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Defining the Roles of Human Carcinoembryonic Antigen-Related Cellular Adhesion Molecules during Neutrophil Responses to *Neisseria gonorrhoeae*

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Symptomatic infection of humans with *Neisseria gonorrhoeae* is characterized by a neutrophil-rich cervical or urethral exudate, suggesting that neutrophils are important both for the clearance of these bacteria and for the pathogenesis of gonorrhea. *Neisseria* interacts with neutrophils through ligation of human carcinoembryonic antigen related-cellular adhesion molecules (CEACAMs) by their surface-expressed Opa proteins, resulting in bacterial binding, engulfment, and neutrophil activation. Multiple CEACAMs are expressed by human neutrophils, and yet their coexpression has precluded understanding of the relative contribution of each CEACAM to functional responses of neutrophils during neisserial infection. In this work, we directly address the role of each CEACAM during infection by introducing individual human CEACAMs into a differentiated murine MPRO cell line-derived neutrophil model. Murine neutrophils cannot bind the human-restricted *Neisseria*; however, we show that introducing any of the Opa-binding CEACAMs of human neutrophils (CEACAM1, CEACAM3, and CEACAM6) allows binding and entry of *Neisseria* into murine neutrophils. While CEACAM1- and CEACAM6-expressing neutrophils bind more bacteria, neisserial uptake via these two receptors unexpectedly proceeds without appreciable neutrophil activation. In stark contrast, neisserial engulfment via CEACAM3 recapitulates the oxidative burst and intracellular granule release seen during human neutrophil infection. Finally, by coexpressing multiple CEACAMs in our model, we show that the expression of CEACAM1 and CEACAM6 potentiate, rather than hinder, CEACAM3-dependent responses of neutrophils, exposing a cooperative role for this family of proteins during neisserial infection of neutrophils. These observations illustrate a divergence in function of CEACAMs in neutrophils and implicate the human-restricted CEACAM3 in the neutrophil innate response to neisserial infection.

Symptomatic gonococcal infection is caused by the human-restricted bacterial pathogen *Neisseria gonorrhoeae* and involves a massive influx of polymorphonuclear neutrophils (PMNs) into the infected urogenital tract. This results in the characteristic PMN-filled urethral or cervical exudate, which is the hallmark of gonorrhea. PMNs are part of a "first line of defense" against bacterial infection through their prompt recruitment and activation at infected sites, where they internalize and neutralize invading pathogens via the production of reactive oxygen species and the release of antimicrobial agents (27). Recognition of bacteria by PMNs can involve specific binding to host-derived opsonins such as serum complement or immunoglobulins that coat the bacteria; however, the interaction between *Neisseria* and PMNs can also be opsonin independent (40). Specifically, these microorganisms can bind to and activate neutrophils directly via their colony opacity-associated (Opa) outer membrane proteins, the majority of which bind members of the human carcinoembryonic antigen-related cellular adhesion molecule (CEACAM) family (10, 44).

To date, four CEACAMs have been shown to act as receptors for the gonococcal Opa proteins: CEACAM1, CEACAM3, CEACAM5, and CEACAM6 (4, 7, 10). Human neutrophils express three of these (CEACAM1, CEACAM3, and CEACAM6), as well as CEACAM4 and CEACAM8, which do not bind Opa (11, 32). Opa-dependent binding to PMNs results in bacterial killing through the ability of neutrophils to capture, internalize, and mount antimicrobial responses upon neisserial infection (28, 43). While the intimate association between *N. gonorrhoeae* and PMNs is well described, coexpression of multiple CEACAMs in PMNs has made it difficult to specifically attribute individual CEACAMs to the bactericidal response. Antibody cross-linking studies have suggested that ligation of CEACAMs, individually (39) or in concert (36), results in neutrophil activation. Any one of the three Opa-binding CEACAMs of PMNs can mediate bacterial engulfment by transfected epithelial cell models (20). However, reflective of their different cytoplasmic domains, studies of these receptors in a number of cell types, including lymphocytes (5), epithelial cells (20), and endothelial cells (26), have shown that they can elicit distinct, and often opposing, cellular responses. For example, ligation of CEACAM1, which contains two immunoreceptor tyrosine-based inhibition motifs (ITIMs; V/L/IxYxxL/V), results in phosphatase recruitment and the suppression of phosphotyrosine-based signaling cascades (8, 17). In contrast, ligation of CEACAM3, which contains an immunoreceptor tyrosine-based activation motif (ITAM; YxxL/Ix$_{6-8}$YxxL/I), results in kinase recruitment and propagation of signaling (21, 30). The effects of engaging the glycosylphosphatidylinositol (GPI)-anchored CEACAM6 remain largely unexplored.

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In the present study, we used a genetic approach to examine the individual roles of CEACAM1, CEACAM3, and CEACAM6 in neutrophils. We show that all three CEACAMs can bind and engulf *N. gonorrhoeae*. However, internalization via CEACAM1 and CEACAM6 proceeds without substantial neutrophil activation, while internalization via CEACAM3 results in stimulation of the PMN oxidative burst and release of intracellular granules in a manner dependent on its cytoplasmic ITAM. Unexpectedly, CEACAM1, which we show can be phosphorylated in PMNs and has been shown to be inhibitory in other systems (5), does not suppress CEACAM3 function in the neutrophil. Instead, we show that CEACAM3 can transduce activating signals upon bacterial CEACAM1 ligation, revealing a previously unrecognized cooperative interaction between CEACAMs in neutrophils. Importantly, our work solidifies the importance of CEACAM3 in the initiation and propagation of signaling in response to neisserial infection of neutrophils.

MATERIALS AND METHODS

Reagents and antibodies. All reagents were from Sigma (Oakville, Ontario, Canada) unless otherwise indicated. The diisopropyl fluorophosphate (DFP) was from BioShop (Burlington, Ontario, Canada). Pansorbin (fixed *Staphylococcus aureus*) was from Calbiochem (La Jolla, CA). The antigonococcal polyclonal rabbit antibody (UTR01) was described previously (21). The rabbit CEACAM-specific polyclonal antiserum and normal rabbit serum were from Dako (Mississauga, Ontario, Canada). The CEACAM pan-specific D14HD11, CEACAM1-specific 4/3/17, CEACAM6-specific 9A6, and CD67 antibodies were from Genovac GmbH (Freiburg, Germany). The anti-phospho Syk, anti-phospho Vav, and anti-Vav antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Syk antibody (Syk-01) was from Abcam (Cambridge, MA), the CEACAM3-specific Col-1 antibody was from Zymed (San Francisco, CA), and the rat anti-mouse CD11b antibody was from BD Biosciences (Mississauga, Ontario, Canada). Fluorescent conjugates were from Jackson ImmunoResearch Laboratories (Mississauga, Ontario, Canada), except for Texas Red-phalloidin, which was from Molecular Probes (Eugene, OR).

Primary neutrophil isolation. Human PMNs were isolated from citrated whole blood taken from healthy volunteers by venipuncture using Ficoll-Paque Plus (Amersham Biosciences, Buckinghamshire, England). Contaminating erythrocytes were removed by dextran sedimentation and hypotonic shock, as described previously (21). Mouse bone marrow neutrophils were taken from 8 to 10-week-old FVB mice that were killed by CO_2 inhalation. Specifically, femurs and tibias were removed and bone marrow was isolated and separated on a discontinuous Percoll gradient (80%/65%/55%). Neutrophils were recovered at the 80%-65% interface.

MPRO cell culture and differentiation to neutrophils. The MPRO cells were purchased from the American Type Culture Collection and were maintained in Iscove modified Dulbecco medium supplemented with 1.25 to 5% conditioned HM-5 supernatant, 20% horse serum (Invitrogen, Burlington, Ontario, Canada), and 1% Glutamax supplement (Invitrogen). The HM-5 granulocyte-macrophage colony-stimulating factor (GM-CSF)-producing cell line was maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum (HyClone, Logan, UT) and was kindly provided by Steven Collins (Fred Hutchinson Cancer Research Center, Seattle, WA). GM-CSF-conditioned medium was prepared as described by Lawson et al. (15). MPRO differentiation was induced with 10 μM all-*trans*-retinoic acid (ATRA) in normal growth medium for roughly 72 h, after which the cells were washed and maintained in Hanks balanced salt solution (HBSS; Invitrogen) supplemented with 10 mM HEPES, adjusted to a pH of 7.4.

Recombinant DNA constructs and establishment of human CEACAM-expressing MPRO cells. Plasmids containing CEACAM1 and CEACAM6 in pRC/CMV were generously provided by Wolfgang Zimmermann (Munich, Germany). Plasmids containing full-length CEACAM3, a naturally occurring isoform of CEACAM3 lacking most of the cytoplasmic domain (1C1), and CEACAM3 mutant cDNAs in pCEP4 were created by A. Popp and kindly provided by T. F. Meyer (Max-Planck-Institut fur Infektionsbiologie, Berlin, Germany). cDNAs were amplified via PCR (5′ primers contained a Kozak sequence, GCC ACC ATG, for protein expression) and subcloned into pMSCVpuro (all cDNAs; Clontech Laboratories, Inc.) or pMSCVblast (a recombinant pMSCVpuro in which the puromycin resistance gene was swapped for the blastocidin resistance gene from pCDNA6 (Invitrogen), the latter used to express CEACAM1 and CEACAM6 cDNAs for creation of the MPRO-CEACAM1+CEACAM3 and MPRO-CEACAM3+CEACAM6 cell lines. These constructs were introduced into undifferentiated MPRO cells using a pantropic retroviral expression system (Clontech Laboratories, Inc.) according to the manufacturer's instructions. Briefly, the packaging cell line, GP-293, was cotransfected with the respective pMSCV vector containing CEACAM cDNA, as well as pVSV-G (to allow viral incorporation of the vesicular stomatitis virus envelope glycoprotein), using FuGene 6 (Roche). After 48 h, virus-containing supernatants were collected and concentrated by ultracentrifugation as described by Zhou et al. (46) at 120,000 $\times$ *g* for 2 h at 4°C, and then undifferentiated MPRO cells were infected with the VSV-G-pseudotyped virus by centrifuging the cells with the concentrated virus preparation for 2 h at 3,000 $\times$ *g* at room temperature. The infected MPRO cells were left overnight at 37°C and selected the following day with 10 μg of puromycin/ml (for pMSCVpuro-containing virus) or 10 μg of blastocidin (Invitrogen)/ml (for pMSCVblast-containing virus). Single drug-resistant cells were cloned to create monoclonal, stable cell lines, which were differentiated to PMNs using ATRA for use in experiments.

