BCG induced neutrophil extracellular traps formation and its regulatory mechanism

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Abstract

Background Intravesical BCG is one of the most effective immunotherapies for bladder cancer. Our previous study showed that BCG could induce the formation of neutrophil extracellular traps (NETs), which play an important role in bladder tumor treatment. To identify how BCG induced NETs formation, in this study, we examined NETs formation induced by BCG in vitro and in mouse model, then analyzed the effects of NETs on BCG, and the relevant regulatory mechanism.

Methods The formation of NETs was visualized by Confocal Laser Scanning Microscope (CLSM) and Scanning Electron Microscopy (SEM). NETs quantitation was evaluated by the strength of extracellular DNA. ROS and NETs formation were examined by co-culturing with the inhibitors of ROS, NADPH oxidase and relevant pathways respectively. FITC-labeled BCG was used to observe the capturing by NETs. Finally, NETs formation was observed in mouse urine and subcutaneous tumor after BCG perfusion.

Results BCG time-dependently induced in vitro NETs formation, as well as in urine and subcutaneous tumor of mouse, which could be inhibited by pretreatment with DNase I and protease. Interestingly, BCG was trapped but not killed in vitro by NETs, which was different from the effect on *S. aureus*. Moreover, ROS was required for BCG-induced NETs formation, which was regulated by star pathways, such as MEK, p38, PI3K and PKC pathways.

Conclusions By exploring how BCG induce the formation of NETs and regulatory mechanism, we concluded a novel immune reaction of neutrophils in the early stages of BCG treatment.

Background

Intravesical administration of Bacillus Calmette-Guerin (BCG), a live attenuated *Mycobacterium bovis* vaccine widely used to prevent tuberculosis, is currently the most common immunotherapy employed for non-muscle invasive bladder urothelial carcinoma (NMIBC). [1] Bladder carcinoma is one of the most widespread cancers, most of which are NMIBC at diagnosis. [2] The precise mechanism of BCG immunotherapy remains undefined, in which Th1-type immunity is considered as the key role. [3] Neutrophils as the first line of defense against invading microbes, accounting for about 75% of the immunocytes recruited by BCG, [4, 5] however, were previously underestimated in mycobacterial infections. Recent studies reveal that neutrophils are required for the efficacy of BCG immunotherapy. [4–6]

A described antimicrobial mechanism of neutrophils is extracellular traps (NETs), which is the mixture of cytoplasm and decondensated chromatin is expelled through the ruptured [cytomembrane](#) upon activation. [7] NETosis is an irreversible cellular process, different from apoptosis or necrosis. [8] NETosis is triggered by a variety of bacteria, eukaryotic parasites, virus and pro-inflammatory factors. [9–14] Remarkably, two strains of mycobacterium (HN37Rv and *M. canetti*) with high or low virulence induce NETs formation. [15, 16] *M. tuberculosis* induced neutrophil and macrophage extracellular traps,

depending on ESX-1/RD1. [17, 18] However, how BCG induces NETs formation and the potential regulatory mechanisms remain unclear.

NETs are fibers composed of chromatin, in association with granular proteins such as neutrophil elastase (NE), cathepsin G, myeloperoxidase (MPO) and cytoplasmic proteins. [9, 14] NETs can prevent microbe dissemination, [14, 15] which represents an important strategy to immobilize and kill invading microorganisms. However, some microorganisms possibly evade NETs entrapment and killing by destroying the backbone (DNA and protein) or inducing immunosuppression. [7] Additionally, NETs are implicated in the innate immune response, and their excessive release can perpetuate sterile inflammation, [14] autoimmune disease and pathologies. [19–21]

So, we hypothesized that BCG could induce NETs formation that mediated immunoreaction in oncotherapy. In our recent published study, we found that BCG could induce the formation of NETs, which could suppress the development of bladder tumor.[22] In this study, we confirmed NETs formation following BCG treatment, and, verified for the first time their roles in trapping BCG, and the regulatory mechanisms involved in BCG-induced NETs formation. The results could help us understand the reactions of neutrophils in the early stages of BCG-related antitumor immunity more clearly.

