Supplementary Information

Exploiting the protein corona: coating of black phosphorus nanosheets enables macrophage polarization via calcium influx

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Experimental Section

Material and cell lines. The BP crystals were purchased from HWRK Chemical Co., Ltd. (Beijing, China), and stored in a N_2 glovebox. N-Methyl pyrrolidone (NMP) was purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). Mouse plasma from BALB/c mice was purchased from the Institute of Hematology, Chinese Academy of Medical Sciences. Mouse breast cancer cells (4T1), mouse melanoma cells (F10), mouse colon cancer cells (CT26) and mouse macrophages (Raw264.7) were purchased from the American Type Culture Collection (Manassas, VA). All the cell lines were not listed by the International Cell Line Authentication Committee as cross-contaminated or misidentified (v8.0, 2016). All of the cell lines were authenticated by STR typing and confirmed to be mycoplasma-free by KeyGEN BioTECH Co., Ltd. (Nanjing, China). All cells were cultured in RPMI 1640 or DMEM with 10% foetal bovine serum (FBS), 100 units/ml penicillin, and 50 units/ml streptomycin at 37 ℃ in a CO_2 incubator (95% relative humidity, 5% CO_2).

Preparation of BPNSs and BPCCs. BPNSs were synthesized according to previous reports.[1] Briefly, 25 mg of BP powder was added to 25 ml of NMP and sonicated in ice bath for 48 h at a power of 600 W. The resulting brown suspension was centrifuged at 1800 g for 8 min to remove the residual unexfoliated particles, and the supernatant containing BPNSs was collected for further use.

For preparation of BPCCs, BPNSs were incubated with mouse plasma concentrations at 37 ℃ for 4 h. To obtain protein corona complexes, the mixed solution was centrifuged to pellet the particle-protein complexes. The precipitate was then resuspended three

times with 500 μl of PBS and centrifuged again for 20 min at 15000 g at 4 ℃.

Protein concentration. 100 μl BPNSs were incubated with plasma at 37 ℃ for 4 h. Then, BPCCs were collected following the above method and resuspended in PBS. Protein concentration were detected and calculated according to BCA protein assay kit (Beyotime).

Characterization of nanomaterials. The morphology of BPNSs and BPCCs were determined by TEM (JEOL JEM-1200EX) and AFM (Bruker MultiMode 8). Energy dispersive X-ray spectroscopy was conducted on the TEM. The zeta potential of nanomaterials in PBS was measured on a Nano-ZS instrument (Malvern Instruments Limited). The size of the nanomaterials in PBS was detected with a DLS instrument (Brookhaven BI-200SM).

LC-MS/MS analysis. Raw264.7 cells were incubated with 15 μg/ml BPCCs for 24 h and treated with SDT buffer (4% SDS, 100 mM DTT, 150 mM Tris-HCl pH 8.0). The cell lysis solution was boiled for 15 min and then centrifuged at 14000 g for 20 min. Protein analysis (200 μg for same sample) was carried out following the FASP procedure reported by Mann et al.[2] Briefly, the detergents were removed by using 200 μl of UA buffer (8 M urea, 150 mM Tris-HCl pH 8.0) for ultrafiltration (Microcom units, 30 KD) by centrifugation. Then, iodoacetamide (0.05 M in UA buffer) was added to block reduced cysteine and incubated in the dark for 20 min. The filter was washed three times with 100 μl of UA buffer and then twice with 100 μl of 25 mM NH_4HCO_3. Finally, the protein suspension was digested with 3 μg of trypsin (Promega) in 40 μl of 25 mM NH_4HCO_3 overnight at 37 °C, and the resulting peptides were collected as a filtrate. The peptide content was estimated by the UV light spectral density at 280 nm by using an extinction coefficient of 1.1 for a 0.1% (g/l) solution, calculated based on the frequency of tryptophan and tyrosine in vertebrate proteins. Each fraction was injected into a Q Exactive mass spectrometer (Thermo Scientific) for LC-MS/MS analysis. The detection method was positive ion, and the ion scanning range was 300-1800 m/z. The primary mass spectrometer resolution was 70,000 at 200 m/z, the automatic gain control (AGC) target was 1e6, the maximum IT was 50 ms, and the dynamic exclusion time was 60 s. The mass/charge ratio of the polypeptide and polypeptide fragments was collected as follows: 20 fragments were acquired after each full scan, the MS2 activation type was HCD, and the isolation window was 2 m/z. The rate was 17,500 at 200 m/z, normalized collision energy was 30 eV, and underfill was 0.1%. All identified proteins were retrieved from the UniProtKB mouse database (Release 2017 _02) in FASTA format. In this study, we used the label-free quantification algorithm for quantification. GO analysis was carried out with Blast2GO. KEGG pathway annotation on the target protein set was performed with KEGG Automatic Annotation Server (KAAS) software. Protein clustering was performed as follows: the quantitative information of the target protein collection was normalized, and then cluster 3.0 software was used to classify the two dimensions of sample and protein expression.

Finally, the hierarchical clustering heat map was generated using Java Trewview software. All experiments were repeated three times to ensure the reproducibility of the results.

For corona identification, the nanoparticle-protein complexes were lysed with lysis buffer. Total protein was reduced by adding 1 M DTT at 60 ℃ for 1 h, and free cysteines were alkylated with 1 M iodoacetamide at room temperature for 10 min in the dark. Then, trypsin was added to the treated protein (mass radio=1:50), and the samples were incubated at 37°C for 20 h. Finally, the resulting peptides after digestion were collected by desalination (Zeba Spin Desalting Columns) and freeze-drying. Before LC-MS/MS analysis, the protein was redissolved in 0.1% formic acid, and then the samples were subjected to LC-MS/MS analysis with Q Exactive (Thermo Fisher) and Easy-nLC 1000 (Thermo Fisher). The identified proteins were retrieved from the UniProtKB database (Release 2017 _02) in FASTA format and then searched against the SwissPort database (mouse) using NCBI BLAST+ software (ncbi-blast-2.2.28+-win 32.exe) to search for homologous proteins for which the functional annotation can be transferred to the studied proteins. In this study, the top 10 blast hits with E-values less than 1e-3 for each query protein were retrieved and loaded into Blast2GO (Version 3.3.5) for GO mapping and annotation. The GO analysis was carried out according to a previous lecture.

Morphological phenotyping analysis. Raw264.7 cells were seeded into 2 cm culture dishes at a density of 2×10^5 cells/ml for 24 h. Corona complexes were obtained as described above and resuspended in 1 ml DMEM media (0% FBS) to prepare for cell culture. Meanwhile, 15 μg of free BPNSs were resuspended in 1 ml of DMEM (0% FBS). In addition, Raw264.7 cells were stimulated with 20 ng/ml IL-4 (Abcam) or 100 ng/ml LPS (sigma) plus 20 ng/ml IFN-γ (PeproTech) for 24 h to generate M2 macrophages or M1 macrophages, respectively. After treatment for 24 h at 37 ℃, the medium was removed from the dish. After rinsing with PBS three times to remove the residual nanomaterials, the cells were immobilized with 1 ml of 4% paraformaldehyde for 15 min. Then, the cell nucleus and F-actin were stained with DAPI (KeyGEN BioTECH Co., Ltd, Nanjing, China) and phalloidin (Beyotime Biotechnology Co., Ltd, Shanghai, China), respectively; the cells were monitored using a fluorescence microscope (ZEISS).

