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Kindlin3-dependent CD11b/CD18-integrin activation is required for potentiation of neutrophil cytotoxicity by CD47-SIRPα checkpoint disruption

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Abstract

The CD47-signal regulatory protein-alpha (SIRPα) immune checkpoint constitutes a therapeutic target in cancer, and initial clinical studies using inhibitors of CD47-SIRPα interactions in combination with tumor-targeting antibodies show promising results. Blockade of CD47-SIRPα interaction can promote neutrophil antibody-dependent cellular cytotoxicity (ADCC) towards antibody-opsonized targets. Neutrophils induce killing of antibody-opsonized tumor cells by a process identified as trogoptosis, a necrotic/lytic type of cancer cell death that involves trogocytosis, the antibody-mediated endocytic acquisition of cancer membrane fragments by neutrophils. Both trogocytosis and killing strictly depend on CD11b/CD18-(Mac-1)-mediated neutrophil-cancer cell conjugate formation, but the mechanism by which CD47-SIRPα checkpoint disruption promotes cytotoxicity has remained elusive. Here, by using neutrophils from Leukocyte Adhesion Deficiency type-III patients (LAD3) carrying *FERMT3* gene mutations, hence lacking the integrin-associated protein kindlin3, we demonstrated that CD47-SIRPα signaling controlled the inside-out activation of the neutrophil CD11b/CD18 integrin and cytotoxic synapse formation in a kindlin3-dependent fashion. Our findings also revealed a role for kindlin3 in trogocytosis and an absolute requirement in the killing process, which involved direct interactions between kindlin3 and CD18-integrin. Collectively, these results identified a dual role for kindlin3 in neutrophil ADCC and provides mechanistic insights into the way neutrophil cytotoxicity is governed by CD47-SIRPα interactions.

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Introduction

The "don't-eat-me" signal CD47 and its receptor, the primarily myeloid cell-restricted inhibitory receptor SIRPα, have been established as an immune checkpoint and *bona fide* therapeutic target for cancer immunotherapy and other indications [1]–[4]. Initial clinical studies using blocking antibodies against CD47 have shown a good safety profile in both solid and hematologic cancers and promising efficacy in combination with cancer-targeting monoclonal antibodies such as rituximab [5][6]. Disruption of CD47-SIRPα interactions is known to act at multiple levels, promoting both adaptive and innate anti-cancer immunity [3]. The latter includes both macrophage-mediated antibody-dependent cellular phagocytosis (ADCP) as well as neutrophil-mediated antibody-dependent cellular cytotoxicity (ADCC)[7][8]. We have established that neutrophil ADCC occurs by a unique mechanism involving trogocytosis, leading to the lytic/necrotic (i.e. "trogoptotic") killing of target cells [7]. Induction of trogoptosis by neutrophils exclusively occurs in the presence of cancer-opsonizing antibodies and requires signaling via Fc-receptors [7][9]–[11]. It strictly depends on cell-cell interactions between neutrophils and tumor cells mediated by the neutrophil β_2-integrin CD11b/CD18, as illustrated by the absence of ADCC by neutrophils from Leukocyte Adhesion Deficiency type I (LAD1) patients and blocking studies with CD11b/CD18 antibodies [7][9][12]. Notably, this CD11b/CD18-integrin dependence is observed both in presence and absence of CD47-SIRPα interactions. Nevertheless, the mechanism(s) by which the CD47-SIRPα checkpoint controls phagocyte, and particularly neutrophil effector function, have remained elusive.

Generally, integrins occur in either an inactive or active state, which involves conformational changes in the extracellular ligand binding domain of the integrin, which affects ligand affinity [13][14]. Integrin activation can be triggered by extracellular stimuli, such as chemoattractants or Fc-receptor signaling [15]. Activation of leukocyte β2-integrins involves the successive binding of talin1 and kindlin3 to the cytoplasmic domain of the CD18-integrin chain, which causes integrin activation and allows association of the integrin with the cytoskeleton [14]. LAD3 patients have mutations in *FERMT3* gene, encoding kindlin3 protein, and generally lack kindlin3 expression [16][17]. Although the mechanisms of integrin activation have been relatively well-established, much less is known about the mechanisms by which integrin activation is controlled [14][18]. Here, we showed how signaling downstream of the CD47-SIRPα axis in neutrophils restricted CD18-integrin activation in a kindlin3-

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dependent manner during neutrophil-tumor cell interactions, while demonstrating a critical role for kindlin3 in neutrophil ADCC.

Materials & Methods

Neutrophil isolation

Neutrophils were isolated from either 5 LAD3 patients [16][17] with confirmed mutations in *FERMT3* or healthy donors. Blood was drawn after written informed consent had been obtained from all patients. The study was approved by the local ethical committee of Sanquin Blood Supply (Amsterdam, The Netherlands), and all experiments involving human blood samples were conducted in accordance to the 1964 Declaration of Helsinki. Blood was collected and transferred at room temperature, and upon arrival (within 24 hours) steps of neutrophil isolation were followed as previously described [19], by density-gradient centrifugation with isotonic Percoll (GE Healthcare; Cat. 17-0891-09) and erythrocyte lysis [19]. The purity of the neutrophils was >95%. After isolation, $5x10^6$ neutrophils/mL were diluted in HEPES medium (7.7 g NACl; Fagron, 4.75 g Hepes; Sigma Aldrich, 450 mg KCl; Merck, 250 mg $MgSO_4$; Merck, 275 mg K_2HPO_4; Merck, H_20; Gibco, pH 7.4 with 10 M NaOH) supplemented with albumin (200 µg/mL; Sanquin Pharmaceuticals), glucose (1 mg/mL; Merck), and calcium (1 M; Cat 208291; Calbiotech), filtered through a 0.2 µm filter, and stimulated either for 4 hours or overnight with recombinant human interferon-γ (IFNγ, 50 ng/mL; Peprotech) and recombinant granulocyte-colony stimulating factor (G-CSF, 10ng/mL; Peprotech).