Materials And Methods

BCG strain and culturing

BCG *Connaught* substrain (ATCC35733) was from the American Type Culture Collection (Manassas, VA). BCG suspension was prepared in Middlebrook 7H9 broth (BD 271310) media and colonies cultured on 7H10 solid media (BD 262710, Difco Laboratories, USA). Viable BCG from the logarithmic growth phase was used for experiments. The number of colony-forming units (CFU) was routinely determined by plating and incubation at 37°C for 4 weeks on solid medium.

Human neutrophil isolation

Neutrophils were isolated using Ficoll-Dextran method from healthy donors' blood. [23] Purity was greater than 98% as confirmed by flow cytometry using CD11b (561015) and CD66b antibodies (561927, BD Biosciences). Trypan blue exclusion showed the viability to be > 95% for all preparations. Cell morphology was inspected microscopically to rule out cell preparations that had become activated during isolation.

Experimental protocol

The main objective of this study was to investigate the ability of BCG-stimulated neutrophils to produce NETs. Briefly, the experiment was divided into three groups: unstimulated group, BCG-stimulation group, and PMA-stimulation group. The formation of NETs at different time points (0, 30, 180 and 360 min) was

observed by optical microscope, Confocal Laser Scanning Microscope (CLSM) and Scanning Electron Microscopy (SEM), and the composition of NETs was analyzed. Then NETs and ROS were quantified by staining extracellular DNA and the cell permeable fluorescent dye respectively. The levels of NETs and ROS were detected after combined BCG stimulated neutrophils with different pathway inhibitors. FITC-labeled BCG was used to observe the capturing by NETs. Finally, two studies were conducted to detect NETs formation in mice. It was observed in mouse urine and subcutaneous tumor after BCG perfusion respectively. To observe NETs in mouse urine, perfusion of BCG into bladder was performed. The formation of NETs in subcutaneous tumor was observed after injection of BCG into tumor.

Stimulation of neutrophils by BCG

5×10^5 – 10^6 cell/mL freshly isolated neutrophils were seeded gently into culture plates on anti-peeling coverslips (S1815–1PAK, Sigma-Aldrich), and allowed to settle in complete RPMI supplemented with 2% FCS (16140071, Gibco BRL) for 2 h, at 37°C, 5% CO₂. Neutrophils were respectively stimulated with 50 nM phorbol 12-myristate 13-acetate (PMA) (79346–1MG, Sigma-Aldrich), BCG (MOI = 10) or left unstimulated, for indicated time. The total amount of DNA/protein extruded by a given number of cells stimulated by BCG/PMA are used for comparison. As a positive control, the potent inducer of NETs-PMA at this concentration induces typical NETosis.

CLSM and SEM visualization of NETs

As film adherent on slips, NETs specimens were gently fixed with 4% paraformaldehyde (P8430, Solarbio), and the percentage of NETs-forming neutrophil was determined by staining DNA with DAPI (D9542–1MG, Sigma). 10 different fields were observed in each triplicate. To evaluate NETs composition, immuno-fluorescence assays were made with respectively antibodies for citrullinated Histone H3 (cit-H3) (ab219407), primary antibodies against NE (ab14188) or isotype IgG (ab27478). Then observed under FV1000 confocal microscope (Olympus, Tokyo, Japan), and calculated using IPP software, as described. [9, 24] Meanwhile, NETs formed in the coculture were observed, after treatment with 50 U/mL DNase I (M6101, Promega) or 50 µg/mL proteinase K (P5568–1ML, Sigma) for 30 min.

The samples were fixed in 2.5% glutaraldehyde and then post-fixed with 1% osmium tetroxide/1% tannic acid. After dehydration with series of ethanol and critical-point drying, the specimens were coated with platinum and analyzed by S2460 N SEM (Hitachi, San Jose, CA).

NETs quantitation

At the indicated time, NETs scaffold in coculture was dismantled by digestion with 250 mU/mL micrococcal nuclease (LS004797, Worthington Biochemical Corp.), which can cleave naked DNA and not act upon histone-attached DNA, ensuring NETs isolation with minimal degradation. Extracellular DNA was then stained with 2.5 µM Sytox Orange (S34861, Molecular Probes) for 10 min at room temperature.

