Tumor cell-released autophagosomes (TRAP) enhance apoptosis and immunosuppressive functions of neutrophils

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ABSTRACT
Our previous studies have confirmed that tumor cell-released autophagosomes (TRAP) could induce the differentiation of B cells into IL-10+ regulatory B cells (Bregs) with suppressive activities on T lymphocytes. However, the mechanism of TRAP-mediated immune suppression is still largely unclear. Herein, we sought to assess the immunomodulatory effect of TRAPs on human neutrophils, a major immune cell type that infiltrates human tumor tissues. We found that TRAPs enriched from malignant effusions or ascites of cancer patients and tumor cell lines were rapidly and effectively phagocytized by neutrophils through macropinocytosis and promoted neutrophil apoptosis via reactive oxygen species (ROS) generation and caspase-3 activation. Moreover, the apoptotic neutrophils that have phagocytized TRAPs inhibited the proliferation and activation of CD4+ T and CD8+ T cells in a cell contact- and ROS-dependent manner. These findings define a novel TRAP-mediated mechanism in neutrophils that potentially suppresses the anti-tumor T cell immunity and highlight TRAPs as an important target for future tumor immunotherapy.

INTRODUCTION
Neutrophils are the most abundant leukocytes in the peripheral blood and classically considered as cells pivotal for the first line of defense against invading pathogens and tissue damage. Although neutrophils exhibit antitumor activity in addition to their traditional antibacterial functions, it is becoming increasingly clear that they can infiltrate a variety of tumors where the tumor-associated neutrophils (TANs) play an important role in cancer biology. The presence of TANs is a poor prognostic factor in several types of tumors, such as renal carcinoma, gastric carcinoma, and hepatocellular carcinoma. Neutrophils can also secrete soluble factors to activate endothelial and parenchymal cells and enhance tumor cell metastasis to distal sites. However, the functions of TANs in tumor-induced immunosuppression and the conditions that promote the development of neutrophils to TANs in the tumor microenvironment are not completely understood.

Recent studies have indicated that there are two major pathways of autophagy: canonical degradative autophagy and unconventional secretory autophagy. Canonical degradative autophagy recycles damaged cellular proteins, lipids, toxic intracellular aggregates and organelles via lysosomal degradation, whereas unconventional secretory autophagy function as a novel protein secretion pathway that enables many cytoplasmic proteins to be exported from the cells. Secretory autophagosomes encompass a broad range of cytoplasmic components. It has been found that autophagosomes play a key role in the secretion of a major pro-inflammatory cytokine, interleukin 1β (IL-1β), which plays a crucial role in the immune response against infections. Insulin-degrading enzyme (IDE) is secreted from astrocytes through an unconventional secretory autophagy pathway in Alzheimer disease (AD), indicating that the regulation of autophagy is a potential therapeutic target in addressing amyloid beta peptide (Aβ) pathology. Furthermore, autophagy underlies an alternative secretory pathway for lysozyme in Paneth cells during bacterial infection, and the disruption of secretory autophagosome formation could significantly decrease the lysozyme secretion and increase the risk for Crohn’s disease in humans.

In our previous study, we have confirmed that tumor cell-released autophagosomes (TRAP) from tumor cell supernatants and malignant effusions and ascites of cancer patients carry damage-associated molecular patterns (DAMPs), such as high-mobility group B1 (HMGB1), and induce the differentiation of B cells into IL-10+ regulatory B cells (Bregs) with suppressive activities on T lymphocytes via the TLR2–MyD88–NF-κB pathway. These findings suggest that TRAPs could be responsible for inhibition of antitumor immune response through...
enhancing the generation of IL-10+ Bregs. In the present study, we investigated the impact of natural TRAPs enriched from tumor cells on neutrophils. We found that TRAPs can be effectively and rapidly phagocytized into neutrophils through macropinocytosis, and induce neutrophil apoptosis via ROS generation and caspase-3 activation. Importantly, we demonstrated that TRAP-treated neutrophils could inhibit the proliferation of CD4+ and CD8+ T cells in a cell contact- and ROS-dependent manner. These findings provide strong evidence that TRAPs are part of the elaborate network of tumor-derived substances that promote immune suppression and should be targeted to improve tumor immunotherapy.