In vitro culture

All cell lines (NB4, SKBR3, A431 and Jurkat T cells) were obtained between 2011 and 2018 (ATCC) and cultured for up to 3 months. Cell lines tested negative for *Mycoplasma* using PCR. The used cell lines and their derivatives were authenticated by flow cytometry using specific markers. NB4 (undifferentiated/differentiated): CD11b, SIRPalpha and FcgR; SKBR3: Her2 and CD47; A431: EGFR and CD47; Jurkat: CD3 and SIRPgamma) as described below, and this was repeated for the most commonly used cell lines (i.e. NB4 and SKBR3) at least every 2 months. In NB4 cells, kindlin-3 was monitored using Western blotting as described below. The time between transduction and freezing was the following:

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NB4 CD18KO: 2 months; Kindlin3KO: 2 months; SIRPαKO: 1.5 months; Kindlin3WT: 2.5 weeks; Kindlin3 W596: 2.5 weeks; SKBR3 CD47KD: 2.5 months.

NB4 is a maturation-inducible cell line derived from an acute promyelocytic leukemia patient [20]. NB4 cells were maintained with IMDM medium (Thermo Fisher Scientific) supplemented with 20% (v/v) fetal bovine serum (FBS), penicillin (100 U/mL; Sigma Aldrich), streptomycin (100 µg/mL; Sigma Aldrich), and L-glutamine (2 mM; Sigma Aldrich), maintained in 5% CO_2 at 37^{o}C and passed twice a week in a 1:10 concentration. NB4 cells were genetically modified by using the CRISPR-Cas 9 genome editing technique, as described below.

SKBR3 is a HER2/neu positive human breast cancer cell line, which we have genetically modified by lentiviral transduction with pLKO.1puro vector (Sigma Aldrich) with a shRNA empty vector as a control (Scr) or shRNA (Sigma Aldrich), resulting in 80- 85% knockdown of CD47 protein (CD47KD)[7], [19]. SKBR3 cells were maintained in IMDM medium supplemented with 20% (v/v) FBS, penicillin (100 U/mL), streptomycin (100 µg/mL), and 2 mM L-glutamine in 5% CO_2, 37^{o}C.

NB4 kindlin3 knockout cells (kindlin3KO)[20] were generated by lentiviral transduction of NB4 cells with pLentiCrispRv2-kindlin3 gRNA. A kindlin3 knockout clone was generated using gRNA sequence 5'gtcactggggagtcgcacat 3'. A CD18 knockout clone was generated using gRNA sequence 5' ctgccgggaatgcatcgagt 3'. A SIRPα knockout clone was generated using gRNA sequence 5' ccgcggcccatggagcccgc 3'. Transduced cells were selected with puromycin (1 µg/mL; Invivogen) and subsequently put on limiting dilution. A total of 2.5x10^{6} cells for each clone were differentiated with all-trans retinoic acid (ATRA; 5 µM; Sigma Aldrich) and examined for kindlin3, CD18, or SIRPα expression and function as indicated below. Kindlin3, CD18, and SIRPα deficiencies were routinely tested by Western blotting (kindlin3) and /or flow cytometry (CD18, SIRPα).

To mimic neutrophil function, 0.5x10^{6} NB4 cells per mL (final volume 5 mL) were stimulated with 5 µM ATRA for 7 days. For reconstitution, the kindlin3 sequence (NM_031471.5) was cloned into pENTR-d-TOPO (Thermo Fisher Scientific). In this construct, using QuickChange Site-Directed Mutagenesis (Agilent Technologies), we modified the gRNA target sequence to 5'gtaacaggcgaaagtcatat 3' and a W596A substitution was also introduced. The constructs were sequence verified. Recombination of these plasmids with pRRL PPT SFFV prester SIN - GFP-Gateway Cassette using LR Clonase II (Thermo Fischer Scientific)

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generated lentiviral GFP-kindlin3 WT/W596A fusion vectors, which were used for lentiviral transduction of NB4 kindlin3 knockout cells. Cells positive for GFP were selected by sorting and confirmed by Western blot. Cells were regularly tested for GFP expression by flow cytometry.

A431 cells were cultured in IMDM supplemented with 20% (v/v) FBS, penicillin (100 U/mL), streptomycin (100 µg/mL), and 2 mM L-glutamine, maintained in 5% CO_2 at 37^{o}C. Jurkat T cells were cultured in RPMI (Thermo Fisher Scientific) supplemented with 10% (v/v) FBS, penicillin (100U/mL), streptomycin (100 µg/mL), and 2 mM L-glutamine, maintained in 5% CO_2 at 37^{o}C.

Antibody-dependent cellular cytotoxicity (ADCC)

Cytotoxic capacity of neutrophils or NB4 cells was evaluated as previously described [7]. In brief, target cells were labelled for 90 minutes with 100 µCi ^{51}Cr (Perkin-Elmer) in their culture medium (described above) and finally diluted to $0.1x10^6$ cells /mL. Neutrophils or NB4 cells ($5x10^6$ per mL diluted in HEPES+ medium, as described above) were pre-incubated with blocking anti-SIRPα (10 µg/mL, clone 12C4) where indicated [7]. Effector and target cells were incubated for 4 hours in a 50:1 ratio (final volume 100 µL). Cytotoxicity was corrected for spontaneous release, normalized to 100% release by either 5% saponin (CAS 8047-15-2; Calbiochem) or 0.1% triton (TX-100; Sigma Aldrich) incubation and assessed by the quantification of ^{51}Cr release into the supernatant (cytotoxicity % = (experimental value - spontaneous release)/ (maximum release - spontaneous release) x 100%) in a gamma counter (Wallac) or a microbeta2 reader (Perkin-Elmer).

Blocking antibodies

Blocking of the CD11b/CD18-integrin was achieved by using either anti-CD18 (IB4 clone; ATCC) or anti-CD18 F(ab')2 (AnCell) where indicated. Effector cells (i.e. neutrophils or NB4 cells) were pre-incubated with 10 µg/mL of each antibody for 10 minutes at room temperature. Afterwards, cells were incubated with target cells as described above. ICAM-1, 2, 3 antagonists were used prior to neutrophil ADCC (clone numbers described below). Tumor cells were pre-incubated with 20 µg/mL for 5 minutes at room temperature.