The cell-impermeable compound SYTOX orange becomes fluorescent only when interacting directly with DNA. Quantification was done using a fluorometer (Synergy H1 Hybrid Reader, [BioTek](#)) every min for up to 300 min. Another way was to calculate percentage of NETotic cells per 100 cells.

Quantification of intracellular ROS

Intracellular ROS production was monitored using the cell permeable fluorescent dye, DCFH-DA (Invitrogen). Neutrophils at 5×10^6 /mL were incubated with 5mM DCFH-DA for 30 min and then harvested. The fluorescence intensity was measured using a reader (488/525 nm, BD Biosciences), every 3 min, for 40 min. At minute 20, neutrophils were infected with non-opsonized BCG at MOI of 10:1, or neutrophils alone as control. Activation with 20 nM PMA was used as an activation control.

Inhibition of ROS, NADPH oxidase and the pathways possibly involved in NETs formation

When indicated, PMNs were pretreated for 30 min and cultured in the presence of 10mM Acetylcysteine (ROS scavenger, Selleck), 20 μ M diphenyleneiodonium (DPI, NADPH oxidase inhibitor; Sigma-Aldrich), 10 mM SB600125 (JNK inhibitor, Cayman), 100 nM Wortmannin (PI3K inhibitor, Cauman), 100 nM Sotrastaurin (PKC inhibitor, Selleck), 10 nM U0126 (MEK inhibitor, Cayman), 10 mM SB203580 (p38 inhibitor, Selleck) for the duration of the experiment.

Capturing and killing by NETs

BCG was FITC-labeled as described previously. [25] Neutrophils were seeded and incubated with BCG at MOI of 10:1, for dedicated time. After fixation with 4% paraformaldehyde, DNA fibers were stained with DAPI. CLSM and SEM were performed to observe them. Using the reported protocols, [15] we examined BCG-killing activity by NETs. Neutrophils were pre-incubated with BCG or PMA for 4 h to induce NETs. The medium was carefully replaced with 10 mg/mL cytochalasin D (Cyt-D), the actin inhibitor (22144-77-0, Sigma-Aldrich). Then, BCG (MOI = 1) was added to incubate for 6 and 24 h, with *S.aureus* (ATCC25923) as the positive control. After CFU counting, survival was determined as percentage of bacteria with NETs to the ones without NETs.

NETs formation in mouse urine and tumor after BCG treatment

6–7-week-old C57BL/6 mice were from HFK Bioscience Co. Ltd., Beijing, China. All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U. K. Animals (Scientific Procedures) Act, 1986. The murine bladder tumor cell line MB49 is a kind gift from M. A. O'Donnell

(University of Iowa, Iowa City, IA). The mice receiving subcutaneous inoculation of 10^6 murine syngeneic MB49 cells were used to evaluate the formation of BCG-induced NETs *in vivo*. Single BCG treatment was performed by perfusion of 10^5 CFU into bladder, or injection of 10^4 CFU into tumor, then urine and tumors were continuously collected for evaluation of NETs formation. Part of mice were sacrificed to track BCG in bladder wall by auramine 'O' staining. Three mice were used once per test.

In freshly collected urine, NETs concentration was determined by fluorescence imaging, after centrifugation and fixation with 2% PFA, staining with DAPI, anti-cit-H3 Ab (ab219407), and anti-NE primary Ab (ab205670). 5 μ m tissue sections were used for routine treatment and blockage with 0.2% horse serum, then incubated with primary antibodies against MPO (ab9535) and NE (ab205670, Abcam), followed by respective Alexa Fluor®488 and Alexa Fluor®555 labeled secondary Ab (A-11008, A-21434, Invitrogen). Lastly, DNA was stained using DAPI, for observation under CLSM.

Statistical analysis

Unless otherwise stated, all data are presented as mean $\pm$ SD of at least three independent experiments. Where appropriate, either two-tailed Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparison test were used. $P < 0.05$ was considered statistical significance. Spss 20.0 software was used for statistical analysis.

