Biochemistry and Biophysics Reports 34 (2023) 101465

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Complement dependent TNFα production in neutrophil-like HL60 cells

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ARTICLE INFO

Keywords:
Calcium
Complement
Integrin
Neutrophil
TNFα

ABSTRACT

Neutrophils develop in the bone marrow (BM) from hematopoietic stem cells (HSCs) through a series of progenitor cells and mature neutrophils play a critical role in the human immune system. Previous studies revealed that tumor necrosis factor α (TNFα) produced by immature neutrophils contributes to HSCs development and vascular regeneration in the BM niche. However, the precise mechanism of TNFα production in immature neutrophils remains unclear. This study aims to assess the relationship between complement C3 activation and TNFα production from immature neutrophils. We investigated the regulatory mechanism of TNFα production by complement components in neutrophil-like HL60 cells. Flow cytometric analysis showed that C3a receptor (C3aR) and C3bi receptor (CR3, Mac-1, CD11b/CD18, integrin αMβ2) are expressed on the surface of neutrophil-like HL60 cells. We found that zymosan-treated human serum leads to TNFα production in neutrophil-like HL60 cells, but not in human polymorphonuclear cells (PMNs). A C3-convertase inhibitor, compstatin suppresses TNFα production. These data suggest that the TNFα production is mediated by complement C3 activation. Furthermore, the TNFα production is enhanced by Ca^{2+} elevating agents, thapsigargin (TG), but is suppressed by treatment with Ca^{2+} chelators, EGTA, or BAPTA-AM. In addition, the soluble TNFα production is suppressed by treatment with immobilized-fibrinogen or -fibronectin. Thus, the TNFα production is enhanced by intracellular Ca^{2+} elevation and is negatively regulated by the interaction between the neutrophil-like HL60 cells and fibrinogen or fibronectin.

1. Introduction

Neutrophils are the most abundant circulating white blood cells and play critical roles in the human immune system. They are produced about 10^{10} to 10^{11} cells per day in the bone marrow (BM) through a series of progenitor cells originating from hematopoietic stem cells (HSCs). In the BM niche, HSCs give rise to multipotent progenitors (MPPs), which produce common myeloid progenitors (CMPs) and then granulocyte-monocyte progenitors (GMPs) [1]. Then, GMPs can differentiate into myeloblasts, promyelocytes, myelocytes, metamyelocytes, band cells, and finally, mature segmented neutrophils [2]. These developmental stages are regulated by various cytokines and adhesion molecules [1,3,4], but their precise mechanisms are still unclear.

The tumor necrosis factor α (TNFα) is a multifunctional pro-inflammatory cytokine that has roles in mammalian immunity and homeostasis [5]. Furthermore, previous studies revealed that TNFα produced by immature neutrophils contributes to HSCs development and vascular regeneration in the BM niche [6–9]. In addition, inflammation-induced TNFα promotes HSCs survival and myeloid differentiation [10]. Therefore, to further elucidate the regulatory mechanisms of hematopoiesis by TNFα in the BM niche, we believe it is important to identify the stimulatory factors that cause TNFα production in immature neutrophils within the BM.

Complement protein C3 plays a central role in the complement system and is activated by C3 convertase to generate the major cleavage fragments C3a and C3b. Microbial surfaces-bound C3b is further cleaved to C3bi. The C3-derived fragments C3a and C3bi are well known to activate neutrophil chemotaxis and phagocytosis, respectively [11]. However, it has not been investigated whether C3 activation is involved in TNFα production in immature neutrophils.

The present study examines whether the complement pathway is involved in TNFα production in neutrophil-like HL60 cells by using zymosan-treated human serum and C3-convertase inhibitor, compstatin. We demonstrate that complement pathway activation is responsible for TNFα production in neutrophil-like HL60 cells. Furthermore, we examine the regulatory mechanisms of TNFα production in neutrophil-like HL60 cells. Our results indicate that the TNFα production is enhanced by intracellular Ca^{2+} elevation and is negatively regulated by

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https://doi.org/10.1016/j.bbrep.2023.101465
Received 26 November 2022; Received in revised form 12 March 2023; Accepted 3 April 2023

the interaction between the neutrophil-like HL60 cells and fibrinogen or fibronectin.

2. Materials and methods

2.1. Reagents and antibodies

Unlabeled-zymosan (#Z-2849) was purchased from Molecular probes (Eugene, OR). Anti-Integrin αM (2LPM19c) FITC monoclonal antibody (#sc-20050) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-TNFα monoclonal antibody (#6945, clone D5G9), and anti-β-actin monoclonal antibody (#8457, clone D6A8) were purchased from Cell Signaling Technology (Danvers, MA). Anti-C3a Receptor (hC3aRZ8) FITC monoclonal antibody (#130-108-108) was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-C5AR1 (P12/1) FITC monoclonal antibody (#MA5-28088) was purchased from Invitrogen (Waltham, MA). Anti-complement C3b/iC3b monoclonal antibody (#846102) was purchased from BioLegend (San Diego, CA). Compstatin (#2585) and compstatin control peptide (#3796) were purchased from Tocris Bioscience (Abingdon, UK). Fibronectin (#063–05591), fibrinogen (#061–03691), and all-*trans*-retinoic acid (ATRA, #188–01113) were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). BAPTA-AM (#B035) was purchased from Dojindo (Kumamoto, Japan). Thapsigargin (#T7459) was purchased from Invitrogen (Waltham, MA). RPMI-1640 was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan, #189–02025). Penicillin-Streptomycin Mixed Solution (100 U/mL penicillin, 0.1 mg/mL streptomycin) was from Nacalai Tesque (Kyoto, Japan, #26253-84). Fetal bovine serum (FBS, #172012, Lot No. 12D165) was from Sigma-Aldrich (St. Louis, MO). Horseradish peroxidase (HRP) conjugated goat anti-rabbit (#1706515) secondary antibodies were from Bio-Rad (Hercules, CA). T-25 flask was obtained from IWAKI (Tokyo, Japan, #3100-025). Nunc 24-well plate was obtained from Thermo Fisher Scientific (Waltham, MA, #142475).

