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BCG-induced formation of neutrophil extracellular traps play an important role in bladder cancer treatment

Kangkang Liu[a,1], Erlin Sun[a,*,1], Mingde Lei[a,1], Limin Li[b], Jingda Gao[c], Xuewu Nian[d], Lining Wang[a]

[a] Tianjin Institute of Urology, Department of Urology, The Second Hospital of Tianjin Medical University, Tianjin 300211, PR China
[b] Department of Urology, The 4th People's Hospital of Hengshui, Hengshui 053000, PR China
[c] Department of Urology, Children's Hospital of Hebei Province, Shijiazhuang 050031, PR China
[d] Department of Urology, Tianjin Nankai Hospital, Tianjin 300100, PR China

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ABSTRACT

Bacillus Calmette-Guerin (BCG) is one of the most effective treatments for bladder cancer. Little attention has been paid to the possible role of neutrophils in BCG immunotherapy. In this study, we examined neutrophil extracellular traps (NETs) formation induced by BCG stimulation, and found that BCG-induced NETs exerted cytotoxicity, induced apoptosis and cell-cycle arrest, and inhibited migration in bladder tumor cells. BCG-activated tumor cells but not non-activated ones elicited NETs formation, in which IL-8 and TNF-α from activated tumor cells both took effect. Moreover, NETs activated peripheral blood mononuclear cells (PBMCs) exhibited a higher expression of CD4 and Th1 cytokines. Additionally, the role of NETs *in vivo* contributed to the recruitment of T cells and monocytes-macrophages and tissue damage, thus preventing tumor growth. NETs proteins mainly caused these effects on tumor and cellular immunity. In conclusion, we demonstrated a novel immunoregulatory role for NETs in the early stages of BCG immunotherapy.

1. Introduction

Bladder urothelial carcinoma (BC) is one of the most widespread cancers, with 81, 190 new cases and 17,240 deaths estimated in the USA, 2018 [1]. The majority of newly diagnosed patients suffer non-muscle invasive BC (NMIBC) and 20–25% of patients have muscle invasive BC (MIBC). [2] Although NMIBC can be successfully identified and removed before becoming invasive, BC still has a high rate of recurrence and progression [3]. Bacillus Calmette-Guerin (BCG) was firstly reported in 1976 to be effective in the prevention and treatment of NMIBC [4]. Intravesical BCG has become the most effective immunotherapy for NMIBC, and effectively reduces postoperative recurrence and tumor progression; [5,6] however, the side effects and intolerance associated with BCG limit its application [7]. Therefore, understanding the mechanism of action by which intravesical BCG affects tumors can provide new strategies for immunotherapy.

Recent studies have revealed that neutrophils are required for the efficacy of BCG immunotherapy [8–10]. Mycobacteria induce a strong local immune response that is characterized by a secretion of large amounts of cytokines and an inflammatory cellular infiltration [11]. Polymorphonuclear neutrophils (PMN) are the first line of defense in human innate immunity and the main leukocyte subpopulation to appear in the urethra following treatment with intravesical BCG [12]. BCG-induced neutrophils release IL-8, GRO-α, and MIP-1 to promote monocyte migration and exploit the accessory function of immune cells to attract T cells [8]. In a mouse orthotopic model, PMN deficiency directly affects the therapeutic efficacy of BCG and survival of mice [8]. In addition, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is induced by BCG treatment, and TRAIL is expressed on PMNs in the urine obtained from patients after intravesical BCG instillation [13]. Furthermore, TRAIL has been confirmed to induce apoptosis of tumor cells *in vitro* and *in vivo* [14,15].

In addition to the well-known bactericidal effect, neutrophils can generate extracellular traps (NETs) in response to certain stimuli. It was

Abbreviations: BC, Bladder urothelial carcinoma; NMIBC, Non-muscle invasive bladder cancer; BCG, Bacillus Calmette-Guerin; PMN, Polymorphonuclear neutrophils; NETs, Neutrophils extracellular traps; PMA, phorbol 12-myristate 13-acetate; PBMCs, Peripheral blood mononuclear cells; DC, Dendritic Cells; NE, neutrophil elastase; MPO, myeloperoxidase; TLR, Toll-like receptor; CLSM, Confocal Laser Scanning Microscope; SEM, scanning electron microscopy; FCM, Flow Cytometry

* Corresponding author at: The 2nd Hospital of Tianjin Medical University, No. 23, PingJiang Road, Hexi District, Tianjin 300211, China.
E-mail address: drelsun@163.com (E. Sun).

[1] Kangkang Liu, Erlin Sun and Mingde Lei contributed equally to this work.

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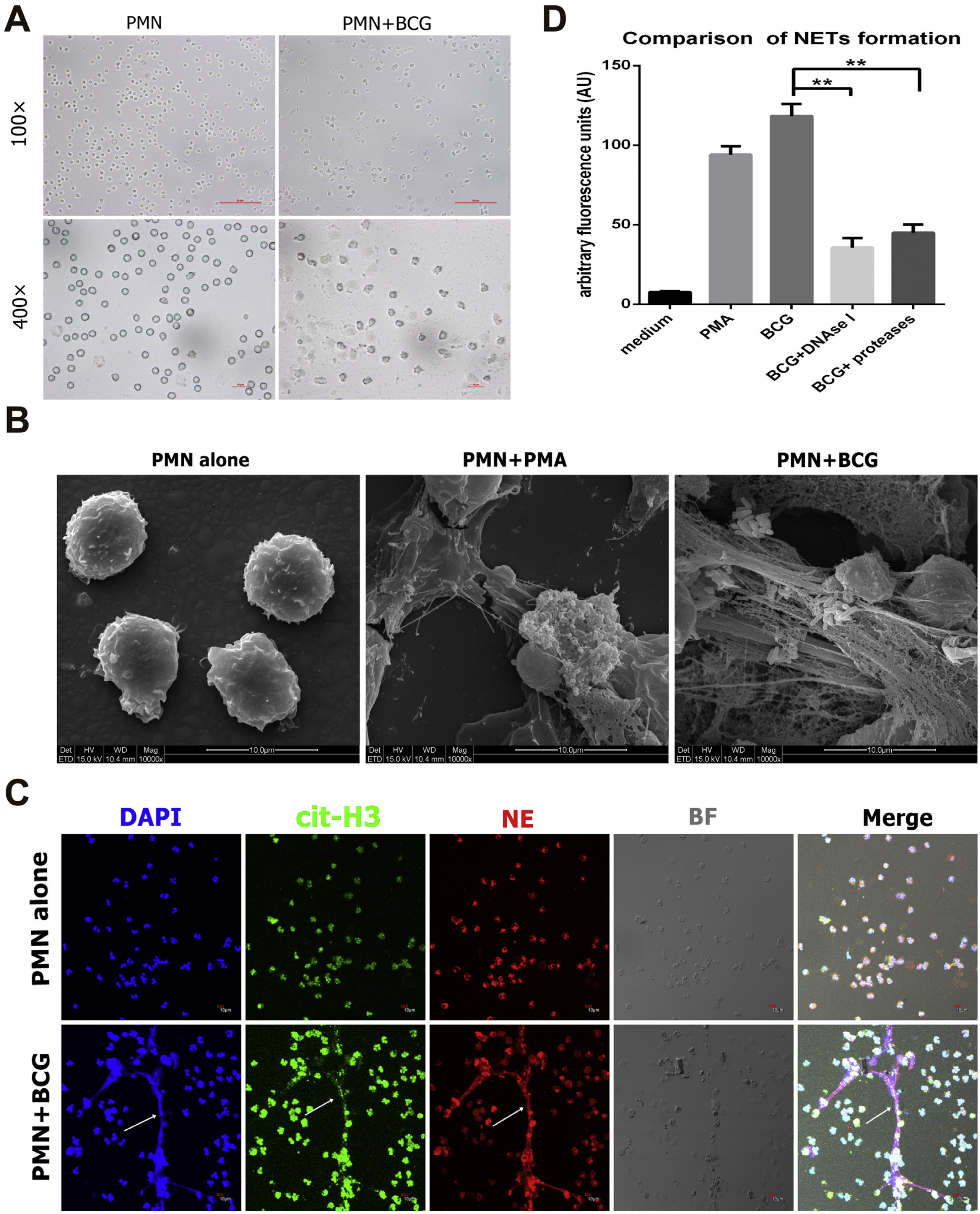