Bacterial strains. All *N. gonorrhoeae* strains used in the present study were derived from the nonpiliated MS11 strain and were kindly provided by T. F. Meyer (Max-Planck-Institut fur Infektionsbiologie, Berlin, Germany) and have been described previously (11, 14). Briefly, the strains used are as follows: the non-CEACAM-binding N302 strain (referred to here as Opa$^-$), the CEACAM1/CEACAM5-binding N306 strain (expressing Opa_{59}; referred to here as Opa_{CCM1}), and the CEACAM1/CEACAM3/CEACAM5/CEACAM6-binding N313 strain (expressing Opa_{57}; referred to here as Opa_{CEA}). Bacteria were subcultured daily using a binocular microscope to monitor colony opacity phenotypes, and grown on solid agar medium (GC + Isovitalex) at 37°C with 5% CO_2.

Bacterial infections for IF microscopy and survival assays. A total of 5×10^5 MPRO PMNs were centrifuged onto washed fetal bovine serum-coated coverslips at 63 $\times$ *g* for 10 min. Where inhibitors were used, the cells were incubated for 20 min at 37°C prior to infection. Cells were then infected at a multiplicity of infection (MOI) of 25 (for binding and internalization studies) or 50 (for F-actin colocalization studies and survival assay) in a volume of 500 μl, recentrifuged for 5 min to facilitate bacterial association with cells, and then incubated at 37°C for 5 min (for F-actin colocalization studies) or 30 min (for internalization and survival studies). Postinfection, immunofluorescence (IF) samples were washed with HBSS and fixed using 3.7% paraformaldehyde. The cells were permeabilized using 0.4% Triton X-100, stained, and observed as described previously (21). For assessment of intracellular versus extracellular bacteria, infected cells were first stained with the rabbit polyclonal antisera against *N. gonorrhoeae*, followed by a Cy5-conjugated secondary; the cells were permeabilized and then stained with the rabbit polyclonal antisera against *N. gonorrhoeae*, followed by a fluorescein isothiocyanate (FITC)-conjugated secondary and Texas Red (TR)-conjugated phalloidin, to localize the cells. Using this method, extracellular bacteria are stained with both the Cy5- and the FITC-conjugated antibodies, whereas intracellular bacteria are only stained with the FITC-conjugated antibody.

Bacterial survival assay. Cells were infected as for IF assay (MOI of 50, 37°C for 30 min) and then washed and incubated with 100 μg of gentamicin/ml for 45 min to kill extracellular bacteria, followed by incubation in 1 mM DFP to inhibit proteases so as to protect intracellular bacteria

during PMN lysis. Cells were then lysed in 0.4% saponin in HBSS for 20 min at 37°C, and then diluted and plated onto GC plus Isovitalex. Colonies were counted the following day.

Immunoprecipitation and Western blotting. Cells (5×10^6 per sample) were infected with *N. gonorrhoeae* at an MOI of 10 for indicated times at 37°C with 5% CO_2. Infections were stopped by centrifugation at 2,400 × *g* for 3 min at 4°C, and the pellets were resuspended in 50 μl of phosphate-buffered saline (PBS)–pervanadate buffer (1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride [PMSF]; 1 μg each of aprotinin, leupeptin, and pepstatin A/ml; 1 mM NaF; 100 μM Na_3VO_4; 10 mM H_2O_2; and 50 mg of soybean trypsin inhibitor/ml in PBS) and then lysed with 50 μl of 2× radioimmunoprecipitation (RIPA) buffer (1× RIPA: 1% Nonidet P-40; 50 mM Tris-HCl; 150 mM NaCl; 1 mM EDTA; 1 mM DFP; 1 mM PMSF; 1 mg each of aprotinin, leupeptin, and pepstatin A/ml; 1 mM NaF; 100 mM Na_3VO_4; 10 mM H_2O_2; and 50 μg of soybean trypsin inhibitor/ml) containing 2% sodium dodecyl sulfate (SDS) (except for coimmunoprecipitation) and then left on ice for 15 min. The lysates were then topped up with 900 μl of RIPA, rotated for 30 min at 4°C, immunoprecipitated with anti-phosphotyrosine antibody (4G10) (for phosphorylation studies) or anti-CEACAM antibody (Dako) (for coimmunoprecipitation studies) for 2 h, incubated with protein A-Sepharose for 1 h, washed two times with RIPA, and boiled for 5 min. Immunoprecipitated samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with 5% milk in TBS with 0.05% Tween for 30 min at room temperature, and incubated with appropriate primary and horseradish peroxidase-conjugated secondary antibodies, and chemiluminescent detection was performed using ECL Plus (Amersham Biosciences).

Syk and Vav phosphorylation assays. A total of 10^6 MPRO PMNs were infected with *N. gonorrhoeae* at an MOI of 10 in 250 μl of HBSS at 37°C with 5% CO_2 for the times indicated. Infections were stopped by centrifugation at 2,400 × *g* for 3 min at 4°C, lysed in boiling SDS sample buffer, and boiled for a further 5 min. The samples were resolved and immunoblotted as described above.

Oxidative burst assays. For dihydrorhodamine (DHR) assay, 10^6 MPRO PMNs were treated with 2 μM dihydrorhodamine-123 (DHR-123) for 20 min at 37°C at a concentration of 10^7 cells/ml prior to agonist treatment. The samples were then treated with agonists (*N. gonorrhoeae* at an MOI of 10, unless otherwise indicated, or phorbol myristate acetate [PMA] at 1 μg/ml) in 500 μl of HBSS for 60 min at 37°C. Infections were stopped by centrifugation at 2,400 × *g* for 3 min at room temperature, and the cell pellets were fixed in 1% paraformaldehyde (PFA) prior to analysis by flow cytometry using a FACSCalibur with CellQuest software (Becton Dickinson, San Diego, CA), gathering the FL-1 signal from a gated sample of 10,000 cells. For the chemiluminescence assay, 5×10^5 cells were incubated with 25 μg of 5-amino-2,3-dihydro-1,4-phthalazinedione (Luminol)/ml in a volume of 100 μl and then treated with agonists in a total volume of 200 μl in triplicate. Infections were allowed to proceed for 60 min at 37°C, after which luminescence was read using a Tecan plate reader with i-control software. Plotted are the data from the 60-min time point, which was consistently the time at which peak fluorescence occurred in the infected samples.

Degranulation assays. For flow-based assays of secondary granule degranulation (CD11b release for murine neutrophils and CD67 release for human neutrophils), 10^6 PMNs were treated with agonists in 500 μl of medium for 30 min at 37°C. Infections were stopped by centrifugation at 2,400 × *g* for 3 min at room temperature. Cell pellets were fixed in 1% PFA and stained with 1.25 μg of phycoerythrin (PE)-conjugated rat anti-mouse CD11b (or 1 μg of anti-CD67 antibody, followed by a PE-conjugated secondary for human neutrophil studies) in a total volume of 50 μl. Flow cytometry was conducted as described above, gathering the FL-2 signal from a gated sample of 10,000 cells. Myeloperoxidase, elastase, and lactoferrin release assays were performed essentially as described previously (1). Briefly, for all three assays, 10^6 PMNs were infected with agonists in a total volume of 500 μl, and infections proceeded for 30 min at 37°C. Cells were then pelleted, and the supernatants were collected. For the myeloperoxidase assay, 50 μl of supernatant was mixed with 150 μl of SureBlue tetremethylbenzidine peroxidase substrate (KPL, Gaithersburg, MD), and the plates were read spectrophotometrically at 650 nm. For the elastase assay, 50 μl of supernatant was diluted 2-fold in PBS, incubated with 100 μl of DQ elastin substrate conjugated to BODIPY FL (from the EnzCheck elastase kit [Molecular Probes]), and then incubated for 24 h at room temperature before the fluorescence was read at 488 nm for excitation and 515 nm for emission. For both myeloperoxidase (MPO) and elastase assays, the percent release is shown, and this was calculated as the amount of the protein in the supernatant divided by the total amount in 10^6 CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}-lysed cells. Lactoferrin release was assayed by enzyme-linked immunosorbent assay (ELISA) as described earlier (23). Because HBSS would often cause spontaneous specific granule release in cells, CD11b and lactoferrin degranulation assays were conducted in Medium 199 (Invitrogen).

Statistical analyses. Immunofluorescence experiments and plating assays were conducted with triplicate samples, and standard deviations (represented as error bars) were calculated from averages between three individual coverslips (25 cells counted per coverslip) from a single representative experiment. Chemiluminescence-based oxidative burst assays, bacterial survival assays, and the MPO, elastase, and lactoferrin release assays were always conducted with triplicate samples, with means and standard deviations presented as error bars. All results depicted here are representative of at least three independent experiments, each conducted on separate days. Where indicated, samples were analyzed by (i) a two-tailed Student *t* test assuming equal variance, taking into account all cells from each condition of a particular experiment, with *P* values of <0.05 being deemed significant, or (ii) one-way analysis of variance (ANOVA) with Tukey's post-test when comparing means between different cell lines treated with the same agonist.

RESULTS

Introduction of human CEACAMs into murine neutrophils is sufficient for neisserial capture and engulfment. To delineate the individual functions of CEACAMs 1, 3, and 6 in the neutrophil, we used a "knockin" approach to introduce human CEACAMs into mouse promyelocytic cells (MPRO) (41). Rather than being immortalized by an oncogenic event, the normal differentiation of MPRO cells is arrested by a dominant-negative retinoic acid receptor alpha (RARα), a defect that can be overcome with supraphysiological doses of retinoic acid. The MPRO model allowed us to circumvent two issues: (i) since the cells are murine, they contain no Opa-binding CEACAMs, and Opa-dependent effects could be specifically attributed to introduced human CEACAMs (Fig. 1a, top; compare with human neutrophils, Fig. 1a, bottom), and (ii) since the cells are promyelocytes, we could create stable cell lines for propagation in culture, which could be differentiated *in vitro* to mature, functional neutrophils (15, 18).