2.2. Cell preparation and differentiation-induction

The human promyelocytic cell line HL60 was maintained culture medium (RPMI-1640 medium containing 8% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin) in 5% carbon dioxide (CO_2) humidified air at 37 °C. HL60 cells were induced to undergo differentiation to neutrophil-like cells by seeding them on 24-well plates (2.5×10^4 to 2×10^5 cells/500 μl/well) in RPMI-1640 differentiation medium (RPMI-1640 medium containing 2.5 μM ATRA, 8% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin) for 1–5 days. The cell morphology was assessed with May-Gruenwald-Giemsa stained cytospin preparations.

2.3. Isolation of human neutrophils

Human polymorphonuclear cells (PMNs) were isolated by Polymorphprep (Abbott Serumwerk Bernburg AG, Bernburg, Germany; #114683) according to the protocol provided by the manufacturer. Briefly, 20 ml of peripheral blood from a healthy female blood donor was divided into 5 ml portions and layered over an equal volume of Polymorphprep in a 15 ml tube. After centrifugation (500×*g*, 45 min, 20 °C), the lower leukocyte band containing PMNs was collected. Isolated 1×10^5 cells of PMNs were washed with PBS, and then resuspended in RPMI-1640.

2.4. Preparation of zymosan-treated human serum and complement-opsonized zymosan

Zymosan particles (5×10^7 particles/ml) were incubated in 50% human serum obtained from a healthy volunteer in RPMI-1640 for 30 min at 37 °C. After the incubation, human serum was separated from zymosan particles by centrifugation at 2000×*g* for 3 min at 4 °C to remove C3b fragments from the serum. The complement-opsonized zymosan particles were washed with PBS, and resuspended in PBS. In the case of inhibition of complement activity, human serum was heated for 30 min at 56 °C, or pre-treated with 250 μM compstatin or control peptide for 30 min at 37 °C, and was then incubated with zymosan particles for 30 min at 37 °C in the presence or absence of compstatin or control peptide. To test the binding ability of C3bi to zymosan, the opsonized zymosan particles were incubated in 10 μg/ml anti-complement C3b/iC3b monoclonal antibody and followed by incubation with 1:100 dilution of Alexa Fluor 488-conjugated goat anti-mouse IgG polyclonal antibodies for 30 min, and finally analyzed by flow cytometry (FACSCalibur; Becton Dickinson, SanJose, CA).

2.5. Cell treatment and sampling for immunoblotting

Neutrophil-like HL60 cells and PMNs (5×10^5 cells/well) were incubated in 250 μl of RPMI-1640 containing 10% zymosan-treated human serum or human serum on 24-well plates. For the negative control, neutrophil-like HL60 cells or PMNs were incubated in day 5-differentiation medium supernatants, or RPMI-1640. In some experiments, cells were pre-incubated in RPMI-1640 containing 1 mM EGTA or 10 μM BAPTA-AM for 30 min at 37 °C, and were then incubated with 10% zymosan-treated human serum for 4 h at 37 °C in the presence of 1 mM EGTA or 10 μM BAPTA-AM. At the time points indicated, the supernatants of human serum-treated or untreated-cell culture were separately transferred from wells to tubes and centrifuged at 800×*g* for 1 min at 4 °C. The 200 μl of cell-free supernatants were collected, and added 100 μl of 3 × SDS-PAGE sample loading buffer (187.5 mM Tris-HCl, pH 6.8; 6% SDS; 15% Glycerol, 15% 2 ME, BPB). After collecting the supernatants, 0.5 ml of PBS was added to each well, and the cells were scraped off the surface of the plate by pipetting and transferred to the corresponding tube. Each well was rinsed with 0.5 ml of PBS to collect any remaining cells, which were transferred to the corresponding tube. The samples were centrifuged at 800×*g* for 1 min at 4 °C. The supernatants were then aspirated, and the cell pellets were resuspended in 37.5 μl of 1 × SDS-PAGE sample loading buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 5% Glycerol, 5% 2 ME, BPB).

2.6. Immunoblotting analyses

Protein samples were separated in SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Merck Millipore, MA, #IPVH00010). The membrane was blocked with 5% skim milk in T-TBS (10 mM Tris-HCl, pH7.5; 100 mM NaCl; 0.1% Tween 20) overnight at 4 °C and then incubated with 1:1000 dilution of mouse anti-TNFα monoclonal antibody or 1:1000 dilution of anti-β-actin monoclonal antibody in T-TBS for 45 min at room temperature (RT). After washing, incubated with 1:1000 dilution of HRP conjugated goat anti-rabbit IgG (H + L) secondary antibodies for 30 min at RT, and specific proteins were detected using an enhanced chemiluminescence immunoblotting system and a WSE-6100H LuminoGraphI (ATTO; Tokyo, Japan).