Conjugate formation

4-hour IFNγ/G-CSF–stimulated neutrophils and tumor cells ($5x10^6$ and $1x10^6$) were labelled for 30 minutes with Cell Trace calcein violet-AM fluorescent dye (Invitrogen, Grand Island,

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NY, USA) and CMTPX/cell-tracker red fluorescent dye (Invitrogen), respectively, as described before [7]. Where indicated, cells were pre-incubated with either intact (ATCC) or F(ab')2 IB4 clone antibodies against β2 integrin (AnCell) at a 10 μg/mL concentration. An ImageStreamX flow cytometer (Amnis Corporation, Seattle, WA, USA) was used for acquisition of the samples, and double-positive events, which indicated conjugate formation, were quantified and analyzed by using IDEAS® data analysis software (Amnis).

Flow cytometry for trogocytosis

The identification of neutrophils or NB4 cells having trogocytosed tumor cell membrane has been thoroughly described [7]. Briefly, target cells were labelled with lypophilic membrane dye DiD or DiO (5 μM, Invitrogen) for 30 minutes at 37^{o}C, and where indicated, cells were pre-incubated for 5 minutes with trastuzumab (10 μg/mL; Roche). Accordingly, effector cells were pre-incubated with blocking anti-SIRPα and either intact (ATCC) or F(ab')2 IB4 clone antibodies against β_2 integrin (AnCell) at a 10 μg/mL concentration. The percentage of tumor cell membrane trogocytosed by the effector cells was assessed using an LSRII flow cytometer (BD Biosciences). Gating strategy was performed using DIVA software analysis, as previously described [7].

Flow cytometry for integrin activation

Neutrophils and tumor cells were incubated (effector to target ratio 5:1) for 1 hour at 37 ^{o}C in absence or presence of 10 μg/mLtrastuzumab and/or 10 μg/mL SIRPα antagonist. Cells were then kept on ice and stained with a human antibody against the activated epitope of CD18 (clone mAb 24; Hycult-Biotech) or an antibody against total integrin expression (clone IB4; ATCC). Goat anti-mouse F(ab')2 Alexa Fluor 633 (Thermofisher) was used as a secondary antibody. Each incubation lasted 20 minutes and was performed on ice. After washing, cells were resuspended in 100 μL of HEPES+ medium (mentioned above), and fluorescence was measured on BD FACsCanto^{TM}II. The ratio of activated to total integrin expression was calculated using DIVA software analysis.

Flow cytometry for surface expression

Expression of different surface markers on neutrophils or NB4 cells was determined by flow cytometry. NB4 cells were labelled with FITC-labelled monoclonal antibodies against: CD16 (clone 3G8; BD Pharmingen), CD32 (clone AT10; BioRAD), CD64 (clone 10.1; BioRAD), CD11b (clone CLB-mon-gran/1-B2; Sanquin Pharmaceuticals), or SIRPα (12C4)[8]. Goat anti-mouse F(ab')2 Alexa Fluor 633 (Thermofisher) was used for the secondary staining.

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Neutrophils, NB4, and Jurkat T cells were stained with a PE-labelled monoclonal antibody against SIRPγ (clone LSB2.20; BioLegend). SKBR3 Scr, CD47KD, or A431 cells were stained with monoclonal antibodies against ICAM-1 (clone 15.2; Sigma-Aldrich), ICAM-2 (clone CBR-IC2/2; Thermofisher), and ICAM-3 (clone CBR-IC3/1; BioLegend). Goat anti-mouse F(ab')2 Alexa Fluor 633 antibody (Thermofisher) was used as secondary antibody where needed. Each incubation lasted 20 minutes and was performed on ice. After thorough wash, cells were resuspended in 100 µl of HEPES+, and fluorescence was measured on BD FACsCanto^{TM}II and analyzed using DIVA software.

Western blotting

$5x10^6$ neutrophils or NB4 cells were washed with ice-cold PBS and incubated with 1 µL of serine protease inhibitor diisopropyl fluorophosphate (DFP; Sigma science) for 10 minutes on ice. Afterwards, cells were centrifuged up to 14000 rpm, and pellets were resuspended in 50 µL cOmplete™ Protease Inhibitor Cocktail (Roche diagnostics, Mannheim, Germany)/EDTA (0.45 M) solution and 50 µL of 2x sample buffer (25mL Tris B, Invitrogen; 20mL 100% glycerol, Sigma Aldrich; 5g SDS, Serva; 1.54g DTT, Sigma Aldrich, St Louis, Mo; 20mg bromophenol blue, Sigma Aldrich; 1.7mL β-mercapro-ethanol, BioRad; H_20 to 50mL, Gibco) for 30 minutes at 95^{o}C with vortexing every 10 minutes. Samples equivalent to $1x10^6$ cells were loaded on 10% SDS-page gels for electrophoresis and ran at 80-120V. Proteins were transferred to a nitrocellulose membrane (GE Healthcare Life Science). The membrane was blocked with 5% ELK (Campina, Amsterdam, The Netherlands) and further stained with antibodies diluted in 2.5% ELK. For kindlin3 detection, rabbit anti-kindlin3 (1:1000 diluted in 3 mL of 2.5% ELK; a kind gift of Prof. Dr. Reinhard Fässler, Max Planck Institute of Biochemistry, Martinsried, Germany) [16] and horseradish peroxidase–coupled goat anti-rabbit HRP-conjugated antibody (4.5 µg diluted in 3 mL of 2.5% ELK; GE Healthcare UK Limited) were used, and protein expression was visualized after incubation with Super Signal West Dura substrate (Thermo Scientific) in a medical film processor (SRX-101A). Loading control was assessed by using mouse anti-GAPDH (1.5 µg diluted in 3 mL of 2.5% ELK; Millipore) and donkey anti-mouse-IgG IRDye 800 (LI-COR) suitable for analysis using Odyssey (LI-COR Biosciences, Lincoln, NE, USA).

