

## **Supplementary Materials**

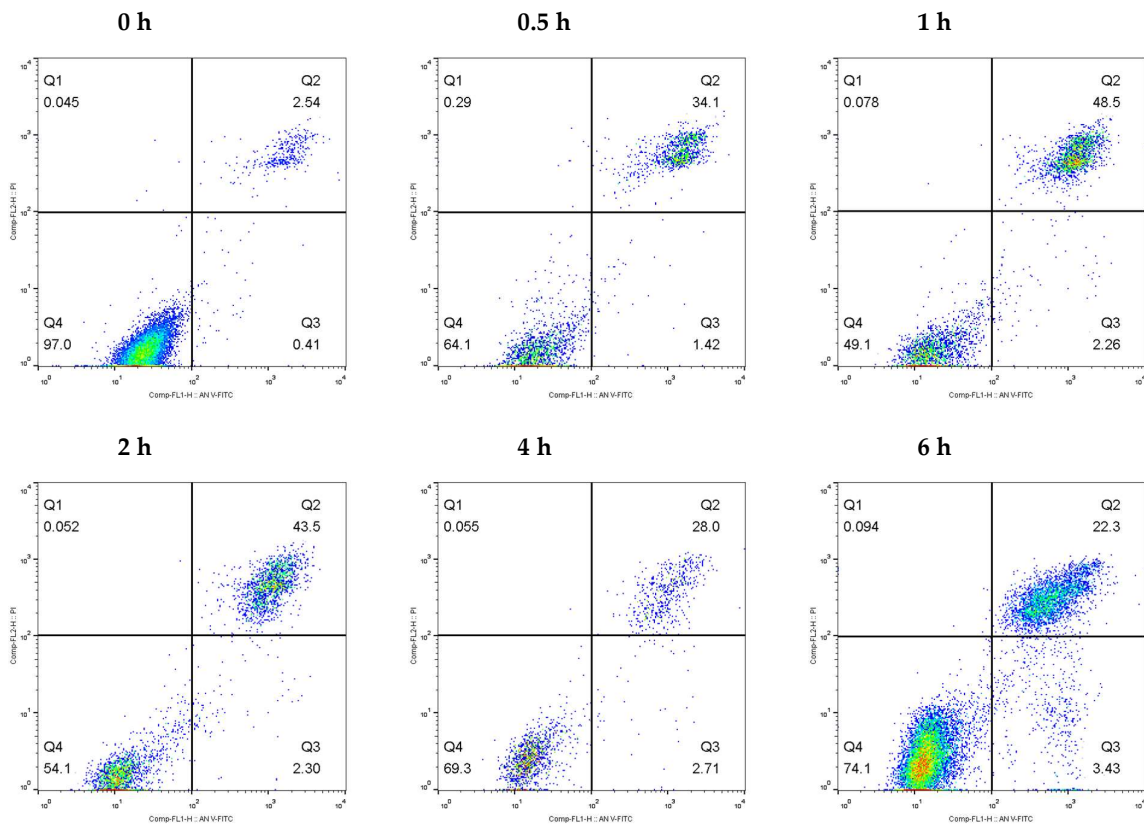
### **Caspase-1–dependent pyroptosis mediates adjuvant activity of platycodin D as an adjuvant for intramuscular vaccines**