Results

TRAPs promote neutrophil apoptosis

To determine whether neutrophils could phagocytize the extracellular autophagosomes of tumor cells, we labeled tumor cell-derived TRAPs17 with carboxyfluorescein succinimidyl ester (CFSE) (Figs. S1A–C) and co-incubated them with highly purified human peripheral blood neutrophils (Figs. S2A and B) for 3 h. Flow cytometric analysis showed that increasing the concentration of CFSE-labeled TRAPs led to an increased percentage and higher mean fluorescence intensity (MFI) of CFSE+ neutrophils (Figs. 1A and B). Time course analysis showed that both the proportion and the MFI of neutrophils that have phagocytized CFSE-labeled TRAPs peaked at 3 h after incubation with 3 μg/ml TRAPs (Figs. 1C and D). There was virtually no internalization of TRAPs at 4°C as compared to 37°C (Figs. 1E and F). Consistently, immunofluorescence analysis demonstrated that TRAPs were effectively internalized by neutrophils after 3 h of incubation (Fig. 1G). Collectively, these results demonstrate a rapid and effective phagocytosis of TRAPs by human neutrophils in vitro.

Neutrophils undergo spontaneous apoptosis to maintain a relatively constant number in healthy individuals.18 To determine whether TRAPs could accelerate neutrophil apoptosis, we co-incubated purified neutrophils with TRAPs. Flow cytometric analysis of neutrophil Annexin V and PI staining showed that TRAPs accelerated neutrophil apoptosis in a dose- and time-dependent manner (Figs. 2A and B). Interestingly, incubation with TRAPs led to significantly more rapid and greater neutrophil apoptosis (22.9% apoptosis at 15 min incubation with TRAPs vs. 24.9% spontaneous apoptosis at 12 h) (Fig. 2B and Fig. S3). To determine whether TRAPs promote neutrophil apoptosis in vivo, we intraperitoneally (i.p.) administered TRAPs isolated from the 4T1 tumor cell line into BALB/c mice. Marked increased apoptosis of peritoneal neutrophils was observed in mice that received 30 μg/ml TRAPs (Fig. S2C and Fig. 2C). Furthermore, autophagosomes enriched from malignant effusions of lung cancer or breast cancer patients or ascites of ovarian cancer patients also potently enhanced the apoptosis of human neutrophils (Figs. S1D–F and Fig. 2D). Chromatin condensation and fragmentation are additional hallmarks of apoptosis. TRAP-treated neutrophils exhibited DNA condensation (Fig. 2E) as well as DNA fragmentation in a dose-dependent manner (Fig. 2F). Additionally, we found that the caspase-3 enzymatic activity increased in TRAP-treated neutrophils in a time-dependent manner (Fig. 2G). Together, these results demonstrate that TRAPs promote neutrophil apoptosis in vitro and in vivo.

The intact membrane structure of TRAPs is required to induce neutrophil apoptosis

To understand the components in TRAPs responsible for accelerating neutrophil apoptosis, we co-incubated neutrophils with either TRAPs or an equal amount of lysate of the tumor cell

Figure 1. TRAPs are rapidly and effectively phagocytized by human neutrophils. The purified human neutrophils were co-cultured with different concentrations of CFSE-labeled TRAPs (0, 0.1, 0.3, 1, 3, 10 or 30 μg/ml) for 3 h (A and B), or with CFSE-labeled TRAPs (3 μg/ml) for 0, 0.25, 0.5, 1, 3, 6, 9 or 12 h (C and D). Flow cytometric analysis was performed to identify the phagocytizing of TRAPs by neutrophils. (E and F) The purified human neutrophils were cultured with CFSE-labeled TRAPs (3 μg/ml) at 4 or 37°C for 3 h. The percentage of CFSE+ neutrophils was assessed by flow cytometry. (G) The purified human neutrophils were cultured with CFSE-labeled TRAPs (3 μg/ml) for 3 h, stained with anti-CD66b-PE antibody, and then analyzed by confocal laser microscopy. Results are the representative of three independent experiments. **p < 0.001 by unpaired t test (F).
from which the TRAPs were isolated. In contrast to TRAPs, the tumor cells lysate failed to increase neutrophil apoptosis (Fig. 3A). While tumor cell culture media significantly triggered neutrophil apoptosis, depletion of TRAPs from the culture media via ultracentrifugation resulted in a significant reduction of neutrophil apoptosis, and the apoptosis-inducing activity was almost exclusively contained in the TRAPs fraction (Fig. S4 and Fig. 3B). Results also revealed that, pretreatment of TRAPs by proteinase K, but not DNase I or RNase A, abolished the apoptosis-inducing activity of TRAPs (Fig. 3C). Furthermore, we found that sonication of TRAPs also impaired their ability to induce neutrophil apoptosis (Fig. 3D). These data indicate that the surface protein component in the intact TRAPs is critical for TRAP-induced neutrophil apoptosis.