2.7. Cell surface expression of complement receptors

The cells were collected in to tube at a density of 1×10^5 cells/500 μl/tube in PBS. After centrifugation (800×*g*, 1 min, 4 °C), these cells were resuspended in 50 μl of PBS containing 2 μg/ml anti-Integrin αM (2LPM19c) FITC monoclonal antibody, 1.1 μg/ml anti-C3a Receptor (hC3aRZ8) FITC monoclonal antibody, or 2 μg/ml anti-C5AR1 (P12/1) FITC monoclonal antibody, respectively, and incubated on ice for 1h. The cells were washed twice with PBS and resuspended in 200 μl of PBS, and finally analyzed by flow cytometry (FACSCalibur; Becton Dickinson, SanJose, CA).

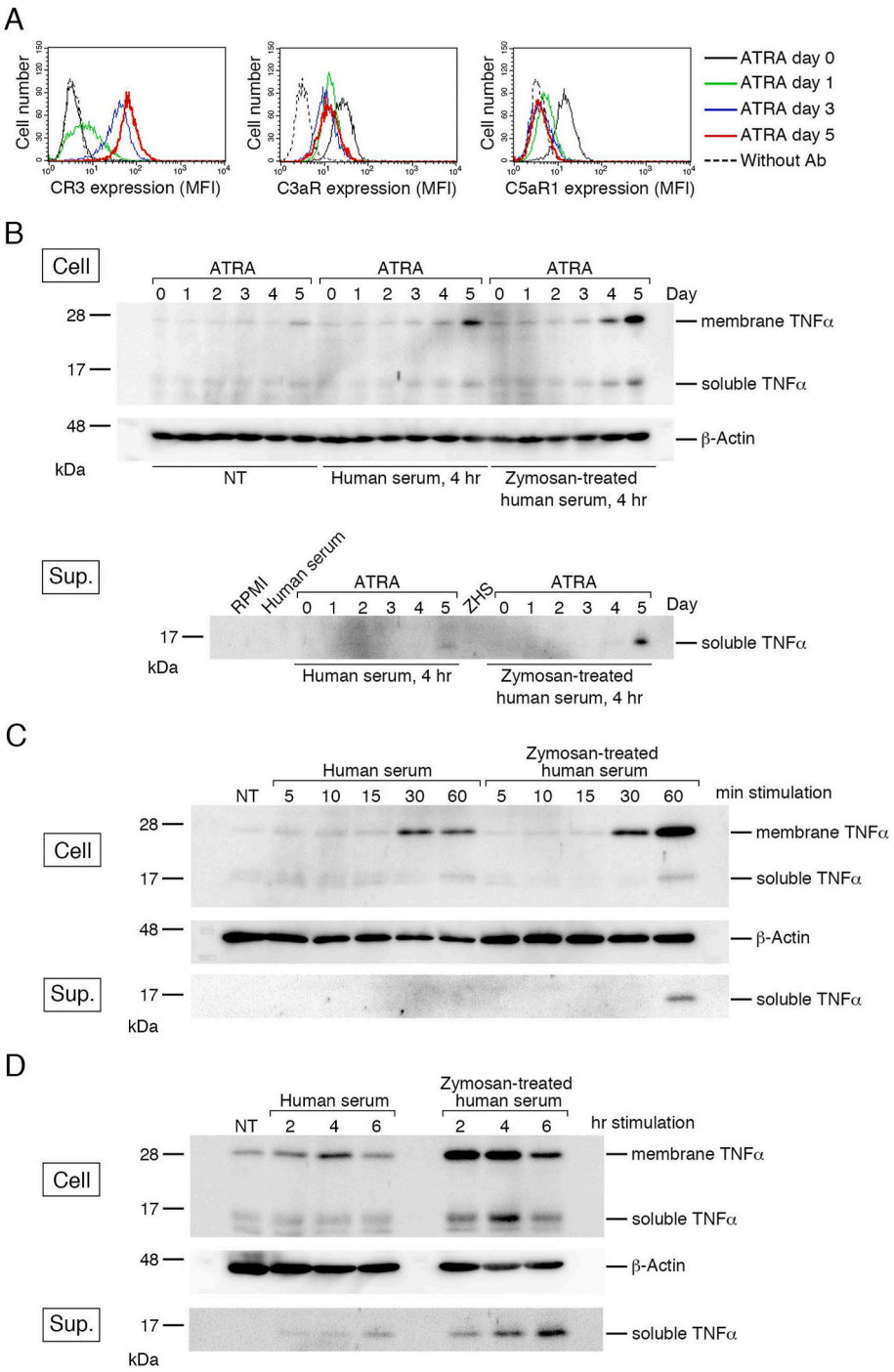

Fig. 1. Zymosan-treated human serum-induced TNFα production in neutrophil-like HL60 cells.
HL60 cells were induced to differentiate into neutrophil-like cells by treatment with 2.5 μM ATRA for 0–5 days. (A) Flow cytometric analysis of the cell surface expression of CR3, C3aR, or C5aR1 in day 0, 1, 3, and 5 ATRA-treated HL60 cells. (B) Day 0–5 ATRA-treated HL60 cells were either untreated (NT) or incubated with 10% human serum or zymosan-treated human serum for 4 h, and the whole cell lysates (Cell) and culture supernatants (Sup.) were then collected. The protein samples were separated in SDS-PAGE, and TNFα and β-actin were detected by immunoblot analysis. RPMI, Human serum, and ZHS in diagonal letters indicate samples of RPMI-1640 medium, RPMI-1640 containing 10% zymosan-treated human serum, or human serum mixed with 1 × SDS-PAGE sample loading buffer, respectively. (C, D) Day 5 ATRA-treated HL60 cells were either untreated (NT) or incubated with 10% human serum or zymosan-treated human serum for indicated time points, and the whole cell lysates (Cell) and culture supernatants (Sup.) were then collected. (E) Day 5 ATRA-treated HL60 cells and PMNs were either untreated (NT) or incubated with 10% human serum (HS) or zymosan-treated human serum (ZHS) for 4 h, and the whole cell lysates (Cell) and culture supernatants (Sup.) were then collected. The protein samples were separated in SDS-PAGE, and TNFα and β-actin were detected by immunoblot analysis. The blot is a representative of 3 independent experiments.

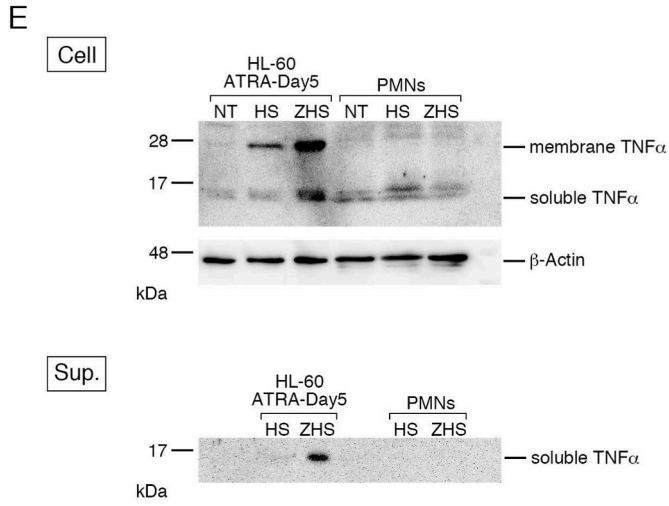

Fig. 1. (*continued*).

2.8. Coating wells

Before neutrophil-like HL60 cells seeding, Nunc 24-well plates were coated with fibrinogen or fibronectin by incubation with 1, 10, or 100 μg/ml solution of these in PBS (0.2 ml/well) for 1 h at 37 °C. The wells were then washed twice with H_2O (0.5 ml/well), and dried.

2.9. Statistical analysis

In some experiments, statistical significance was determined by the Student *t*-test.

Ethics approval

