Multi-walled carbon nanotubes act as a chemokine and recruit macrophages by activating the PLC/IP3/CRAC channel signaling pathway

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The impact of nanomaterials on immune cells is gaining attention but is not well documented. Here, we report a novel stimulating effect of carboxylated multi-walled carbon nanotubes (c-MWCNTs) on the migration of macrophages and uncover the underlying mechanisms, especially the upstream signaling, using a series of techniques including transwell migration assay, patch clamp, ELISA and confocal microscopy. c-MWCNTs dramatically stimulated the migration of RAW264.7 macrophages when endocytosed, and this effect was abolished by inhibiting phospholipase C (PLC) with U-73122, antagonizing the IP3 receptor with 2-APB, and blocking calcium release-activated calcium (CRAC) channels with SK&F96365. c-MWCNTs directly activated PLC and increased the IP3 level and [Ca2+]i level in RAW264.7 cells, promoted the translocation of the ER-resident stromal interaction molecule 1 (STIM1) towards the membranous calcium release-activated calcium channel modulator 1 (Orai1), and increased CRAC current densities in both RAW264.7 cells and HEK293 cells stably expressing the CRAC channel subunits Orai1 and STIM1. c-MWCNTs also induced dramatic spatial polarization of KCa3.1 channels in the RAW264.7 cells. We conclude that c-MWCNT is an activator of PLC and strongly recruits macrophages via the PLC/IP3/CRAC channel signaling cascade. These novel findings may provide a fundamental basis for the impact of MWCNTs on the immune system.

Carbon nanotubes (CNTs), a one-dimensional nanomaterial with unusual mechanical, electrical and chemical properties, have applications in many fields such as material engineering, drug delivery1-3, scaffolding for neuronal growth4-7 and bone cell proliferation8. The potentially extraordinary and unexpected impact of CNTs is increasingly focused on organisms or cells, including for biosecurity. Emerging studies have shown that CNTs are broad-spectrum potassium channel inhibitors9,10 and inflammation inducers, and exposure to CNTs can lead to organ injuries11-13.

The inflammation-inducing effects of CNTs suggest that they may have an impact on immune cells. It has been found that multi-walled carbon nanotubes (MWCNTs) increase the production of H2O2 and reactive oxygen species (ROS)14 in RAW264.7 macrophages; inhalation of MWCNTs causes nonmonotonic systemic immuno-suppression characterized by reduced T-cell-dependent antibody response and T-cell proliferative ability and decreased NK cell function15. Meng et al.16 reported that MWCNTs activated RAW264.7 macrophages into a M1

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M2 mixed status and stimulated macrophages to secrete a large amount of MIP-1α and MIP-2 to recruit macrophages and produce angiogenesis-related cytokines. However, these findings actually depicted the downstream events of activated macrophages in response to MWCNT exposure. The upstream signaling by which MWCNTs activate the macrophages is still poorly understood, and this is the major focus of the present study.

Research has revealed that calcium mobilization is the essential event that initiates immune cell activation. Increase of cytoplasmic Ca2+ in immune cells can lead to changes of several physiological functions, and the level and duration of cytoplasmic Ca2+ determines the strength and pattern of immune cell reactions to antigen stimuli. As with other cells, the calcium mobilization process in immune cells involves Ca2+ influx through certain Ca2+ channels such as the calcium release-activated calcium (CRAC) channel, and Ca2+ release from the endoplasmic reticulum (ER) via the Ca2+-releasing channel inositol 1,4,5-trisphosphate receptor (IP3R).

The CRAC channel is composed of the pore-forming calcium release-activated calcium channel modulator 1 (Orai1) subunit at the plasma membrane (PM) and the endoplasmic reticulum (ER)-resident stromal interaction molecule-1 (STIM1) auxiliary subunit, and it plays a crucial role in the calcium mobilization of immune cells. ER Ca2+ release is mediated by the calcium-releasing channel IP3R in non-muscle cells, and Ca2+ release often cross-interact. Ca2+ influx can induce Ca2+ release through the calcium-induced calcium release (CICR) mechanism, and Ca2+ release can also induce Ca2+ influx by the store-operated calcium entry (SOCE) mechanism. Ca2+ influx through the CRAC channel is a typical example of SOCE. STIM1 is a Ca2+ sensor of the ER. After detecting ER depletion, STIM1 translocates from the ER to the ER-PM junctions to reconstitute the CRAC channel to introduce Ca2+ influx. We noticed in the early stage of the present study that exposure of RAW264.7 macrophages to c-MWCNTs leads to activation of CRAC channels and dramatic cell migration, and this finding inspired us to explore the action mechanisms of c-MWCNTs in recruiting macrophages.