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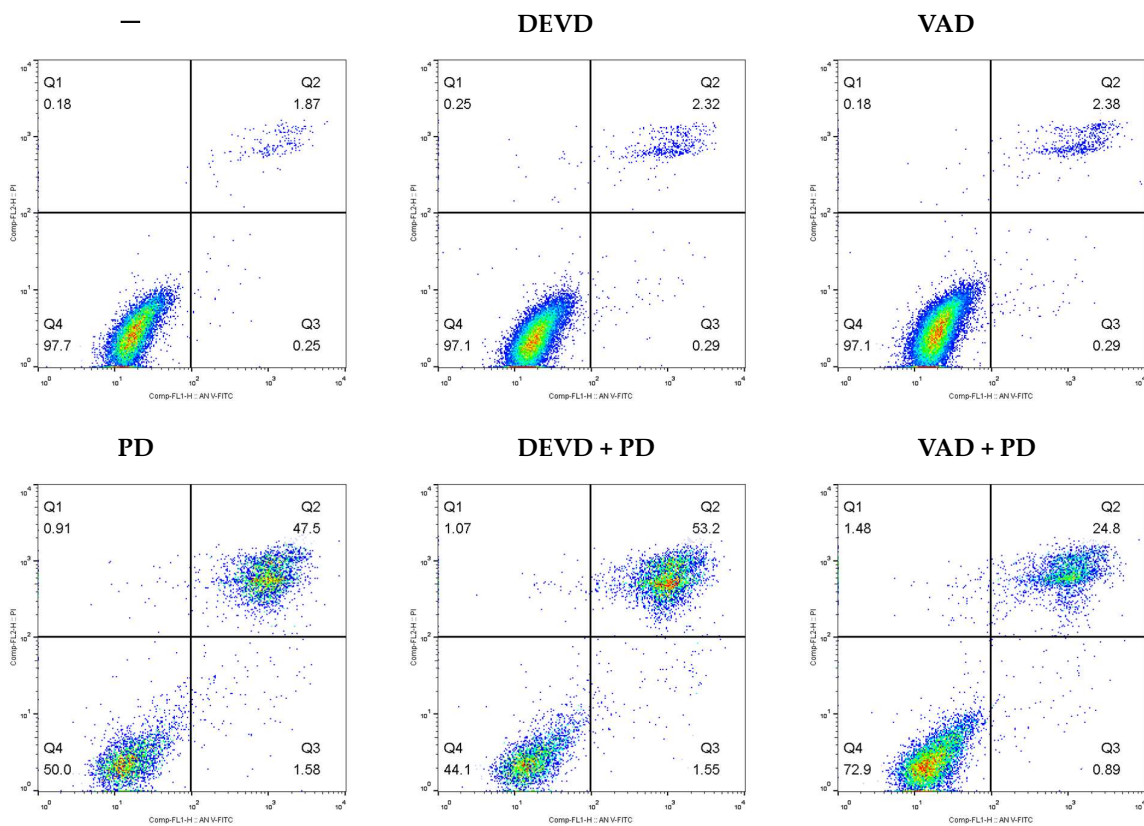
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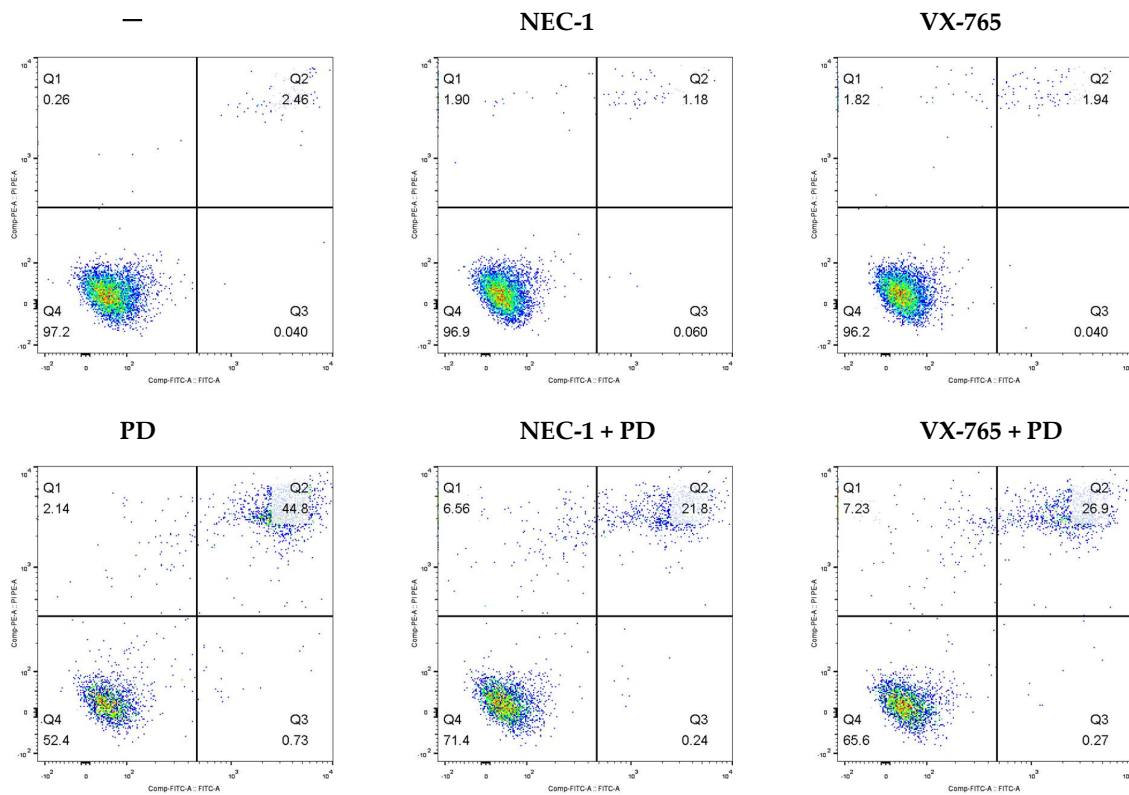
**A**