This study included the experiments using human subjects. Therefore, this study was approved by the ethics committee of Himeji Dokkyo University based on the Declaration of Helsinki and the ethical guidelines for medical and health research involving human subjects by Ministry of Education, Culture, Sports, Science and Technology of Japan. Human polymorphonuclear cells and serum were obtained from the peripheral blood of healthy volunteers after informed consent.

3. Results

3.1. Zymosan-treated human serum induces TNFα production in neutrophil-like HL60 cells

For induction of differentiation into neutrophil-like cells, we treated HL60 cells with ATRA. Consistent with our previous study [12], flow cytometric analysis showed that the cell surface expression of C3bi receptor (CR3, CD11b/CD18, integrin αMβ2) was detected in ATRA-treated HL60 cells (Fig. 1A). The cell surface expression of CR3, which was 'without Ab' level in undifferentiated HL60 cells (day 0 ATRA-treated HL60 cells), was elevated from 1 to 5 days after ATRA treatment. As described below, we treated the cells with zymosan-treated human serum containing complement components such as C3a and C5a. Therefore, we also analyzed the cell surface expression of complement C3a receptor (C3aR) and complement C5a receptor 1 (C5aR1). The cell surface expression of C3aR and C5aR1, which was detected in undifferentiated HL60 cells, was decreased from 1 to 5 days after ATRA treatment. Notably, the cell surface expression C3aR remained, but C5aR1 was decreased to 'without Ab' level in ATRA-treated HL60 cells (Fig. 1A). These results suggest that ATRA-treated HL60 cells are expressing the receptor for C3bi (CR3) and C3a (C3aR), but few receptors for C5a (C5aR1).

We examined whether TNFα production is observed in HL60 cells from 1 to 5 days after ATRA treatment by 4 h incubation with zymosan-treated human serum (Fig. 1B). We analyzed the amount of soluble and membrane TNFα protein in whole cell lysates and culture supernatants by immunoblotting. Soluble and membrane TNFα protein in whole cell lysate was increased by incubation of zymosan-treated human serum with day 4 and 5 ATRA-treated HL60 cells. Furthermore, soluble TNFα protein in the culture supernatant was increased by incubation of zymosan-treated human serum with day 5 ATRA-treated HL60 cells. These results indicate that day 5 ATRA-treated HL60 cells are appropriate for observing the production and release of TNFα protein in response to the complement component in zymosan-treated human serum. Although soluble and membrane TNFα production was slightly observed in the cells incubated with untreated human serum, this TNFα production appears to be due to the spontaneous activation of the alternative complement pathway in human serum samples.