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Fig. 1. BCG induced NETs formation.
A, a significant morphologic change induced by BCG was shown in neutrophils after a 3-h incubation under an optical microscope. Cell membranes became matted, and resembled a burr-like morphology. B, Under SEM, many fibers with different diameters and lengths were produced from BCG-treated neutrophils. The fibers aggregated and nearly covered the entire surface. C, Under CLSM, an extracellular co-localization was displayed of DNA fibers (blue), cit-H3 (green), and NE (red). D, after pretreatment by DNAse I and proteases, NETs formation was inhibited significantly, which demonstrated that DNAs and proteins were important components of NETs ($P < .001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

first reported that activated PMNs could release NETs to kill pathogenic bacteria [16]. Unlike apoptosis or necrosis, NETosis is a kind of cell death process that is triggered by a variety of bacteria, eukaryotic parasites, viruses, and pro-inflammatory factors [17–19]. NETs are involved in the innate immune response, and the excessive release of NETs can perpetuate sterile inflammation, [19] autoimmune activity and pathologic changes. [20–22] Moreover, NETs might injure alveoli, intestinal epithelium, and endothelium. [18,23,24] Some studies have suggested that protein components, particularly histones are responsible for NETs-mediated cytotoxicity. Interestingly, a possible role for NETs released by tumor-associated neutrophils in pediatric Ewing sarcoma has been indicated [25], and microvascular NETs deposition may be involved in cancer progression and metastasis, and systemic infection [26,27]. Neutrophils have been reported to have a role in cancer progression or suppression in a context-specific manner [28]. Thus, we hypothesize that BCG induce NETs that mediate immunoreactions during oncotherapy.

The objective of this study was to confirm NETs formation by neutrophils following BCG stimulation. We verified the role of NETs in in excercising antitumor effects *in vitro* and in a mouse model of bladder cancer. The findings in this study will help us understand the role of neutrophils in the early stages of BCG-related antitumor immunity better.

2. Materials and methods

2.1. Human neutrophil isolation

Neutrophils were isolated from fresh blood of healthy volunteers using the Ficoll-Dextran method [29]. The purity of neutrophils was > 95%, as confirmed using CD11b (561015) and CD66b antibodies (561927, BD Biosciences) on flow cytometry. The viability was > 95% for all preparations, as indicated by trypan blue exclusion. The cell preparations that had become activated during isolation were ruled out by inspecting cell morphology microscopically. All researches involving human samples were approved by the Ethics Committee of the Second Hospital of Tianjin Medical University (No: KY2016K010) and in accordance with the Helsinki declaration. Informed consents were obtained from all individual participants included in our study.

2.2. BCG cultures

The BCG *Connaught* substrain (No: ATCC35733) was obtained from the American Type Culture Collection (Manassas, VA). BCG suspension was cultured in Middlebrock 7H9 broth media (BD 271310), and 7H10 solid media (BD 262710, Difco Laboratories USA) for colony growth. The number of colony-forming units (CFUs) was routinely determined by plating and incubation on solid medium at 37 °C for 4 weeks. Viable BCG selected from the logarithmic growth phase were used for experiments.

2.3. Induction of NETs formation

Freshly isolated PMNs (5×10^5/mL) were gently added into culture plates on anti-peeling coverslips (Sigma-Aldrich) and cultured in complete RPMI supplemented with 2% FCS (Gibco BRL) at 37 °C in 5% CO_2 for 2 h. PMNs were stimulated with 50 nM PMA (Sigma-Aldrich), BCG (MOI = 10), or left unstimulated respectively. As a positive control, neutrophils stimulated with 50 nM PMA (79346-1MG, Sigma-Aldrich) formed typical NETs [16].

2.4. Visualization of NETs by confocal laser scanning microscope (CLSM) and scanning electron microscopy (SEM)

NETs specimens were gently fixed with 4% paraformaldehyde (P8430, Solarbio) and NETs-forming neutrophils were determined by staining DNA with DAPI (D9542-1MG, Sigma). To evaluate NETs composition, immuno-fluorescence assays were applied with primary antibodies against citrullinated Histone H3 (cit-H3) (ab219407) and NE (ab14188) or isotype IgG (ab27478), then observed by CLSM (FV1000, Olympus, Japan), as previously described [17,30]. In addition, the samples were fixed in 2.5% glutaraldehyde then 1% osmium tetroxide/1% tannic acid. After dehydration with an ethanol series and critical-point drying, the specimens were coated with platinum and visualized by SEM (Hitachi, CA).