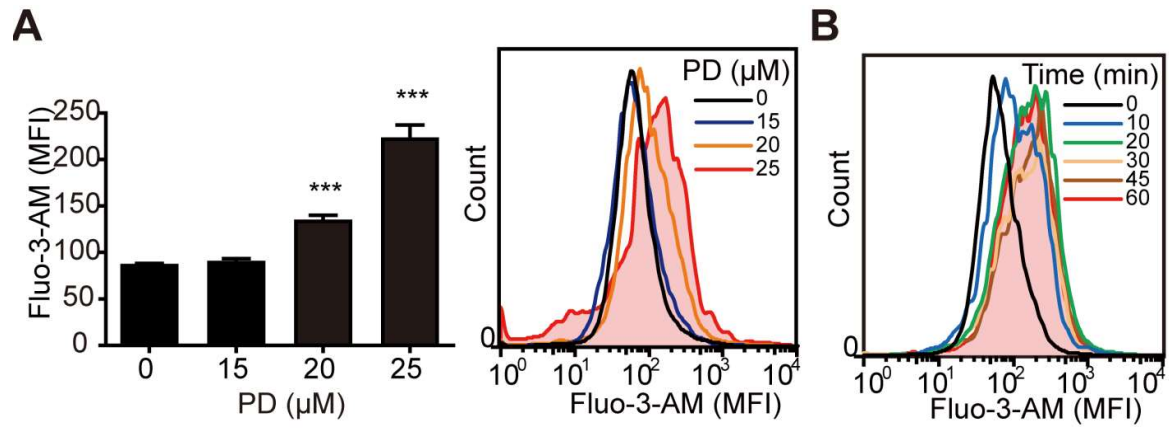
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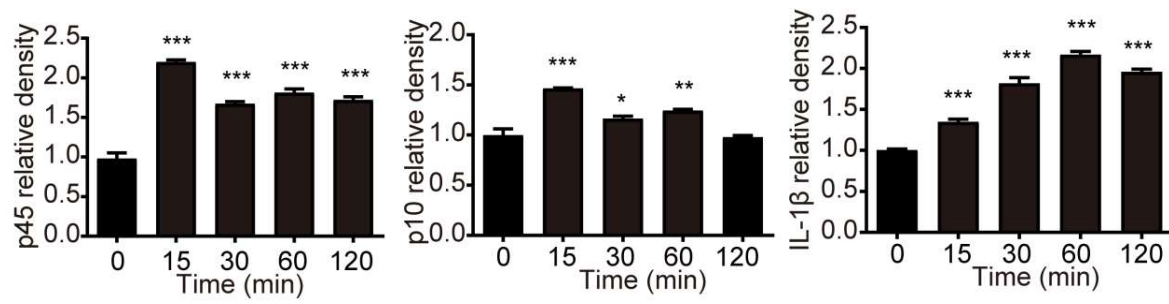
C



**Figure S1.** The flow diagram of proportion of different staining states in C2C12 cells using Annexin V-FITC/PI staining. Q1: necrotic cells; Q2: necrotic-like cells; Q3: early apoptotic cells; Q4: intact cells. (A) C2C12 cells were stimulated with PD (25  $\mu$ M) at indicated time. (B,C) C2C12 cells were pretreated with indicated inhibitors before PD (25  $\mu$ M) stimulation for 1 h.