We introduced cDNAs encoding human CEACAM1 ("1"), CEACAM3 ("3"), and CEACAM6 ("6"), as well as the empty vector ("puro") into MPRO cells using retroviral transduction. In both the promyelocytes and the neutrophils differentiated from the promyelocytes all three human CEACAMs were expressed in much the same manner as in human neutrophils, with CEACAM1 and CEACAM6 in larger quantities than CEACAM3 (Fig. 1b and c). In all of the experiments performed here, we used neutrophils differentiated from the CEACAM-expressing promyelocytes. As with normal mouse bone marrow neutrophils (Fig. 1a, top), MPRO-puro cells were unable to substantially bind or engulf *N. gonorrhoeae*, whereas the introduction of CEACAM1, CEACAM3, or CEACAM6 permitted the binding and engulfment of *N. gon-*

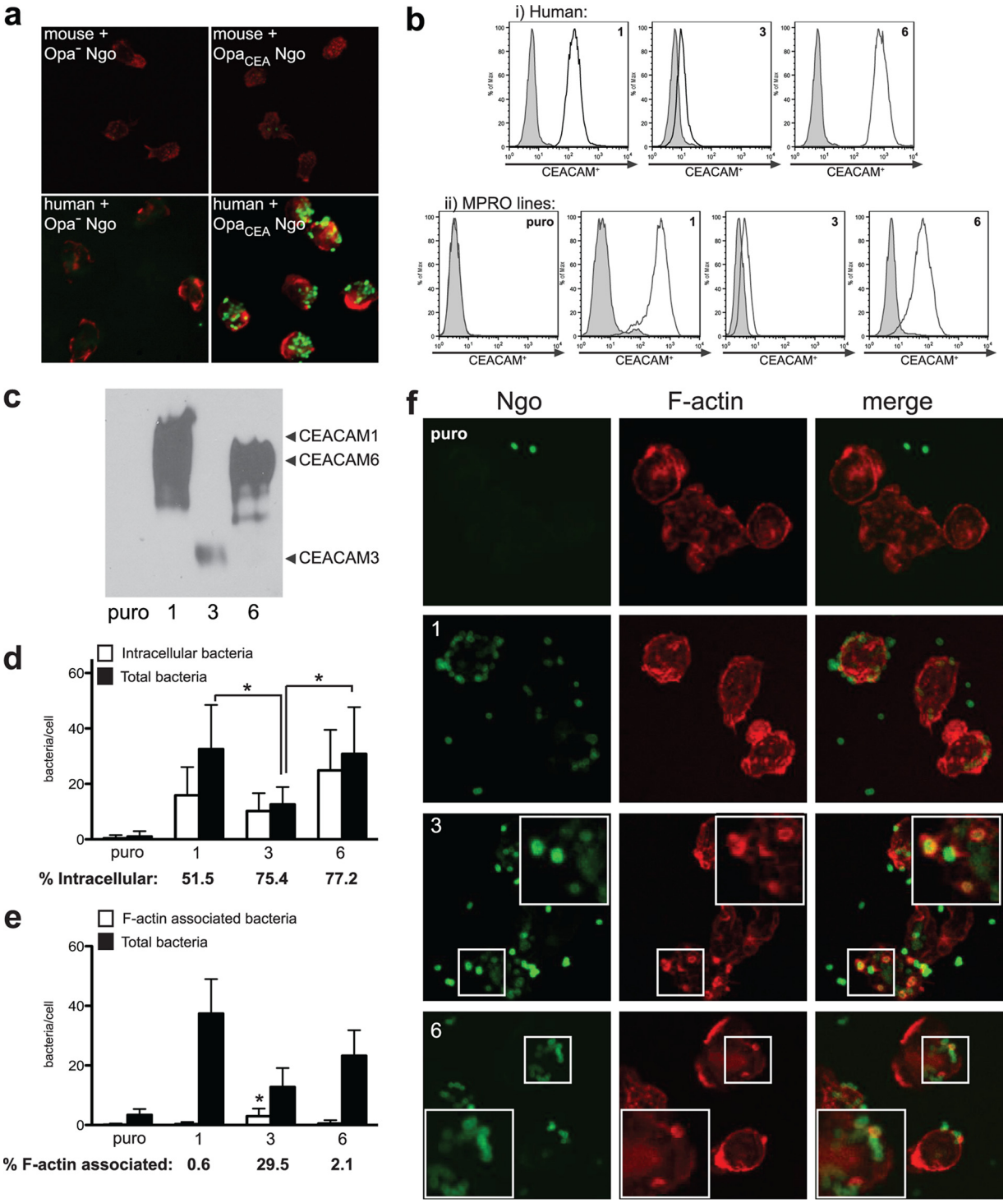

FIG 1 Human CEACAM expression in murine neutrophils permits the binding and engulfment of *N. gonorrhoeae*. (a) Murine neutrophils do not bind *N. gonorrhoeae*, and human neutrophils bind *N. gonorrhoeae* in an Opa_{CEA}-dependent manner. Neutrophils from mouse bone marrow (top) or human blood (bottom) were infected with *N. gonorrhoeae* either lacking (Opa^-) or expressing an Opa adhesin that binds all of the CEACAMs used in the present study (Opa_{CEA}). Cells were visualized by staining with phalloidin (red), and bacteria are shown in green. (b) Human CEACAMs are expressed in MPRO neutrophils. (i) Human neutrophils were fixed and stained with antibodies specific for CEACAM1 (4/3/17), CEACAM3 (Col-1), or CEACAM6 (9A6) and then analyzed by flow cytometry. Shaded histograms correspond to the isotype control. (ii) MPRO PMNs were fixed and stained with the same antibodies used in subpanel i. (c) Western blot analysis of cell lines shown in panel b. (d) All human neutrophil-expressed CEACAMs are capable of binding and internalizing Opa-expressing *N. gonorrhoeae*. Differentiated MPRO cells were infected on glass coverslips with *N. gonorrhoeae* expressing Opa_{CEA} for 60 min at 37°C at an MOI of 25, followed by fixation with paraformaldehyde. Intra- and extracellular bacteria were differentially stained as described in Materials and Methods and quantified via immunofluorescence microscopy. Total bacteria bound or internalized per cell, for a total of 25 cells counted, were averaged and plotted. (e) F-actin dynamics during bacterial internalization by CEACAM1, CEACAM3, and CEACAM6. Cells were infected as in panel d, except for a shorter duration (5 min at 37°C) and with an MOI of 50. "F-actin associated" refers to the number of bacteria colocalizing with F-actin, divided by the total number of bound bacteria per cell. 25 cells were counted, and the average is shown. In panels d and e, two-tailed *t* tests were conducted for relevant samples; an asterisk denotes *P* values of <0.05. (f) MPRO-CEACAM3 (and to a lesser extent, MPRO-CEACAM6) neutrophils internalize *N. gonorrhoeae* with reorganization of F-actin at sites of bacterial attachment. Images from panel e are shown.

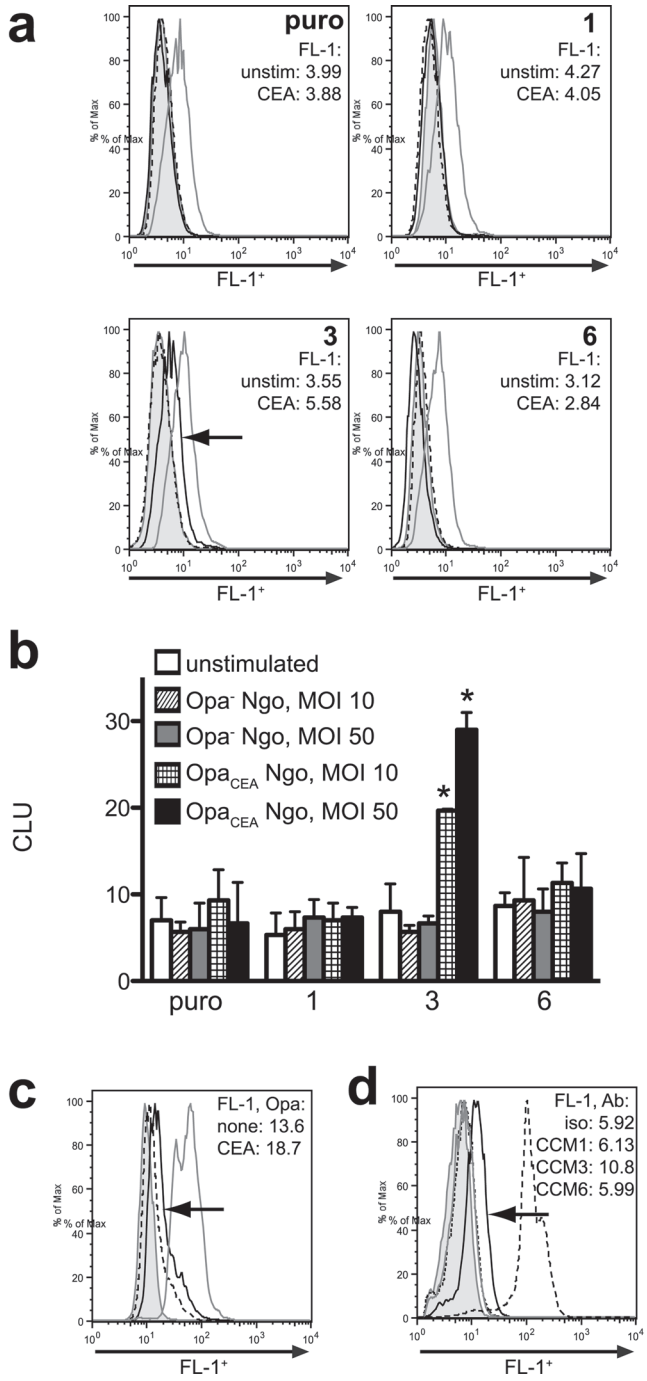

FIG 2 CEACAM3 expression in mouse neutrophils recapitulates the oxidative burst response of human PMNs to *N. gonorrhoeae.* (a) CEACAM3-dependent oxidative burst response to *N. gonorrhoeae* infection in murine neutrophils. MPRO neutrophils were treated with the oxidative burst reagent DHR-123 (which fluoresces upon oxidation), followed by infection with Opa$^-$ *N. gonorrhoeae* (dashed line), Opa_{CEA}-expressing *N. gonorrhoeae* (black line), PMA (gray line), or left uninfected (tinted). The bacterial MOI was 10. After 60 min at 37°C, the cells were fixed, and the FL-1 signal was collected from a gated sample of 10,000 cells by flow cytometry to monitor the oxidative burst response. The geometric mean of the FL-1 signal is shown for uninfected cells ("unstim") or for Opa_{CEA}-infected samples ("CEA"). In all panels, an arrow denotes a CEACAM-dependent (c) or CEACAM3-dependent (a and d) event. (b) CEACAM3-dependent chemiluminescent response to luminol. MPRO neutrophils were preincubated with luminol and then infected with the indicated strains of *N. gonorrhoeae* at an MOI of 10 or 50 or left uninfected. Luminescence was read after 60 min at 37°C. Relevant samples were analyzed by one-way ANOVA with Tukey's post-test. An asterisk denotes that sample is significantly different than the corresponding samples from the other cell lines. (c) Human neutrophils undergo oxidative burst when infected with CEACAM-binding *N. gonorrhoeae.* Human neutrophils were treated with DHR-123, infected, and analyzed as in panel a. The geometric mean of the FL-1 signal is shown for cells infected with Opa$^-$ bacteria ("none") or Opa_{CEA}-expressing bacteria ("CEA"). (d) Human neutrophils exhibit oxidative burst when infected with CEACAM-binding Pansorbin particles. Human neutrophils were treated with DHR-123 and infected as in panel a with fixed *S. aureus* particles (Pansorbin) coated with anti-CEACAM1 (4/3/17; gray line), anti-CEACAM3 (Col-1; black line), anti-CEACAM6 (9A6; dotted line), isotype (shaded), or PMA (dashed line).

orrhoeae by the mouse neutrophils (Fig. 1d). More bacteria consistently adhered to cells expressing CEACAM1 or CEACAM6, relative to CEACAM3. Strikingly, bacterial association with MPRO-CEACAM3 cells frequently resulted in the appearance of F-actin-rich structures surrounding the bound bacteria (Fig. 1e and f). Such structures were infrequently apparent in infected MPRO-CEACAM6 cells and were never observed during neisserial infection of MPRO-CEACAM1 cells. These observations suggested that these receptors were signaling differently in response to engagement by *N. gonorrhoeae*, arguing for different functional outcomes upon bacterial infection.