Results

DNA and proteins both are required for NETs formation

An obvious increase of extracellular DNA fibres produced by human neutrophils exposed to BCG was monitored. Under fluorescence microscope, the modest increase was shown in extracellular DNA staining after exposure of human neutrophils to BCG and PMA (MOI = 10), and clusters of neutrophils were visualized, similarly to the typical structure of NETs (Fig 1A). [8–11] Under SEM, BCG-stimulated neutrophils firmly attached to the slide and aggregated, with fibrillar material extruded extracellularly. Compared with intact neutrophil round and regular shape, BCG-treated neutrophils produced lots of fibers in varying diameter and length and decorated with globular granules, moreover, the fibers clumped and covered nearly the entire surface. In contrast, NETotic cells induced by PMA scattered with fine fibers connected with each other (Fig 1B). The CLSM demonstrated extracellular co-localization of NE and histone H3 superimposed with DNA, confirming the presence of NETs (Fig 1C). Notably, fresh BCG (MOI = 10) stimulated more efficiently than senile (More than 2 weeks of platform period of the growth cycle) and dead one (autoclaved), even than 100nM PMA. To explore the composition of NETs, Dnase I and proteases were added. Following either Dnase I or proteases pre-treatment, neutrophils did not produce NETs in response to BCG ($P < 0.01$; Fig 1D). Therefore, DNA and protein structures are required for NETs formation. Additionally, the amount of DNA and proteins in BCG-induced NETs were significantly higher than those in PMA-induced ones ($P < 0.001$; Fig 1E).

BCG induce in vitro NETs in a time-dependent manner

Indeed, it was observed that NETs began to be released from some neutrophils after BCG-stimulation for a few minutes. The morphological changes of neutrophils induced by BCG included immediate cell flattening, increased adherence, pseudopod extension, and aggregation, compared with resting neutrophils (Fig 2A). Neutrophils attached to the slips and flattened, with decondensed chromatin mixed with the cytoplasmic content. Progressively, most nuclei lost round shape and their lobules with a homogeneous staining, as well as many neutrophils lost plasma membrane integrity and released cellular constituents. In contrast, the nuclei of the unstimulated controls were still lobulated and NE signal clearly depicted cytosol. (Fig 2B-C). The percentage of NETosis ratio increased gradually and reached about 65% at 12h (Fig 2D). In addition, the increases in cell-impermeable SYTOX orange can reflect of both NETosis and the following cell death (Fig 2E-F). Taken together, we summarised that BCG could induce NETs formation in a time-dependent manner, which was mainly composed of DNA and proteins expelled by activated neutrophils.

ROS was required for BCG-induce NETs, which was regulated by MEK, p38 MEAK, PI3K and PKC pathways

After inhibiting neutrophil phagocytosis by pretreatment with Cyt-D, NETs did not decrease, indicating their formation does not depend on phagocytosis BCG. However, in the presence of Acetylcysteine (ROS scavenger), NETs formation induced by BCG was not observed. The same happened to DPI (NADPH oxidase inhibitor) (Fig 3A). Highly similar results were found to exist between NETs and ROS levels after the same treatment. (Fig 3B-C) So, we concluded that ROS production was necessary for BCG-induced NETs formation.

Many inhibitors were used to find out the potential pathways that played essential roles in NETs formation. Compared with the results of no inhibitor, BCG-induced NETs formation was lower after pretreated with U0126 (MEK inhibitor) and SB203580 (P38 inhibitor), as well as after wortmannin (PI3K inhibitor), sotrastaurin (PKC inhibitor) pretreatment (Fig 3D). Consistent with this result, ROS production was reduced after pretreated with these inhibitors (Fig 3E). Moreover, NETs did not obviously change and ROS was slightly decreased after addition of SB600125 (JNK inhibitor). In summary, these results suggested that MEK, p38, PI3K and PKC pathways, but not JNK, were required for BCG-induced NETs formation.