Neutrophil apoptosis requires TRAP internalization by macropinocytosis

When neutrophils were co-incubated with CFSE-labeled TRAPs, those that have phagocytized TRAPs underwent increased apoptosis (Fig. 4A), suggesting a requirement of TRAP internalization in the acceleration of neutrophil apoptosis. Four distinct types of pinocytosis have been documented that mediate the internalization of macromolecules and particles into cells: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis.19 To determine which form of pinocytosis is involved in TRAP internalization and induction of apoptosis, neutrophils were pre-treated with the appropriate concentration of a cytoskeletal rearrangement inhibitor cytochalasin D (CD), a clathrin-mediated endocytosis inhibitor chlorpromazine (CPZ) or a caveolae-mediated endocytosis inhibitor filipin III (Figs. S5A–C), followed by incubation with TRAPs. Pre-treatment with CD, but not CPZ or filipin III, significantly inhibited the internalization of TRAPs (Fig. 4B) and concomitantly decreased TRAP-induced apoptosis of neutrophils without affecting their spontaneous apoptosis (Fig. 4C). The results suggest that cytoskeletal rearrangement is particularly important for TRAP internalization into neutrophils. Macropinocytosis is an active cellular process that requires
cytoskeletal rearrangement and organization of intracellular vesicles. To ascertain the involvement of macropinocytosis in TRAP internalization, neutrophils were pre-treated with the appropriate concentration of a macropinocytosis inhibitor 5-(N,N-Dimethyl)-amiloride (EIPA) (Fig. S5D), followed by incubation with TRAPs. Pre-treatment with EIPA significantly inhibited the internalization of TRAPs (Fig. 4D) and concomitantly decreased TRAP-induced apoptosis of neutrophils.

Figure 3. The intact membrane structure of TRAPs is required to induce neutrophil apoptosis. (A) The purified human neutrophils were co-cultured with TRAPs (3 μg/ml) or with an equal amount of tumor cell lysate. The apoptosis of neutrophils was assessed by flow cytometry. (B) Neutrophils were co-cultured with tumor cell culture media, TRAP-depleted tumor cell culture media (TRAP-depl.) or TRAPs, respectively and the apoptosis of neutrophils was assessed by flow cytometry. (C) TRAPs were pre-treated with Proteinase K (55 °C for 2 h), DNase I (37 °C for 1 h) or RNase A (37 °C for 3 h) followed by co-incubation with neutrophils for 6 h. The apoptosis of neutrophils was assessed by flow cytometry. (D) TRAPs were subjected to 20 cycles of sonication (30 sec sonication, 15 sec ice) and then they were co-incubated with neutrophils for 6 h. The apoptosis of neutrophils was assessed by flow cytometry. Results are representative of at least three independent experiments. *p < 0.01 by unpaired t test (A–D).

Figure 4. Neutrophil apoptosis requires TRAP internalization by macropinocytosis. (A) Neutrophils were co-incubated with CFSE-labeled TRAPs (3 μg/ml) for 6 h and stained with Annexin V-Alexa Fluor 647. The percentage of CFSE− neutrophils and Annexin V− neutrophils were analyzed by flow cytometry. (B) Neutrophils were pre-treated with CD (10 μg/ml), CPZ (4 μg/ml) or filipin III (12 μg/ml) for 30 min, and then co-cultured with TRAPs (3 μg/ml) for 3 h. The phagocytizing of TRAPs by neutrophils was detected by flow cytometry. (C) Neutrophils were pre-treated with CD (10 μg/ml), CPZ (4 μg/ml) or filipin III (12 μg/ml) for 30 min, co-incubated with or without TRAPs (10 μg/ml) for 6 h, and then the apoptosis of neutrophils was assessed by flow cytometry. (D) Neutrophils were pre-treated with EIPA (20 μM) for 30 min, and then co-cultured with TRAPs (3 μg/ml) for 3 h. The phagocytizing of TRAPs by neutrophils was analyzed by flow cytometry. (E) Neutrophils were pre-treated with EIPA (20 μM) for 30 min, co-incubated with or without TRAPs (10 μg/ml) for 6 h, and then the apoptosis of neutrophils was assessed by flow cytometry. Results are representative of at least three independent experiments. *p < 0.01 and **p < 0.001 by unpaired t test (C and E).
without affecting their spontaneous apoptosis (Fig. 4E). These results suggest that macropinocytosis mediates TRAP internalization into neutrophils prior to neutrophil apoptosis.

