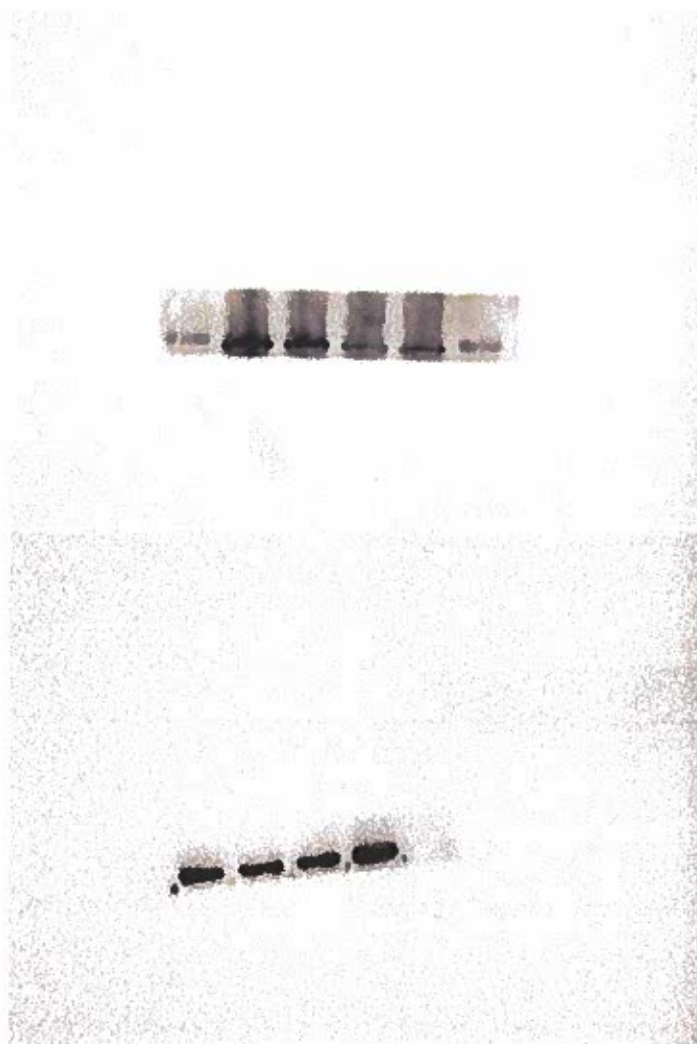
CD206(D14 D14 PAP-1 PAP-1)





Fig7

Nlrp3(D14 D14 PAP-1 PAP-1)



Caspase-1(D14 D14 PAP-1 PAP-1)



II-1b(d14 d14 pap-1 pap-1 d14 d14 pap-1 pap-1)