Flow cytometry analysis. Raw264.7 cells were seeded into 6-well culture plates at a density of 5×10^5 cells/ml for 24 h. After treated with BPNSs (15 μg/ml), BPCCs (15 μg/ml), IL-4 (20 ng/ml) and LPS (100 ng/ml) plus IFN-γ (20 ng/ml) foe 24 h, the cells were collected, washed and incubated with anti-CD80 antibody (Invitrogen) for 1 h. Then the cells were further washed, and analysed by flow cytometry (BD FACSCalibur).

Ca^{2+} signal measurement. Macrophages were first treated with 15 μg/ml BPNSs or BPCCs for 24 h at 37 ℃, and the cytosolic Ca^{2+} concentration was determined with Fluo-4 AM (Beyotime Biotechnology Co., Ltd, Shanghai, China) according to the manufacturer's procedure. The Ca^{2+} signals were detected by a microplate reader (Tecan Infinite M1000 PRO) and a fluorescence microscope

(ZEISS).

To confirm the interaction between BPCCs and the STIM2 protein, 10 μM BTP2 was added after the treatment of BPCCs. Then, the Ca^{2+} signals were measured with Fluo-4 AM by fluorescence microscopy (ZEISS).

Quantitative RT-PCR. The total RNA of Raw264.7 with or without treatment was extracted using an RNA Extraction Kits (OMEGA) according to the manufacturer's procedure. Then, 2 μg of total RNA was used in a reverse transcription reaction with the Fasting RT Kit (TIANGEN Biotech Co., Ltd, Beijing, China). Quantitative RT-PCR was carried out with primers reported previously on the ABI StepOnePlus Real-Time PCR System (Applied Biosystems) using TB Green Premix Ex Taq (Takara).[3-5] mRNA expression levels were determined by the comparative Ct method.[6]

Co-culture model. To evaluate the chemotactic effects of BPCCs on macrophages, 4T1 cancer cells and Raw264.7 cells were co-cultured in Transwell systems with 3 μm-sized microporous membranes (Corning), which allow macrophages to translocate across microporous membranes[7]. Macrophages (1.0×10^6 cells) were collected and pre-labelled with Dio (Beyotime Biotechnology Co., Ltd., Shanghai, China) following the manufacturers' procedure. Dio-labelled macrophages were plated onto Transwell inserts, and 1.0×10^6 4T1 cancer cells were seeded into the bottom wells of the Transwell systems. Then, 15 μg/ml BPNSs or BPCCs were added into the bottom chamber. Two types of cells were co-cultured with nanomaterials for 24 h at 37 ℃. Then, the cells in the bottom chamber were rinsed with PBS twice. Dio-positive macrophages in the bottom chamber were detected and counted with a fluorescence microscope (Zeiss).

To evaluate the cytotoxicity of macrophages, different types of cancer cells and Raw264.7 cells were co-cultured in Transwell systems with 0.4 μm-sized microporous membranes (Corning), which do not allow macrophages to migrate across the microporous membranes. Cancer cells were seeded into the bottom wells of the Transwell systems at 8×10^4 cells/ml for 24 h, and Raw264.7 cells were simultaneously seeded into the insert chamber of the Transwell systems at 1.6×10^2 cells/ml. Then, 15 μg/ml nanomaterials were added into the insert or bottom chamber for 24 h at 37 ℃. After that, the caspase 3/7 activity of the cancer cells in the bottom chamber was determined with a Caspase 3/7 Detection Kit (KeyGEN BioTECH Co., Ltd., Nanjing, China).

Macrophage phagocytosis. To visualize phagocytosis, 1×10^5 4T1 cells were first plated into 2 cm culture dishes for 24 h and then labelled with DAPI (KeyGEN BioTECH Co., Ltd., Nanjing, China). Then, 5×10^5 Dil-labelled (Beyotime Biotechnology Co., Ltd, Shanghai, China) macrophages and 15 μg/ml BPCCs or BPNSs were added to the dishes, and the co-culture was incubated for an additional 24 h at 37 ℃. Cells were fixed with 4% formaldehyde for 15 min, washed three times with PBS and analysed with a fluorescence microscope (ZEISS).

Cytokine secretion assay. To analyse cytokine secretion by Raw 264.7 cells, the cells were seeded into 96-well plates at 1.0×10^5 cells/well for 24 h. BPNSs and BPCCs were resuspended in 1 ml of DMEM (0% FBS) and added into each well at different concentrations for 6 h. Cells incubated with plasma proteins were set as controls. Then, the supernatant was collected, and the cytokines were measured using an ELISA Kit (R&D Systems). In addition, the cytokine concentration in the culture medium from the co-culture model (described in the previous paragraph) was also measured by ELISA.

Western blot analysis. Raw264.7 cells were first treated with BPCCs or BPNSs for 24 h, and then the cell lysates were obtained with Whole Cell Lysis Assay (KeyGEN BioTECH Co., Ltd. Nanjing, China). The protein concentration was determined by the Super-Bradford Protein Assay Kit (CWBiotech, Inc., Beijing, China). The phosphorylation levels of MAPK members were determined by Western blot according to previous literature. Briefly, the extracts were first separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad, CA, USA). The membrane was blocked with 5% BSA in TBST at 25 ℃ for 1 h and then incubated with antibodies at 4 ℃ overnight. The expression of β-actin was used as the internal standard. Primary antibodies against the following proteins were used: β-actin (13E5, 1:1000 for WB), p38 MAPK (8690, 1:1000 for WB), p-p38 MAPK (4511, 1:1000 for WB), p-Erk1/2 (4370, 1:1000 for WB), JNK (9252, 1:1000 for WB), p-JNK (4668, 1:1000 for WB) and Erk1/2 (4695, 1:1000 for WB) from CST. The appropriate secondary antibodies (1:1000 for WB) were purchased from CST.

Immunofluorescence. Raw264.7 cells were first treated with BPCCs or BPNSs for 24 h and then immobilized with 4% paraformaldehyde for 15 min. After washing three times with PBS, cells were permeabilized with PBS containing 0.2% Triton X-100 for 10 min. The cells were blocked with 1% BSA (Sigma) and 22.52 mg/ml glycine in PBST (PBS + 0.1% Tween-20) for 30 min and then incubated with antibodies at 4 ℃ overnight. Finally, the cell nucleus was stained with DAPI (Beyotime Biotechnology Co., Ltd.), and the cells were monitored using a fluorescence microscope (ZEISS). The primary antibody against NF-κB p65 (15095, 1:1000 for IF) and appropriate secondary antibodies (1:1000 for IF) were obtained from CST.

Statistical analysis. All the dates are expressed as the mean ± standard deviation. Differences between different experimental groups were analysed by two-tailed Student's t test. One-way analysis of variance (ANOVA) was used in multiple group comparisons. Statistical analysis was performed using SPSS statistical program version 13 (SPSS Inc., Chicago, IL). Differences with $P < 0.05$ (*) or $P < 0.01$ (**) were considered statistically significant.