NETs capture but do not Kill BCG

After neutrophils were stimulated with FITC-labeled BCG, green signals together with DAPI signals were found. NETs were released from activated neutrophils and became abundant gradually with time, meanwhile, more BCG were trapped in the fluorescent extracellular DNA (Fig 4A). Longer incubation time

led to more NETs and therefore more BCG captured by the DAPI⁺ structures (Fig 4B). SEM showed BCG trapped in NETs and localized the latters (Fig 4C). Fewer bacteria were trapped when coverslips were treated with Dnase I. Notably, after inhibiting phagocytosis, BCG was not confined intracellular, and lots of aggregated or individual BCG could be seen free (Fig 4D). An *in-vitro* killing assay was made to determine whether NETs induced by BCG or PMA are associated with bacterial killing, then found that BCG- and PMA-induced NETs eliminated > 60% of *S. aureus* independently of phagocytosis at 24h of incubation, but almost no BCG ($P<0.05$; Fig 4E) Our results suggested that NETs formed after stimulation could capture but did not kill BCG, which was different from our general understanding of NETs.

NETs formation in mouse urine and tumor after BCG treatment

In the absence of infectious or inflammatory stimuli, there are relatively few neutrophil and NETs in tumor and no NETs in urine. After intravesical perfusion, BCG count in mouse urine and bladder wall peaked at 3h (Fig 5A). Notably, the maximal recruitment of neutrophils and a significant increase of NETs in urine appeared at 24h and 12h respectively. But at the moments, the amount of BCG in urine was few, although BCG in bladder wall was still abundant (Fig 5B). At 3 h after BCG perfusion, some neutrophils began with nuclei de-lobulated, and co-localization of extracellular chromatin with NE (Fig 5C). After BCG-treatment, considerable increase of fluorescence was observed in tumors of mice, and the shapes of NETs were more compact than those formed *in vitro*, with higher packing density of the globular domains and co-localization of DNA as reported. [26] The observable intra-tumor NETs showed that the summit occurred in 12 to 24h after injection of BCG (Fig 5D-E). And the kinetics of NETs in mice tumor was similar to those in urine.

Discussion

Neutrophils constituting 60% of circulating leukocytes, are the first immune cells recruited to inflamed tissue as the first line of defense against microbes, and direct the activation of adaptive immune cells. [27] Mature neutrophils are terminally differentiated and short-lived, with enzymes and proteins stored in granules. [28] *In-vitro* NETs are neutrophil-derived web-like structures made of chromatin decorated with antimicrobial proteins, released extracellularly in response to bacteria, fungi, viruses, and some inflammatory cues. [29] NETs *in vivo* has been found in a variety of infectious, inflammatory and autoimmune diseases. [9] The co-localization of granular proteins/histones, with the DNA is critical in discriminating DNA released during cell necrosis from that specific to NETs formation. This study demonstrated the BCG-induced web-like structure decorated with globule, of which the components were verified by co-localization of DNA, histone and NE, further by quantitation of dsDNA and proteins. Importantly, DNA strands rather than proteins constituted the backbone of NETs. Moreover, BCG induced subcellular changes leading to NETs formation time-dependently, as reported. [30] The viable BCG induced more than the senile and dead ones.

NETs formation is demonstrated in skin, gingival tissues, fasciitis, pneumonia, appendicitis, and breast tumor in mouse or human. [7, 20, 31, 32] Our results showed the nuclei decondensed and delobulated, the fluorescence profile of co-localization of DNA and granular proteins in mouse urine and tumor, suggesting that BCG promoted NETs formation *in vivo*. NETs detected in urine formed with rapid kinetics, within 3 h of intravesical BCG perfusion, following BCG adhesion to and neutrophil infiltration in bladder wall. In contrast to *in-vitro* human NETs appearing as outspread web-like structures, *in-vivo* NETs showed a more compact structure with higher packing density of the globular domains as reported, [26] likely due to the physical constraints of surrounding tissue [33] or the absence of long strands of chromatin. [34]

Previous reports have shown that neutrophils combat microbes intra-cellularly by phagocytosis or extra-cellularly by NETs. [28, 29] NETs are web-like structures composed of chromatin in complex with over 30 proteins, which play important roles in host defense through physically capturing [14, 33, 35] or killing microbes. [31, 36] In this study, confocal and scanning electron imaging demonstrated clusters of BCG embedded within DNA webs, indicating that NETs could bind and ensnare BCG. Three covalently linked structures in mycobacterial cell wall might be involved in NETs attachment. [15, 37] Nevertheless, NETs could not *in vitro* kill BCG as efficiently as they killed other bacteria, as reported in *M.tuberculosis*. [15] It seems that the high lipid composition and structural features of mycobacterial envelope [7] or nuclease [38] confer an effective protection.