To explore the temporal change of the TNFα production in day 5 ATRA-treated HL60 cells, we analyzed the amount of TNFα protein in whole cell lysates and culture supernatants from 5 min to 1 h after incubation with zymosan-treated human serum (Fig. 1C). Immunoblot analysis showed that soluble TNFα in culture supernatants and membrane TNFα in whole cell lysates were increased by 1-h incubation with zymosan-treated human serum. In contrast, soluble TNFα in whole cell lysates was slightly increased under the same condition. Furthermore, to explore an extended period of time, we performed the same analysis with prolonged incubation time (Fig. 1D). Immunoblot analysis showed that soluble TNFα in culture supernatants was increased from 2 to 6 h after incubation with zymosan-treated human serum. Following incubation with zymosan-treated human serum, membrane TNFα in whole cell lysates was increased from 2 to 4 h and then declined at 6 h. Interestingly, soluble TNFα in whole cell lysates was increased to its maximum at 4 h and then declined again to a comparable level of 2 h at 6 h. These results suggest that zymosan-treated human serum induces TNFα synthesis followed by TNFα release in neutrophil-like HL60 cells.

3.2. PMNs did not induce TNFα production by incubation with zymosan-treated human serum

To examine whether mature neutrophils produce TNFα, we treated PMNs with zymosan-treated human serum for 4 h (Fig. 1E). Immunoblot analysis showed that the soluble TNFα in culture supernatants and membrane TNFα protein in whole cell lysates of PMNs were not detected. Furthermore, compared to HL60 cells, the soluble TNFα protein level in whole cell lysates of PMNs was not increased 4 h after incubation with zymosan-treated human serum. These results suggest that mature