NADPH oxidase activity

The release of hydrogen peroxide was measured by using the Amplex Red kit (Molecular probes), as earlier described[19]. $0.25x10^6$ cells were stimulated with *E.coli* (OD625=0.2, strain ML-35; $0.25x10^9$ per mL) in the presence of 2 mM azide, platelet-activation factor

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(PAF; 100 nM; Sigma-Aldrich)/N-formylmethionine-leucyl-phenylalanine (fMLP; 30nM; Sigma Aldrich, phorbol 12-myrisatate 13-acetate (PMA)(100 ng/mL, Sigma Aldrich), serum-treated zymosan (STZ)[21], or unopsonized zymosan (1mg/mL; MP Biomedicals) for 30 minutes at 37^{o}C. Every 30 seconds, fluorescence was assessed with a Genios plate reader (Tecan). Maximal slope of H_2O_2 release was measured at 2-minute intervals at an excitation wavelength of 535 nm and an emission wavelength of 595 nm. Analysis is shown as the maximal slope in relative fluorescence units/minute (RFU/min).

Adhesion

An adhesion assay was performed as previously described [19]. In short, 5x10^6 cells/mL were incubated with calcein-AM (1 µM; Molecular Probes, Eugene, OR, USA) for 30 minutes at 37^{o}C. Adhesion was measured in an uncoated 96-well MaxiSorp plate (Nunc, Wiesbaden, Germany). 2x10^5 calcein-labelled cells were stimulated with PMA (100 ng/mL; Sigma Aldrich), dithiothreitol ([DTT]; 10 mmol/L; Sigma Aldrich, St Louis, Mo), Pam3Cys (20 mg/mL; EMC Microcollections), C5a (10 nmol/L; Sigma Aldrich), and TNFα (10 ng/mL; Peprotech) and incubated for 30 minutes at 37 ^{o}C, 5% CO_2 in an uncoated Maxisorp plate (Nunc). Adhesiveness of the cells was determined in a Genios plate reader after lysis in 0.5% (w/v) Triton X-100 (Sigma Aldrich) for 5 minutes at room temperature at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Statistics

Statistical significance was assessed with GraphPad Prism version 8.0 (GraphPad software, San Diego, CA, USA). Data were evaluated by one-way ANOVA or t-test, and significance was set at $p<0.05$ for all comparisons. Where indicated, correction for multiple comparisons using the methods of Dunnet or Sidak was performed.

Results

Our previously reported findings show that neutrophil ADCC towards cancer cells is dependent on CD11b/CD18-integrin, both in presence or absence of CD47-SIRPα interactions [7][8]. By using a monoclonal antibody against an activation epitope of CD18, we evaluated the ratio of activated-to-total integrin expression in the context of neutrophil ADCC. We observed that integrin activation is enhanced when CD47-SIRPα interactions are

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disrupted (Fig. 1A), whereas total integrin expression remains unaltered (Suppl. Fig. S1A). The conformational change of the integrins from the inactive to the active high-affinity ligand binding state is, at least in hematopoietic cells, mediated primarily by two cytosolic proteins, talin1 and kindlin3 [13]. To dissect the mechanism by which SIRPα downstream signaling limited integrin activation, we used neutrophils from LAD3 patients, which specifically lack kindlin3 expression but have normal CD11b/CD18 expression (Suppl. Table S1, Suppl. Fig. S1B-C)[16]. We used imaging flow cytometry to measure the percentage of conjugate formation between neutrophils and trastuzumab (Tmab)-opsonized SKBR3 breast cancer target cells [7]. Antibody opsonization of target cells triggered conjugate formation to some extent, as indicated previously [7], and this was further promoted by disruption of CD47-SIRPα interactions (Fig. 1B). Both of these effects were CD11b/CD18-dependent under all conditions tested, as conjugate formation was abrogated after incubation with blocking anti-CD11b and/or anti-CD18 (Fig. 1B). In neutrophils from LAD3 patients, the enhancement of conjugate formation in the absence of SIRPα downstream signaling (using SKBR3-CD47 knock down cells (SKBR3-CD47KD) as target cells) was not observed (Fig. 1B), although a normal level of conjugate formation occurred in the presence of functional CD47-SIRPα interactions in CD47-expressing SKBR3 cells (Fig. 1B). As we have previously described[7][8], disruption of CD47-SIRPα interaction by either blocking antibodies against CD47 or SIRPα [8], or the use of SKBR3-CD47KD cells [7] results in enhancement of trogocytosis and subsequently ADCC. In contrast, the lack of kindlin3 led to significantly decreased trogocytosis (Fig. 1C), and subsequent neutrophil cytotoxicity was abolished in LAD3 patients or by use of CD11b/CD18 blocking antibodies (Fig. 1D, Suppl. Fig. S1D). Collectively, the effect on conjugate formation indicated that signaling downstream of SIRPα controlled integrin activation in a kindlin3-dependent manner. Overall, these data demonstrated a dual role for kindlin3 in neutrophil ADCC: i) mediating the enhanced effector-target cell conjugate formation upon SIRPα-CD47 disruption and ii) showing a requirement for killing.

In order to confirm the above studies performed in freshly isolated human neutrophils, which are short-lived and therefore cannot be manipulated by standard genome editing methods, we explored whether promyelocytic NB4 cells differentiated into neutrophilic cells with ATRA *in vitro* could provide a valuable alternative for studying the role of kindlin3 in neutrophil function [20]. First, we evaluated NB4 wild-type (NB4 WT) cell cytotoxicity against SKBR3 Scr or CD47KD cells and assessed the role of CD47-SIRPα interaction (Suppl. Fig. S2A). We

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showed that like neutrophils, ATRA-differentiated NB4 cells can efficiently kill antibody-opsonized tumor cells in absence of CD47-SIRPα interactions. Blockade of the SIRPα receptor or absence of CD47 ligand resulted in similar cytotoxicity, whereas addition of a SIRPα antagonist did not further enhance ADCC against CD47KD cells. By generating SIRPα knockout (SIRPαKO) cells, we confirmed that absence of either SIRPα on NB4 effector cells or CD47 on SKBR3 tumor cells showed similar ADCC responses (Suppl. Fig. S2B). By confirming the absence of expression of SIRPγ [22][23] we excluded a role for the only other known CD47-binding SIRP family member in this context (Suppl. Fig. S2C).