NETs are thought to prevent bacterial dissemination and uncontrolled infection. [26, 39] And impaired NETs formation result in host high susceptibility to infection. [31] Along these lines, we postulate that NETs formation limits the serious side effects caused by mycobacterial dissemination, and provides high concentrations of local mycobacteria as spatially restricted repositories, so that abnormal NETs induction might influence local retention of BCG and antitumor efficiency. Our recent study have confirmed that BCG-induced NETs could inhibit the vitality of bladder tumor cells and suppress tumor growth in mice.[22]

Regarding the mechanism, most studies demonstrated that NETosis occurred depending on NADPH oxidase-mediated ROS generation. But, ROS-independent way also was reported, *eg.* stimulation with staphylococcus aureus. [40] Another example was cold shock/rewarming-induced NETs, in which neither ROS nor NETs generation was significantly affected by DPI. But DPI could completely inhibit the formation of canonical NETs, including PMA-stimulated NETs. [33] Ramos-Kichik *et al*/ reported that the release of NETs was preceded by the production of ROS during mycobacterial infection. [15] Therefore, the requirement of NETs formation varies depending on the stimuli, [41] and the molecular mechanisms by which ROS drives this process remain poorly understood. In our study, generation of ROS was necessary for neutrophil to form NETs when stimulation with BCG. This process was dependent on the activity of NADPH oxidase, as evidenced by the fact that inhibition of NADPH oxidase reduced ROS and NETs formation.

In the process of infection with pathogens, a series of signaling pathways involved in cell life activities, *eg.* PI3K/Akt, PKC, Ras/MAPK (ERK, JNK/C - Jun, P38 and ERK5), are regulated. For instance, activation of the Raf-MEK-ERK and p38 MAPK pathway mediates PMA-induced NETs release from human

neutrophils. [42, 43] In our study, pharmacological inhibition of MEK and p38 MAPK activity blocked BCG-induced NETs formation to different extent, and ROS production was lower than the control. Reportedly, several enzymes that regulate Nox2 activity such as protein kinase C (PKC) isoforms and MAPK kinases have been implicated in PMA-induced NETosis process. [42, 44] Remijsen *et al*/reported, [8] blockade of PI3K with wortmannin inhibited autophagy, and pretreatment of PMA-stimulated neutrophils with wortmannin prevented chromatin decondensation. Similarly, the current evidence in our study suggested that PKC and PI3K also involved in BCG-induced NETosis. However, the formation of NETs was non-significantly influenced by inhibition of JNK.

Conclusions

In this study, we firstly corroborated BCG-induced NETs formation both *in vitro* and in mouse model. Moreover, our results verified their roles in trapping or killing BCG, and proposed that NETs act as physical barriers to localize BCG, thus preventing spread. Our study confirmed that, generation of ROS was necessary for neutrophil to form NETs when stimulation with BCG, and this process was dependent on the activity of NADPH oxidase. The current evidence in our study suggests that MEK, p38, PI3K and PKC pathways play important roles in this process, and locate in the upstream of regulation of ROS generation. So, it is speculated that NETs as a novel neutrophil-dependent mechanism participate in BCG immunity. Further experiments will be necessary to clarify the mechanisms, and examine their roles in oncotherapy or pathological responses.

Abbreviations

BCG, Bacillus Calmette-Guerin; PMN, polymorphonuclear neutrophil; NETs, neutrophil extracellular traps; BC, bladder cancer; NMIBC, non-muscle invasive bladder cancer; PMA, phorbol 12-myristate 13-acetate; cit-H3, Citrullinated-Histone H3; NE, neutrophil elastase; MPO, myeloperoxidase, SEM, scanning electron microscopy; CLSM, Confocal Laser Scanning Microscope; FCM, Flow Cytometry; ELISA, Enzyme-linked immunosorbent assay

Declarations

Ethics approval and consent to participate

All researches involving human samples were in accordance with the Helsinki Declaration of 1975, and have been approved by the Ethics Committee of the second Hospital of Tianjin Medical University (KY2016K010) and written informed consents were obtained from all participants.