Results

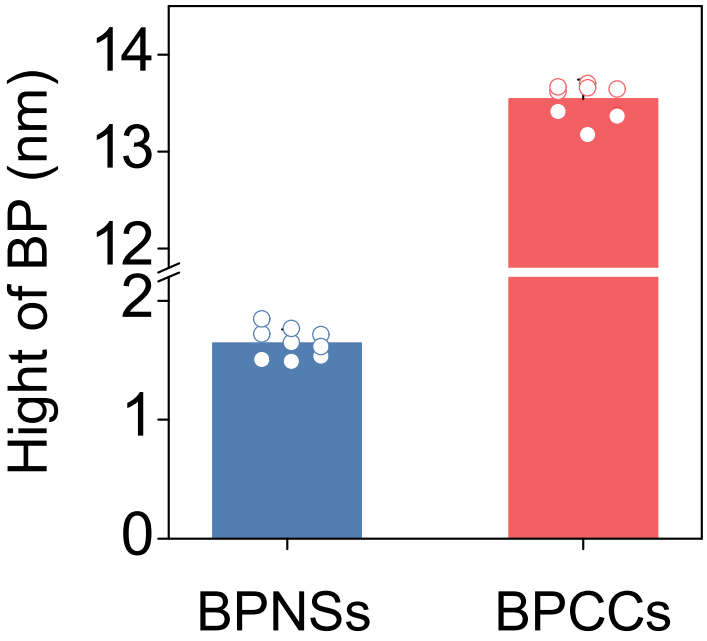

Fig. S1 Statistical analysis of the height of 10 nanomaterials determined by AFM.

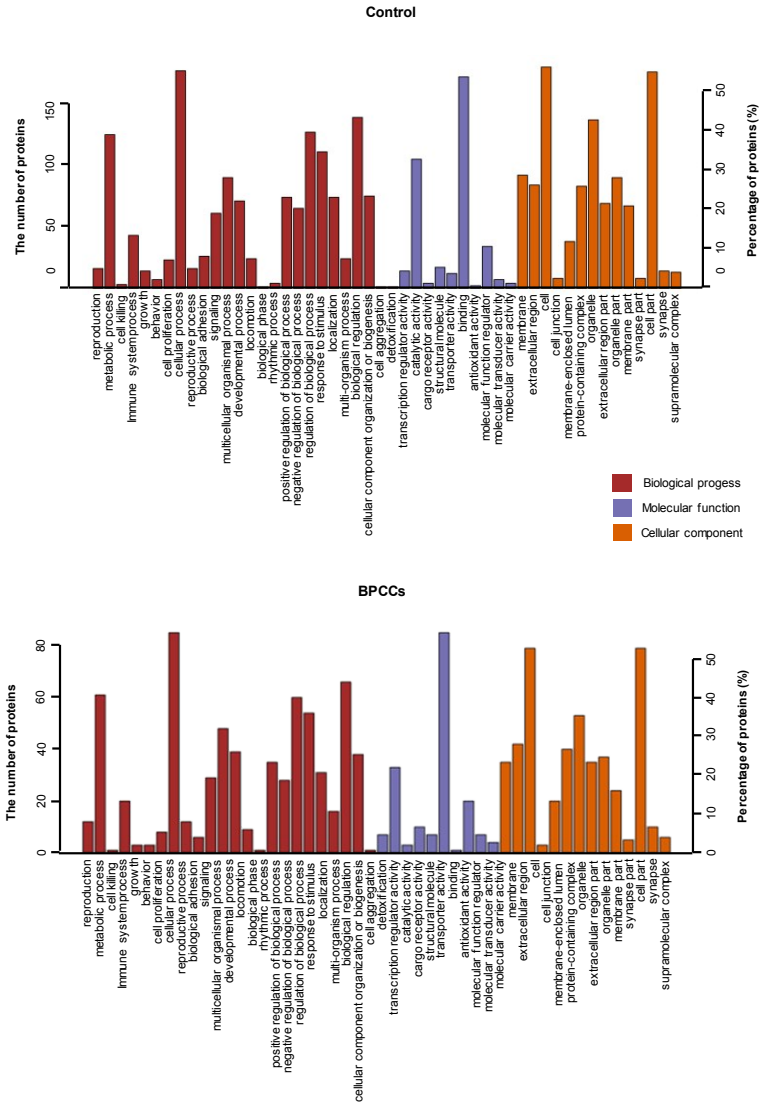

Fig. S2 Gene ontology analysis of corona proteins classified according to their Biological Process, Molecular Function and Cellular Component, as analyzed by LC-MS/MS.

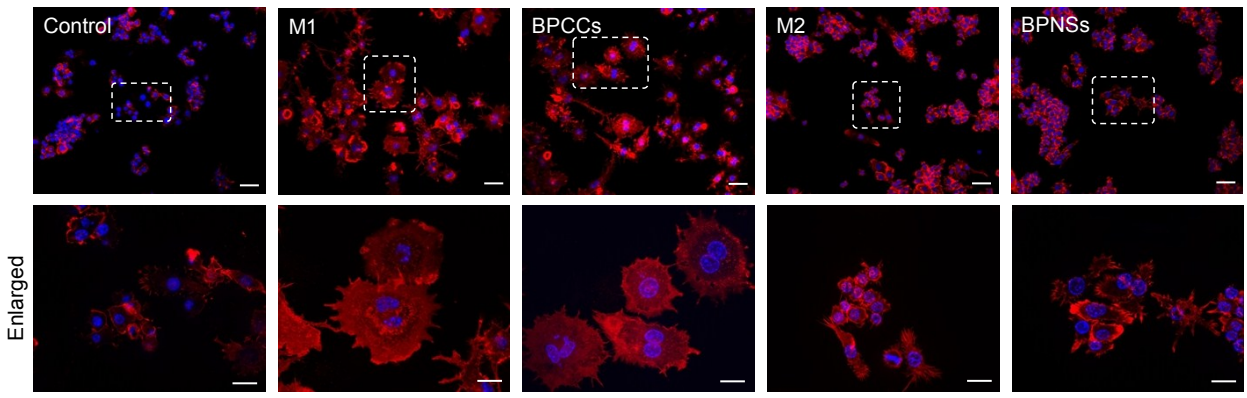

Fig. S3 Morphological phenotyping analysis of Raw264.7 macrophages by fluorescence microscopy. Red, actin; blue, nucleus. Scale bars: (top) 20 μm and (bottom) 50 μm.

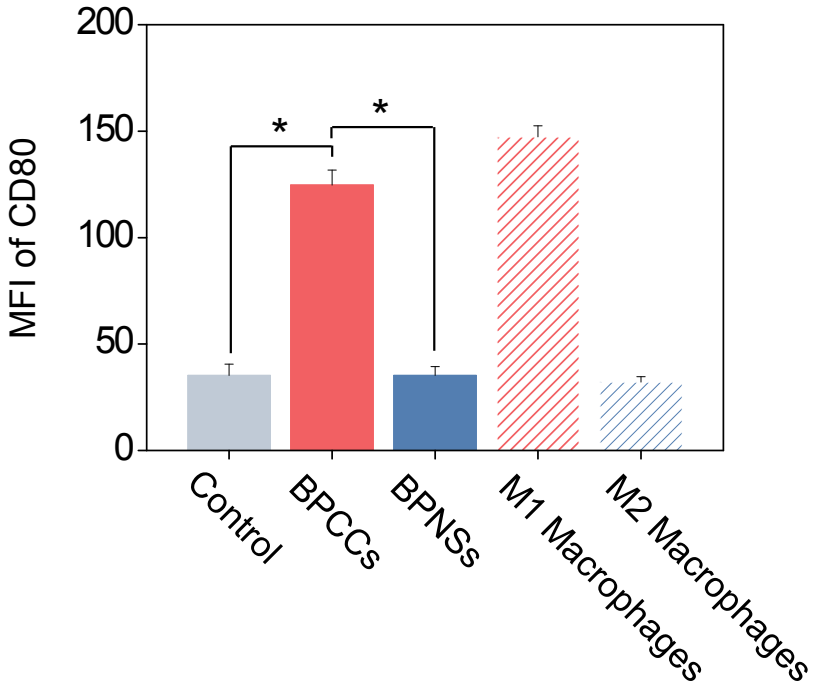

Fig. S4 Flow cytometry analysis of CD80 expression on macrophages after 24 h treatment with nanomaterials. All values are expressed as the means ± SDs of triplicates. Statistical significance is assessed by Student's t test. *p<0.05, **p<0.01.