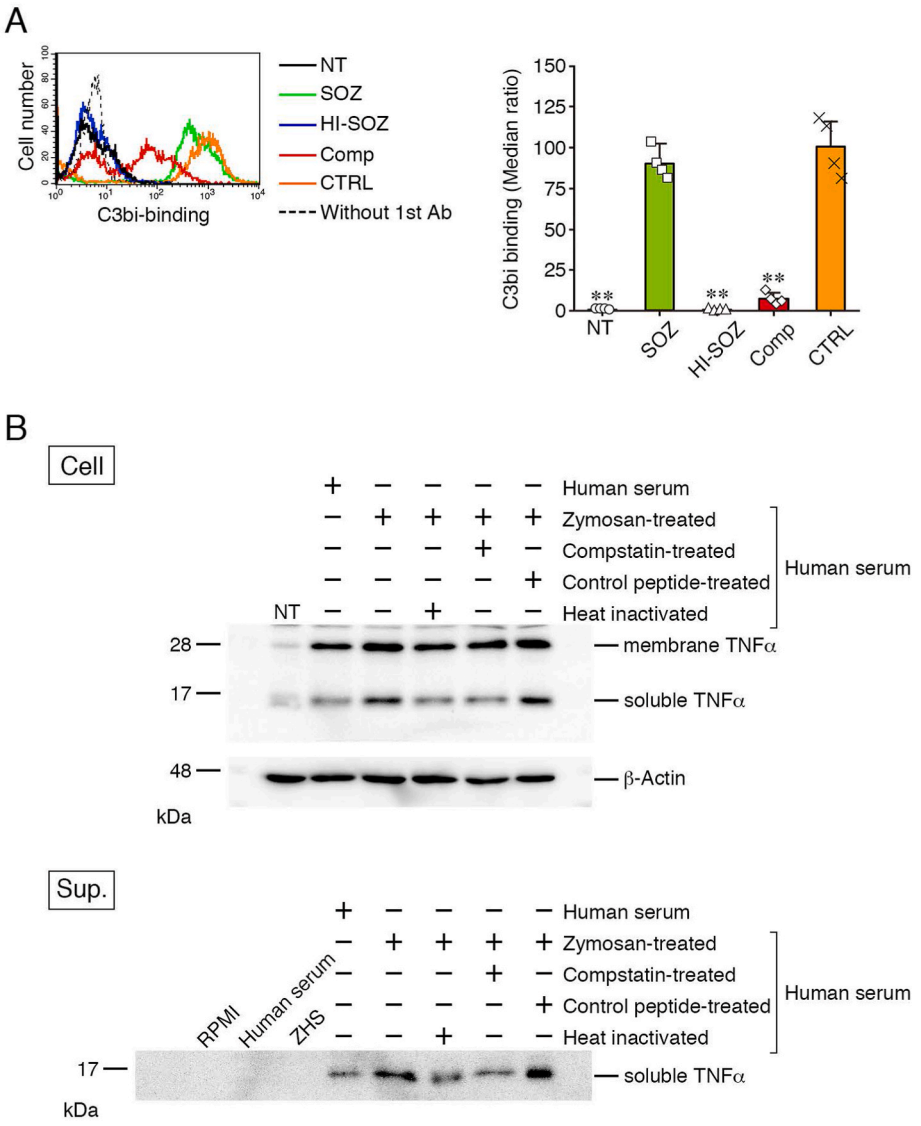

Fig. 2. Complement-mediated TNFα production in neutrophil-like HL60 cells.
(A) Zymosan particles were incubated in human serum that was either non-treated (SOZ) or heat-inactivated (HI-SOZ) or pre-treated with compstatin (Comp) or control peptide (CTRL). Non-treatment (NT) indicates zymosan particles that were not treated with human serum and any reagent. Binding of C3bi to zymosan was analyzed by flow cytometry with anti-C3bi antibody. Representative histograms (left) and bar graph of Median ratio = Median fluorescence intensity for each sample/Median fluorescence intensity for Without 1st Ab (right) at the indicated conditions are presented. Data show the means ± SD derived from 4 independent experiments ($n = 4$). p values were calculated using a two-tailed unpaired Student's t-test. ** <0.01. (B) Day 5 ATRA-treated HL60 cells were incubated with 10% human serum pre-treated under the conditions indicated for 4 h, and the whole cell lysates (Cell) and culture supernatants (Sup.) were then collected. Non-treatment (NT) indicates cells that were not treated with human serum and any reagent. TNFα and β-actin in samples were detected by immunoblot analysis, as shown in Fig. 1. RPMI, Human serum, and ZHS in diagonal letters indicate samples of RPMI-1640 medium, RPMI-1640 containing ZHS, or HS with 1 × SDS-PAGE sample loading buffer, respectively. The blot is a representative of 3 independent experiments.

neutrophils do not produce TNFα after incubation with zymosan-treated human serum.

3.3. Complement pathway is involved in TNFα production in neutrophil-like HL60 cells

To examine whether the complement pathway is required for TNFα production in neutrophil-like HL60 cells, we treated human serum with C3-convertase inhibitor, compstatin, before adding of zymosan. We first tested the inhibitory effect of compstatin on the binding of C3bi to zymosan caused by opsonization with human serum (Fig. 2A). Flow cytometric analysis showed that the binding of C3bi to zymosan was increased by opsonization with non-treated (SOZ) or control peptide-treated (CTRL) human serum. In contrast, the binding of C3bi to zymosan was significantly suppressed by opsonization with compstatin-treated (Comp) or heat inactivated (HI-SOZ) human serum.

Next, we examined the effect of compstatin on zymosan-treated human serum-induced TNFα production in day 5 ATRA-treated HL60 cells (Fig. 2B). Zymosan-treated human serum-induced TNFα production was not changed by treatment with control peptide. However, the TNFα production was suppressed by treatment with compstatin. No differences in TNFα levels were observed among compstatin-treated, heat inactivated, and untreated human serum. These results indicate that the complement pathway is critical for TNFα production in neutrophil-like HL60 cells.

3.4. Intracellular Ca^{2+} regulates zymosan-treated human serum-induced TNFα production

Previous studies have reported that C3a-dependent intracellular Ca^{2+} elevation in human neutrophils [13,14], and Ca^{2+} influx is involved in cytokine production in mouse neutrophils [15,16]. Therefore, we hypothesized that intracellular Ca^{2+} levels control TNFα production in neutrophil-like HL60 cells. We examined the effect of Ca^{2+} chelators and Ca^{2+} elevating agents on zymosan-treated human serum-induced TNFα production in neutrophil-like HL60 cells (Fig. 3). Immunoblot analysis showed that the TNFα production was suppressed by treatment with EGTA or BAPTA-AM. In contrast, the TNFα production was enhanced by treatment with thapsigargin (TG). Furthermore, we confirmed that treatment with TG alone did not induce TNFα production in neutrophil-like HL60 cells (data not shown). These results suggest that intracellular Ca^{2+} elevation potentiates TNFα production by C3a fragments in neutrophil-like HL60 cells.

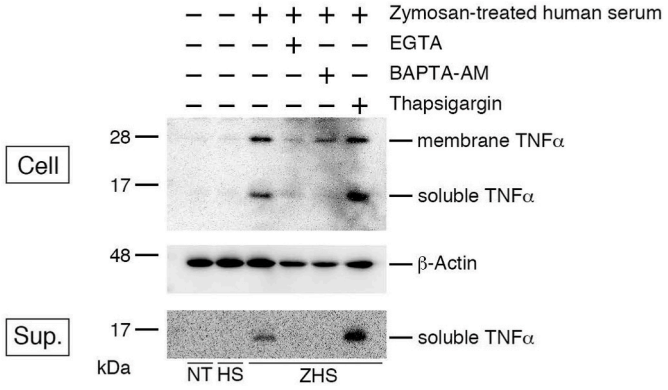

Fig. 3. Effect of intracellular Ca^{2+} levels on complement-mediated TNFα production in neutrophil-like HL60 cells.
Day 5 ATRA-treated HL60 cells pre-incubated in the absence or presence of DMSO, 1 mM EGTA, 10 μM BAPTA-AM, or 10 nM Thapsigargin for 30 min. Following pre-incubation, the cells were either untreated (NT) or incubated with 10% human serum (HS) or zymosan-treated human serum (ZHS) for 4 h. The ZHS-incubated cells were co-incubated with DMSO, 1 mM EGTA, 10 μM BAPTA-AM, or 10 nM Thapsigargin until the end. The whole cell lysates (Cell) and culture supernatants (Sup.) were collected. TNFα and β-actin in samples were detected by immunoblot analysis, as shown in Fig. 1. The blot is a representative of 3 independent experiments.