When macrophages sense the concentration gradients of a chemokine, they start to migrate towards the chemokine by changes of cell polarity, linear motion apparatus and steering response to the outside chemokine gradients. Macrophage migration is the premise of many immunological processes and can be considered a behavioral signal of macrophage activation. Now that c-MWCNTs act as a chemokine, the question is how c-MWCNTs initiate the migration process. Using several approaches including transwell assay, patch clamp recording, confocal microscopy and enzyme-linked immunosorbent assay (ELISA), we constructed a live picture illustrating the upstream signaling by which c-MWCNTs recruit macrophages, and it may suggest a fundamental basis to the impact of CNTs on macrophages and even other immune cells.

Results

**c-MWCNTs induce dramatic migration of RAW264.7 macrophages, and this effect was abolished by inhibitors of PLC, IP3R or the CRAC channel.** In transwell studies (Fig. 1A through 1J), quiescent RAW264.7 macrophages (control) showed weak migration behavior (Fig. 1B). Carboxylated MWCNTs (c-MWCNTs) at 50 μg/ml for 24 h in the lower chamber of the transwell, which could penetrate the PET membrane pores of the upper chamber and contact the RAW264.7 cells, induced a dramatic (≥9-fold) migration of RAW264.7 cells (Fig. 1C) compared with the control (Fig. 1B). Pretreatment of RAW264.7 cells with phospholipase C (PLC) inhibitor U-73122 (Fig. 1E), IP3R antagonist 2-APB (Fig. 1F), or CRAC channel blocker SK&F96365 (Fig. 1G) almost totally abolished the migration induced by c-MWCNTs. As a well-known chemokine, monocyte chemoattractant protein-1 (MCP-1) (10 ng/ml at the lower chamber) induced significant RAW264.7 cell migration (Fig. 1D), and this effect was also abolished by CRAC channel blocker SK&F96365 (Fig. 1H). A statistical summary of the RAW264.7 cell migration under different conditions is shown in Fig. 1I. U-73122, 2-APB or SK&F96365 themselves did not induce significant migration of RAW264.7 cells (not shown).

To determine whether RAW264.7 macrophages can phagocytize c-MWCNTs, we performed a transmission electron microscopy (TEM) study on the macrophages collected from the lower face of the PET membrane of the transwell insert (representing the migrated RAW264.7 cells). The TEM images (Figure S1) show that c-MWCNTs were present inside the RAW264.7 cells exposed to c-MWCNTs for 24 h, and most of them were likely wrapped by a vesicle-like structure, but a few of them directly pierced the cytoplasm. This result clearly shows that RAW264.7 macrophages can phagocytize c-MWCNTs in our experimental system.

**c-MWCNTs activate PLC and increase the IP3 level in RAW264.7 macrophages.** As the macrophage-recruiting effect of c-MWCNTs can be abolished by PLC inhibitor U-73122 as shown in Fig. 1E, we speculated that PLC may be the initial molecule of the signaling chain by which c-MWCNTs stimulate macrophage migration. Consistent with this speculation, the ELISA assay showed that c-MWCNT exposure for 6 h significantly increased the mRNA expression of PLC (Figure S2).