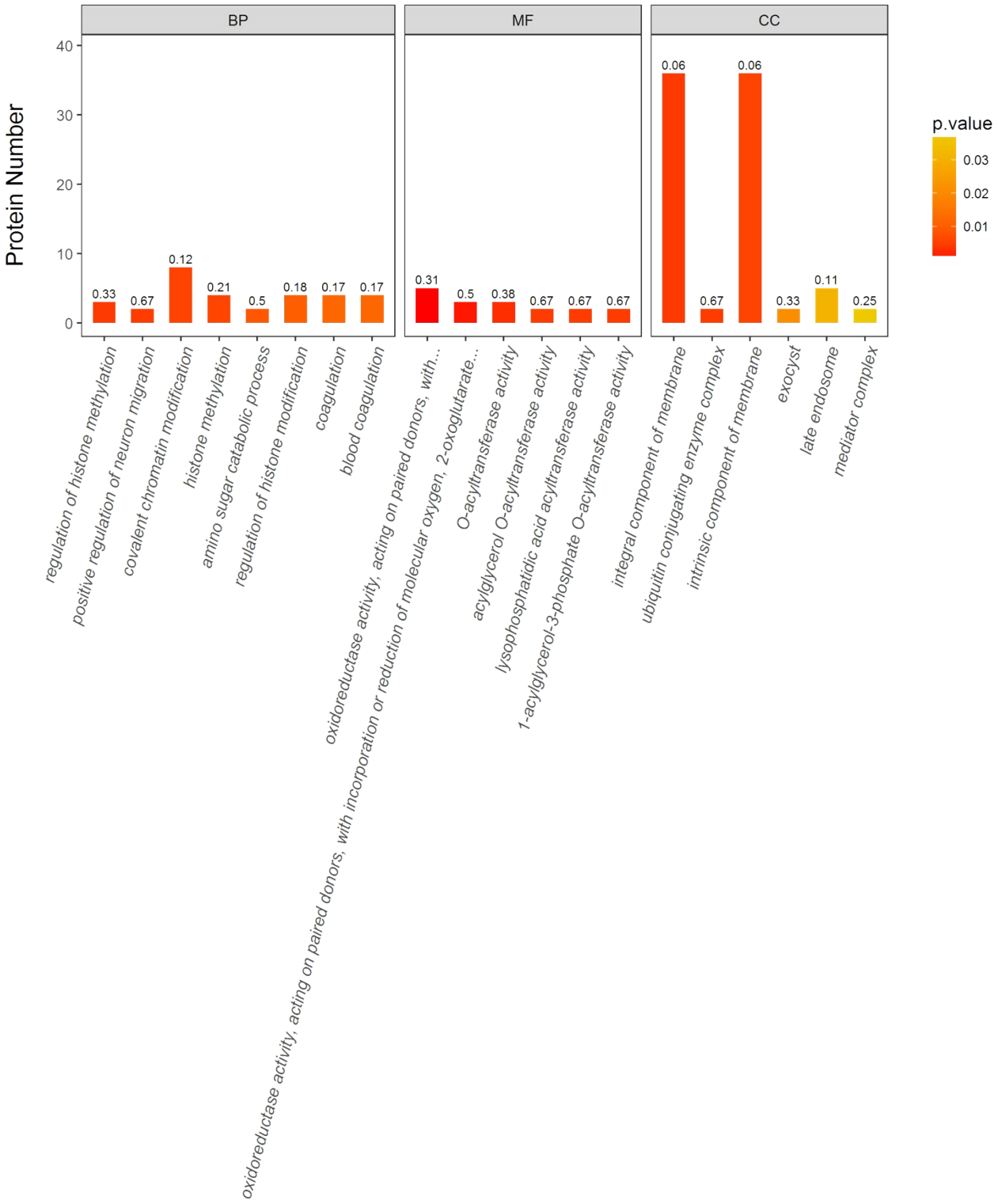

Fig. S5 Gene ontology analysis of Proteins classified according to their Biological Process (BP), Molecular Function (MF) and Cellular Component (CC), as analyzed by LC-MS/MS.

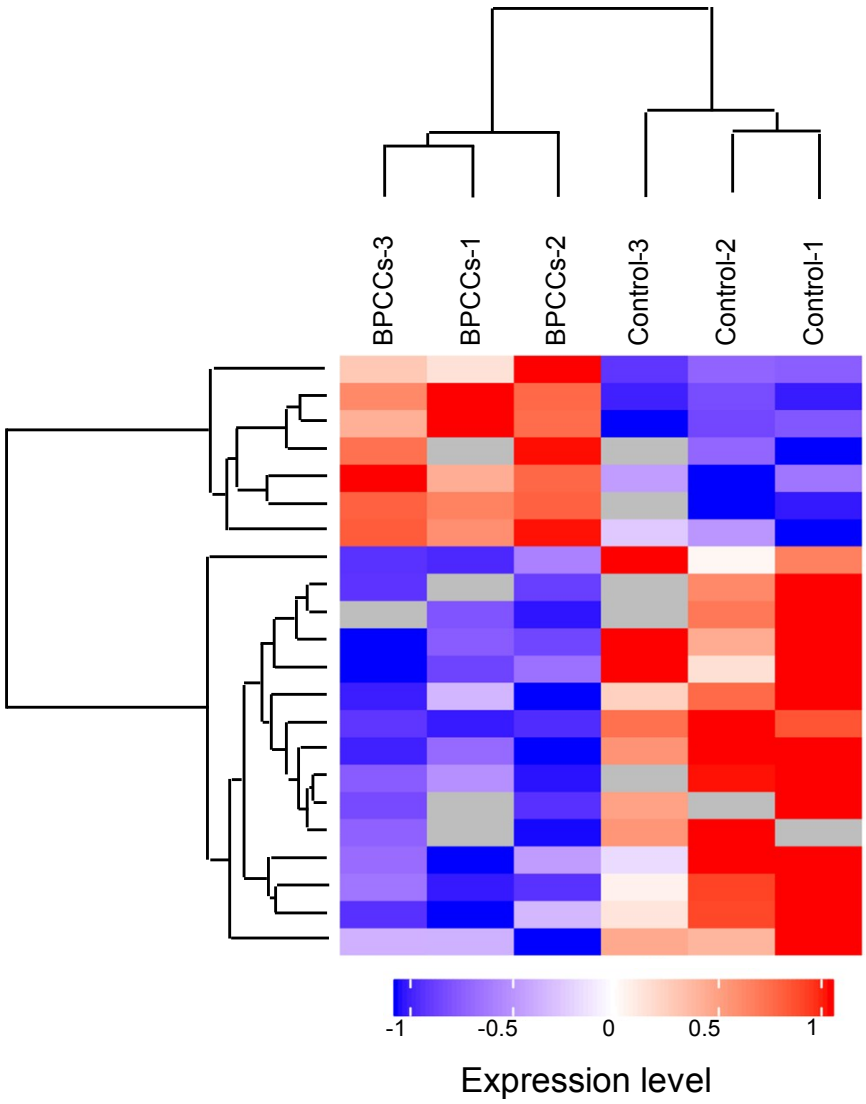

Fig. S6 Heat maps of the 20 most significantly different proteins by label-free quantitative analysis ($P < 0.05$).