We then created NB4 cells lacking kindlin3 or CD18 protein. First, we confirmed the LAD3-like phenotype in NB4 kindlin3 knockout (kindlin3KO) cells (Fig. 2A), which is characterized by impairments in adhesion and respiratory burst [16][17](Fig. 2B-C). Both LAD3 neutrophils and NB4 kindlin3KO cells showed a comparable defect in CD11b/CD18-dependent adhesion in response to various stimuli known to be dependent on inside-out integrin activation [16][17], including the TLR2 agonist Pam3Cys, TNFα, and PMA(Fig. 2B). No differences were observed by direct extracellular activation of integrins by DTT, indicating that integrin expression and function were unaltered. Similarly, these cells were unable to induce an oxidative burst in response to unopsonized zymosan as reported before [16][17](Fig. 2C). Whereas the other stimuli tested did not show differences in oxidative burst between WT and kindlin3-deficient cells, a slight enhancement of the response was seen with PAF/fMLP in NB4 cells. We then evaluated NB4 kindlin3KO cells in ADCC and trogocytosis assays, and the results recapitulated the findings observed in LAD3 patient neutrophils. Specifically, an impaired trogocytosis response was detected, with CD18-dependent residual trogocytosis still remaining (Fig. 2D, Suppl. Fig. S2D) and a complete defect in cytotoxicity (Fig. 2E), as seen in LAD3 patient neutrophils (Fig. 1B-D).

Kindlin3 interacts via its F3 domain with the cytoplasmic tail of CD18, which is known to be critically dependent on the kindlin3 W596 residue [24]. To investigate whether such direct kindlin3-CD18 interactions during neutrophil ADCC were required for the observed effects, we re-introduced WT kindlin3 or a kindlin3 W596A mutant (Fig. 2A) into NB4 kindlin3KO cells and confirmed efficient cell differentiation by evaluating the expression of various maturation markers (Suppl. Fig. S3A-B). Again, first we evaluated the effect of W596 mutation on the established functions impaired in neutrophils kindlin3 deficiency. Whereas WT kindlin3 reconstituted the adhesion and NADPH oxidase activity defects in NB4 kindlin3KO cells, no restoration was seen with the kindlin3 W596A mutant-expressing NB4

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cells (Suppl. Fig. S3C-D). Next, we evaluated the kindlin3 W596 mutant cells for their trogocytic capacity, which appeared almost exclusively effective when CD47-SIRPα interactions were disrupted. Disruption of the kindlin3-CD18 interaction significantly impaired trogocytosis of antibody-opsonized target cells in absence of CD47-SIRPα interactions (Fig. 3A, Suppl. Fig. S3E). Similar to the findings in LAD3 patient neutrophils and in NB4 kindlin3KO cells, ADCC towards cancer cells was abolished when kindlin3 was unable to bind CD18 (Fig. 3B).

In an effort to identify possible ligands of CD18 on SKBR3 cells, we considered ICAM-1, -2, and -3 as possible candidates, and we explored the surface expression of each using flow cytometry. We used SKBR3 Scr, CD47KD, or A431 cells as control, and our preliminary exploration identified only ICAM-1 on SKBR3 cells, with no changes in expression upon CD47 knockdown in these cells (Suppl. Fig. S4A). Blocking of neither ICAMs during neutrophil ADCC significantly decreased killing efficacy, suggesting that ICAM-1 was not the principal ligand for CD11b/CD18 during killing (Suppl. Fig. S4B).

In conclusion, these data suggest that kindlin3 binding to the CD18 cytoplasmic tail positively regulated integrin activation and therefore mediated neutrophil trogocytosis and ADCC in the absence of CD47-SIRPα interactions.

Discussion

Although the CD47-SIRPα checkpoint is now an established target in cancer immunotherapy [1], detailed insights with respect to the underlying mechanisms by which CD47-SIRPα interactions restrict antitumor immunity have remained limited. Our current findings provide compelling evidence, based on experiments with kindlin3-deficient neutrophils from LAD3 patients, that CD47-SIRPα interactions controlled integrin activation and CD11b/CD18-integrin-dependent neutrophil-tumor conjugate (‘cytotoxic synapse’) formation during neutrophil ADCC, which could also explain the enhanced destruction of antibody-opsonized tumor cells upon CD47-SIRPα checkpoint disruption (Fig. 4A-B). Our findings provide evidence for an essential role of kindlin3 in trogocytosis and killing during the process, which are both strictly CD11b/CD18 integrin-dependent. This indicated a dual role of kindlin3 in neutrophil ADCC. On one hand, kindlin3 was required for the enhanced conjugate formation between neutrophils and cancer cells that was selectively observed upon disruption of CD47-SIRPα interactions, but kindlin3 was clearly dispensable for conjugate formation under

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conditions where such interactions were intact. On the other hand, there appeared to be an absolute requirement for kindlin3 in the killing process, which occurred both in the absence and presence of CD47-SIRPα interactions. The latter was also shown to be dependent on the previously established interaction of the kindlin3 *FERM* domain and the critical W596 residue therein with the cytoplasmic region of the integrin CD18-chain [24]. It seems likely that the enhanced conjugation was primarily driven by the kindlin3-dependent change in integrin ligand binding capacity on neutrophils, which was controlled by inhibitory signaling downstream of SIRPα. We are further investigating this in more detail. Regardless, the data clearly suggest that SIRPα, and potentially signaling by other inhibitory immunoreceptors on neutrophils, may negatively affect integrin activation, which, to our knowledge, has not been demonstrated before [18]. A study recently reported [25] has indicated that the CD47-SIRPα checkpoint may also control the macrophage CD11b/CD18 integrin, although it is not clear whether that mechanism also involves kindlin3 as it does in neutrophils.