2.5. Quantification of NETs

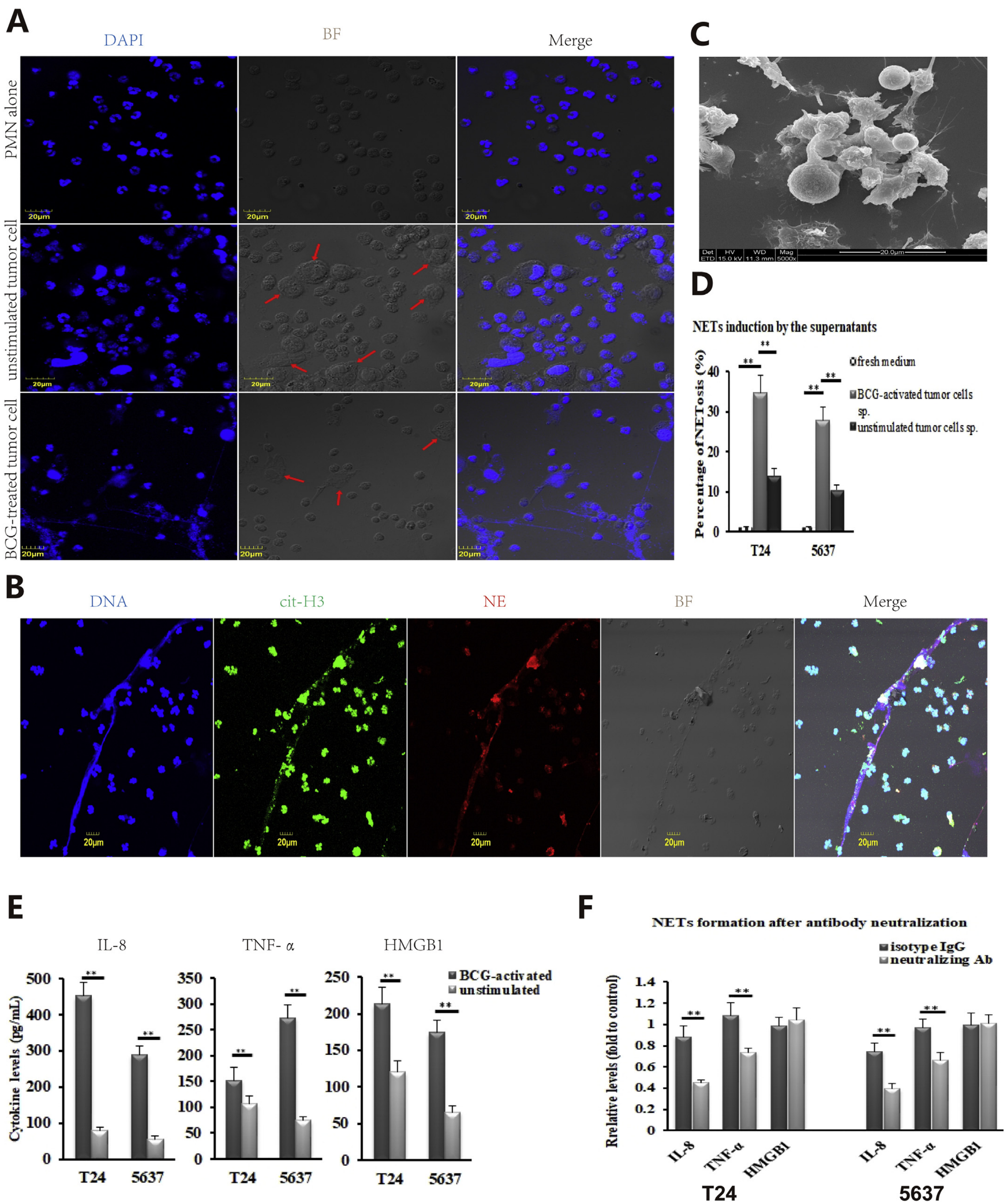
NETs scaffolds in coculture were digested with 250 mU/mL of micrococcal nuclease (LS004797, Worthington Biochemical Corp.) and then stained with 2.5 μM Sytox Orange (S34861, Molecular Probes) for 10 min at room temperature. Quantification was measured using a fluorometer (Synergy H1 Hybrid Reader, BioTek) every min for up to 300 min.

2.6. Preparation of cell-free NETs and quantification of DNA and protein

Neutrophils (1×10^5/mL) were seeded in 6-well culture plates with 2 mL of phenol red–free RPMI-1640 (11835030, Invitrogen). Following stimulation with BCG (MOI = 10) for 4 h, the medium was removed and cells were gently washed. After addition of 1 mL RPMI (phenol red–free) to the adherent film and vigorous agitation followed by centrifugation at 220 $\times g$ for 5 min, the supernatant was collected, as reported [23]. DNA and protein were quantified using Picogreen dsDNA kit (P11495, Invitrogen) and Micro-BCA protein assay kit (PI23235, Pierce Biotechnology) respectively, according to the instructions. Additional micrococcal nuclease digestion and centrifugation at 2200 $\times g$ for 10 min were required for obtaining cell-free NETs. Less than 3 cells/HPF and a BCG count < 5 CFU/mL, as detected by microscopy and plate culture, respectively, was necessary to exclude residual effects in the following tests.

2.7. Co-culture of BCG-stimulated cancer cells with neutrophils

The human urinary BC lines, T24 (ATCC HTB-4) and 5637 (ATCC HTB9), were adopted. Cells were cultured in cc RPMI-1640 (21875091, Invitrogen) supplemented with 10% FCS at 37 °C in a humidified atmosphere and 5% CO_2. T24 and 5637 cells at 70% -80% confluence were stimulated with BCG (MOI = 10) for 6 h. The supernatants were collected by centrifugation at 6000 $\times g$ for 10 min and used for the stimulation experiments and cytokine detection. Neutrophils (1×10^5 cells/mL) were incubated for 3 h, in 1 mL of the prepared supernatants or fresh medium. Intact neutrophils (1×10^5 cells/mL) incubated with BCG-treated or untreated cancer cells (1×10^4 cells/mL) were washed repeatedly to remove all traces of agents and NETs formation was observed.



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2.8. Measurement of viability, cell cycle, apoptosis, migration and invasion of tumor cells

The BC cell lines, T24 (ATCC HTB-4) and 5637 (ATCC HTB9), seeded at a density of 1×10^5 cells/mL to reach 90% confluency, were treated with different amounts of NETs (0.5, 1, and 2 ×), and the supernatant with no NETs served as a negative control. The morphologies were noted and the following tests were performed.

Fig. 2. NETs formation was induced by BCG-activated tumor cells.
A, as shown by CLSM after DAPI staining, NETs formation was abundant in the neutrophils co-cultured with tumor cells pre-activated by BCG, but limited in the ones co-cultured with non-stimulated tumor cells (red arrows indicated tumor cells). B, representative images showed that the supernatants of BCG-activated tumor cells induced NETs that contain chromatin and granule proteins, as confirmed by extracellular multi-fluorescence co-localization of DNA, cit-H3, and NE. C, the supernatants of BCG-activated tumor cells induced neutrophil morphologic changes to liberation of fibrous material, that was fine and sparse, resembling the material induced by PMA (reference to Fig. 1B). D, the supernatants of BCG-activated tumor cells induced more NETs than the non-activated ones did. E, the levels of IL-8, TNF-α and HMGB1 were determined with ELISA, and shown to be markedly increased in the supernatants of tumor cells stimulated by BCG compared with those in the non-stimulated supernatants. F, administration of anti-IL-8 and anti-TNF-*α* antibodies in the supernatants of BCG-activated tumor cells significantly reduced NETs formation, with non-stimulated neutrophils served as the control. $^{*}P < .05$, $^{**}P < .01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

After NETs treatment, 100 μL of CCK-8 (CK04, Dojindo) was added to the cell suspension with media containing only NETs as the blank. Following incubation, absorbance at 450 nm was determined using a microplate reader (Multiskan EX, Thermo, USA). The growth inhibition rate (%) was calculated as follows: $(1 - \text{Abs}_{test}/\text{Abs}_{con}) \times 100\%$.