CEACAM3 is distinct in its ability to elicit neutrophil oxidative burst and degranulation responses to *N. gonorrhoeae.* Once we had established that human CEACAMs were functionally expressed in the murine background, we sought to study the contribution of the different CEACAMs to other neutrophil functions. Human neutrophils respond to Opa-expressing *N. gonorrhoeae* by triggering an increased consumption of oxygen, resulting in the production of oxygen radicals in the cell (the "oxidative burst" response), as well as by releasing granule components to the cell surface or into the newly formed phagosome ("degranulation"). Using both a single cell-based assay (oxidation of DHR) (Fig. 2a) and a chemiluminescence-based protocol measuring the oxidation of luminol (Fig. 2b), we observed that MPRO-CEACAM3 cells could stimulate the oxidative burst upon neisserial infection, whereas the CEACAM1- and CEACAM6-expressing cell lines did not lead to appreciable oxidative burst activation in the cells. This did not result from a difference in bacterial association, since all three CEACAM-expressing MPRO lines were able to bind and internalize *Neisseria* (Fig. 1d). The magnitude of the response measured with DHR reflected that observed in human neutrophils (Fig. 2c). Using fixed *Staphylococcus aureus* particles (Pansorbin) coated with anti-CEACAM monoclonal antibodies to infect human neutrophils, we observed that only those Pansorbin particles coated with CEACAM3-specific antibody were able to stimulate an oxidative burst response compared to Pansorbin particles coated with the isotype control or CEACAM1- or CEACAM6-specific antibodies (Fig. 2d). These experiments clearly implicate CEACAM3, and not CEACAM1 or CEACAM6, as being sufficient to signal the oxidative burst upon neisserial infection.

To monitor neutrophil degranulation in response to neisserial infection, we first used a single cell-based flow cytometric assay measuring degranulation of the specific granule protein CD11b. As with the oxidative burst experiments, we observed that MPRO-CEACAM3 cells initiated this response upon neisserial infection, while the mouse neutrophils lacking human CEACAMs, or those

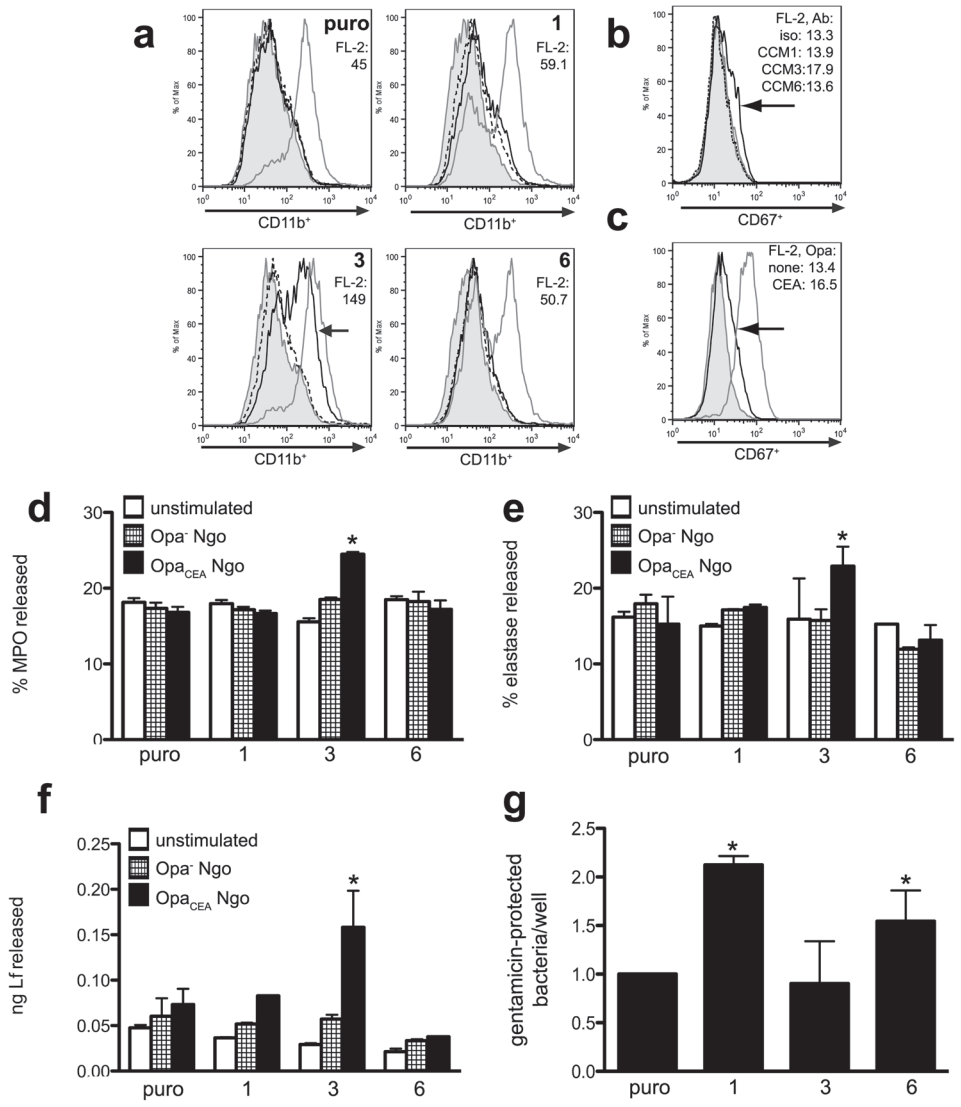

FIG 3 CEACAM3 expression by mouse neutrophils allows primary and secondary granule release in response to *N. gonorrhoeae* infection. (a) CEACAM3-dependent degranulation of the specific granule protein CD11b in response to *N. gonorrhoeae* infection of murine neutrophils. Cells were infected with Opa$^-$ *N. gonorrhoeae* (dashed line), Opa_{CEA}-expressing *N. gonorrhoeae* (black line), PMA (gray line), or left uninfected (tinted). The bacterial MOI was 10. After 30 min at 37°C, the cells were fixed and stained with a PE-conjugated rat anti-mouse CD11b antibody. The FL-2 signal was collected from a gated sample of 10,000 cells by flow cytometry to monitor the oxidative burst response. The geometric mean of the FL-2 signal is shown for Opa_{CEA}-infected cells. In all panels, an arrow denotes a CEACAM3-dependent (a and b) or CEACAM-dependent (c) event. (b) Human neutrophils degranulate the secondary granule protein CD67 when infected with CEACAM-binding Pansorbin particles. Human neutrophils were infected with Pansorbin particles coated with anti-CEACAM1 (4/3/17; gray line), anti-CEACAM3 (Col-1; black line), anti-CEACAM6 (9A6; dashed line), or isotype antibody (shaded), and analyzed as in panel a, except with an antibody to CD67 followed by a PE-conjugated secondary. (c) Human neutrophils degranulate CD67 when infected with CEACAM-binding *N. gonorrhoeae*. Human neutrophils were infected and analyzed as in panel b. Histograms are labeled as follows: Opa$^-$ *N. gonorrhoeae*, shaded; Opa_{CEA}-expressing *N. gonorrhoeae*, black line; and PMA, gray line. The geometric mean of the FL-2 signal is shown for cells infected with Opa$^-$ bacteria ("none") or Opa_{CEA}-expressing bacteria ("CEA"). (d, e, and f) CEACAM3-specific degranulation in response to neisserial infection. Cells were preincubated with cytochalasin B and infected as shown with *N. gonorrhoeae* at an MOI of 50 (d and e) or 1 (f), for 30 min at 37°C. Postinfection, the supernatants were collected and analyzed for myeloperoxidase (d), elastase (e), or lactoferrin (f) release as described in Materials and Methods. (g) *N. gonorrhoeae* engulfed by CEACAM3 are less able to survive the infection compared to those engulfed by CEACAM1 and CEACAM6. MPRO PMNs were infected with Opa_{CEA}-expressing *N. gonorrhoeae* at an MOI of 50 in 24-well tissue culture plates (5×10^5 cells per well). Bacteria were allowed to interact with the neutrophils for 30 min and then treated with gentamicin to kill extracellular bacteria for 45 min 37°C. Cells were then treated with the cell-permeable protease inhibitor DFP (1 mM) for 15 min to prevent killing of viable intracellular bacteria during lysis and lysed with 0.4% saponin in HBSS to release viable bacteria for dilution plating. The CFU were counted the following day. Plotted are the results of four experiments, with puro set at "1" to allow normalization of the samples. The samples in panels d to g were analyzed by one-way ANOVA with Tukey's post-test. An asterisk indicates that a sample is significantly different than the corresponding samples from the other cell lines.

expressing CEACAM1 or CEACAM6, did not (Fig. 3a). We performed a similar assay in human neutrophils, measuring the degranulation of the specific granule protein CD67, and observed that only CEACAM3-specific Pansorbin particles and not those with isotype or antibodies to CEACAM1 or CEACAM6 could stimulate degranulation of CD67 (Fig. 3b). The magnitude of the

degranulation response to CEACAM3-binding Pansorbin particles reflected that seen with pan-CEACAM-binding bacteria (Opa_{CEA}; Fig. 3c). We further confirmed the CEACAM3-dependent degranulation responses in MPRO PMNs using an ELISA-based assessment of degranulation in the population: by measuring degranulation of two azurophilic granule proteins, MPO (Fig. 3d) and elastase (Fig. 3e), and by measuring the release of a specific granule protein, lactoferrin (Fig. 3f).

Because *N. gonorrhoeae* is particularly susceptible to the azurophilic granule protein cathepsin G (34), we reasoned that degranulation responses to CEACAM3 ligation should also result in neisserial killing. To test this, we performed gentamicin protection assays with MPRO PMNs. We observed that the amount of gentamicin-protected bacteria recovered from MPRO-CEACAM3 PMNs reflected that of MPRO-puro (i.e., background levels). In contrast to this, MPRO-CEACAM1 and MPRO-CEACAM6 PMNs contained an increased number of viable internalized bacteria (Fig. 3g). Because MPRO-CEACAM3 PMNs are fully capable of internalizing bacteria (Fig. 1d), this result suggests that those bacteria that are internalized via CEACAM3 do not survive the infection. These results, as well as those in the previous section, suggest that ligation of CEACAM3 elicits signals that lead to both neisseria-induced PMN activation and may also result in subsequent bacterial killing.