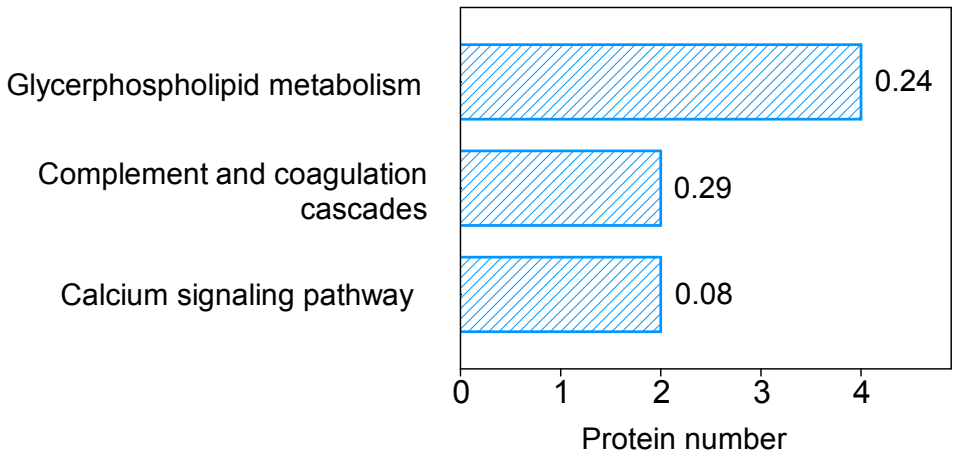

Fig. S7 KEGG pathway database screening for specific pathways. Statistical significance was assessed by Student's test.

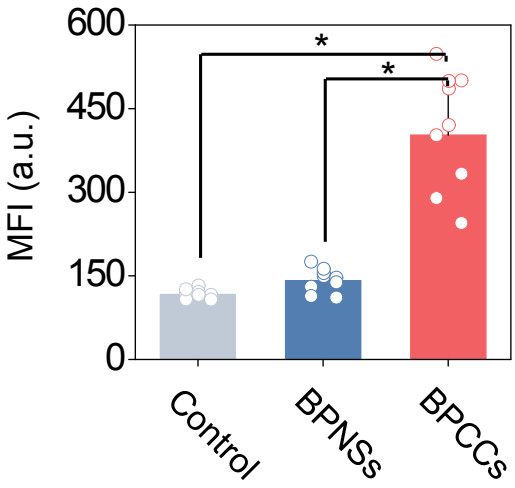

Fig. S8 The MFI of Fluo-4 AM in macrophage cells detected by fluorescence microscopy. The cells were treated with 15 μg/ml BPNSs orBPCCs for 12 h. All values are expressed as the mean ± SD of triplicates. Statistical significance was assessed by Student's t test. *p<0.05, **p<0.01.

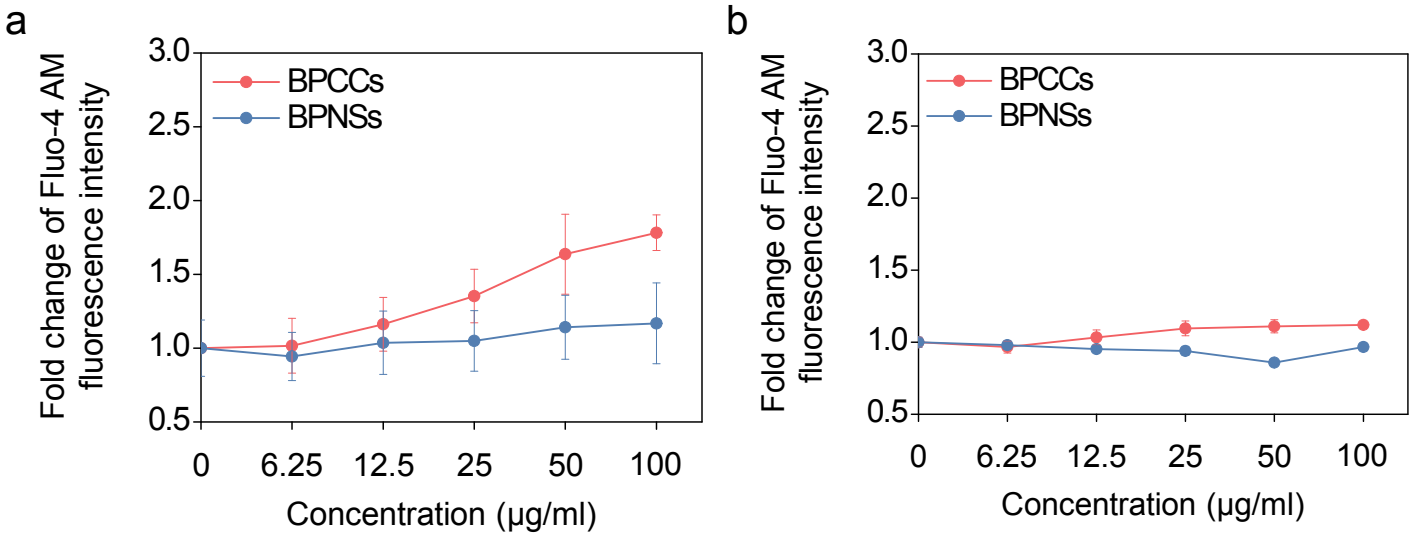

Fig. S9 Fold Change of Fluo-4 AM fluorescence intensity elicited in the following conditions: (a) Ca^{2+}-contain culture medium and (b) Ca^{2+}-free culture medium. All values are expressed as the means ± SDs of triplicates.

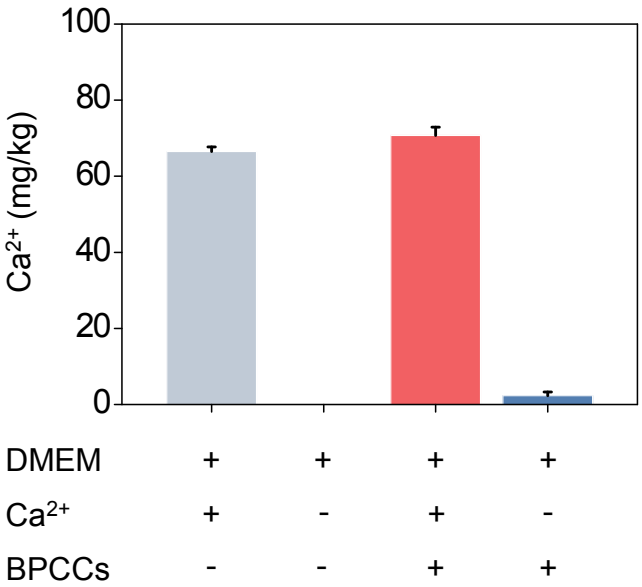

Fig. S10 Detection of Ca^{2+} concentration in different culture medium. All values are expressed as the means ± SDs of triplicates. Statistical significance is assessed by Student's t test. *p<0.05, **p<0.01.