**c-MWCNTs activate CRAC channels and induce calcium mobilization in RAW264.7 macrophages.** Based on the calcium mobilization hypothesis of c-MWCNTs mentioned above and the finding that blockage of CRAC channels abolished the macrophage-recruiting effect of c-MWCNTs as shown in Fig. 1G, we examined the potential effects of c-MWCNTs on the CRAC channel currents in RAW264.7 macrophages.
The c-MWCNTs significantly increased the $I_{\text{CRAC}}$ densities in HEK293 cells stably expressing Orai1 and STIM1 (Fig. 2A and B) and in RAW264.7 cells (Fig. 2C and D). We first observed that c-MWCNTs given extracellularly did not significantly affect the $I_{\text{CRAC}}$ of RAW264.7 cells within 10–20 min (not shown), suggesting that c-MWCNTs act intracellularly. In HEK293 cells, c-MWCNTs (50 μg/ml) directly applied into the cytoplasm via the pipette solution soon increased $I_{\text{CRAC}}$ (Fig. 2A), the peak current density ($I_{\text{peak}}$) was increased from the control value of $-3.66 \pm 0.30$ pA/pF ($n = 5$) to $-21.54 \pm 6.42$ pA/pF ($n = 5$) ($P < 0.05$) (Fig. 2B), and the steady state current density ($I_{\text{ss}}$) was increased from the control value of $-2.06 \pm 0.36$ pA/pF ($n = 5$) to $-9.50 \pm 2.28$ pA/pF ($n = 5$) ($P < 0.05$) (Fig. 2B). As a positive control drug, thapsigargin (TG) (1 μmol/L, exposure for 30 min) increased $I_{\text{CRAC}}$, increased $I_{\text{peak}}$ from the control value of $-3.66 \pm 0.30$ pA/pF ($n = 6$) to $-19.41 \pm 2.16$ pA/pF ($n = 5$) ($P < 0.05$) (Fig. 2B). As a positive control drug, thapsigargin (TG) (1 μmol/L, exposure for 30 min) increased $I_{\text{CRAC}}$, increased $I_{\text{peak}}$ from the control value of $-3.66 \pm 0.30$ pA/pF ($n = 6$) to $-19.41 \pm 2.16$ pA/pF ($n = 5$) ($P < 0.05$) (Fig. 2B). Similar to HEK293 cells, in RAW264.7 cells, c-MWCNTs given intracellularly through the pipette solution also quickly and significantly increased $I_{\text{CRAC}}$. The peak $I_{\text{CRAC}}$ densities ($I_{\text{peak}}$) were increased from the control value of $-2.56 \pm 0.48$ pA/pF to $-16.77 \pm 1.94$ pA/pF, and the steady-state $I_{\text{CRAC}}$ densities ($I_{\text{ss}}$) were increased from the control value of $-2.22 \pm 0.16$ pA/pF to $-12.88 \pm 1.93$ pA/pF (Fig. 2C and D). TG exposure for 30 min also significantly increased the $I_{\text{CRAC}}$ density in RAW264.7 cells (Fig. 2C and D).

As DMEM was used to disperse c-MWCNTs, we also observed whether the pipette solution containing 50 μl/ml DMEM (the final concentration used for dispersing c-MWCNTs in the pipette solution) without c-MWCNTs would exert an effect on $I_{\text{CRAC}}$. The result showed that 50 μl/ml DMEM did not significantly affect $I_{\text{CRAC}}$ compared with the pipette solution without DMEM (Figure S3).
The results of confocal microscopy showed that c-MWCNTs significantly increased the \([\text{Ca}^{2+}]_i\) level in RAW264.7 cells (Fig. 3). As a positive control drug, TG also increased the \([\text{Ca}^{2+}]_i\) level in RAW264.7 cells (Fig. 3).

We also examined whether c-MWCNTs would exert an effect on the production of reactive oxygen species (ROS) in RAW264.7 macrophages. The result (Figure S4) showed that c-MWCNTs (50 μg/ml) moderately increased the ROS level. As a positive control agent in inducing ROS, \(\text{H}_2\text{O}_2\) (0.5 mmol/L) strongly increased the ROS level in RAW264.7 macrophages.

c-MWCNTs stimulate the translocation of STIM1 to the PM in HEK293 cells and RAW264.7 cells. Using confocal microscopy, we demonstrated the translocation of STIM1 upon c-MWCNT exposure in HEK293 cells stably expressing EGFP-STIM1 and CFP-Orai1 and in RAW264.7 cells. In HEK293 cells, Orai1 was consistently in the PM (Fig. 4), and STIM1 was mainly distributed in the cytoplasm before exposure to c-MWCNTs (STIM1 is theoretically ER-resident at resting condition). There was a very low level of STIM1 in the PM in resting HEK293 cells, but it was diffusely distributed in the cytoplasm (Fig. 4, upper row). Upon c-MWCNT (50 μg/ml) stimulation for 24 h, STIM1 were obviously translocated to the PM area, as the STIM1 fluorescence signal in the PM area was significantly increased (Fig. 4, middle row). As a known ER-depleting agent, TG (1 μmol/L) exposure for 30 min strongly redistributed the STIM1 to the PM area (Fig. 4, lower row).