Cancer cells were treated with NETs, 1% Triton X-100 (high control) or untreated (low control). LDH release was assessed using a cytotoxicity detection kit (11,644,793,001, Roche Applied Science) according to the manufacturer's instructions and represented as the average absorbance at 490 nm. Cytotoxicity (%) was calculated as follows: (NETs − low control)/(high control − low control) × 100%.

Quantification of apoptosis and the cell cycle was performed on a FACSCalibur Flow Cytometer (Becton Dickinson, USA), as described [31]. NETs-treated and untreated cancer cells were harvested, treated with 20 μg/mL of RNase A and 50 μg/mL of PI (R4642-10MG, 81845-25MG, Sigma-Aldrich) after fixation with 70% ethanol, or stained with FITC-Annexin V and PI (556547, BD Pharmingen), then analyzed immediately.

For the scratch wound healing assay, cancer cells (5×10^4 cell/mL) were incubated overnight. Migration was calculated according to the measurements at time 0 and 8 h after making a scratch and adding 0.5 × NETs. In addition, 24-well Transwell chambers (cat# 3422, Corning) with 8 μm-pore insets were used. Cells (2×10^5 cell) in serum-free media were planted on the inset for 6 h incubation in the migration assay, and plated on the inset coated with Matrigel (354234, BD Biosciences) for 48 h incubation in the invasion assay. RPMI-1640 containing 0.5 × NETs was added on to the inset and media containing 10% FCS was in the lower compartment as a chemoattractant. Cells invading the lower surface of the filter were stained with 0.1% crystal violet, and then counted in 5 random fields. The stained crystal violet was extracted using 33% acetic acid to obtain the absorbance at 570 nm.

2.9. CCK-8 and FCM analysis of NETs-induced PBMC

Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated from a Ficoll-Paque density gradient (17-5442-02, GE Healthcare). PBMCs (1×10^5) were stimulated with cell-free NETs (0.5, 1, and 2 ×), in 96-well plates. The media served as a negative control. After 24 h, 48 h and 72 h of culture, supernatants were used to measure cytokine levels and cells were analyzed by CCK-8 for survival.

The cells were stained using FITC-labeled anti-CD4 (130-092-358, Miltenyi Biotech) and PerCP-Cy5.5- labeled anti-CD8 antibodies (565310, BD Biosciences), then analyzed on FCM and processed using FlowJo 7.6.4. The isotype IgG served as the control.

2.10. Analysis of cytokine levels with ELISA

Cytokine levels of IL-8 (D8000C, R&D Systems), TNF-α (BMS223HS, eBioscience) and HMGB1 (ST51011, IBL International GmbH) in supernatants of BCG-treated cancer cells and IFN-γ (cat# 430107, BioLegend), TNF-*α*, IL-12 and IL-2 (BMS223HS, BMS238TEN, BMS221HS, eBioscience) in supernatants of NETs-treated PBMCs, were measured using the corresponding human ELISA kits, according to the manufacturers' instructions.

2.11. Mouse tumor models treated by BCG-induced NETs

C57BL/6 mice (6–7-weekold) were purchased from HFK Bioscience Co., Ltd. (Beijing, China). All animal experiments were approved by the institute Ethics Committee. Murine bladder tumor cells (MB49) were obtained from M.A. O'Donnell (University of Iowa, IA). The mice were inoculated subcutaneously with 10^6 syngeneic MB49 cells to evaluate the role of BCG-induced NETs *in vivo*.

Mouse marrow was taken for isolating neutrophils by centrifugation on a Ficoll-Hypaque density gradient. Neutrophils were purified using a mouse Neutrophil Isolation Kit (130-097-658, Miltenyi Biotec) for preparation of cell-free BCG-induced NETs. On day 10 following tumor implantation, the mice with tumors of similar size were treated intratumorally every 3 day with NETs, DNase- or heat-treated NETs, or medium as a control, for 4 times with 8 mice in each group. The mice were accessed for tumor weight, survival, and terminal morbidity. Tumor volume was calculated according to the following formula: major axis × minor axis^2 × 0.52.

NETs-treated mice were sacrificed on day 24 following tumor implantation, and the tumors were resected for pathologic observation after hematoxylin and eosin (H&E) staining. For immunochemistry (IHC), sections of paraffin-embedded samples were incubated with primary antibodies against mouse CD3 (555,273, BD Biosciences, Germany) or CD14 (ab182032, Abcam), then with peroxidase-conjugated secondary antibodies to quantify T cells or mononuclear cells. The number of infiltrating immune cells, expressed as the average number/high-power field (HPF), was determined based on 10 randomly fields at ×400 magnification.

TUNEL assays were conducted with the *in Situ* Cell Death Detection Kit (cat# 11684817910, Roche Applied Science), according to the manufacturer's protocol. Slides were counterstained with hematoxylin to identify the nuclei.

2.12. Statistical analysis

SPSS.20.0 software was used for statistical analysis and graphing. Unless otherwise stated, all data are presented as the mean ± SD of at least three independent experiments. A two-tailed Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparison test was used, as indicated. A $P < .05$ was considered statistically significant.

3. Results

3.1. BCG induces NETs formation, which mainly consist of DNA and proteins

After stimulation by BCG for 2 h, the contour of neutrophils changed. Specifically, the cell membranes were shown to be matted under an optical microscope (Fig. 1A). The formation of NETs was confirmed by staining and visualized by CLSM and SEM. Under SEM, BCG-stimulated neutrophils were shown to be firmly attached to the slide and aggregated. In addition, the cellular morphology was characterized by flattening of the plasma membrane with extracellular extrusion of fibrillar material, which was consistent with the cellular morphology following PMA stimulation (Fig. 1B). CLSM demonstrated