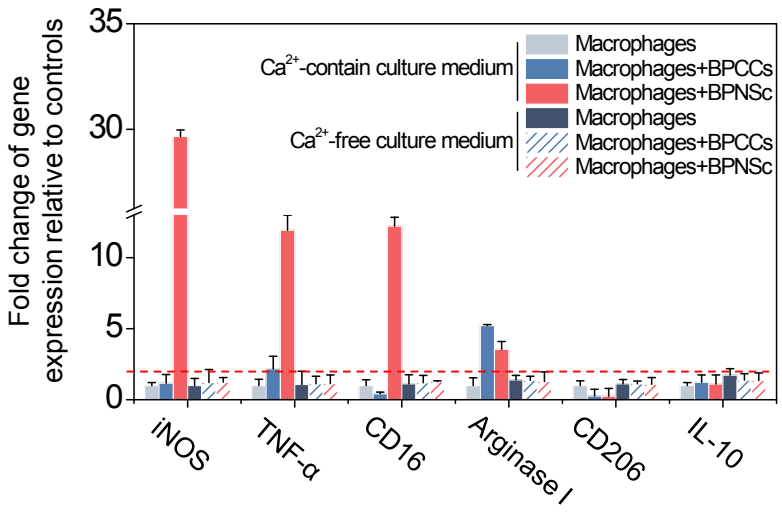

Fig. S11 Mark genes of M1 or M2 macrophages were measured in Raw264.7 with or without nanomaterials treatment. Cells were cultured in Ca^{2+}-contain culture medium and Ca^{2+}-free culture medium.

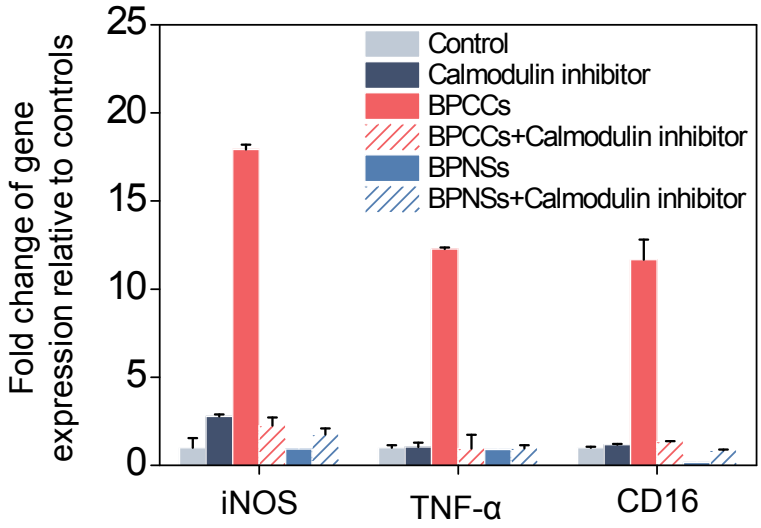

Fig. S12 RT-PCR analysis of mark genes of M1 macrophages were measured in Raw264.7 with or without nanomaterials treatment. Cells were cultured in Ca^{2+}-contain culture medium and Ca^{2+}-free culture medium.

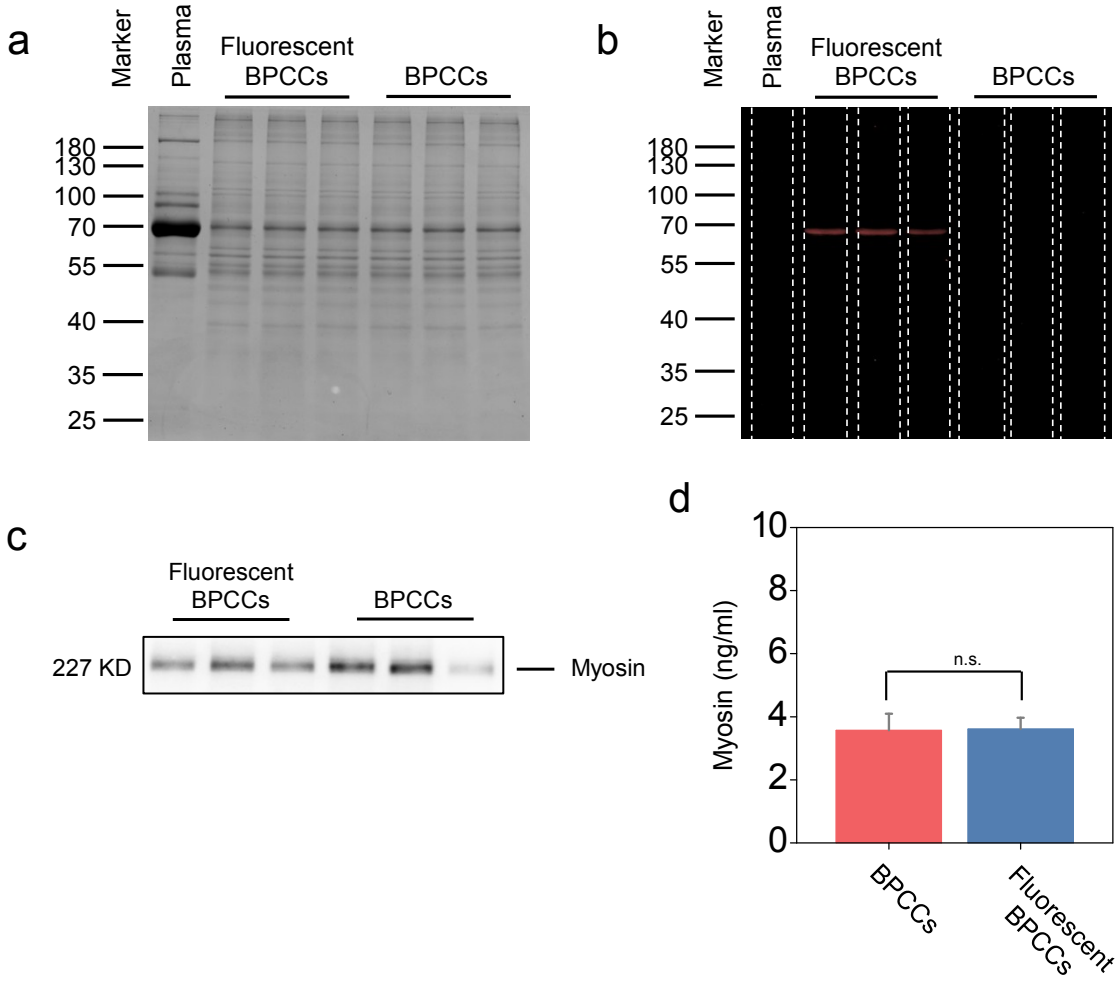

Fig. S13 Analysis of fluorescent BPCCs. (a) SDS-PAGE analysis of BPCCs and fluorescent BPCCs. (b) . (c) Western blot analysis of myosin from BPCCs and fluorescent BPCCs. (d) ELISA analysis of myosin from BPCCs and fluorescent BPCCs. All values are expressed as the mean ± SD of triplicates. Statistical significance was assessed by Student's t test. *p<0.05, **p<0.01.