**Macropinocytosed TRAPs trigger ROS production and caspase-3 activation**

After 3 h of incubation of neutrophils with CFSE- or pHrodo-labeled TRAPs, respectively, the majority of TRAPs was found to co-localize with lysosomes (Figs. 5A and B), suggesting the modulations of lysosome function by TRAPs in neutrophil apoptosis. In light of phagolysosomal NADPH oxidase-derived ROS playing an important role in neutrophil apoptosis, we evaluated whether TRAP-induced neutrophil apoptosis was associated with the generation of ROS. Following 30 min of incubation of neutrophils with various concentrations of TRAPs, there was a rapid and robust ROS production in neutrophils (Fig. 5C). When neutrophils were pre-treated with an optimal concentration of the NADPH oxidase inhibitor DPI or the antioxidant NAC (Figs. S5E and F), TRAP-induced apoptosis was significantly attenuated (Fig. 5H).

Figure 5. Macropinocytosed TRAPs trigger ROS production and caspase-3 activation. (A and B) Neutrophils were co-incubated with CFSE-labeled TRAPs (3 μg/ml) or pHrodo-labeled TRAPs (3 μg/ml) for 3 h and stained with Lyso-Tracker Red. Confocal laser microscope system was used to demonstrate the intracellular localization of CFSE-labeled TRAPs. The yellow puncta in cells were quantified, and the yellow puncta/green puncta ratio was calculated. The green puncta that co-localized with lysotracker were counted from at least 20 cells. (C) DCFH-DA was used to detect NADPH oxidase-derived ROS in the cells. Neutrophils were co-incubated with different concentrations of TRAPs (0, 1, 3, 10 or 30 μg/ml) for 30 min and treated with DCFH-DA for 15 min. The intracellular ROS was detected by flow cytometry. (D and E) Neutrophils were pre-treated with DPI (20 μM) or NAC (7.5 mM) for 30 min and co-incubated with or without TRAPs (10 μg/ml) for 30 min. The intracellular ROS (D) and apoptosis (E) of neutrophils were detected by flow cytometry. (F) Neutrophils were pre-treated with CD (10 μg/ml) or EIPA (20 μM) for 30 min and co-incubated with or without TRAPs (10 μg/ml) for 30 min. The intracellular ROS was determined by flow cytometry. (G) Neutrophils were pre-treated with CD (10 μg/ml), EIPA (20 μM) or DPI (20 μM) for 30 min and then co-incubated with TRAPs (10 μg/ml) for 24 h. Caspase-3 activities were analyzed as described in Materials and Methods Section. (H) Neutrophils were pre-treated with zVAD-fmk (50 μM) for 30 min and co-incubated with TRAPs (10 μg/ml) for 6 h. The apoptosis of neutrophils was detected by flow cytometry. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001 by unpaired t test (C–H).
neutrophil ROS production (Fig. 5D) and neutrophil apoptosis (Fig. 5E) were significantly diminished. Consistent with a macropinocytosis-dependent mechanism of TRAPs internalization revealed earlier, pre-treatment of neutrophils with CD or EIPA dramatically inhibited TRAP-induced ROS production (Fig. 5F). Furthermore, pretreatment of neutrophils with CD, EIPA or DPI prior to TRAP incubation resulted in a significant inhibition of caspase-3 activity (Fig. 5G). To further determine whether caspase activity is necessary for TRAP-induced neutrophil apoptosis, the pan-caspase inhibitor zVAD-fmk was used to pre-treat neutrophils. Flow cytometric analysis showed that pre-treatment with zVAD-fmk significantly reduced TRAP-induced neutrophil apoptosis (Fig. 5H). Taken together, these results suggest that macropinocytosed TRAPs trigger NADPH oxidase-mediated ROS production and caspase-3 activation to promote neutrophil apoptosis.