Similar to the HEK293 cells, in resting (control) RAW264.7 cells, STIM1 was diffusely distributed in the cytoplasm (Fig. 5, upper row). As the ER could not be clearly recognized under a confocal microscope, it is assumed that STIM1 was diffusely distributed in the ER membrane in this condition. Exposure of RAW264.7 cells to c-MWCNTs (50 μg/ml for 24 h) led to a dramatic translocation of STIM1 to the PM area, and STIM1 distribution in the PM presented a discrete punctate manner (Fig. 5, lower row). The co-localization images of STIM1 and nuclei (DAPI stained) also support the translocation of STIM1 to the PM (Fig. 5). The above results suggest that c-MWCNTs stimulate the translocation of diffusely ER-resident STIM1 to the ER-PM junctions in HEK293 cells and RAW264.7 cells.

c-MWCNTs induce spatial polarization of the KCa3.1 channel in RAW264.7 cells. In the control RAW264.7 cells (before exposure to c-MWCNTs), most of the KCa3.1 channels were uniformly and diffusely distributed in the cytoplasm and some were likewise distributed in the PM (Fig. 6, upper row). However, after exposure to c-MWCNTs, the KCa3.1 channels were strikingly assembled to one pole of the RAW264.7 cells (Fig. 6, lower row). These results suggest that c-MWCNTs activate the KCa3.1 channels and induce spatial polarization of these channels, a phenomenon suggesting a role of the KCa3.1 channel in the process of polarity and steering behavior during macrophage migration.
**Figure 3.** Confocal microscopy showing the calcium-mobilizing effects of c-MWCNTs (*upper row*) and the positive control drug TG (*lower row*) in RAW264.7 macrophages. Note that both c-MWCNTs and TG increased the intracellular free calcium level ([Ca$^{2+}$]$_i$) of RAW264.7 cells.

**Figure 4.** Confocal microscopy showing the cellular localization of Orai1 and STIM1 in the transfected HEK293 cells before and after treatment with c-MWCNTs (50μg/ml, 24h) and TG (1μmol/L, 30min). Note that Orai1 is uniquely located in the plasma membrane (PM). STIM1 distributed mainly in the cytoplasm and slightly in the PM at baseline (control), while it significantly translocated to the PM after treatment with c-MWCNTs or TG.
Discussion

The present study focused on the impact and mechanism of c-MWCNTs on macrophage migration. Based on the results of transwell assays, we found that c-MWCNTs act as a chemokine and strongly stimulate the migration of RAW264.7 macrophages. We further outlined the underlying upstream signaling pathways (summarized in Fig. 7), i.e., after internalization, c-MWCNTs first activated PLC, thus leading to elevation of the IP3 level in the macrophages; IP3 induced Ca\(^{2+}\) release from the ER by activating the Ca\(^{2+}\)-releasing channel IP3R and thus led to ER depletion; the ER-resident STIM1 sensed the ER depletion and then translocated from the ER to the ER-PM junctions, where it reconstituted the complete CRAC channels with the membranous Orai1 and led to the
et al c-MWCNTs induced macrophage migration by targeting PLC as the most upstream signaling molecule. Here, we demonstrated that α and MIP-2 that can recruit RAW264.7 macrophages. We thought that the chemokine secretion expected enzyme-like activities (nanozymes) and have been considered the next-generation artificial enzymes. In recent years, some nanomaterials, including carbon nanomaterials, have been found to exhibit unprecedented enzyme-like activities and have been considered the next-generation artificial enzymes.

The question remains: What is the first targeting signaling molecule of c-MWCNTs at the intracellular side of macrophages? We speculated and experimentally demonstrated that it is PLC, an enzyme that is the most upstream signaling molecule in controlling the Ca\(^{2+}\) mobilization in non-muscle cells, especially for explicating the action mechanism of c-MWCNTs, as c-MWCNTs are unlikely to be equipped with specific membrane receptors that react to extracellularly acting agonists. We showed that PLC inhibitor U-73122 almost totally inhibited the c-MWCNT-induced macrophage migration, a result strongly supporting the above speculation. Some other investigators have provided clues to the effects of CNTs on PLC signaling. For example, Guidetti et al. showed that MWCNTs stimulate the secretion of some chemokines such as macrophage inflammatory protein (MIP)-1α and MIP-2 that can recruit RAW264.7 macrophages. We thought that the chemokine secretion they observed was actually the downstream signaling in the event of MWCNT-induced migration of RAW264.7 cells. The present study mainly depicted the upstream signaling by which c-MWCNTs induced migration of RAW264.7 macrophages release cytokines including chemokines, further stimulating macrophage migration. In addition, the K\(_{Ca3.1}\) channel is activated by [Ca\(^{2+}\)]\(_{i}\) elevation and mediates K\(^{+}\) efflux, thus resulting in repolarization of the membrane potential (V\(_{m}\)) and providing a feedback signal to the activated macrophages.