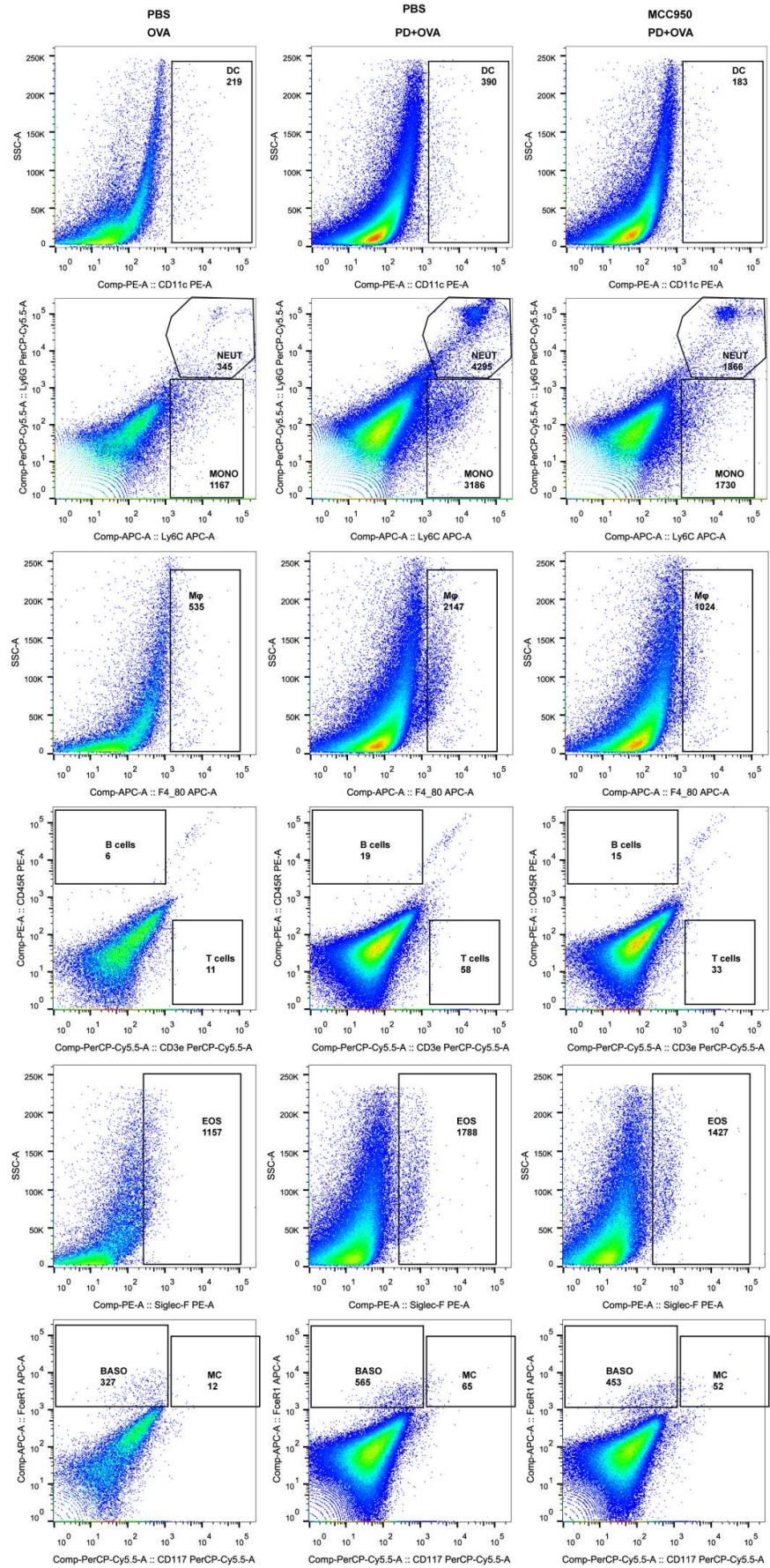


**Figure S2.** The levels of intracellular free calcium in C2C12 cells treated with PD at 0–25  $\mu\text{M}$  for 20 min (**A**) and at 25  $\mu\text{M}$  for 0–60 min (**B**) by flow cytometry. The histograms shown were representative of three independent experiments.