CEACAM3 is phosphorylated and recruits murine Syk upon *N. gonorrhoeae* infection. Because CEACAM3 encodes a cytoplasmic ITAM that is phosphorylated upon ligation (21, 29), we speculated that this motif was responsible for the functional responses we were observing. To assess CEACAM phosphorylation in response to infection, we infected the various MPRO neutrophils with Opa-expressing *N. gonorrhoeae* and immunoprecipitated either total CEACAM or phosphotyrosine-containing proteins, which we then probed with a pan-specific antibody that recognizes all of the CEACAMs used in the present study. Consistent with past work using human-derived cell lines (17, 21, 29), the CEACAMs containing tyrosines in their cytoplasmic tails (CEACAM1 and CEACAM3) were phosphorylated upon neisserial infection (Fig. 4a). CEACAM6 phosphorylation was not observed, which was expected since it is a GPI-anchored protein lacking a cytoplasmic domain. We considered whether the phosphorylated CEACAMs could activate mouse Syk, a tyrosine kinase known to be centrally involved in signaling for oxidative burst and degranulation by neutrophils (42). To test for this, we monitored the phosphorylation state of Syk and a downstream effector, Vav, in infected whole-cell lysates using phospho-specific antibodies. Upon neisserial infection, CEACAM3-expressing cells showed an increase in Syk and Vav phosphorylation; this did not occur in CEACAM1- or CEACAM6-expressing cells or in CEACAM3-expressing cells that were uninfected or infected with Opa^- bacteria (Fig. 4b and c). Furthermore, treatment of MPRO-CEACAM3 PMNs with either the Src family kinase inhibitor PP2 or the Syk inhibitor piceatannol inhibited Vav phosphorylation upon neisserial infection (Fig. 4d), confirming that the majority of the Vav phosphorylation seen was dependent on both the phosphorylation of the CEACAM3 ITAM and the subsequent recruitment and activation of Syk, as observed previously in human neutrophils (29). Finally, coimmunoprecipitation of Syk with CEACAM3 confirmed that these two proteins became transiently associated upon infection with Opa-expressing *N. gonorrhoeae* (Fig. 4e). Thus, human CEACAM3 ligation is connected to Syk downstream signaling responses in this murine neutrophil system.

CEACAM3-specific activation of neutrophils is ITAM dependent. Using an epithelial cell-based model, we previously observed that Syk recruitment to CEACAM3 is critically dependent on the presence of both phosphorylatable tyrosines in the cytoplasmic ITAM (26). We therefore reasoned that if CEACAM3-dependent recruitment of Syk was required for PMN activation in response to neisserial infection, then the intact ITAM would be required for this as well. To directly test for the requirement of the ITAM in the response to neisserial infection by neutrophils, we constructed MPRO lines expressing CEACAM3 proteins with either one (MPRO-CEACAM3-Y230F or MPRO-CEACAM3-Y241F) or both (MPRO-CEACAM3-Y230F/Y241F) ITAM tyrosines mutated to phenylalanine (and therefore unable to be phosphorylated), as well as a natural splice variant of CEACAM3 lacking the ITAM and most of the cytoplasmic domain (MPRO-CEACAM3-1C1). The various mutant alleles were expressed at levels similar to that of the wild-type CEACAM3 (WT) protein in MPRO cells (Fig. 5a). Each mutant CEACAM3 could bind and internalize Opa_{CEA}-expressing *N. gonorrhoeae* when expressed by the neutrophils (Fig. 5b), highlighting that the cytoplasmic domain was not essential for bacterial uptake. The CEACAM3 mutant proteins were not, however, able to induce oxidative burst (Fig. 5c) or degranulation (Fig. 5d) responses upon neisserial infection. Thus, an intact ITAM is not required for gonococcal uptake by PMNs. The ITAM is, however, required for activation of PMNs in response to neisserial infection.

We confirmed that infection of cells expressing mutant CEACAM3 proteins did not lead to Syk or Vav activation (Fig. 6a). Consistent with the role of Syk activation in the CEACAM3-specific PMN responses, inhibition of Syk function using the pharmacological inhibitor piceatannol effectively abolished the ability of MPRO-CEACAM3 cells to mount an appreciable oxidative burst (Fig. 6b) or degranulation response (Fig. 6c and d) upon neisserial infection, with the response becoming indistinguishable from that of cells expressing the ITAM-less CEACAM3-1C1. Taken together, these data demonstrate that CEACAM3-dependent neutrophil responses require an intact ITAM to allow the recruitment and activation of Syk and its effectors. Importantly, these data also reiterate that these responses are not simply the result of CEACAM-dependent binding or bacterial uptake but rather require the initiation of ITAM-dependent signaling as a result of CEACAM3 ligation by Opa.

CEACAM3 transmits CEACAM1-dependent signals for neutrophil activation. Our results to this point show that MPRO-CEACAM3 neutrophils function remarkably similarly to human neutrophils. CEACAM1 encodes a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) with the potential to oppose ITAM activation signals through the recruitment of inhibitory phosphatases in some cell types (5, 17). Because CEACAM3 is always coexpressed with CEACAMs 1 and 6 in the human neutrophil, we sought to determine whether either of these CEACAMs could modulate CEACAM3 function. Importantly, we observed that coexpression of CEACAM 1 or 6 with CEACAM3 did not affect CEACAM3 function in neutrophils. Specifically, MPRO-CEACAM1 plus CEACAM3 ("1 + 3") and MPRO-CEACAM3 plus CEACAM6 ("3 + 6") neutrophils mounted oxidative burst and degranulation responses to *N. gonorrhoeae* indistinguishable from those seen in MPRO-CEACAM3 neutrophils (Fig. 7a and b).

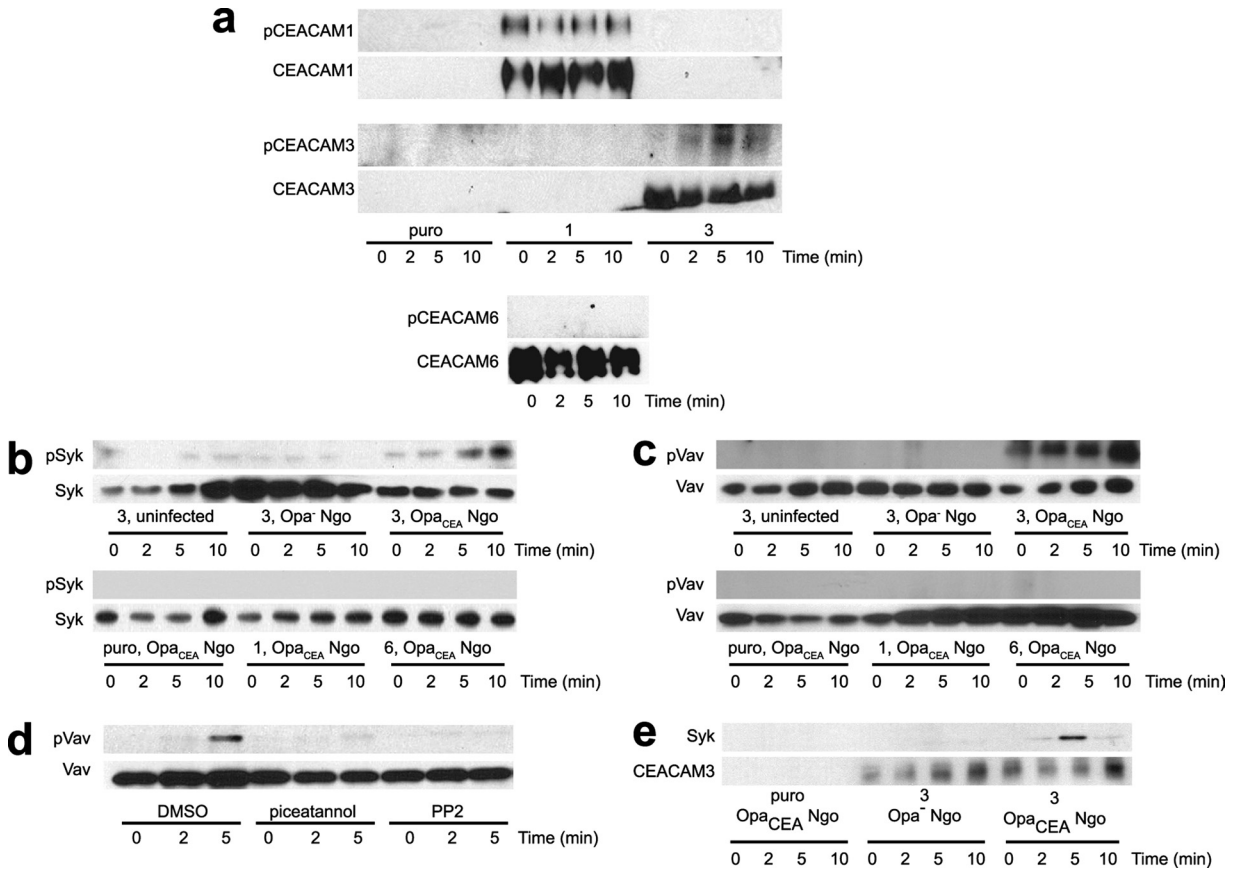

FIG 4 CEACAM3 ligation by *N. gonorrhoeae* results in phosphotyrosine-based signaling in mouse neutrophils. (a) CEACAM1 and CEACAM3 are phosphorylated on tyrosine upon *N. gonorrhoeae* infection. MPRO-puro ("puro"), MPRO-CEACAM1 ("1"), MPRO-CEACAM3 ("3"), and MPRO-CEACAM6 ("6") neutrophils were infected with Opa_{CEA}-expressing *N. gonorrhoeae* for the indicated times and then lysed, followed by immunoprecipitation of phosphotyrosine-containing proteins (top panels) or total CEACAM (bottom panels). Immunoprecipitates were resolved by SDS-PAGE and transferred, and all membranes were probed for CEACAM. (b) Syk phosphorylation upon *N. gonorrhoeae* infection of MPRO-CEACAM3 neutrophils. The cells were infected as shown (MPRO-puro, MPRO-CEACAM1, and MPRO-CEACAM6 with Opa_{CEA}-expressing bacteria, MPRO-CEACAM3 PMNs with Opa^- and Opa_{CEA} bacteria, or uninfected), followed by lysis of the cell pellet, resolution of lysates by SDS-PAGE and probing for a phosphorylated form of Syk (pSyk). Total Syk is shown as a loading control. (c) Vav phosphorylation upon *N. gonorrhoeae* infection of MPRO-CEACAM3 neutrophils. Experiment was conducted in as panel b, except the lysates were probed for a phosphorylated form of Vav (pVav) with total Vav shown as a loading control. (d) Inhibition of Src and Syk kinase activity abolishes Vav phosphorylation upon neisserial infection of MPRO-CEACAM3 PMNs. Cells were treated with the Src- and Syk-specific inhibitors PP2 and piceatannol (both at 10 μM), or carrier (dimethyl sulfoxide) for 30 min at 37°C. Cells were then infected and probed for pVav as in panel c. (e) Syk coimmunoprecipitates with CEACAM3 upon infection of MPRO-CEACAM3 PMNs with Opa_{CEA}-expressing bacteria. MPRO-puro and MPRO-CEACAM3 PMNs were infected as shown for indicated times and then lysed, followed by immunoprecipitation of the CEACAMs. Immunoprecipitates were resolved by SDS-PAGE and transferred, and the membranes were probed for Syk (top) or total CEACAM (bottom).