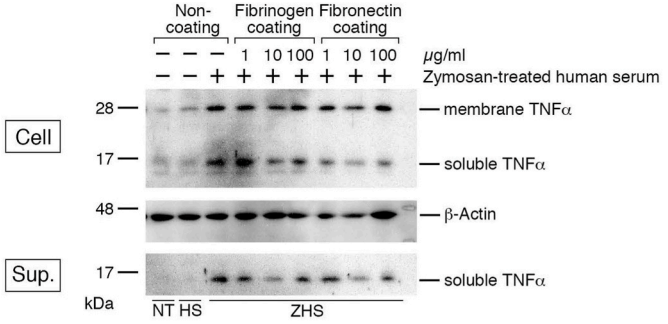

Fig. 4. Effect of interaction with fibrinogen or fibronectin on complement-mediated TNFα production in neutrophil-like HL60 cells.
Day 5 ATRA-treated HL60 cells were either untreated (NT) or incubated with 10% human serum (HS) or zymosan-treated human serum (ZHS) for 4 h on fibrinogen or fibronectin coated-well that indicated concentrations. The whole cell lysates (Cell) and culture supernatants (Sup.) were collected. TNFα and β-actin in samples were detected by immunoblot analysis, as shown in Fig. 1. The blot is a representative of 3 independent experiments.

3.5. Zymosan-treated human serum-induced soluble TNFα production is negatively regulated by immobilized-fibrinogen or -fibronectin

Beta2 integrins are heterodimeric adhesion molecules that comprise CR3 and activate outside-in signaling in leukocytes [17,18]. Several previous studies have reported that β2 integrins of neutrophils interact with fibrinogen or fibronectin, as well as C3bi [19–23]. Therefore, we hypothesized that zymosan-treated human serum-induced TNFα production in neutrophil-like HL60 cells is controlled by interaction with fibrinogen or fibronectin. We examined whether zymosan-treated human serum-induced TNFα production in neutrophil-like HL60 cells is affected by immobilized-fibrinogen or -fibronectin (Fig. 4). Immunoblot analysis showed that the membrane TNFα protein levels in whole cell lysates were not affected in wells coated with fibrinogen or fibronectin at any concentration. In contrast, compared to non-coating wells, soluble TNFα protein levels in whole cell lysates and culture supernatants were suppressed in wells coated with fibrinogen or fibronectin at 10 μg/ml. The interaction of neutrophil-like HL60 cells with fibrinogen or fibronectin alone did not result in TNFα production (data not shown). These results suggest that immobilized-fibrinogen or -fibronectin negatively regulates zymosan-treated human serum-induced TNFα production in neutrophil-like HL60 cells.

4. Discussion

In this study, we concluded that complement components induce TNFα production in neutrophil-like HL60 cells. Previous studies have shown that TNFα produced by immature neutrophils contributes to HSCs development and vascular regeneration in the BM niche [6–9]. Accordingly, our results suggest that complement-dependent TNFα production in immature neutrophils is involved in HSCs development and vascular regeneration in the BM niche.

HL60 cells have been commonly used as a substitute model to study neutrophil functions [24–28]. As shown in our and others' previous studies [12,29], day 5 ATRA-treated HL60 cells exhibit a significant increase in the cell surface expression of CR3. We also found that the cell surface expression of C5aR1 was 'without Ab' level, but C3aR was observed in day 5 ATRA-treated HL60 cells (Fig. 1A). These results suggest that C3a-C3aR and C3bi-CR3 signaling simultaneously regulate neutrophil activity.

We showed that soluble TNFα is increased in the culture supernatant of day 5 ATRA-treated HL60 cells after incubation with zymosan-treated human serum. In contrast, soluble TNFα was not observed in the culture supernatant of the cells after incubation with untreated human serum (Fig. 1B). Our previous studies have used zymosan particles for complement activation in human serum [12,30,31]. Therefore, we considered that the complement pathway is involved in TNFα release from neutrophil-like HL60 cells.

Time course analysis showed that TNFα synthesis and release are maximal at different times (Fig. 1C and D). Interestingly, soluble TNFα is transiently increased in whole cell lysate after incubation with zymosan-treated human serum. This result suggests that soluble TNFα protein may be transiently stored in some kind of intracellular organelles within the cells.

In the present study, we showed that TNFα production is observed in neutrophil-like HL60 cells but not in human PMNs by incubation with zymosan-treated human serum (Fig. 1E). Notably, no studies have implicated TNFα production in neutrophils mediated by complement pathway. Hence, our data represent the first evidence that endogenous complement components induce TNFα production in neutrophils.

The present study showed that the TNFα production is suppressed to a negative control level by either compstatin or heat-inactivation (Fig. 2B). As shown in Fig. 1, ATRA-treated HL60 cells express C3aR. Taken together, it is possible that C3a fragments, which are generated by complement activation in human serum, induce TNFα production in neutrophil-like HL60 cells.