CRAC channel opening and Ca\(^{2+}\) influx; Ca\(^{2+}\) release and influx resulted in Ca\(^{2+}\) mobilization and macrophage activation, then triggering the subsequent downstream signaling and driving the macrophages to undergo certain immunological activities such as migration and secretion. At the same time, the c-MWCNT-induced Ca\(^{2+}\) mobilization also activated the K\(_{Ca3.1}\) channel and accordingly led to K\(^{+}\) efflux and a certain degree of membrane hyperpolarization, and this effect might restrict the macrophage activities to an appropriate level to avoid excessive immune responses. These findings might provide a fundamental basis for the impact of MWCNTs on macrophages and potentially even other immune cells and imply that the chemokine-like action of MWCNTs is important for further nanobiomedical practices utilizing carbon nanomaterials, both for curative and adverse effects evaluations.

Now that c-MWCNTs can recruit macrophages, we wondered what the first targeting signaling molecule for c-MWCNTs would be to initiate the migration event. By observing the reaction time course of RAW264.7 macrophages to c-MWCNT stimulation, we realized that c-MWCNTs must enter the macrophages to act. For example, in the patch clamp study, extracellular application of c-MWCNTs could not increase I\(_{CRAC}\) within the observed 20 min, while direct intracellular application of c-MWCNTs via the pipette solution soon increased I\(_{CRAC}\). We have observed similar phenomena in studying the effects of c-MWCNTs on the Kv4.2/4.3 channels in HEK293 cells and rat ventricular myocytes. Extracellular application of c-MWCNTs for a longer time (for example >30 min) can increase I\(_{CRAC}\), suggesting that the time latency is the time needed for phagocytosis of c-MWCNTs. We also noticed in a previous study that c-MWCNTs in the PC12 cells also needed a longer time to significantly reduce the current densities of three types of K\(^{+}\) channels. These studies suggest that c-MWCNTs act at the intracellular side, and of course internalization of c-MWCNTs needs time.

An additional important question emerges: Is c-MWCNT an activator of PLC or a nanozyme with PLC activ-
Although the abovementioned studies suggest that CNTs may activate PLC, the researchers did not measure the activities of PLC of cell lysates in their studies, and thus could not answer the above question. In the present study, we showed that U-73122, a PLC inhibitor, almost totally abolished the c-MWNT-induced migration of RAW264.7 cells (see Fig. 1E). We further identified that incubation of RAW264.7 macrophages with c-MWNTs for 6h directly increased the PLC activity by up to fourfold (shown in Fig. 1J) in the supernatant of cell lysates in which c-MWNTs had been removed by centrifugation at 12,000 rpm, and the absence of c-MWNTs in the supernatant was confirmed by light microscopy and TEM. These studies strongly suggest that c-MWNT is an activator of PLC, rather than an artificial nanzyme possessing the PLC activity. Overall, we identified for the first time that c-MWNTs induce macrophage migration by activating the PLC/IP3/CRAC channel signaling cascade in RAW264.7 macrophages, and PLC is the most upstream target signaling molecule for CNTs to induce macrophage migration, and importantly, c-MWNT is an activator of PLC. We speculate that CNTs are possibly universal PLC activators, although it still needs to be investigated whether all types of CNTs are PLC activators. This PLC-activating character of c-MWNTs may have broad use in many industrial fields, including enzymatic biosensoring, and microenvironmental modeling of tumors through macrophage recruitment. In addition, the potential for killing tumor cells using tumor-targeting MWNTs to recruit certain type(s) of macrophages in the tumor sites merits investigation, although most recent studies on the role of macrophages in tumor development focus on the tumor-promoting effects of tumor-associated macrophages.

Except for the finding of the PLC/IP3/CRAC channel signaling cascade as a “forward” pathway by which c-MWNTs induce macrophage migration, we also identified the involvement of the KCa3.1 channel, which may potentially act as a “feedback” signal in the c-MWNT–induced behavioral event of macrophages, as the function of the KCa3.1 channel is to introduce K+ efflux when activated by Ca2+, and it thus provides a feed-forward effect on the initial influx of Ca2+ and preserves the negative membrane potential required for sustained Ca2+ influx. In addition, KCa3.1 not only acts as a feedback signal for the behaviors of macrophages, but it may also facilitate the migration of macrophages cooperating with other channels such as Kv1.3 and CRAC channels. The dramatic cellular polarization of KCa3.1 in RAW264.7 macrophages that we observed in response to c-MWNT exposure suggests the activation of the KCa3.1 channel and its certain roles in the migration process of macrophages, potentially including the formation of protruding and cell polarity. KCa3.1 has been found to promote the migration of other types of cells, including human lung mast cells, human fibrocytes and mouse neuroblasts in vivo. The present study further identified the involvement of KCa3.1 in the c-MWNT-induced migration of RAW264.7 macrophages. However, the exact role of KCa3.1 in c-MWNT-induced macrophage migration needs further investigation.