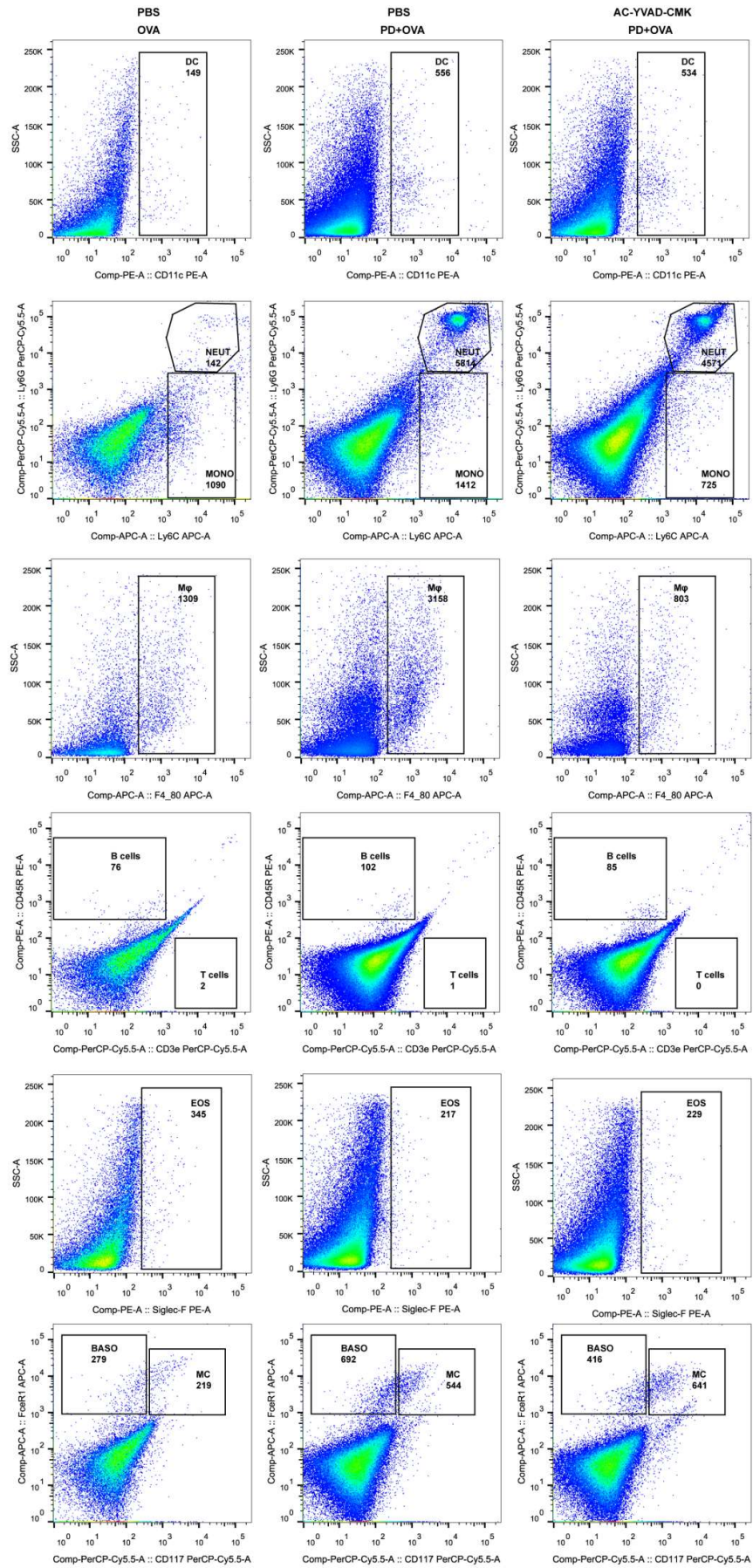
**Figure S3.** The effects of PD on the protein expression levels of caspase-1 and IL-1 $\beta$  in C2C12 cells. C2C12 cells were stimulated with PD (25  $\mu$ M) at the indicated time, and the activated caspase-1 and mature IL-1 $\beta$  protein levels were analyzed by Western blotting. p45, pro-caspase-1; p10, activated caspase-1. Quantitative results of mature IL-1 $\beta$ , p45, and p10 in Figure 6C ( $n = 3$ , 'n' is the number of replicates). Data were presented as mean  $\pm$  SD. (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$ , (\*\*\*)  $p < 0.00$ .