**Discussion**

Autophagy has emerged to play a key role in regulating tumorigenesis, tumor-stroma crosstalk, cancer progression, and cancer therapy. Although it occurs at a low basal level under normal conditions, autophagy is rapidly induced in tumor cells by starvation, hypoxia, stress, and damaging stimuli. In established tumor model, it was demonstrated that autophagy could support tumor cells survival and growth. In addition to their degradative function, autophagy has recently been

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**Figure 6.** TRAP-treated neutrophils suppress T cell proliferation in a cell contact- and ROS-dependent manner. (A and B) CFSE-labeled PBMCs were stimulated in 48 well plate pre-coated with anti-CD3/anti-CD28 and then they were co-cultured with or without neutrophils pre-incubated with TRAPs at the indicated concentrations (0, 3, 10 or 30 μg/ml) at ratio of 1:2 for six days. CFSE dilution was determined by flow cytometry. (C and D) Neutrophils were co-incubated with TRAPs (30 μg/ml) for 30 min. PBMCs were co-incubated with plates pre-coated anti-CD3/anti-CD28 and then they were co-cultured with or without neutrophils at ratio of 1:2 for three days. The percentage of IFN-γ+ T cells was determined by flow cytometry (C) and the IFN-γ in the supernatant was detected by ELISA (D). (E and F) Neutrophils were pre-treated with NAC (7.5 mM) for 30 min and then they were co-incubated with TRAPs (30 μg/ml) for 30 min. CFSE-labeled PBMCs were co-incubated with plates pre-coated anti-CD3/anti-CD28 and then they were co-cultured with or without neutrophils at ratio of 1:2 for six days. CFSE dilution was determined by flow cytometry. (G and H) In the trans-well experiment, neutrophils and CFSE-labeled PBMCs were added in the upper and lower chambers, respectively. Six days later, CFSE dilution was determined by flow cytometry. Results are representative of at least three independent experiments. p < 0.05, **p < 0.01, and ***p < 0.001 by unpaired t test (B–D, F and H).
implicated in various secretory pathways. Triggering of autophagy by starvation remarkably enhances the secretion of a proinflammatory cytokine IL-1β in autophagosome via the unconventional secretory pathway. Secretry autophagy is also triggered in intestinal Paneth cells by bacteria-induced endoplasmic reticulum stress, where the Paneth cells limit bacterial invasion using autophagosomes that carry the anti-microbial molecule lysozyme. We previously demonstrated that, upon stress induction with either lysosomal or proteosomal activity inhibitors, a broad spectrum of defective ribosomal products (DRiPs) are sequestered in autophagosomes and released by tumor cells as DRiPs-containing blebs form (known as Dribbles). Dribbles are carriers of tumor antigens for cross-presentation by dendritic cells (DCs) and stimulate T cell activation, leading to an anti-tumor efficacy in different tumor models. Furthermore, we found that Dribbles can be uptaken by B cells, resulting in B cell activation, antibody secretion, and cytokine production. Natural TRAPs enriched from the supernatant of tumor cells without inhibition of the lysosomal or proteosomal degradation pathway, or collected TRAPs directly from malignant effusions or ascites samples of cancer patients effectively induced B cells to differentiate into IL-10+ Bregs with suppressive activity on T cell activation via the TLR2-MyD88–NF-κB signaling pathway, and the DAMP molecule HMGB1 on the intact TRAP surface plays a critical role in this process. These findings indicate that TRAPs could inhibit anti-tumor immune response by enhancing the generation of regulatory immune cells.

Neutrophils are the most abundant leukocytes in the human circulation, and they are critical effectors in inflammation, tissue injury, and host defense against infection. These cells also infiltrate various tumor tissues and have profound influence on the tumor microenvironment. TANs promote immunosuppression and inflammation in the tumor microenvironment by producing cytokines and chemokines that recruit and activate inflammatory cells. Meanwhile, the factors released from the tumor microenvironment also play an important role in modulating neutrophil function. For example, the exposure of neutrophils to tumor cell culture supernatant, which contains hyaluronan (HA) fragments, causes the neutrophils to acquire pro-tumorigenic functions. Upon priming with breast cancer cell-conditioned supernatants or co-culture with breast cancer cells, neutrophils produced large amounts of oncostatin M (OSM), and neutrophil-derived OSM only induced VEGF expression by cancer cells but also increased their invasive capability. Moreover, tumor-derived exosomes could induce the formation of NETs from neutrophils to promote cancer-associated thrombosis. In this study, we found that TRAPs were rapidly phagocytized by neutrophils, effectively accelerated their apoptosis and endowed them with immunosuppressive properties on the proliferation of CD8+ T and CD4+ T cells, revealing a new mechanism of tumor-neutrophil crosstalk (Fig. 7).