Similarly, we confirmed that CEACAM3, as well as Syk and Vav, could be phosphorylated in the presence of CEACAM1 (Fig. 7c to e). Our results imply that the mechanisms of inhibition by CEACAM1 are receptor and/or cell type specific, and the lack of inhibition of CEACAM3 by CEACAM1 ligation may reflect the special "wiring" of biochemical pathways within the neutrophil.

It is known that of the hundreds of Opa variants potentially encoded by *N. gonorrhoeae*, the majority bind CEACAM1 (44), whereas only a small proportion bind to CEACAM3. Based upon work such as that described here, this specificity may be attributed to selective pressures against CEACAM3 binding *in vivo*. Importantly, Opa proteins that bind CEACAM3 always bind CEACAM1, while numerous Opa proteins exist that bind CEACAM1 and not CEACAM3 (4, 7, 11). As such, we deemed it important to determine the outcome of a CEACAM1-binding strain on cells expressing CEACAM1 in the presence or absence of CEACAM3, the former being the normal situation in human neutrophils. To address this, we infected MPRO cells expressing CEACAM1 or CEACAM3, or both together, with an *N. gonorrhoeae* MS11 strain that binds to CEACAM1 but not CEACAM3 (hereafter referred to as Opa_{CCM1} to distinguish it from our regular strain, Opa_{CEA}, which binds all CEACAMs in the present study). At a low MOI, we observed that degranulation of MPRO-CEACAM1+CEACAM3 neutrophils were not stimulated by *N. gonorrhoeae* expressing the CEACAM1-specific Opa variant, in contrast to the response apparent when these cells were infected with the isogenic strain that instead expressed the CEACAM1- and CEACAM3-bispecific Opa_{CEA} (Fig. 8a, top). Therefore, it seemed the CEACAM1-binding strain was evading the CEACAM3 response. Unexpectedly, however, when a higher MOI was used, the MPRO-CEACAM1+CEACAM3 cells were observed to degranulate in response to specific CEACAM1 binding

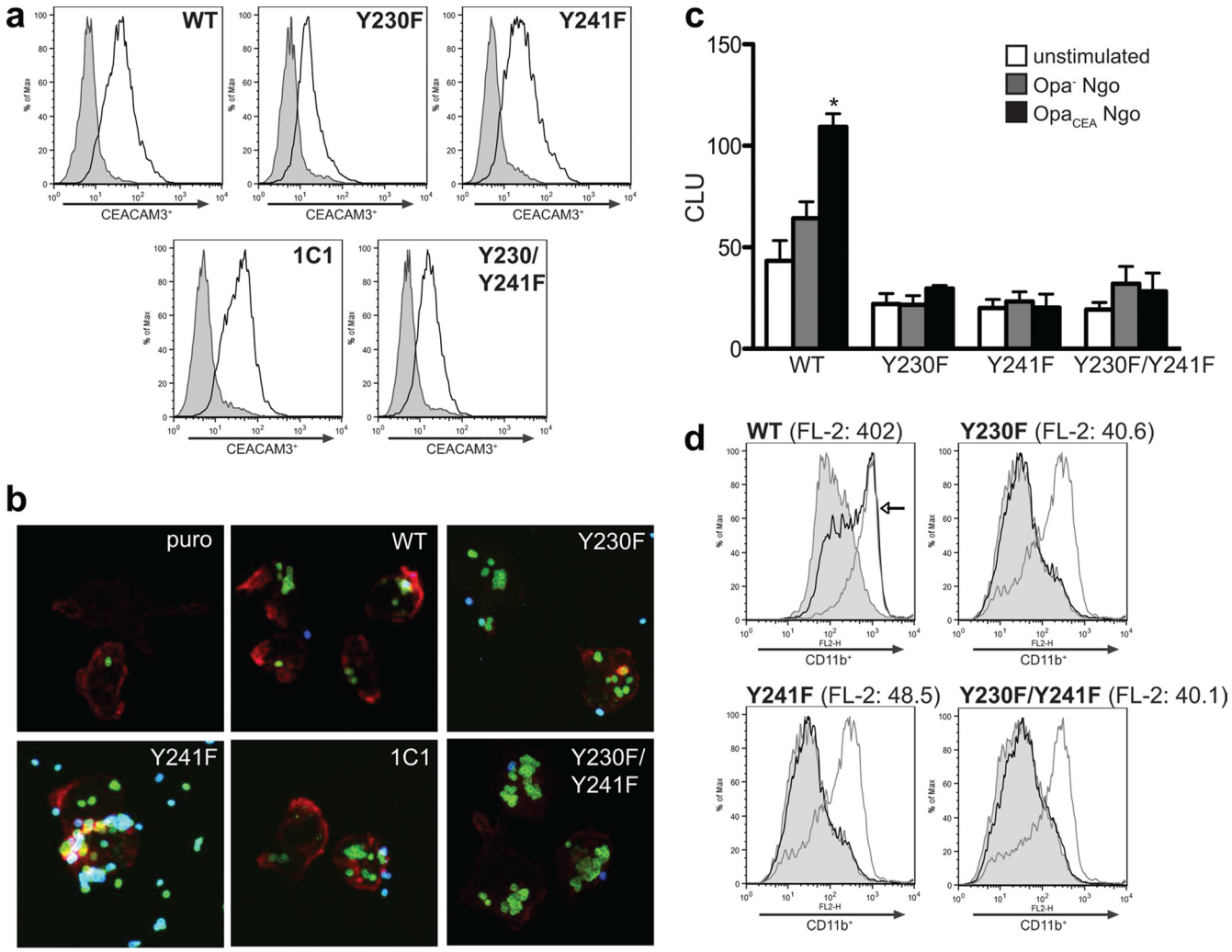

FIG 5 Mutation of critical tyrosines in CEACAM3 ITAM does not impede bacterial internalization but affects oxidative burst and degranulation. (a) Expression of CEACAM3 mutants in MPRO cells. "WT" denotes wild-type CEACAM3, "Y230F" is CEACAM3 with the first ITAM tyrosine mutated to phenylalanine, "Y241F" is the second tyrosine to a phenylalanine, "Y230F/Y241F" contains both mutations, and "1C1" is a natural splice isoform of CEACAM3 lacking the ITAM. Cells were fixed and stained for CEACAM3 expression using the Col-1 antibody, followed by a PE-conjugated secondary. FL-2 signal was acquired by flow cytometry as described in Materials and Methods. (b) CEACAM3 mutants are able to bind and internalize Opa_{CEA}-expressing *N. gonorrhoeae*. The cells were plated on glass coverslips, followed by neisserial infection for 30 min at 37°C, fixation, and processing for immunofluorescence microscopy as described in Materials and Methods. F-actin is stained with phalloidin (red) (to see the cell shape), total bacteria are in green, and extracellular bacteria appear blue. (c) Mutation of either tyrosine in the CEACAM3 ITAM is sufficient to lose oxidative burst upon CEACAM3 binding. Shown are chemiluminescence unit (CLU) counts from cells infected for 60 min. Relevant samples were analyzed by one-way ANOVA with Tukey's post-test. An asterisk indicates that a sample is significantly different than the corresponding samples from the other cell lines. (d) CD11b degranulation to the cell surface does not occur when CEACAM3 ITAM is mutated. The MOI was 10. Postinfection, cells were fixed and stained with a PE-conjugated rat anti-mouse CD11b antibody. The FL-2 signal was collected from a gated sample of 10,000 cells by flow cytometry. The geometric mean of the FL-2 signal is indicated. An arrow denotes a CEACAM3-dependent event. Histograms are labeled as follows: Opa_{CEA}-expressing *N. gonorrhoeae*, black line; PMA, gray line; or uninfected, shaded.

(Fig. 8a, bottom). This required CEACAM3, as MPRO-CEACAM1 neutrophils did not degranulate in response to either gonococcal strain. When the same two strains were used to infect human neutrophils, a similar phenomenon was observed; at low MOIs, the CEACAM1-binding strain (Opa_{CCM1}) elicited a response indistinguishable from that to the non-CEACAM binding (Opa^-) strain, while at a higher MOI it behaved like the pan-CEACAM binding strain (Opa_{CEA}) (Fig. 8b). Furthermore, the Opa_{CCM1} strain, as well two other non-CEACAM3-binding strains (expressing Opa_{50} ["50"], which binds proteoglycan receptors on cells, leading to cross-linking of β1 integrins known to activate Syk [22]; and Opa_{54} ["54"], which also binds CEACAM1) were able to stimulate Syk and Vav phosphorylation in infected human neutrophils (Fig. 8c). These data suggest that CEACAM3 can transduce signals from other CEACAMs, albeit less effectively than through direct CEACAM3 engagement. This previously unappreciated interaction lends credence to a previous suggestion that CEACAMs may exist as a complex on the activated PMN surface (36).