We demonstrate that increased intracellular Ca^{2+} levels are critical for TNFα production in neutrophil-like HL60 cells (Fig. 3). C3aR signaling increases intracellular Ca^{2+} levels mediated by G protein in human neutrophils [13,14]. However, it is unclear how C3aR signaling controls TNFα production in neutrophils. Previous studies have reported that Ca^{2+} influx is required to activate NF-κB and TNFα production in mouse neutrophils [15,16]. Therefore, we speculate that the TNFα production in neutrophil-like HL60 cells is controlled by C3aR signaling with increased intracellular Ca^{2+} levels. We treated the cells with C3aR agonist, [Trp63, 64]-C3a (63–77), the cells exhibited spread morphology, but TNFα production was not observed (data not shown). This contradictory result may be due to differences between the biological activity of endogenous complement components and synthetic agonists. The present study has not directly identified the specific complement component responsible for TNFα production in immature neutrophils. Further studies are required to elucidate the mechanism of TNFα production in immature neutrophils.

In this study, we showed that immobilized-fibrinogen or -fibronectin

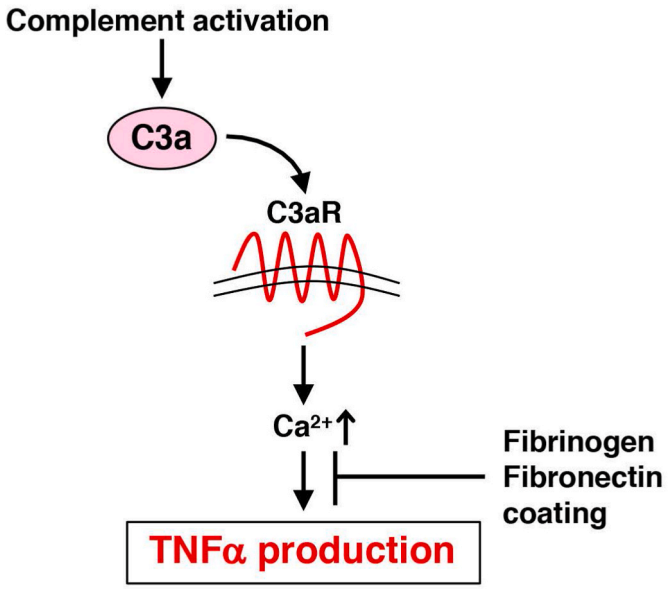

Fig. 5. Schematic diagram of the hypothesis that complement-induced TNFα production in neutrophil-like HL60 cells.
The complement activation product C3a binds to C3aR and induces TNFα production in neutrophil-like HL60 cells. The TNFα production is enhanced by intracellular Ca^{2+} elevation and is negatively regulated by fibrinogen or fibronectin coating.

negatively regulates complement pathway-induced soluble TNFα production in neutrophil-like HL60 cells (Fig. 4). We did not find out why soluble TNFα levels were maximum reduced in wells coated with 10 μg/ml fibrinogen or fibronectin. In the BM niche, β1 integrins expressed in HSCs interact with fibrinogen or fibronectin in the extracellular matrix (ECM) to control maintenance, survival, proliferation, and fate decisions of HSCs [4]. Similarly, β2 integrins expressed on immature neutrophils may interact with their ligands within the BM niche and regulate TNFα production. Thus, we hypothesize that the mechanism by which β2 integrins suppress TNFα production in immature neutrophils may contribute to the appropriate regulation of TNFα supply to the BM niche. Both intracellular Ca^{2+} elevation and β2 integrin signaling activate NF-κB in neutrophils [15,32–34]. The effect of crosstalk between these signaling pathways on TNFα production in immature neutrophils is not yet known. Additional studies need to delineate how β2 integrin signaling regulates TNFα production.

We hypothesized that C3a fragments generated in the bone marrow induce TNFα production by immature neutrophils. Previous studies have shown that TNFα inhibits stromal cell-derived factor-1 (SDF-1, CXCL12) production in bone stromal cells [35], and SDF-1 contributes to migration, adhesion, and maintenance of HCSs [36,37]. These findings suggest that C3a fragments may indirectly regulate the dynamics of HSCs in the BM niche by inducing TNFα production in immature neutrophils. Immature neutrophils are regulated by a variety of factors within the bone marrow. Therefore, there are limitations to reconstruct the bone marrow environment, and to examine the regulatory mechanisms of TNFα production in immature neutrophils. In the present study, we found that the complement pathway induced-TNFα production in immature neutrophils.

5. Conclusion

This study has shown that TNFα production in neutrophil-like HL60 cells is induced by complement activation. Furthermore, the TNFα production is enhanced by intracellular Ca^{2+} elevation and is negatively regulated by fibrinogen or fibronectin coating. (Fig. 5).

Authorship contribution statement

Hiroyuki Tabata: Investigation, writing - original draft, and writing - review & editing. **Hiroyuki Morita:** Investigation. **Kenichi Kouyama:** Investigation. **Yumi Tohyama:** Conceptualization, writing - review & editing, project administration, supervision, and funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported in part by Grant-in-Aid for Scientific Research (KAKENHI; 18K07432) from Japan Society for the Promotion of Science.

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Abbreviations

ATRA: all-*trans*-retinoic acid
BM: bone marrow
C3aR: C3a receptor
C5aR: C5a receptor
CMPs: common myeloid progenitors
CR3: complement receptor 3
FCS: fetal calf serum
GMP: granulocyte-monocyte progenitors
HSCs: hematopoietic stem cells
MPPs: multipotent progenitors
NF-κB: nuclear factor kappa B
PMNs: polymorphonuclear cells
TBS: Tris-buffered saline
TG: thapsigargin
TNFα: tumor necrosis factor α