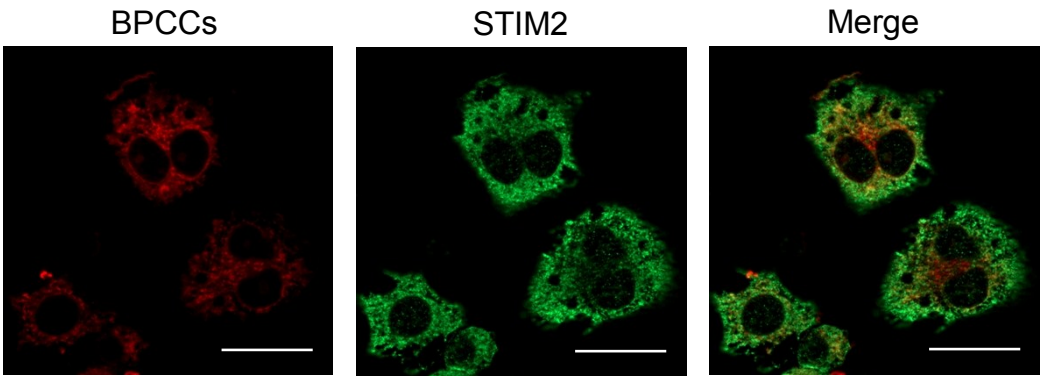

Fig. S14 Cellular image of BPCCs and STIM2 location. Red, fluorescent BPCCs; Green, STIM2. Scale bar: 20 μm. Fluorescent BPCCs were obtained by incubating BPNSs with plasma and SA-RBITC. The cells were treated with 15 μg/ml fluorescent BPCCs for 12 h and visualized using fluorescence microscopy.

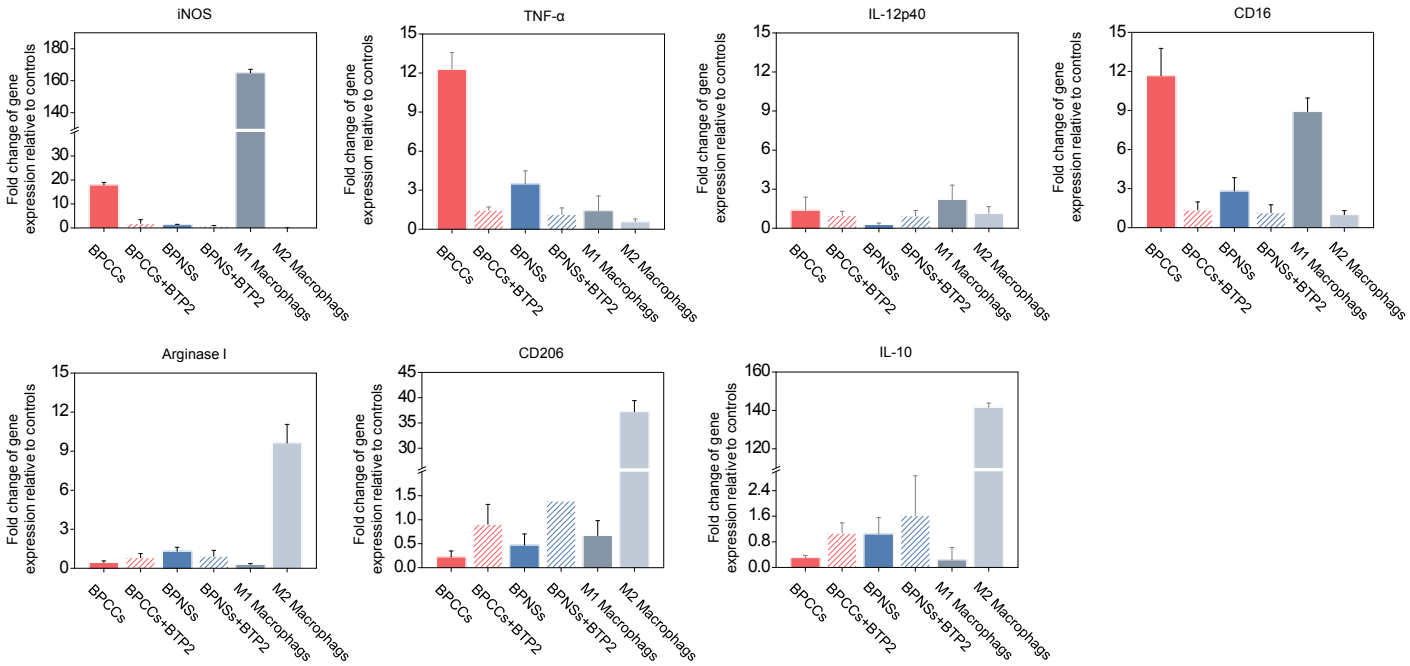

Fig. S15 Mark genes of M1 or M2 macrophages were measured in Raw264.7 with or without nanomaterials treatment by quantitative RT-PCR.

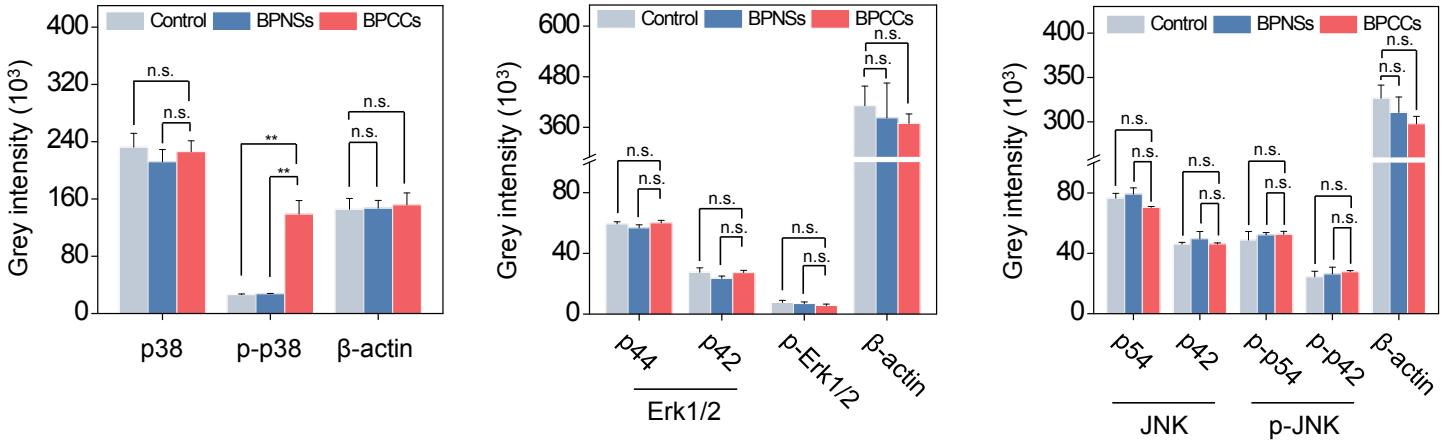

Fig. S16 Quantitative analysis of western blots from Figure 4e.