CD11b/CD18 is known as a promiscuous integrin family member that interacts with a range of endogenous and pathogenic ligands [26][27]. At present, the putative ligand(s) for CD11b/CD18-integrin on tumor target cells has not been established. Preliminary investigation of candidate-cellular CD11b/CD18-ligands on SKBR3 cells showed that none of the ICAM ligands (ICAM-1, -2, and -3) were significantly crucial for neutrophil ADCC, although more studies are needed to shed light on this interaction.

For the moment, we can only speculate about the strict requirement of kindlin3 for trogocytosis and killing. First, both trogocytosis and the lytic/necrotic killing of tumor cells (i.e. trogoptosis) by neutrophils is a mechanical process that requires proper anchorage to the target cell and active pulling forces to be generated by the neutrophils. It seems reasonable to assume that only properly activated integrins can provide optimal anchorage to the tumor, thereby explaining, at least in part, the kindlin3-dependence of these processes. Neutrophil trogocytosis and trogoptosis induction likewise require an efficient linkage of the CD11b/CD18 to the cytoskeleton in order to relay the forces of actino-myosin contraction that would govern these processes within neutrophils. Kindlin3 may perhaps additionally act to stabilize integrin-actin cytoskeletal interactions instrumental in this. Kindlins are known to act as a hub for a variety of proteins, including paxillin and those of the Arp2/3 complex [14]. The exact contributions of these and other relevant components will have to be uncovered in future studies.

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It deserves to be highlighted that apart from these mechanistic insights, our work also described an assay based on the use of neutrophil-like differentiated human promyelocytic leukemic NB4 cells [20][28], which can be used to study, via CRISPR-Cas9 gene editing [29], the mechanistic aspects of neutrophil ADCC, including both trogocytosis and trogoptosis induction in cancer cells and particularly their modulation by inhibition of CD47-SIRPα interactions and potentially other relevant innate immune checkpoints. This is particularly useful as there is no way to genetically manipulate fresh human short-lived neutrophils.

Clearly, the physiological relevance of our findings can ultimately only be established by *in vivo* tumor models using mice in which kindlin3 is deleted or mutated, preferably in a neutrophil-specific fashion. However, the extravasation capacity of the neutrophils from such mice will be affected as well, as unequivocally reported previously [30]. It will probably be difficult to clearly associate any of the observed effects to the alterations in the process of cytotoxicity that we reported here.

Based on the collective current results and the existing literature described, an overall picture emerges related to the role of the CD11b/CD18 and its control by CD47-SIRPα interactions during neutrophil ADCC (Fig. 4A-B). Initial recognition of antibody-opsonized target cells by neutrophil Fc-receptors results in an intermediate cell-cell adhesion to the tumor cells that is largely mediated by inactive or incompletely active CD11b/CD18. This incomplete integrin activity is maintained by inhibitory signaling via the CD47-SIRPα axis that limits kindlin3 function, thereby preventing full integrin activation. Consequently, upon prevention of CD47-SIRPα interactions by appropriate checkpoint blockers, increased integrin activation occurs resulting in enhanced interaction with the cancer target cells and ultimately trogocytosis and killing. At the same time kindlin3 plays an essential role in maintaining integrin activation and/or anchorage of CD11b/CD18-integrin to the cytoskeleton and is therefore essential to both processes that lead to the destruction of the cancer cells.

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Figure legends

Figure 1. CD47-SIRPα checkpoint blockade controls neutrophil integrin activation, conjugate formation, trogocytosis and killing during ADCC in a kindlin3-dependent manner. A. Neutrophil CD11b/CD18 integrin-activation evaluated by flow cytometry. Healthy neutrophils pre-cultured for 4h with G-CSF and IFNγ were incubated for 1h with SKBR3 control or CD47KD cells in presence or absence of trastuzumab (Tmab) and then stained with antibodies against activated (mAb24) and total (IB4) CD18. Corresponding ratios of mean fluorescent intensities (MFIs) of active/total protein are shown (data derived from 10 independent experiments). **B.** Conjugate formation analysis of healthy or LAD3 patient-derived neutrophils incubated with trastuzumab-opsonized SKBR3 control or CD47KD cells (data shown are from 5 independent experiments, with 2 different patients). Where indicated, cells were pre-incubated with intact or F(ab')2 blocking anti-CD18 **C.** Trogocytosis determined by the percentage of DiD-positive neutrophils from healthy controls or LAD3 patients after a 90-minute incubation with SKBR3 control or CD47KD cells opsonized with trastuzumab and/or CD18 blocking antibody (data shown are from 6 independent experiments with 4 different patients). **D.** Percentage of healthy or patient-derived neutrophil cytotoxicity determined by ^{51}Cr release assay with SKBR3 control or CD47KD tumor cells (data shown are from 5 independent experiments with 4 different patients). Note the complete absence of cytotoxicity for LAD3 patient neutrophils. Bars indicate mean±SEM; statistical significance assessed with one-way ANOVA and corrected for multiple comparison with the Sidak method; *p<0.05, **p<0.01, ****p<0.0001.

Figure 2. Kindlin3 deficiency in NB4 cells recapitulates the impaired effector function of LAD3-derived neutrophils. A. Generation of kindlin3 mutants in NB4 neutrophil-like cells. Western blot with fresh human neutrophils, WT NB4, and NB4 kindlin3KO cells reconstituted with kindlin3 W596A mutant or kindlin3 WT and stained for kindlin3. Staining for GAPDH was used as a loading control (one representative example is shown). **B-C.** Evaluation of (B) adhesion and (C) NADPH oxidase activity in neutrophils from healthy donors (white) or LAD3 patients (red) and NB4 WT (white dotted) or kindlin3KO (red dotted) cells in presence of the indicated stimuli (data from 5 independent experiments with 4 different patients). Dithiothreitol: DTT, platelet activating factor: PAF, formylated Met-Leu-Phe peptide: fMLP. **D.** Percentage of trogocytosed DiD-labelled SKBR3 control or CD47KD cells after a 90-minute incubation (n≥3). **E.** Killing of ^{51}Cr-labelled SKBR3 control or CD47KD cells by the NB4 cells was assessed by the ^{51}Cr release into the supernatant (n≥4).