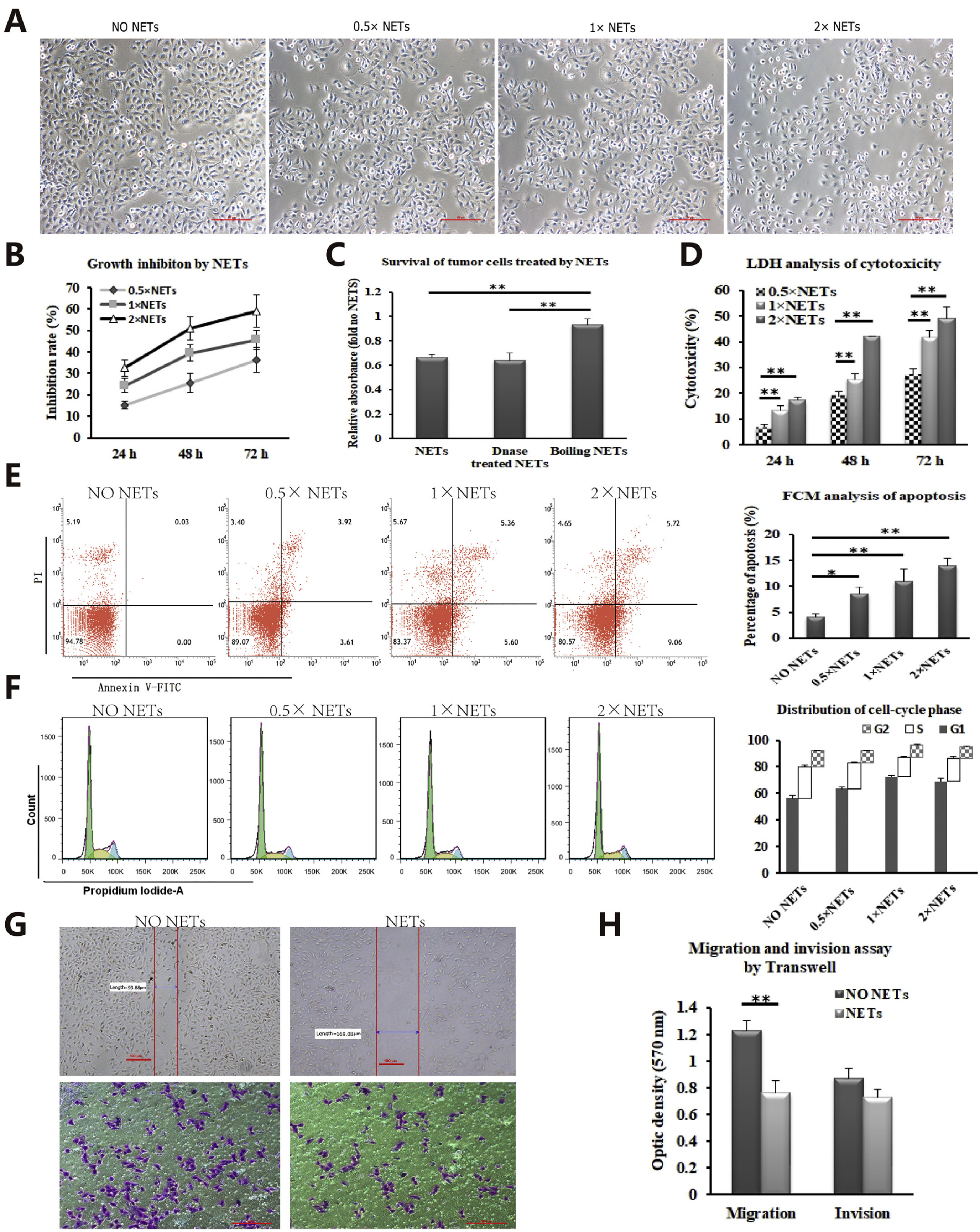

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Fig. 3. NETs prevented the viability of BC cell lines.
A, representative images of tumor cells (T24) treated with NETs for 24 h showed a dose-dependent influence on morphology. B, inhibition ratios of T24 cells, which were determined by CCK-8, increased with time and dose of NETs. C, at 48 h of incubation, the extent of growth did not decrease after boiled-NETs treatment, but significantly decreased after DNase-digested NETs treatment, with NETs one as the control. D, NETs exerted a cytotoxic effect on tumor cells, and the effect was dose- and time-dependent in the range used here. E, after treatment with 1 × and 2 × NETs for 48 h, the percentages of annexin V positive tumor cells increased compared with that of no NETs. F, representative images showed that the percentage of cells in the G0/G1 phase increased after a 48 h incubation, suggesting a contribution to the growth inhibitory effects of NETs (*denote comparision with no-NETs). G, migration assay with plates and transwell chambers both indicated that NETs reduced migration. H, the results of migration and invasion assays, detected by optical density of stained crystal violet extracted from the cells invading the lower surface of the transwell filter, showed an obvious reduction in migration, and the results of T24 invasion were not significantly different. Scale bar = 100 μm; *$P < .05$, **$P < .01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

extracellular fluorescence of NE and cit-H3 superimposed with DAPI, indicating co-localization, therefore confirming the presence of NETs (Fig. 1C). Following DNAse I or protease treatment, neutrophils could not produce NETs in response to BCG (Fig. 1D). Taken together, we concluded that BCG induced neutrophils to form NETs, which were mostly consisted of DNA and proteins.

3.2. BCG-activated tumor cells also elicit NETs formation, in which IL-8 and TNF-α from activated tumor cells both have an effect

An increase of extracellular fluorescence in co-cultures of BCG-activated cancer cells with neutrophils suggested that NETosis was triggered (Fig. 2A). The major components of the extracellular lattices were chromatin and granule protein, as demonstrated by staining DNA, histones and NE (Fig. 2B). SEM revealed that dispersed filaments, characterized the morphology of NETs following supernatant stimulation, which was consistent with that from PMA stimulation (Fig. 2C). In addition, an abundance of NETs appeared in neutrophils activated by the supernatants from BCG-treated tumor cells, but few existed in neutrophils activated by the untreated ones (Fig. 2D). We then showed that cytokines in the supernatants of BCG-stimulated bladder cancer cells, including IL-8, TNF-α, and HMGB1, were significantly increased compared to cytokines from unstimulated cells ($P < .05$; Fig. 2E). The corresponding antibodies were used to neutralize these cytokines. Inhibition of IL-8 and TNF-α, but not HMGB1, markedly diminished NETs formation ($P < .05$; Fig. 2F).

3.3. NETs inhibit bladder cancer lines by preventing viability, the cell cycle and migration, as well as inducing apoptosis

To analyze the effects of NETs on bladder tumor alone, *in vitro* prepared cell-free NETs were used in the following experiments. NETs induced significant cell floating and shrinking in a dose-dependent fashion (Fig. 3A). As detected by CCK-8, inhibition of cell proliferation by NETs treatment was dose- and time-dependent ($P = .001$; Fig. 3B). Moreover, the inhibition with NETs was largely abolished by protein inactivation, but not by DNA digestion (Fig. 3C). Additionally, BCG-NETs induced time- and dose-dependent cytotoxicity on tumor cells ($P < .05$and $P < .001$ respectively; Fig. 3D). In addition, NETs exposure dose-dependently induced apoptosis and G0/G1 phase arrest in BC cell (Fig. 3E–F), which partly accounted for the growth inhibitory effects of NETs. Similar results were observed in the other cell line used; representative data from the T24 cell lines were shown. Given the dose-dependent cytotoxicity and growth inhibition, we used NETs at a low dose (0.5 ×) to analyze migration and invasion, and found an obvious reduction in migration through filters and on plates ($P < .01$; Fig. 3G–H), but no obvious decrease in invasion of T24 cells ($P > .05$; Fig. 3H).