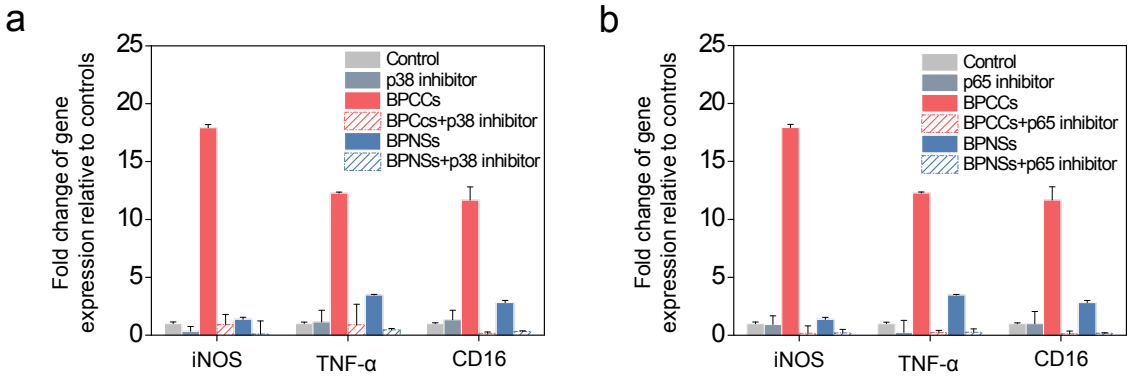

Fig. S17 The mRNA levels of mark genes of M1 macrophages were analyzed by RT-PCR in Raw264.7 that had been pretreated with 10 μM (a) MAPK p38 inhibitor or (b) NF-κB p65. All values are expressed as the means ± SDs of triplicates.

Table S1. List of distinct proteins in Raw264.7 cells treated with BPCCs compared with un-treated cells by label-free quantitative proteomics analysis. Statistical significance was determined by Student's test. $P < 0.05$.

Uniport accession number	Protein ID	Unique PepCount	MolWeight	Average LFQ intensity of control group	Average LFQ intensity of BPCCs group	BPCCs/Control	p value
Q5F2E7	Nuclear fragile X mental retardation-interacting protein	6	75.656	312130333.3	987870000	3.164928	0.01531
Q8K2T8	RNA polymerase II-associated factor 1 homolog	4	60.518	29436000	73641500	2.50175	0.01847

A0A077S9N161	Lysozyme	3	16.794	342450000	849100000	2.479486	0.04841
F6VQ62H5	Heterogeneous nuclear ribonucleoprotein D-like (Fragment)	4	35.534	143912000	305590000	2.12345	0.02779
Q9CQF639	Prenylcysteine oxidase	2	56.466	30087000	63476666.67	2.109771	0.00059
Q3U5U664	Heme oxygenase	12	30.197	3305700000	6848933333	2.071856	0.00142
A5CVE465	Stromal interaction molecule 2	3	83.924	1233566667	2499600000	2.026319	0.00566
Q8K4X766	1-acyl-sn-glycerol-3-phosphate acyltransferase delta	6	43.81	113933000	56537666.67	0.496236	0.03224
Q5SV0267	DNA repair protein RAD50	8	153.54	85520666.67	41908000	0.490034	0.01117
Q91YX068	Protein THEMIS2	6	74.377	66237500	32447500	0.489866	0.02083
Q8JZV769	N-acetylglucosamine-6-phosphate deacetylase	8	43.5	454970000	198810000	0.436974	0.04811
Q6116470	Transcriptional repressor CTCF	7	68.349	44972000	19565000	0.435048	0.01878
Q8BXZ1	Protein disulfide-isomerase TMX3	8	51.847	747760000	319690000	0.42753	0.00114
Q8VEB4	Group XV phospholipase	2	47.307	61892333.33	26151666.67	0.422535	0.00248
Q9D3B1	Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 2	2	28.402	72587000	30309666.67	0.417563	0.04255
V9GXJ9	Protein KRI1 homolog	5	68.631	111074666.7	45552333.33	0.410106	0.02001
Q91VC9	Growth hormone-inducible transmembrane protein	5	37.275	338633333.3	132710666.7	0.391901	0.02327
B7ZC46	Septin-8	3	50.895	141160000	52817000	0.374164	0.00269
E9Q0Z7	Sulfatase-modifying factor 1	3	25.827	16919666.67	4962800	0.293315	0.00022
Q9D0J4	ADP-ribosylation factor-like protein 2	2	20.864	64941500	17813000	0.274293	0.01488

UniquePepCount: the total number of unique peptides associated with the protein group (i.e. these peptides are not shared with another protein group)

Table S2. Top 30 most abundant corona proteins on BPNSs.

No	Protein ID		
	BPCCs-1	BPCCs-2	BPCCs-3
1	Complement C3	**Myosin-9**	**Myosin-9**
2	Fibronectin	**Myosin-11**	Actin, cytoplasmic 1
3	**Myosin-9**	Uncharacterized protein	Complement C3
4	Fibrinogen beta chain	Uncharacterized protein	Uncharacterized protein
5	Inter-alpha trypsin inhibitor, heavy chain 2	Complement C3	Filamin, alpha
6	Apolipoprotein B-100 (Fragment)	Uncharacterized protein	Fibrinogen beta chain
7	Antithrombin	Actin, aortic smooth muscle	Fibronectin
8	Heparin cofactor 2	Heat shock protein HSP 90-alpha	Apolipoprotein B-100 (Fragment)
9	Ceruloplasmin	Antithrombin-III	Thrombospondin 1
10	Inter-alpha-trypsin inhibitor heavy chain H1	Moesin	Complement C4-B
11	Complement C5	Apolipoprotein B-100 (Fragment)	Complement C5
12	**Myosin-9**	Uncharacterized protein	Antithrombin
13	Uncharacterized protein	Fibrinogen, B beta polypeptide, isoform CRA	Vitronectin
14	Gelsolin	Heat shock protein HSP 90-beta	Carboxypeptidase N catalytic chain
15	Prothrombin	14-3-3 protein zeta/delta (Fragment)	Fibrinogen alpha chain
16	Complement component 4B (Childo blood group)	MCG140959, isoform CRA	Gelsolin
17	Predicted gene 20547 (Fragment)	Vitronectin	UTP--glucose-1-phosphate uridylyltransferase
18	Plasminogen	Transthyretin	Inter-alpha trypsin inhibitor, heavy chain 2
19	Albumin 1	Carboxypeptidase N catalytic chain	Proteasome subunit alpha type
20	Serotransferrin	Chondroadherin	Serine (Or cysteine) peptidase inhibitor, clade D, member 1
21	Fibrinogen gamma chain	Calpain small subunit 1	Coagulation factor V
22	Pregnancy zone protein	Talin-2	Heat shock protein HSP 90-alpha
23	Alpha-2-macroglobulin-P	Titin	Alpha-actinin-1
24	Inter-alpha-trypsin inhibitor heavy chain H3	Eno3 protein	MCG140959, isoform CRA

25	Proteasome subunit alpha type-6	Complement C4-B	Heat shock protein 84b
26	Coagulation factor X	**Myosin regulatory light polypeptide 9**	Pregnancy zone protein
27	Haptoglobin	Uncharacterized protein	Alpha-2-macroglobulin-P
28	Serum paraoxonase/arylesterase 1 (Fragment)	**Myosin-7**	Uncharacterized protein
29	Transthyretin	Filamin, alpha	Vinculin
30	Vitronectin	Uncharacterized protein	Transthyretin

All experiments were conducted twice to ensure the reproducibility of the results.

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