DISCUSSION

In the present study, we have used a murine neutrophil model to describe the contribution of individual human CEACAMs on neutrophil responses to neisserial infection. We show that *N. gon-*

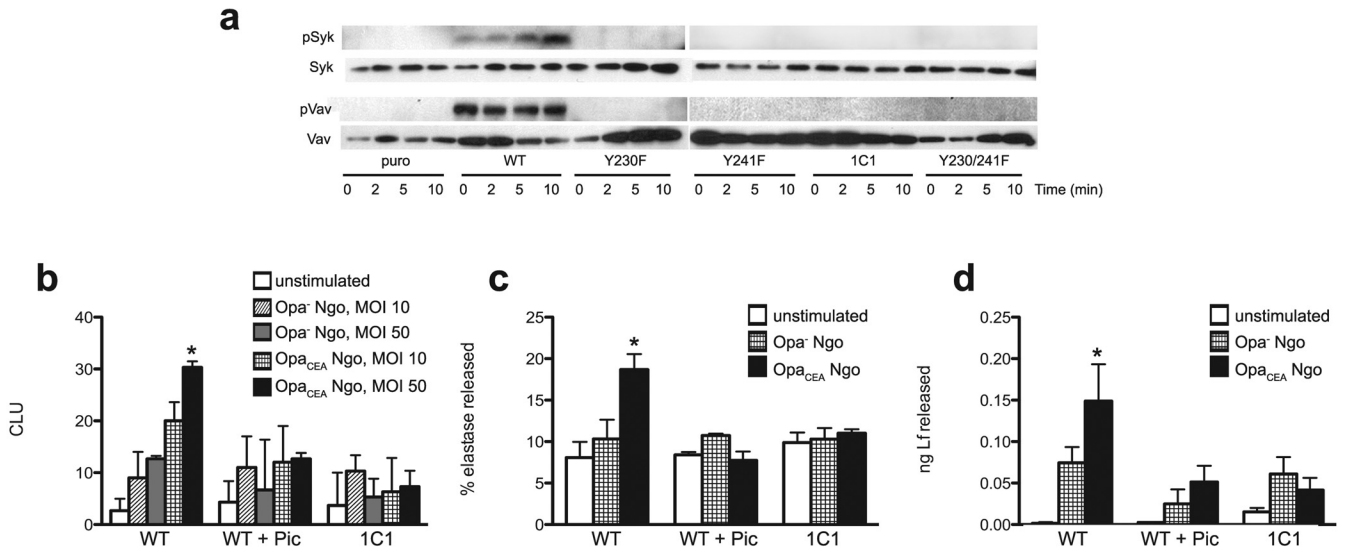

FIG 6 CEACAM3-dependent Syk activation is responsible for the PMN oxidative burst and degranulation responses to *N. gonorrhoeae* infection. (a) Mutation of either tyrosine in the CEACAM3 ITAM ablates Syk and Vav phosphorylation upon *N. gonorrhoeae* binding. Cells were infected as shown and then lysed, followed by the resolution of whole-cell lysates by SDS-PAGE and probing for activated forms of Syk (pSyk) and its downstream effector, Vav (pVav). The bottom panels show total Syk and Vav, confirming equal loading of samples. (b, c, and d) Disruption of Syk function using the pharmacological inhibitor piceatannol ("Pic"), or the use of an ITAM-deficient CEACAM3 ("1C1"), abolishes CEACAM3-mediated oxidative burst and degranulation in response to *N. gonorrhoeae* infection. Cells were infected as shown and then analyzed via luminol-dependent chemiluminescence for oxidative burst (b), or supernatants were assayed for elastase (c) or lactoferrin (d) release as described in Materials and Methods. Relevant samples were analyzed by one-way ANOVA with Tukey's post-test. An asterisk indicates that a sample is significantly different than the corresponding samples from the other cell lines.

orrhoeae, which normally cannot bind murine cells, can be captured and internalized by any one of the Opa-binding CEACAMs expressed by human PMNs (CEACAM1, CEACAM3, or CEACAM6). Strikingly, however, only CEACAM3-expressing mouse PMNs elicited an oxidative burst and the degranulation response upon neisserial infection. Accordingly, we observed that the Src family kinase-dependent phosphorylation of tyrosine residues within the CEACAM3 cytoplasmic ITAM was required for these effects, apparently due its ability to recruit and activate the protein tyrosine kinase, Syk. Indeed, when Syk function was inhibited in CEACAM3-expressing PMNs, oxidative burst and degranulation responses were lost, mirroring the effects of expressing an ITAM-less CEACAM3 in PMNs. These results reveal that CEACAM-dependent neisserial uptake in PMNs is ITAM independent, whereas activation of the neutrophil oxidative burst and degranulation are ITAM dependent. Finally, we show that the coexpression of CEACAM1 and CEACAM6 with CEACAM3 in mouse PMNs potentiates the neutrophil response to neisserial infection; this unexpected finding contrasts with the response observed in lymphocytes, in which CEACAM1 engagement has been shown to inhibit T cell receptor-mediated ITAM-based signaling. Although CEACAM1 is the only CEACAM expressed by lymphocytes, our data suggest that CEACAMs may act cooperatively in the neutrophil, allowing high-density bacterial CEACAM1 or CEACAM6 binding to elicit activating signals via CEACAM3.

The central importance of neutrophils in the pathogenesis of gonorrhea has prompted focused research seeking to characterize molecular mechanisms of neutrophil-expressed CEACAM functions in response to *N. gonorrhoeae* infection (3, 20, 31). This work has largely been conducted in transfected epithelial cell lines in an effort to study individual CEACAMs in the absence of coexpressed CEACAMs and/or other potential receptors on the neutrophil (3, 20, 32). While helpful, this approach does not permit the study of specialized PMN functions, including oxidative burst, degranulation, and cytokine responses, which are centrally important to the biology of these potently bactericidal cells. Furthermore, epithelial cells lack signaling proteins, such as the tyrosine kinase Syk, that are critical for the function of immunoreceptors (29). As such, we felt that studying these receptors in the natural context of the neutrophil was critical to understanding their authentic function.

In order to express human CEACAMs in mouse neutrophils to demonstrate distinct functions for neutrophil-expressed CEACAMs that bind neisserial Opa proteins, we exploited a developmentally arrested murine promyelocyte cell line (MPRO) that has been shown to be differentiated into functional neutrophils *in vitro*. MPRO cells were created by transducing bone marrow cells *ex vivo* with a dominant-negative form of the retinoic acid receptor alpha (RARα), a neutrophil differentiation factor, which resulted in cells arrested at the promyelocyte stage of neutrophil differentiation (41). Addition of supraphysiological concentrations of retinoic acid is sufficient to overcome the arrest, likely stimulating endogenous RARα proteins that are not associated with the dominant-negative form. These cells are, therefore, completely normal PMNs once differentiation is complete, not transformed or defective as are leukemic cell lines. Indeed, it has been shown that HL-60 cells (6) lack the ability to undergo chemotaxis in response to f-Met-Leu-Phe (37), and mis-sort secondary granule proteins such as CD11b/CD18 (12, 16), whereas these and other functions are normal in MPRO-differentiated PMNs (9, 15, 18).

There are some technical issues that must be kept in mind when discussing results from the MPRO cells. We find that these cells are less robust in their stimulation, particularly with respect to oxidative burst. This reflects what is seen with other cultured

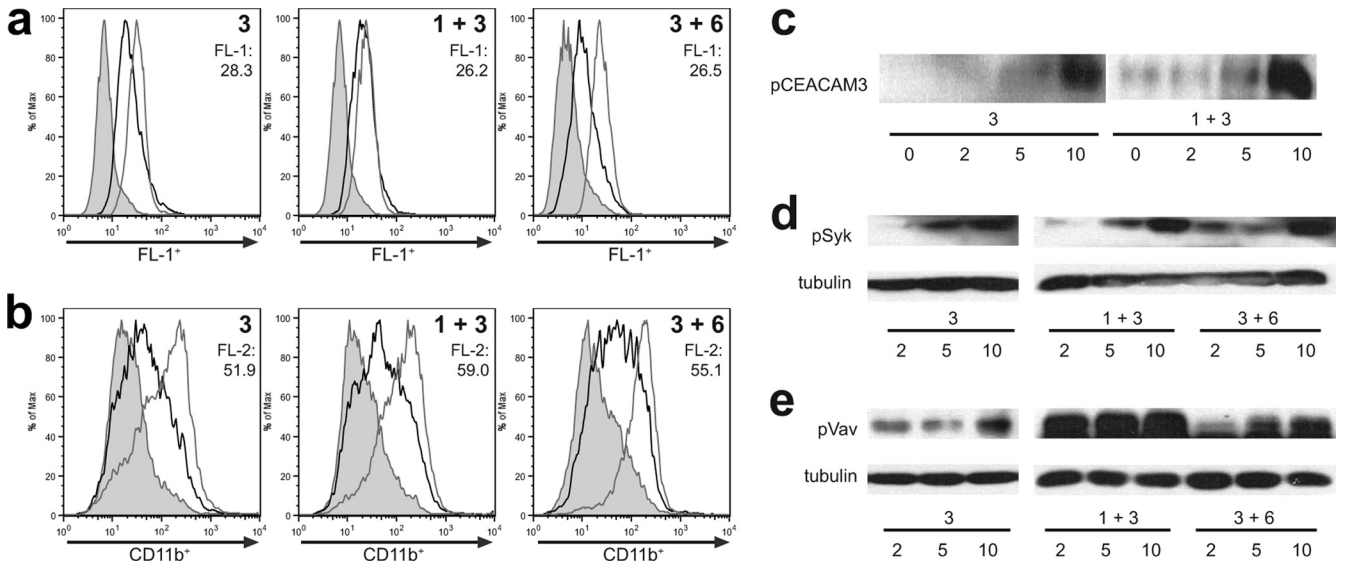

FIG 7 Coexpression of CEACAM1 or CEACAM6 with CEACAM3 does not affect CEACAM3-mediated signaling or functional effects of PMNs. (a) CEACAM1 or CEACAM6 coexpression with CEACAM3 does not hinder CEACAM3 oxidative burst activation. CEACAM1 or CEACAM6 were introduced into MPRO-CEACAM3 cells to create two double-CEACAM-expressing cell lines: MPRO-CEACAM1+CEACAM3 ("1 + 3") and MPRO-CEACAM3+CEACAM6 ("3 + 6"). Cells were differentiated for 72 h and then treated with DHR-123 and infected as described in Fig. 2a. Histograms: uninfected, tinted; Opa_{CEA}-expressing *N. gonorrhoeae*, black line; PMA treated, gray line. (b) Introduction of CEACAM1 or CEACAM6 into MPRO-CEACAM3 does not inhibit degranulation of CD11b in response to *N. gonorrhoeae* infection. Cells were infected as in Fig. 3a. Histograms are labeled as in panel a. (c) CEACAM3 is phosphorylated in the presence of CEACAM1. MPRO-CEACAM3 ("3") and MPRO-CEACAM1+CEACAM3 ("1 + 3") neutrophils were infected with Opa_{CEA}-expressing *N. gonorrhoeae* for indicated times and then lysed, followed by immunoprecipitation of phosphotyrosine-containing proteins. Immunoprecipitates were resolved by SDS-PAGE, transferred, and probed for CEACAM. (d) Syk phosphorylation upon *N. gonorrhoeae* infection of MPRO-CEACAM3, MPRO-CEACAM1+3, and MPRO-CEACAM3+6 neutrophils. Cells were infected with Opa_{CEA}-expressing bacteria, followed by lysis of the cell pellet, resolution of lysates by SDS-PAGE, and probing for a phosphorylated form of Syk (pSyk). Tubulin is shown as a loading control. (e) Vav phosphorylation upon *N. gonorrhoeae* infection of MPRO-CEACAM3, MPRO-CEACAM1+3, and MPRO-CEACAM3+6 neutrophils. The experiment was conducted as described for panel d, except the lysates were probed for a phosphorylated form of Vav (pVav) with tubulin shown as a loading control.

phagocytes, including the widely used RAW mouse macrophage-like cells, which display a considerably less robust oxidative burst compared to primary mouse macrophages (19). MPRO cells may also have a lower capacity to degranulate in response to stimulation, particularly in the case of the primary granules. Whether this is a generalized property of the cells themselves or may depend upon the nature of the stimulus remains to be determined.