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Bars indicate mean±SEM; statistical significance was assessed with t-test or one-way ANOVA and corrected for multiple comparison with the Sidak method; *p<0.05.

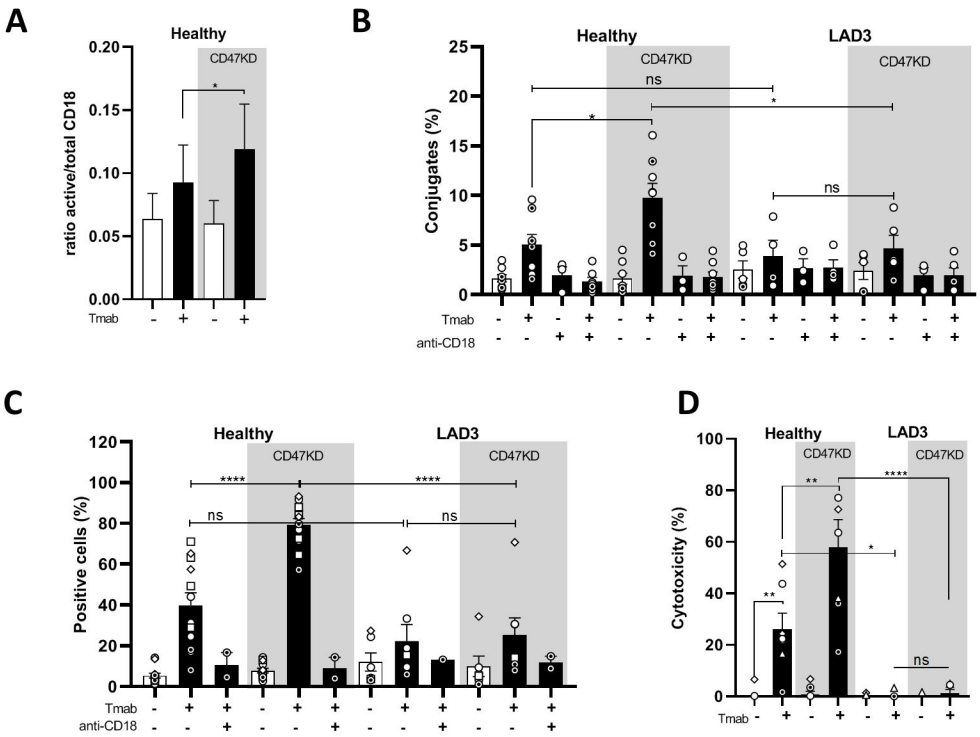
Figure 3. Interaction of kindlin3 with the CD18-integrin chain is required for trogocytosis and killing of antibody-opsonized target cells. A. Percentage of trogocytosed DiD-labelled SKBR3 control or CD47KD cells after a 90-minute incubation (n≥3). **B.** Killing of ^{51}Cr-labelled SKBR3 control or CD47KD cells by the NB4 cells was assessed by the ^{51}Cr release into the supernatant (n≥4). Bars indicate mean±SEM; statistical significance was assessed with one-way ANOVA and corrected for multiple comparison with the Sidak method; *p<0.05.

Figure 4. The role of kindlin3 during neutrophil ADCC and its regulation by the CD47-SIRPα checkpoint.

A. Established model for the regulation of integrin activity by inside-out signaling by talin and kindlin3. Intracellular signaling, including that triggered by Fc-receptors, promotes the subsequent association of talin and kindlin3 to the β-chain of the integrin causing i) a conformational change in the extracellular region of the integrin that enhances ligand binding and ii) the association of the integrin to the cytoskeleton [13][14]. **B.** Proposed model for the regulation of antibody-dependent destruction of tumor cells by neutrophils by kindlin3 and its modulation by the CD47-SIRPα interactions. Whereas Fc-receptor signaling provides the stimulatory signals for integrin activation as mentioned above, inhibitory signaling downstream of SIRPα triggered by interactions between tumor cell CD47 and neutrophil SIRPα controls CD11b/CD18-integrin activation via kindlin3. Disruption of the CD47-SIRPα checkpoint thus mediates integrin activation and promotes cytotoxic synapse formation/neutrophil-tumor conjugate formation, trogocytosis, and cytotoxicity by cancer cell trogoptosis [7]. At the same time, kindlin3 plays an essential role in trogocytosis and in particular the trogoptotic killing process, possibly by stabilizing anchorage of the integrin with the actin cytoskeleton, which allows neutrophil-triggered fulling forces to be transmitted to the tumor cell surface that are necessary for its lytic destruction. Therefore, kindlin3 plays a dual role in the neutrophil killing process.