A



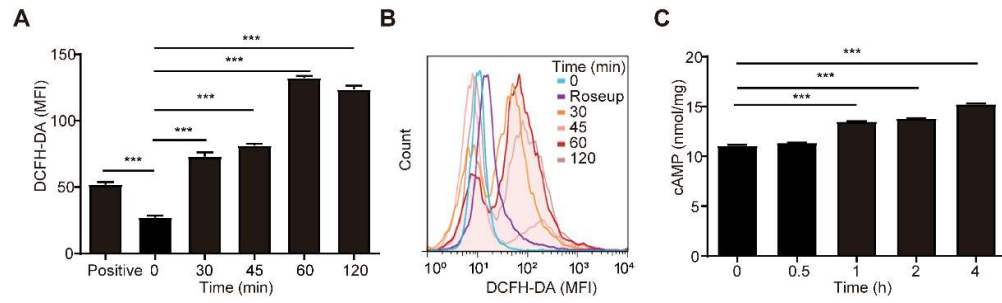


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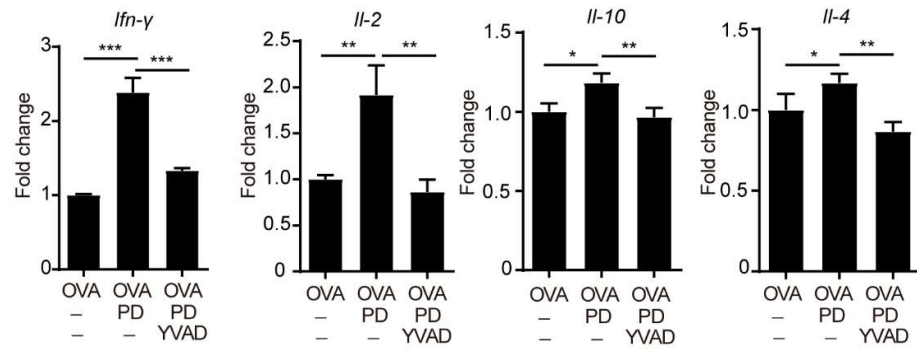


**Figure S4.** The flow cytometry gating strategy of recruited immune cells into the quadriceps muscles. Mice were injected *i.m.* with MCC950 (**A**, 3 mg/kg, 30 min) or AC-YVAD-CMK (**B**, 1 mg/kg, 1 h) and with PD (50 µg) in quadriceps muscles at the indicated time interval. After 24 h, the quadriceps muscle tissues were collected and assayed for the recruitment of various immune cells. DC, dendritic cell; MONO, monocyte; NEUT, neutrophil; Mφ, macrophage; EOS, eosinophil; BASO, basophil; MC, mast cell.





**Figure S5.** Effects of PD on the levels of intracellular ROS and cAMP in C2C12 cells. **(A, B)** The cells were incubated with PD at 25  $\mu$ M for 0–120 min, and then collected to detect the levels of ROS using 2',7'-dichlorofluorescein diacetate (DCFH-DA) by flow cytometry. The histograms were representative of three independent experiments. **(C)** The cells were incubated with PD at 25  $\mu$ M for 0–4 h, and then collected to detect the levels of cAMP using ELISA kit. Data were presented as mean  $\pm$  SD ( $n = 3$ ). (\*\*\*)  $p < 0.001$ .