Although we demonstrated the key upstream signaling by which c-MWNTs triggered the macrophage migration, other effects of CNTs on the macrophages, such as the enhancement of PLC gene transcription and ROS production that we showed, might be involved in the migration event or other activities of macrophages.

In summary, the present study demonstrated that c-MWNTs act as a chemokine and strongly stimulate the migration of RAW264.7 macrophages by activating the PLC/IP3/CRAC signaling pathway. In particular, PLC serves as the most upstream targeting signaling molecule of CNTs, and c-MWNT is an activator of PLC. The KCa3.1 channel is also involved in this event. These findings may shed light on the biomedical impact of CNTs at both the theoretical and practical level.

**Methods**

**Reagents.** Reagents were purchased from the following suppliers: monocyte chemotactic protein-1 (MCP-1), SK&F96365, 2-aminophosphorylborinate (2-APB), NP-40, Cs aspartate, CsOH, EGTA, HEPES, anti-beta actin antibody, 4',6-diamidino-2-phenylindole (DAPI), thapsigargin (TG) and mouse anti-KCa3.1 antibody (Sigma, USA); ryanoide and Dylight 650-conjugated donkey anti-mouse secondary antibody (Abcam, UK); rabbit anti-Orai1 antibody (Alomone Labs, Israel); mouse anti-STIM1 antibody (Abnova, USA); bovine serum albumin (BSA) (Bovogen, Australia); anti-fluorescence quenching agent (Dako, Denmark).

**Preparation and characterization of MWNTs.** We used the carboxylated MWNTs (c-MWNTs) in this study, as this type of CNT has a good hydrophilic property and can disperse well in the aqueous phase. The c-MWNTs were developed from the pristine MWNTs (p-MWNTs). The p-MWNTs were purchased from Chengdu Organic Chemical Co. Ltd. (Chengdu, China) and the c-MWNTs were prepared as previously described. In brief, the c-MWNTs were synthesized based on the p-MWNTs through a combined oxidation procedure and probe sonication. The as-received MWNTs were dried at 50 °C in a vacuum oven overnight and then were suspended in a 3:1 (volume) mixture of concentrated H2SO4/HNO3 and sonicated with a probe sonicator. The as-received MWNTs were dried at 50 °C in a vacuum oven over night and then were suspended in a 3:1 (volume) mixture of concentrated H2SO4/HNO3 and sonicated with a probe sonicator. The as-received MWNTs were dried at 50 °C in a vacuum oven over night and then were suspended in a 3:1 (volume) mixture of concentrated H2SO4/HNO3 and sonicated with a probe sonicator. The as-received MWNTs were dried at 50 °C in a vacuum oven overnight and then were suspended in a 3:1 (volume) mixture of concentrated H2SO4/HNO3 and sonicated with a probe sonicator.
ranged from 300 nm to 1.5 μm, and the average length was 926–945 nm depending on the manufacturing batch. The FTIR spectrum of c-MWCNTs showed that the characteristic absorption of the carboxylic group at 1720 cm⁻¹ is substantially decreased in the c-MWCNT spectrum, while an absorption peak at 1630 cm⁻¹ appeared.

**Evaluation of potential endotoxin contamination in the MWCNTs.** To inactivate the potentially contaminated endotoxin in the c-MWCNT samples, the above prepared c-MWCNTs were routinely autoclaved (temperature >250°C) and were again heated in an oven at 250°C for 45 min prior to the experiment, with the suggested procedures of the International Pharmacopoeia (Sixth Edition, 2016) as a reference. The potential presence of endotoxin in the c-MWCNT solution was further evaluated using the limulus amebocyte lysate (LAL) gel clot assay kit (sensitivity 0.125 EU/ml) (Hounshii Company, Xiamen, China). To perform the assay, c-MWCNTs were added into the endotoxin-free water, vortexed and shaken for 15 min, followed by centrifugation at 12,000 × g for 10 min. The supernatant was collected and added to the LAL assay solution (v/v = 1:1). The mixture was incubated at 37°C for 1 h. Then, the tube was inverted to observe gel clot formation. Negative results were obtained with the c-MWCNT solution, demonstrating that the c-MWCNTs we used were endotoxin-free. The endotoxin of 0.25 EU/ml was set as a positive control which could induce gel clot formation. Because c-MWCNTs produced a black solution, we did not use a colorimetric test to examine endotoxin contamination in the c-MWCNT solution, out of concern that it may yield a false-positive result.