One impetus for studying the individual CEACAMs in neutrophils was to consider the relative effect of CEACAM1 and CEACAM3 phosphotyrosine-based signals in response to extracellular ligation (38). That CEACAM3 is responsible for the neutrophil responses that we observed makes biological sense given the presence of the activating ITAM. Considering the potent response of neutrophils to bacterium-derived products, it was unexpected that neutrophils that bind and engulf *N. gonorrhoeae* by CEACAM1 and CEACAM6 do not initiate a bactericidal degranulation and oxidative burst response. The absence of response upon CEACAM1 ligation does not seem to emanate from its coinhibitory function, which can oppose activating signals when bound by neisserial Opa proteins in other (non-neutrophil) cell types via the phosphorylation of its ITIM tyrosines and subsequent recruitment of phosphatases (17). Our finding that coexpression of CEACAM1 and CEACAM3 does not suppress the activating effect of the latter suggests that this coinhibitory activity does not occur in neutrophils. We suspect this may be attributed to the special characteristics of the PMN, since these cells are short-lived and their activation is reigned in through apoptosis and clearance via surface phosphatidylserine recognition by macrophages (33), they may not require intricate coinhibitory signaling seen in other cell types.

While engulfment by CEACAM3 appears to occur via a process that reflects that by phagocytic immunoglobulin-specific Fc receptors (13, 29, 3, 30), the mechanism by which *N. gonorrhoeae* are engulfed upon CEACAM1 and CEACAM6 binding remains uncharacterized. In this regard, it is of interest that we observed F-actin-rich regions surrounding some bacteria being internalized via CEACAM6, something that has not been observed in epithelial cell models (3); this may suggest a neutrophil-specific binding partner for CEACAM6. Alternatively, because CEACAM6 is a GPI-anchored protein that has been shown to reside in cholesterol-rich membrane microdomains (24), its inclusion in such regions of the plasma membrane may promote association with signaling factors that themselves reside in these regions (35). Further studies characterizing proteins involved in CEACAM-mediated uptake processes in neutrophils will be useful to determine how these processes differ from those observed in epithelial cells.

Another unexpected result emanating from this work was the capacity for a CEACAM1-binding Opa protein to elicit CEACAM3-dependent PMN activation. This effect was not apparent at low bacterial densities, suggesting that it is less efficient than direct CEACAM3 engagement. Whether it reflects a CEACAM-specific cooperative response (as suggested previously [36]) or the result of nonspecific clustering of receptors on PMNs, such as through presence in similar membrane microenvironments, remains to be determined. In considering the lack of acti-

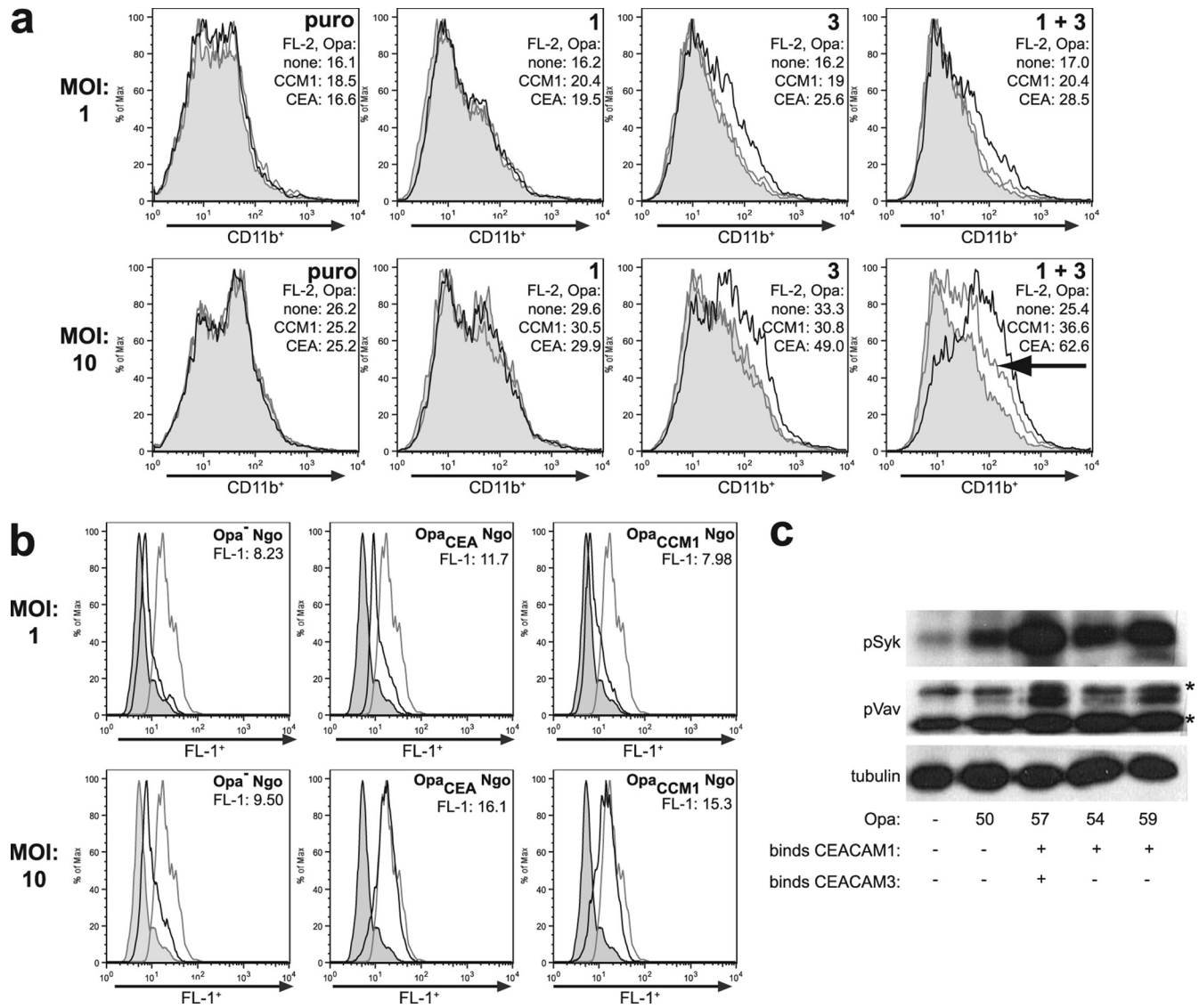

FIG 8 Cooperative CEACAM signaling in response to *Neisserial* infection. (a) Activation of CEACAM3-dependent signaling with a CEACAM1-binding *Neisseria* strain. We used a *N. gonorrhoeae* strain expressing a CEACAM1-specific Opa variant (Opa_{CCM1}) to determine whether bacteria could evade the CEACAM3-dependent neutrophil response. Differentiated MPRO-puro, MPRO-CEACAM1, MPRO-CEACAM3, and MPRO-CEACAM1+CEACAM3 cells were infected with Opa_{CCM1}-expressing *N. gonorrhoeae* (gray line) or Opa_{CEA}-expressing *N. gonorrhoeae* (black line) as shown with specific MOIs or left untreated (tinted). The cells were then fixed, stained for surface appearance of the granule marker CD11b, and then analyzed by flow cytometry for FL-2 signal. (b) Human neutrophils undergo oxidative burst in response to infection by a CEACAM1-binding *Neisseria* strain, at a high MOI. Human PMNs were treated with DHR-123 and then infected with Opa^- *N. gonorrhoeae* (left panels, black histogram), Opa_{CCM1}-expressing *N. gonorrhoeae* (middle panels, black histogram), or Opa_{CEA}-expressing *N. gonorrhoeae* (right panels, black histogram) as shown with specific MOIs. Untreated (tinted; FL-1, 6.16) and PMA-treated (gray histogram; FL-1, 19.3) are superimposed on all histograms for comparison. Geometric means of the FL-1 signal are shown for the *N. gonorrhoeae* strains. (c) CEACAM3 effectors are activated when human PMNs are infected with *Neisseria* that bind CEACAM1 and not CEACAM3. A total of 10^6 human PMNs were infected with *N. gonorrhoeae* strains expressing the following Opa proteins: "−" (no Opa), "50" (binds heparan sulfate proteoglycans and cross-links integrins), "57" (OpaCEA); "54" (binds CEACAM1 but not CEACAM3), and "59" (OpaCCM1). The MOIs were 10; cells were infected at 37°C as shown, followed by lysis of the cell pellet, resolution of lysates by SDS-PAGE, and probing for a phosphorylated form of Syk (pSyk) and Vav (pVav). Tubulin is shown as a loading control. An asterisk denotes nonspecific bands.

vating signals upon exposure to CEACAM1-specific bacteria at a low MOI (Fig. 8a and b), it is interesting to consider that there is variability in PMN responses to gonococci bearing different Opa variants (2, 11). The ability of different Opa variants to activate PMNs had been speculated to be a product of the Opa proteins themselves, as a result of variable sequence conferring different affinities for a single receptor (2). Our data support this proposal, since the variability may reflect the specific outcome of binding to CEACAM3 directly versus indirect CEACAM3-engagement via Opa protein binding to CEACAM1 and/or CEACAM6.

While the number of physiological functions attributed to the CEA family is vast, ranging from cell adhesion, insulin signaling, and angiogenesis, the only known role for CEACAM3 in human tissues is as a receptor for the Gram-negative, human-restricted

pathogens *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Moraxella catarrhalis*, and *Haemophilus influenzae* (30). This peculiar observation has been postulated by ourselves and others as a function of coevolution between pathogen and host. While CEACAM1, CEACAM5, and CEACAM6 are useful for colonization (25, 45) and immune evasion (5), CEACAM3 is an evolutionarily "new" receptor that is clearly deleterious for bacteria. As such, CEACAM3 represents a "molecular mimic" that allows neutrophil-dependent clearance of pathogens that target other CEACAM family receptors. The striking parallels between the response of CEACAM3-expressing murine neutrophils and primary neutrophils from humans reflects the ability of human CEACAM3 to engage downstream mouse effectors, as seen here. By directly engaging Syk kinase, the CEACAM3-dependent signals converge with those from immunoreceptors, including the phagocytic immunoglobulin Fc domain-specific receptors and Dectin-1, eliciting an evolutionarily conserved and highly effective bactericidal response to these highly adapted human pathogens.

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