It has been reported that neutrophils undergo apoptosis following phagocytosis of bacteria and viruses, a process known as phagocytosis-induced cell death (PICD) and associated with the generation of ROS by NADPH oxidase. Here, we found that TRAPs were rapidly internalized by neutrophils and effectively accelerated their apoptosis. Mechanistically, macrophagocytosis was responsible for TRAP internalization by neutrophils, and facilitated TRAP-induced apoptosis which was dependent on ROS production and caspase-3 activation. These results are consistent with other studies showing that ROS-dependent caspase-3 activation plays an important role in the apoptosis of neutrophils.

Mounting evidences suggest that TANs play an important role in the progression, metastasis and angiogenesis of cancer. TANs or their myeloid precursors, myeloid-derived suppressor cells (MDSCs), can directly inhibit T cell activities via arginase-1, PD-L1 or ROS. Furthermore, neutrophils can mediate cancer cell metastasis through inhibiting the anti-tumor activity of cytotoxic CD8+ T cells. Consistent with previous reports, our results showed that neutrophils phagocytizing TRAPs could significantly inhibit the proliferation of CD4+ T and CD8+ T cells in a cell contact- and ROS-dependent manner. Interestingly, TRAPs can induce rapid and robust ROS production by neutrophils upon internalization. Therefore, these findings support that TRAPs could be a critical factor in the tumor microenvironment that promotes the development of pro-tumorigenic TANs as well as pro-tumorigenic IL-10+ Bregs, and highlight TRAPs as an important therapeutic target to enhance tumor immunotherapy and reduce tumor-induced immune suppression.

Materials and methods

Patients

Malignant effusions or ascites were obtained from patients with pathologically confirmed lung, ovarian, and breast carcinomas. All experimental protocols were approved by the Ethics Committee for Human Studies of Southeast University (No:
Tumor cell lines and TRAPs preparation and characterization

Human breast (MDA-MB-231) and mouse breast (4T1) carcinoma cells were cultured in complete DMEM (Gibco) medium. The complete DMEM medium were supplemented with 10% heat-inactivated FBS (Gibco), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Beyotime, C0222). TRAPs were harvested from breast carcinoma cell lines as previously described.52 Briefly, culture supernatant was harvested and centrifuged at 5000 rpm to remove dead cells and debris. The supernatant was then centrifuged at 12000 rpm to harvest the TRAPs secreted by tumor cells. A similar method was used to harvest autophagosomes from cancer patients’ malignant effusions or ascites. TRAPs were then stained with anti-LC3 (Novus Biological, NB600-1384AF488) and the purity of TRAPs was analyzed using flow cytometry. The LC3 protein in TRAPs was also conducted by Western blot. The primary antibody was rabbit anti-LC3 (Novus Biological, NB100-2322) and the secondary antibody was HRP-conjugated affinity pure goat anti-rabbit IgG (H+L) (Proteintech, SA00001-2).

Electron microscopy

Briefly, according to routine techniques, the samples were fixed in 100 mM sodium cacodylate, 2.5% glutaraldehyde, 1.6% paraformaldehyde, 0.064% picric acid, 0.1% ruthenium red, post-fixed with osmium tetroxide plus potassium ferrocyanide and embedded in Epon. The pictures were taken on a JEM-1011 transmission electron microscope (TEM).

Isolation and identification of human neutrophils

Human neutrophils were isolated from peripheral venous blood of healthy donors following the standard laboratory procedures.33 The blood was diluted with PBS-0.5% BSA and subjected to Histopaque 1.077 (Sigma, 10771) and Histopaque 1.119 (Sigma, 11191). Briefly, the blood samples were centrifuged at 700 g, 22 °C for 30 min. Then, PBMCs and neutrophils were collected from the plasma-1.077 interface and 1.077-1.119 interface, respectively. After removing RBCs, purified neutrophils were identified using Wright’s staining and flow cytometric analysis. The purity of neutrophils was more than 98%.