**Cell culture and transfection.** The RAW264.7 macrophage-like murine cell line (ATCC Number TIB-71; American Type Culture Collection, US) was purchased from the Cell Center, Peking Union Medical College. Cells were cultured at 37°C and 5% CO₂ humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 unit/ml penicillin, 100 mg/ml streptomycin. Cultured RAW264.7 cells were used for the transwell assay, confocal microscopy and patch clamp study.

Plasmids encoding mCFP-Orai1 and STIM1-EYFP were constructed according to the method described by Zeng et al. Transfected HEK293 cells (Invitrogen) were routinely cultured and transfected with the above two plasmids to obtain stable expression of Orai1 and STIM1 in this cell line. Transfected HEK293 cells were grown in a DMEM complete culture medium with 200 μg/ml zeocin and 1 μg/ml tetracycline to induce Orai1 expression.

**Transwell assay.** Transwell assays were performed to evaluate the effect of c-MWCNTs on the migration capacity of RAW264.7 cells. The transwell apparatus (Millipore Co., USA) is composed of an upper chamber (Millipore insert) and a lower chamber. The bottom of the upper chamber is a polycarbonate teflon plate (PET membrane) with 8-μm pores that allow cells to penetrate/migrate. The lower chamber is a well of a 24-well culture plate. Cultured RAW264.7 cells were collected and suspended in DMEM medium, and then were counted and adjusted to a concentration of approximately 10⁵ cells/ml DMEM. Cell suspensions (200 μl) were seeded onto the upper chamber and cultured in DMEM with 10% FBS at 37°C for 24 h to allow cells to fully grow and adhere to the upper face of the PET membrane so that they would be ready to migrate. The lower chamber was filled with 700 μl DMEM. c-MWCNTs or other agents (such as MCP-1, U-73122, etc.) were delivered into the DMEM solution contained in the lower chamber. Cells that migrated across the pores of the PET membrane would adhere to the lower face of the PET membrane of the Millipore insert. Note that although c-MWCNTs were placed in the lower chamber, the 8-μm pores of the PET membrane equipped in the bottom of the Millipore insert also allowed c-MWCNTs to penetrate the pores and diffuse from the lower chamber to the upper chamber driven by the c-MWCNT gradient between the two chambers, therefore providing a chance for RAW264.7 cells in the upper chamber to be exposed to c-MWCNTs and thus trigger cell migration. After the 24-h migration, the Millipore insert was removed and the DMEM medium was discarded. The insert (including the PET membrane) was washed with PBS three times, and cells adhering to the lower face of the PET membrane (indicating the migrated cells) were fixed with 4% paraformaldehyde for 15 min, and then were stained with 0.1% crystal violet for 20 min, followed by a PBS wash to remove excess stain. Cells on the upper face of the PET membrane (cells not migrated) were removed by a gentle wipe with a cotton swab. Cells adhering to the lower face of PET membrane (cells migrated) were counted under a microscope (200× objective). Five fields were randomly chosen and cells were captured and counted with Image-pro Plus 6.0 with the amount of normal control cells standardized to 1. The same experiments were performed in triplicate in three wells.

**Transmission electron microscopy (TEM)**

TEM was performed to evaluate the phagocytosis of RAW264.7 macrophages on c-MWCNTs according to our recent report with modifications. Briefly, after the transwell experiments, RAW264.7 cells were collected from the lower face of the PET membrane of the insert (representing the migrated cells). The collected RAW264.7 cells in DMEM were centrifuged at 1000 rpm for 4 min. The cell pellets were fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.2) and stored at 4°C until TEM was performed. To perform the TEM, RAW264.7 cells were washed with phosphate buffer and were further fixed with 1% phosphate buffered osmium tetroxide, rewashed with phosphate buffer, dehydrated by graded ethanol and acetone, infiltrated with epoxy resin and acetone, and finally were embedded in epoxy resin. Ultra-thin sections were cut with an RMC MT-X ultrathin microtome, mounted on copper grids and then stained with uranyl acetate and lead citrate and washed with distilled water. Ultrastructural images of RAW264.7 cells were shot with a Gatan digital camera (Gatan, USA) equipped in a transmission electron microscope (JEOL-1011, Japan). RAW264.7 cells without exposure to c-MWCNTs served as the control.