**Figure S6.** Effects of PD on the mRNA expression levels of Th1/Th2 cytokines in OVA-stimulated splenocytes from the OVA-immunized mice. Mice were injected *i.m.* with Ac-YVAD-CMK at the dose of 1  $\mu\text{g/g}$  for 1 h before immunization. Splenocytes were prepared 2 weeks after the secondary immunization, and then stimulated with OVA (20  $\mu\text{g/ml}$ ) for 18 h at 37  $^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . The collected cells were measured for the mRNA expression levels of *Il-2*, *Il-4*, *Il-10*, and *Ifn-γ* by RT-qPCR. Data were presented as mean  $\pm$  SD ( $n = 3$ ). (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$ , (\*\*\*)  $p < 0.001$ , ns not significant.

**Table S1.** Sequences of primers used for RT-qPCR

<b>Gene</b>	<b>Forward (5'- 3')</b>	<b>Reverse (5'- 3')</b>	<b>Product (bp)</b>
<i>Gapdh</i>	AGCCTCGTCCCGTAGACAA	AATCTCCACTTTGCCACTGC	104
<i>Il-6</i>	ACAACCACGGCCTTCCCTACTT	CACGATTTCAGAGAACATGTG	129
<i>Il-1<math>\beta</math></i>	TTGACAGTGATGAGAATGACCTG	GCTCTTGTTGATGTGCTGCT	137
<i>Ccl-3</i>	ATTCTGCCACCTGCATAGCT	AGTCCCTCGATGTGGCTACTTG	68
<i>Cxcl2</i>	CTGAACAAAGGCAAGGCTAA	GCACATCAGGTACGATCCAG	125
Caspase-1	CGTACACGTCTTGCCCTCAT	AACTTGAGCTCCAACCCTCG	72
<i>Il-18</i>	TCAGCTGGGAAAACCTCAGGA	TGGGAACAGCCAGTGTTTCCAG	119
<i>Ptgs2</i>	GCAGATGACTGCCCAACTC	CAGGGATGAACTCTCTCCGT	103
<i>Nlrc4</i>	AGAATCGCTATGCTCTGGGC	AAGGTCGGCTTGCTGATGAA	188
<i>Nlrp3</i>	TGGACCAGGTTCACTGTGTT	CGGTTGGTGCTTAGACTTGA	118
<i>Aim-2</i>	AAATGCTGTTGTTGACCGGC	GAGTGTGCTCCTGGCAATCT	101
Caspase-3	GTCATCTCGCTCTGGTACGG	CACACACACAAAGCTGCTCC	169
Caspase-8	GCAAGACTGGAGCATGAATCTG	CACCCCATTTCTGCTGACTCAC	143
Caspase-9	TTGGGCGCAAAGGTTAAAGC	GGAGCCTTCAACAATCACAGC	139
<i>Bax</i>	CTCAAGGCCCTGTGCACTAA	CACGGAGGAAGTCCAGTGTC	73
<i>Bcl-2</i>	CCACCTGTGGTCCATCTGAC	CAATCCTCCCCCAGTTCACC	175
<i>Mlkl</i>	CCGGACAGCAAAGAGCACTA	TCCAGTGGAATTTCCCAGA	145
<i>Ripk1</i>	GGTCAAATTCAGAACAACCTGGA	CACACTGCGATCATTTCTCGT	122
<i>Ripk3</i>	ACACGGCACTCCTTGGTATC	CCGAACTGTGCTTGATCATA	168
<i>Il-2</i>	CCCAAGCAGGCCACAGAATTGAAA	AGTCAAATCCAGAACATGCCGCAG	81
<i>Ifn-<math>\gamma</math></i>	TCTTGAAAGACAATCAGGCCATCA	GAATCAGCAGCGACTCCTTTTCC	233
<i>Il-4</i>	CAAACGTCCTCACAGCAACG	CTTGGACTCATTCATGGTGC	203
<i>Il-10</i>	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG	105