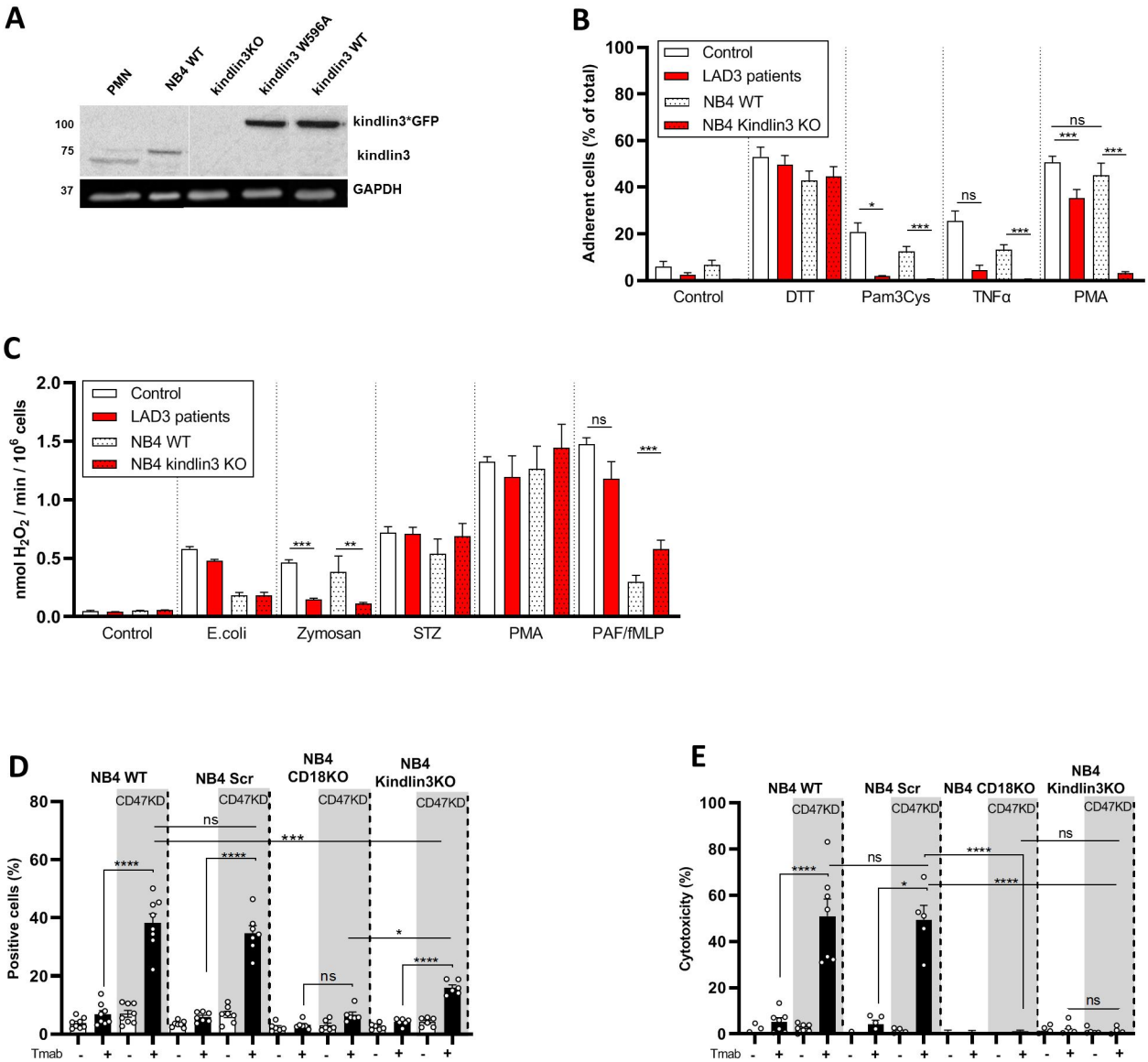
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Figure 1



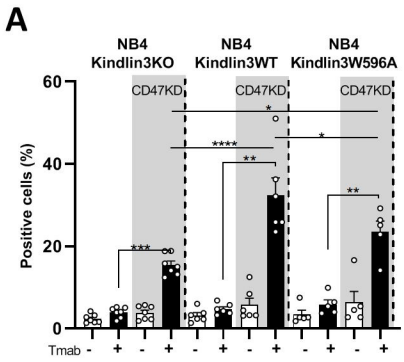
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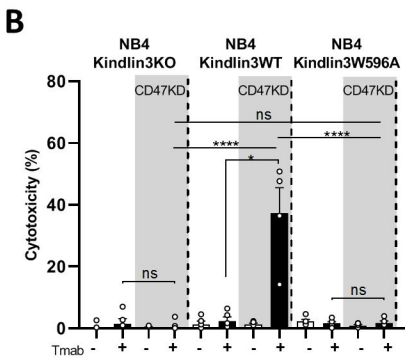
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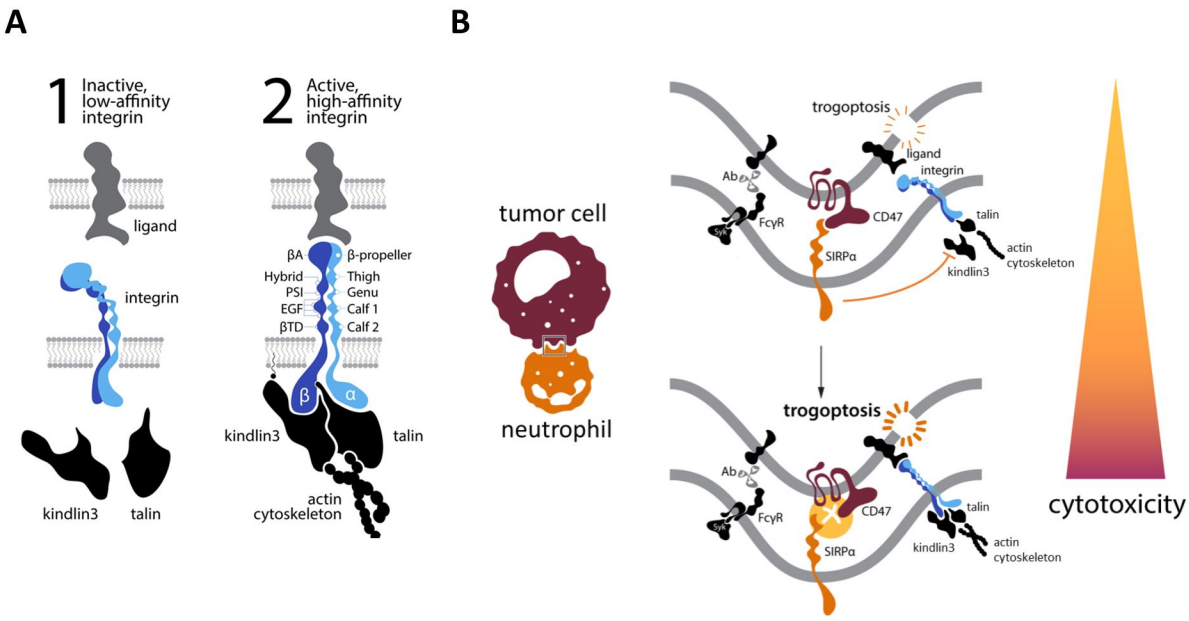
Figure 3





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Kindlin3-dependent CD11b/CD18-integrin activation is required for potentiation of neutrophil cytotoxicity by CD47-SIRPα checkpoint disruption

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