3.4. BCG-induced NETs are potent activators of PBMCs, in which NETs' protein components play a key role

To determine whether cellular immunity was induced, we exposed PBMCs to NETs prepared from the same donor neutrophils. NETs induced IFN-γ, IL-2, and TNF-α in a negative dose-dependent manner ($P < .05$; Fig. 4A), achieving a maximum effect using 0.5 × NETs and minimum induction or even inhibition with 2 × NETs. The changes in IL-12 were not statistically significant. Furthermore, heat-inactivation remarkably decreased the responses to near the level of no stimulation ($P < .01$), and DNase reduced part of the responses (Fig. 4B). Similar to the secretion profiles, NETs showed a positive effect on PBMC survival at a low dose, but inhibition at a high dose (Fig. 4C). Additionally, NETs at a high dose induced apoptosis and death of PBMCs ($P < .05$; Fig. 4D).

Unlike the above responses, different doses of NETs promoted CD4 expression in co-cultured PBMCs ($P < .001$), with no significant difference between high and low NETs (Fig. 4E). However, CD8 expression and the percentage of $CD8^+$ cells were not significantly upregulated by 0.5 × NETs ($P > .05$; Fig. 4F).

3.5. BCG-induced NETs inhibit tumors in mice by attacking tumor cells and increasing immunocyte infiltration, and the effects are mainly mediated by NETs proteins

Prepared NETs were applied to preliminary analyze the NETs only effect on tumors. Subcutaneous tumors rather than an orthotopic model were suitable for administration of NETs. All tumor-bearing mice survived with metastases until the mice were sacrifice. The average tumor weight and volume were significantly lower in NETs-administered mice than the shams ($P < .01$; Fig. 5A–C). Importantly, the effect of NETs was nearly eliminated by inactivation of proteins, indicating that the protein components were mostly responsible for the inhibition (Fig. 5B). Moreover, NETs administration resulted in increased necrosis, and significant apoptosis or death of tumor cells (Fig. 5D–E). NETs strongly increased intra-tumor $CD3^+$ and $CD14^+$ infiltration ($P < .001$), which was reduced by boiling but not DNase pre-treatment of NETs (Fig. 5F–G). Together these results suggest a therapeutic effect of NETs on bladder cancer by direct damage and induction of the cellular immune cascade.

4. Discussion

Recent studies suggested that NETs might lead to tissue damage, [18,21] activation of lymphocytes [20], dendritic cells (DC) and macrophages [22], as well as autoimmune [19] and chronic inflammatory diseases [23,24]. Additionally, several studies have demonstrated that cancers predispose neutrophils to release NETs, [27,32] which are involved in tumor-associated thrombosis, then possibly contribute to tumor progression and metastases by degrading the extracellular matrix [25,33,34]. In addition, the cytotoxicity of NETs on endothelial, epithelial, and neuronal cells has been reported [19,23]. After NETs treatment, tumor cell proliferation was significantly inhibited in the current study, and a similar observation was noted with respect to cytotoxicity. Both BC cell lines exhibited shrinkage and a decline in adherent cell numbers after NETs treatment, suggesting a non-specific mechanism underlying cell damage. Also, BCG-induced NETs promoted G0/G1 phase arrest and apoptosis of tumor cells in a dose- and time-dependent fashion. In addition, our study confirmed that BCG-induced NETs inhibited mouse tumor growth. More tissue destruction was observed in NETs-treated tumors than the sham treatment. Therefore, we