Availability of data and materials

All data used during the current study available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Competing interests

None

Funding

None

Authors' contributions

ELS designed the experiments. ELS, KKL, and MDL analyzed the data and wrote the manuscript. LNW and KKL performed the experiments in vitro. MDL and JDG were major contributors in mouse experiment. XWN and LML prepared reagents and materials. All authors read and approved the final manuscript.

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Figures

Figure 1

Fluorescent and scanning electron micrograph showed NETs formation induced by BCG. A) The extracellular DNA staining after exposure of human neutrophils to BCG and PMA was visualized by fluorescence microscope ($\times 200$, Scale bar= $50\mu\text{m}$). B) SEM showed the structures of BCG-treated neutrophils ($\times 5000$, Scale bar= $20\mu\text{m}$). C) CLSM demonstrated that extracellular co-localization of NE (red) and cit-H3 (green) with DNA fibers (blue) of PMN alone, BCG stimulation, and DNase I treatment

(Scale bar = 10µm). D) Measurement of extracellular fluorescence, expressed using arbitrary fluorescence units (AU). E) The quantification of DNA and proteins in NETs produced from neutrophils (1×10^5 cell/mL) exposed to BCG, PMA and US (unstimulated neutrophils), when incubated for 3h. The time was adopted because NETs induced by BCG for longer time were too thick easy to fall off.

Figure 2

Time-course of morphology change of BCG-activated neutrophils. A) Isolated neutrophils (1×10^5 cell/mL) monitored by live cell imaging using differential interface contrast ($\times 200$, Scale bar = $20 \mu\text{m}$). B&C) CLSM (Scale bar = $5 \mu\text{m}$) and SEM (Scale bar = 20 and $50 \mu\text{m}$) showed the structures of NETs after BCG stimulation with different times. D) The percentage of NETosis in the co-culture with BCG changed with time, and the kinetic analysis showed rapid increase at about 3h. E) Fluorescence microscopy of Sytox orange-stained neutrophil showed extracellular DNA, NETotic and dead cells (Scale bar = $10 \mu\text{m}$). F) Sytox orange-fluorescence staining from 3 independent experiments demonstrated DNA release with time, from neutrophils stimulated with BCG, 1% Triton X-100 (lysed cells as positive control) or PBS (negative control).

Figure 3

. ROS and the pathways involved in the regulation of BCG-induced NETs. A) CLSM showed that the formation of NETs was inhibited after pretreatment by DPI (NADPH oxidase inhibitor) and Acetylcysteine (ROS scavenger) (Scale bar = 10μm). B&C) DPI and Acetylcysteine inhibited BCG-induced NETs and ROS production ($P < 0.001$). D&E) After pretreatment by U0126, SB203580, Wortmannin, and Sotrastaurin, NETs were reduced significantly ($P < 0.001$), but didn't obviously change after addition of SB600125.

Figure 4

BCG-induced NETs could trap but not kill BCG in vitro. A) After incubation and DAPI-staining DNA (blue), CLSM showed the neutrophils and BCG (green) (Scale bar = 10µm). B) The result of bacterial count showed that BCG bacteria captured in NETs increased with time, during 6 hours. C) SEM analysis revealed, the NETs formed when incubating with BCG, trapped and localized the latters. D) After inhibiting

phagocytosis (right), the green staining was not confined intracellular. E) The survivals of BCG and *S. aureus* after be treated by BCG- and PMA-induced NETs.

Figure 5

Kinetics of NETs formation in urine and tumor tissue of BCG-treated mouse models. A) After 12 h of BCG intravesical perfusion, the mycobacteria in bladder wall and urine appeared green or yellow green, when stained with auramine ‘O’. B) The NETs formation and neutrophils in urine were observed in the summit at

12h and 24h after perfusion. C) Immunofluorescence stained neutrophils in the urine of mice at 3h and 24h (Scale bar = 10 μ m). D) Representative confocal images demonstrated the co-localization of MPO (green) with NE (red) and DAPI (blue) in BCG-treated subcutaneous tumor, where the formation of NETosis (yellow arrow) and NETs (inset 1, 2) was evident. (Scale bar = 20 μ m). E) NETs formation in tumor with different time.