Phagocytosis of TRAPs

TRAPs from tumor cells culture supernatant were labeled with CFSE (5 μM; Invitrogen, C1157). Neutrophils were firstly cultured in RPMI-1640 (Gibco) medium containing 10% FBS at a density of 1 × 10⁶ cells/ml, and then co-incubated with different concentrations (0.1, 0.3, 1, 3, 10 or 30 μg/ml) of CFSE-labeled TRAPs for 3 h or with 3 μg/ml CFSE-labeled TRAPs for 0, 0.25, 0.5, 1, 3, 6, 9 or 12 h. The percentage of CFSE⁺ neutrophils was assessed by flow cytometry. In some experiments, neutrophils were co-incubated with 3 μg/ml CFSE-labeled TRAPs at 4 or 37 °C for 3 h. The percentage of CFSE⁺ neutrophils was assessed by flow cytometry. For detection of effective phagocytosis of TRAPs efficiency, confocal microscopy was used to detect neutrophils phagocytizing TRAPs. The cultured neutrophils were co-incubated with CFSE-labeled TRAPs (10 μg/ml) for 3 h, washed with ice-cold PBS, fixed in 4% paraformaldehyde, and then they were stained with anti-CD66b-PE (ebioscience, 12-0666). Cytochalasin D (CD) (Merck Millipore, 250255), chlorpromazine (CPZ) (Sigma, C8138), filipin III (Sigma, F4767) and 5-(N,N-Dimethyl)-amiloride (EIPA) (Sigma, A4562) were used to analyze the mechanisms of neutrophils phagocytosis. For lysosome tracking, the treated neutrophils were stained with Lyso-Tracker Red (Beyotime, C1046) without fixing. The stained cells were visualized under the confocal laser microscope system, and the co-localization of the TRAPs with Lyso-Tracker Red was imaged.

Assessment of neutrophils apoptosis

Neutrophils apoptosis were quantified using Annexin V-FITC (Miltenyi Biotec, 130-092-052) to detect externalized phosphatidylserine and PI (Miltenyi Biotec, 130-092-052) to detect plasma membrane disruption. Briefly, neutrophils (1 × 10⁶ cells/ml in complete medium) were co-incubated with TRAPs (3 μg/ml) for 0, 3, 6, 12 or 24 h, or co-incubated with TRAPs (0, 1, 3, 10 or 30 μg/ml) for 6 h, the apoptotic neutrophils were assessed with Annexin V-FITC and PI in binding buffer for 15 min prior to analysis using flow cytometry. Thirty thousand cells were collected for each group and the data were analyzed using FlowJo 7.6 software. To detect neutrophil phagocytosis and apoptosis synchronously, neutrophils (1 × 10⁶ cells/ml in complete medium) were firstly co-incubated with CFSE-labeled TRAPs (3 μg/ml) for 6 h. Then the apoptotic neutrophils were assessed with Annexin V-Alexa Fluor 647 (Fcmacs, FMSAV647-100) in binding buffer for 15 min prior to analysis using flow cytometry. Thirty thousand cells were collected for each group and the data were analyzed using FlowJo 7.6 software.

Preparation of peritoneal neutrophils

Female BALB/c mice were purchased from the Comparative Medicine Center, Yangzhou University (Jiangsu, China). Six to eight-week-old female mice were lightly anesthetized by inhalation anesthesia. 1 ml of 1% sterile glycerol solution was injected intraperitoneally (i.p.). Four hours after glycerogen inoculation, mice were injected i.p. with murine TRAPs (0, 10, 30 or 100 μg/ml) in 2 ml normal saline for 30 min, and then mice were euthanized and peritoneal lavage samples were collected. The purity of collected samples were assessed with anti-Ly6G-APC (BD Pharmingen, 560599) by flow cytometry. To assess the apoptosis of neutrophils in vivo, four hours after glycerogen inoculation, mice were injected i.p. with murine TRAPs (0, 10, 30 or 100 μg/ml) in 2 ml normal saline for 30 min, and then mice were euthanized and peritoneal lavage samples were collected. The apoptosis of Ly6G-gated cells were assessed with Annexin V-FITC and PI in binding buffer for 15 min prior to analysis using flow cytometry.

ROS production assay

Intracellular ROS were measured by flow cytometry using Reactive Oxygen Species Assay Kit (Beyotime, S033). Briefly,
neutrophils (1 × 10^6 cells/ml in complete medium) were treated with various concentrations of TRAPs (0, 1, 3, 10 or 30 μg/ml) for 30 min and washed with PBS. DCFH-DA was added for 15 min to measure the intracellular ROS. In some experiments, neutrophils were pretreated with Diphenyleneiodonium chloride (DPI) (Sigma, D2926) or N-acetyl-L-cysteine (NAC) (Beyotime, S0077) for 30 min before adding TRAPs for ROS generation.