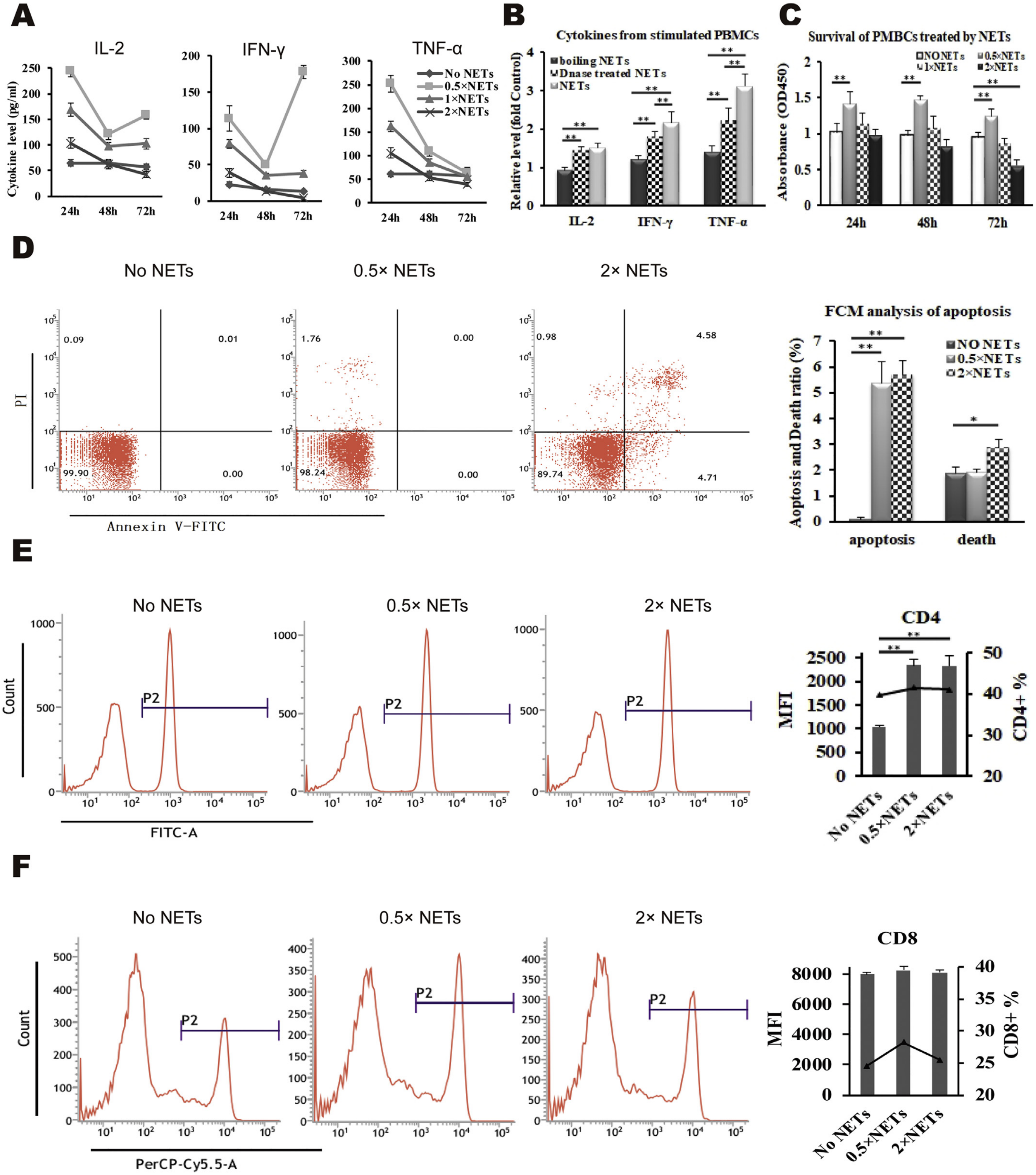

Fig. 4. NETs influenced PBMCs proliferation and activation *in vitro*.

A, in contrast to the control (no NETs), the production of IFN-γ, IL-12, and TNF-*α* were up-regulated in PBMCs exposed to 0.5 × and 1 × NETs. Interestingly, 2 × NETs increased the levels of IFN-γ, IL-2 and TNF-*α* at 24 h, but inhibited the levels of them after 24 or 48 h. Thus, increased Th1-type cytokines secretion from PBMCs showed a profound effect with 0.5 × NETs, and a mild to moderate increase or even inhibition at higher doses, compared with the control. B, DNase-digestion somewhat reduced NETs-induced secretion; however, heat-inactivation remarkably decreased the responses to nearly the levels of no induction. C, 1×10^5 PBMCs significantly proliferated after stimulation with lower doses of NETs (0.5× and 1×), whereas no increase or even an obvious inhibition was observed when treated with a high dose (2×) of NETs. Thus, the proliferation percentage was negatively dependent on NETs dose, and declined with time. D, the representative results of FCM analysis indicated that the induced apoptosis and death of PBMCs was significantly higher in 2× NETs after a 24 h incubation. E, overnight NETs upregulated the mean fluorescence intensity (MFI [column]) of the markers CD4, but not the percentage of CD4+ cells (% [line]). F, the marker on cytotoxic lymphocytes, CD8 was non-significantly upregulated in MFI and the percentage of positive cells treated with 0.5 × NETs. $^*P < .05$, $^{**}P < .01$.

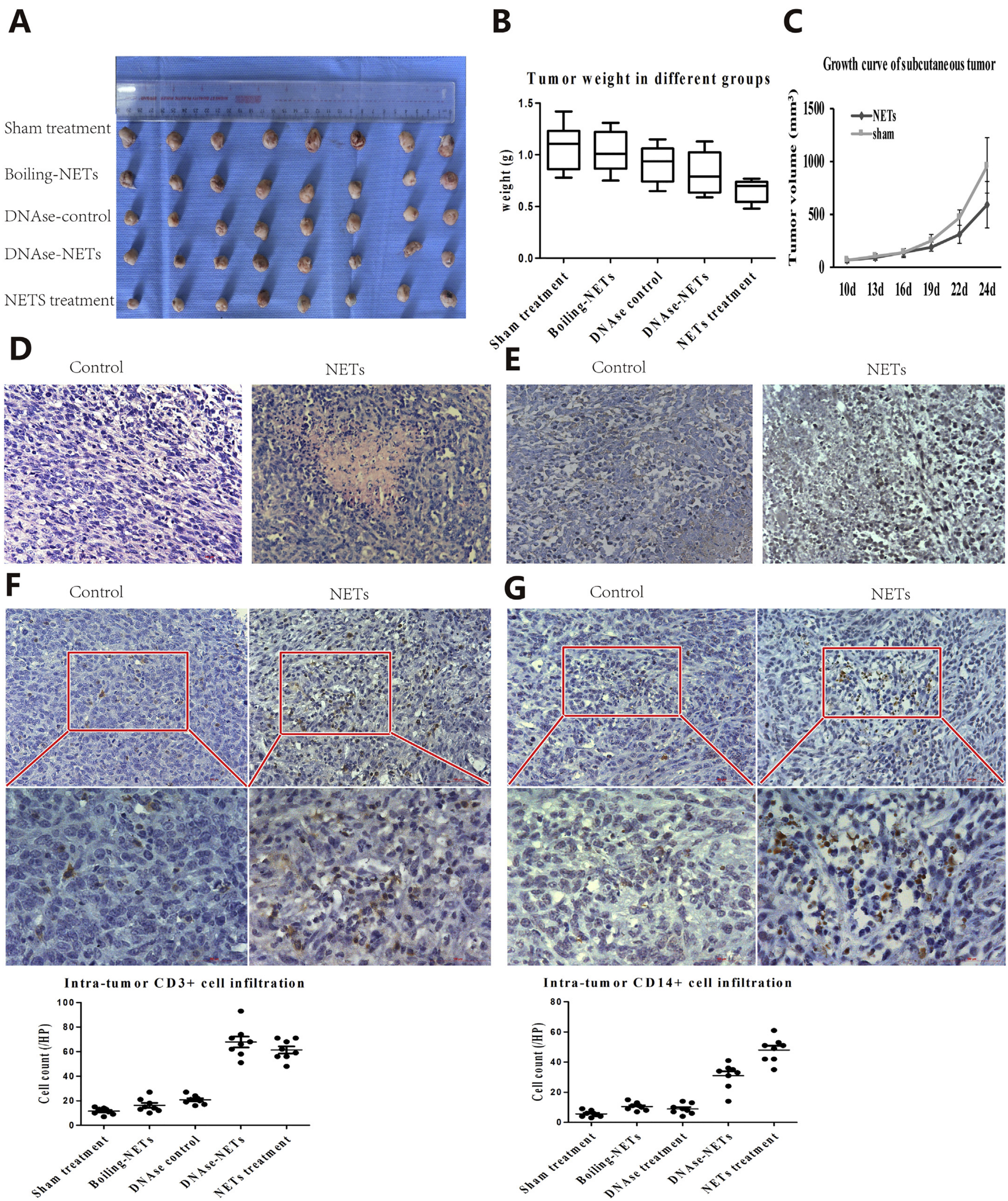

Fig. 5. Antitumor effects of administration of BCG-induced NETs.
A & B, comparison of differently treated subcutaneous tumors showed that the weights were significantly reduced in NETs and DNase-pretreated NETs group, but non-significantly in the boiling-inactivation group (* denote comparision to sham). C, the growth-time curve showed that tumor volume in the NETs group and the sham group increased with time. D, after administration of NETs, necrosis was evident with population by leukocytes. E, *in situ* cell death detection of the tumor tissue showed increased apoptosis or death cell in the NETs group than the sham group. F, after IHC staining, infiltrating inflammatory cells were observed in the tumor. CD3+ counting was higher in the NETs group than the sham or boiling-NETs group. G, the counting results of CD14+ were also higher in the NETs group; however, the increase was not apparent between the boiling-treated NETs group and the sham one. $^*P < .05$, $^{**}P < .01$.

concluded that BCG-induced NETs inhibited tumors *via* cytotoxicity, induction of cell-cycle arrest and apoptosis.