**Patch clamp.** Whole-cell patch clamp experiments were performed to record the CRAC currents (I_{CRAC}) of cultured RAW264.7 cells and HEK293 cells with stable expression of CRAC channels (Oria1 and STIM1 subunits) at room temperature using an EPC-10 amplifier (HEKA, Germany). Pipette resistance was controlled to 2–4 MΩ. To record I_{CRAC}, a 200-ms step pulse was delivered from a holding potential of 0 mV to −120 mV. The
bath (external) solution consisted of (in mmol/L): NaCl 155, KCl 4.5, CaCl₂ 22, MgCl₂ 1, D-glucose 10, HEPES 5 (pH 7.4 with NaOH). The pipette (internal) solution consisted of (in mmol/L): Cs aspartate 140, MgCl₂ 3.01, CaCl₂ 0.66, EGTA 11.68 (free [Ca²⁺]i, was approximately 10 nM), HEPES 10 (pH 7.2 with CsOH). Current data were analyzed using Clampfit 10.1 software, and current tracing generation was performed by OriginPro8.0 software. The c-MWCNTs were added to the pipette solution to obtain the desired concentration (50 μg/ml), thus yielding an intracellular application of c-MWCNTs to observe the “immediate” effect of c-MWCNTs on I_{CRAC}, as the effect of extracellular c-MWCNTs on I_{CRAC} emerged very slowly (>30 min).

Confocal microscopy. Confocal microscopy was used to visualize the subcellular localization of Orai1 and STIM1 both in RAW264.7 cells and HEK293 cells stably expressing Orai1 and STIM1, and KCa3.1 in RAW264.7 cells. After incubation with c-MWCNTs (dispersed in DMEM) for 24 h, both types of cells were fixed with 4% paraformaldehyde, washed with PBS, blocked with BSA, and then incubated with antibodies, respectively, against Orai1 (1:500), and STIM1 (1:500) or KCa3.1 (1:500) at 4 °C overnight. Cells were then washed thrice with PBS, followed by incubation with DyLight650-conjugated donkey anti-mouse secondary antibody for 1 h. Cells were finally washed with PBS, stained with DAPI (10 μg/ml) for 10 min, washed with PBS, and cover sealed with anti-fluorescence quenching agent. Visualization and a photo shot of the fluorescence for Orai1, STIM1 and KCa3.1 were performed under a confocal microscope (Olympus, Japan). The excitation/emission wavelengths (nm) for DAPI, CFP, EYFP and DyLight650 were, respectively, 358/461, 433/476, 513/527 and 654/673.

Confocal microscopy was also used to evaluate the calcium mobilizing effect of c-MWCNTs in RAW264.7 cells. Briefly, RAW264.7 cells were incubated with Fluo-3/AM (3 μmol/L) in the dark for 1 h, washed with PBS three times, and then placed into DMEM containing c-MWCNTs. Cell fluorescence change was continuously monitored under a confocal microscope until the Ca²⁺ transient appeared.

Measurement of ROS level. To determine whether c-MWCNTs induce production of reactive oxygen species (ROS) in RAW264.7 cells, intracellular ROS was measured using an ROS assay kit (Jiancheng Biotechnology, China). Briefly, RAW264.7 cells were washed and then were lysed. Cell lysates were collected and centrifuged at 12,000 rpm to acquire the supernatant free of MWCNTs. The absence of c-MWCNTs in the supernatant was confirmed by light microscopy and TEM. ELISA was performed to measure the activity of PLC and the level of IP3 in the cell lysate supernatant according to the manufacturer’s instructions. Optical absorbing values were read at 450 nm and corrected by 570 nm. The data were analyzed using SoftMax Pro4.8 software.

Statistical analysis. The data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using Student’s t-test for paired data and analysis of variance followed by Newman-Keuls multiple comparison for multiple group data. P < 0.05 was considered statistically significant.

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Author Contributions
H.L. and J.C. designed the research. B.Z. performed the plasmds and performed cell transfection. J.M. and H.X. prepared the MWCNTs, performed the TEM study and evaluated endotoxin contamination. H.L., X.T. and L.Y. performed the main research. H.L., X.T., L.Y. and J.C. analyzed the data. J.C. wrote the manuscript. All authors reviewed and approved the manuscript.

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