**Hoechst 33258 staining**

The basis of nuclear morphology changes of apoptotic cells were chromatin condensation and fragmentation. Neutrophils (1 × 10^6 cells/ml in complete medium) were co-incubated with TRAPs (10 μg/ml) for 12 h, fixed in 4% paraformaldehyde at 22 °C for 30 min, and stained with Hoechst 33258 (Beyotime, C1017) for 30 min in the dark. Neutrophils were then examined immediately under the confocal laser microscope system after three times of washing by PBS.

**DNA fragmentation detection**

DNA fragmentation was detected as previously described. The neutrophils DNA was obtained using TaKaRa MiniBEST Universal Genomic DNA Extraction Kit (Takara, 9765). After co-incubation with TRAPs (0, 3, 10 or 30 μg/ml) for 30 h, neutrophils were harvested and lysed with 180 μl buffer GB containing 20 μl Proteinase K and 10 μl RNase A (10 mg/ml) at 56 °C for 10 min. The DNA was precipitated with 200 μl ethanol, dried and resuspended in 20 μl deionized water. Then the samples were separated by 1.8% agarose gel electrophoresis at 50 V for 1.5 h. Separated DNA fragments were stained with ethidium bromide (EB) and viewed under UV light.

**Caspase-3 activity assay**

The activity of caspase-3 was determined using caspase-3 activity kit (Beyotime, C1115). Briefly, after co-incubation with TRAPs (10 μg/ml) for 0, 3, 6, 12 or 24 h, neutrophils were collected and lysed with 100 μl lysis buffer. Lysate were incubated with caspase-3 substrate (Ac-DEVD-pNA) at 37 °C for 2 h. The OD value of each sample was measured at an absorbance of 405 nm. In some experiments, neutrophils were pretreated with CD, EIPA or DPI for 30 min before adding TRAPs for caspase-3 activation.

**T cell proliferation and activation assay**

PBMCs were collected from the plasma-1.077 interface as described above, adjusted to 1 × 10^8 cells/ml in RPMI-1640 containing 100U/ml human IL-2, labeled with CFSE (5 μM; Invitrogen, C1157), and then they were cultured in a 48 well plate that had been pre-coated with anti-CD3 (2 μg/ml; BD Pharmlingen, 550367) and anti-CD28 (2 μg/ml; BD Pharmlingen, 555726). After co-incubation with TRAPs (0, 3, 10 or 30 μg/ml) for 30 min, neutrophils were harvested and co-cultured with CFSE-labeled PBMCs at a ratio of 2:1. Six days later, proliferation of CD4^+ T or CD8^+ T cells was evaluated by flow cytometry. To assess the mechanisms of inhibitory effect of TRAP-incubated neutrophils, NAC was used to suppress ROS generation. In the transwell experiment, neutrophils and CFSE-labeled PBMCs were added in the upper and lower chambers, respectively. Six days later, proliferation of CD4^+ T or CD8^+ T cells was evaluated by flow cytometry. After 3 days coculture, cells were stained with CD4, CD8 and surface markers: anti-CD69-FITC (Biolegend, 310904), anti-CD137-PE (Biolegend, 309804), anti-CD1-FITC (Biolegend, 329904) or anti-CTLA4-PE (Biolegend, 349906). Activation and exhaustion of CD4^+ T or CD8^+ T cells were evaluated by flow cytometry. Intracellular staining for IFN-γ was performed following surface staining, fixation, and permeabilization of the cells with Cell Stimulation Cocktail (ebioscience, 00-4975-03). Intracellular staining for Foxp3 was also performed following surface staining, fixation, and permeabilization of the cells. Stained samples were analyzed on a flow cytometer (BD, FACS Calibur).

**IFN-γ ELISA**

IFN-γ production in the supernatant was measured by an ELISA kit (ebioscience, 88–7316) according to the manufacturers’ recommendations.

**Statistical analysis**

Data were derived from at least three independent experiments and analyzed for statistical significance using SPSS 20.0 software. The mean values ± standard deviation (s.d.) were compared using unpaired Student’s t test for two groups and ANOVA test for more than 2 groups followed by Student-Newman-Keuls test. P-value less than 0.05 was deemed as statistically significant.

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**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Author contributions**

R.G., X.J.Z., N.P. and L.X.W. designed this study. R.G., J.M., Z.F.W., P.Y.Y., and L.X.W. performed the experiments. J.J.Z., M.X., Y.Q.C. and N.P.
provided experimental support. All authors analyzed and discussed the data. R.G. and L.X.W. prepared the Figs. and wrote the manuscript. M.A. and H.M.H. contributed to writing the manuscript.

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