In the current study, BCG-activated tumor cells induced more NETs than non-activated ones. Stimulation with the supernatant of activated cells significantly increased neutrophil adhesion and NETs release, indicating an important role for cytokines. Certain cytokines have already been identified from epithelium or malignant cells treated by BCG [8,35]. NETs also formed in response to proinflammatory stimuli [29,36,37], including HMGB1, IL-8, and TNF-α that significantly increased after BCG stimulation in our study. IL-8 is a potent inducer of NETs, which is the strongest chemokine for PMNs, and involved in NETs formation mediated by activated endothelial cells [18,29]. HMGB1, a danger-associated molecular pattern mediator released from dying and activated cells, mediates neutrophil recruitment, reportedly promotes some proinflammatory cytokines [38] and induces NETs [21,37]. TNF-α, an important cytokine associated with tumors, is also an inducer of NETs [39,40]. This study demonstrated that IL-8 and TNF-α mediated NETs induction by bladder cancer cell, at least in part, following BCG stimulation.

A murine model of systemic infection demonstrated that microvascular NETs deposition and consequent trapping of systematically administered circulating lung carcinoma cells, increased metastasis [33]. It cannot be overlooked, however, that systemic inflammation induces a multitude of effects on numerous cell types, which may contribute to tumor adhesion and metastasis. In this study, we directly used cell- and bacteria -free NETs to rule out the roles of activated neutrophils and other active components. Addition of NETs resulted in an inhibition of migration and invasion of tumor cells.

CD4 is mainly expressed on the surface of Th cells. CD4+ T cells are of central importance in BCG therapy, [8] based on IFN-γ production or activation of CD8+ T and NK cells [11]. Neutrophils are essential for T-cell trafficking to the bladder after BCG perfusion [8]. Furthermore, it has been reported that NETs primed T cells, [20] function as remarkable DC activators and type I interferon inducers [21]. PBMCs mainly consist of T cells, DCs, and monocytes, and the latter influenced NETs-mediated T cell activation [20]. To confirm that NETs, but not neutrophils, mediated this cellular immunity, cell-free NETs were used. We found that lower doses but not high doses of NETs led to proliferation of PBMCs and Th1 cytokine secretion. The negative correlation might be the result of activation and accompanying cytotoxicity of NETs [41]. Also, we observed that NETs treatment increased CD4 expression *in vitro*, and CD3+ and CD14+ cells in tumors, indicating immune up-regulation. The local presence of Th1 cells, monocytes, and cytotoxic T-lymphocytes (CTLs) is generally associated with tumor regression and a favorable prognosis.

Previous study has shown that the toll-like receptor (TLR) 2 ligand, such as HP-NAP could inhibit the growth of BC by activating a cytotoxic Th1 response [42]. Therefore, combined with the results of our study, we suggested that NETs might play a role in tumor progression by activating PBMCs, Th1 cytokine secretion, and CD4 expression. Nevertheless, the activation cannot compensate for the inhibition of survival by high dose of NETs. Given that the density of neutrophils that accumulated in tissues cannot reach the levels in blood, the NETs in BCG-administrated tissues possibly mediated an anti-tumor effect by inhibiting tumors and activating the immune cascade. Our findings represented a novel mechanism of how NETs contribute to anti-tumor immunity.

Notably, neutrophils have either pro- or anti-tumor activity, depending on factors, such as cancer type and cytokine profile in the microenvironment [9,30]. It has been demonstrated that efficient release of myeloperoxidase (MPO) and NE, which are important components of NETs, is important for the antitumor effect of neutrophils. At high concentrations, these factors are cytotoxic to tumor cells, but reducing the release might result in the conversion of neutrophil function from anti-tumor to pro-tumor [43,44]. Thus, we postulated that NETs might play different predominant roles according to the differences of stimulators, neutrophil sources, component patterns and action sites, such as neutrophils from tumors or the circulation, formation in vessels or tissues, and induction with BCG or others. Clarification of these roles await further exploration.

The protein components are mainly responsible for tumor inhibition and cellular immunity. In the current study, the inhibition phenotype on cancer cells was reversed by disrupting NETs with boiling, but not DNase, indicating that NETs proteins were involved in cytotoxicity. NETs contained different proteins [17], however, investigation of all the proteins involved in NETs-mediated cytotoxicity was beyond the scope of this study. The damaging effect of NETs has been attributed, at least in part, to histones, MPO and NE [23,45,46]. Although endothelial cell damage during extended co-culture with neutrophils was prevented by destroying the DNA backbone of NETs, [18] and DNase therapy has an improvement in airflow obstruction and respiratory exacerbations in partial cystic fibrosis patients, but digestion of DNA was not sufficient to abolish NETs cytotoxicity. As expected, denaturing NETs proteins reduced the cytokine promotion, while degradation of DNA reduced cytokine promotion in part. The possible immune effects of residual CpG or the nucleic acid protected by conjunct proteins after DNase-digestion cannot be ruled out. It has been reported that NETs activated DCs through Toll-like receptor 9 (TLR9), an intracellular receptor recognizing DNA, [47] but the role of TLR9 in NETs-mediated T cell priming was not demonstrated [20]. Together, the effects of inhibiting tumor cells and stimulating immunity are mainly dependent on NETs proteins.

5. Conclusion

In current study, we first corroborated that BCG activation induced neutrophils to form NETs. Then, BCG-induced NETs exerted cytotoxicity, induced apoptosis and cell-cycle arrest, and inhibited migration in bladder tumor cells. Moreover, NETs activated PBMCs, as indicated by the higher expression of CD4 and Th1 cytokines. Our observations suggested the complex *in vivo* effects, including direct effects of NETs and indirect effects from NETs-stimulated immunity. NETs contributed to recruitment of T cells and monocytes-macrophages, and tissue damage, thus preventing tumor growth. Further experiments are warranted to clarify the mechanisms and examine the roles of NETs in pathologic responses.

Author contributions

K Liu, E Sun and M Lei were responsible for the coordination of the project and contributed to study design. The writing team consisted of K Liu, M Lei and E Sun. L Li, J Gao, X Nian, and L Wang performed experiments and data analysis. All authors read and approved the final manuscript.

Declarations of interest

None.

Conflict of interest

The authors declare no potential conflicts of interest.

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