

6. Bonder MJ, Luijk R, Zhernakova DV, Moed M, Deelen P, Vermaat M, et al. Disease variants alter transcription factor levels and methylation of their binding sites. *Nat Genet* 2017;49:131-8.
7. Croteau-Chonka DC, Qiu W, Martinez FD, Strunk RC, Lemanske RF, Liu AH, et al. Gene expression profiling in blood provides reproducible molecular insights into asthma control. *Am J Respir Crit Care Med* 2017;195:179-88.
8. Schedel M, Michel S, Gaertner VD, Toncheva AA, Depner M, Binia A, et al. Polymorphisms related to ORMDL3 are associated with asthma susceptibility, alterations in transcriptional regulation of ORMDL3, and changes in TH2 cytokine levels. *J Allergy Clin Immunol* 2015;136:893-903.e14.
9. Millstein J, Chen GK, Breton CV. cit: hypothesis testing software for mediation analysis in genomic applications. *Bioinformatics* 2016;32:2364-5.

Available online January 31, 2018.
<http://dx.doi.org/10.1016/j.jaci.2017.11.057>

Human T_H17 cell development requires processing of dendritic cell-derived CXCL8 by neutrophil elastase

To the Editor:

IL-17-producing effector CD4⁺ helper T cells (T_H17 cells) have been identified as crucial cells in the defense against

pathogens such as bacteria and fungi, which are effectively challenged through amplification of neutrophil activity.¹ Indeed, patients with inborn errors in IL-17 expression or the IL-17Rs suffer from severe mucocutaneous candidiasis.² On the other hand, T_H17 cells are implicated in the pathogenesis of various chronic inflammatory diseases, including autoimmune diseases. Although in mice the factors for initiation (IL-6/TGF- β) and stabilization (IL-21/ICOSL/IL-1 β /IL-23) of T_H17 cells from naive CD4⁺ precursors are known, the factors that mediate human T_H17 cell development are still under debate.³

The development of effector T cells is directly driven by pathogen-primed dendritic cells (DCs), while innate effector cells may affect DC function and amplify polarization into specific T-cell subsets.⁴ Therefore, we hypothesized that neutrophils, the innate effector cell associated with T_H17 cell responses present in peripheral tissues and lymph nodes during infections,⁵ may amplify or initiate the development of DC-driven T_H17 cells from naive human CD4⁺ T cells. In line with this hypothesis, we found that patients with impaired neutrophil function (for details, see the [Methods](#) section in this article's Online Repository at www.jacionline.org) had no or

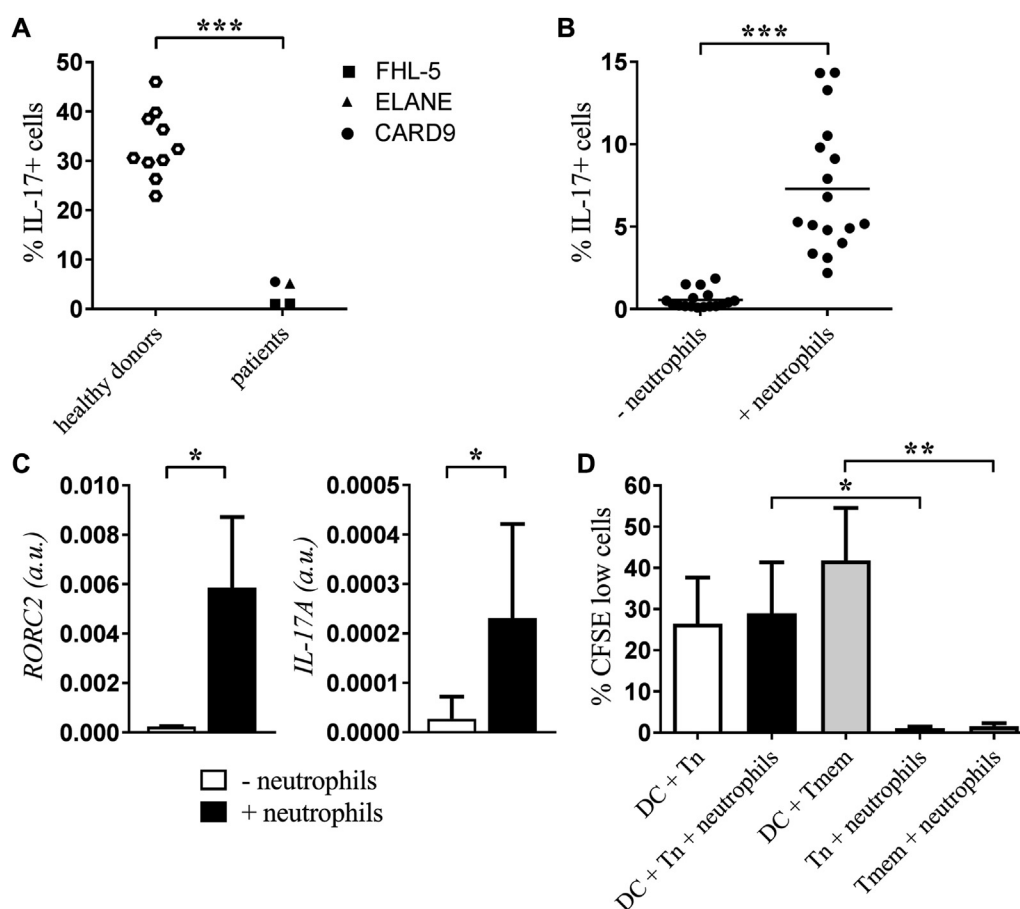


FIG 1. Neutrophils are essential for the induction of human T_H17 cells. **A**, Percentage of *C albicans*-specific memory T_H17 cells analyzed after coculture with *C albicans*-activated DCs. Cells were isolated from 4 patients with different functional neutrophil defects. **B** and **C**, Highly pure human CD4⁺CD45RA⁺CD45RO[−] naive T cells were cocultured with *C albicans*-activated DCs with or without neutrophils and restimulated after a resting phase. Shown are pooled data of the percentage IL-17-expressing cells (healthy donors, Fig 1, **B**), and RORC2 and IL-17A mRNA expression in antigen-specific T cells sorted on day 13 (Fig 1, **C**, n = 9, mean ± SEM). **D**, T-cell proliferation (measured by CFSE dilution) in different culture conditions. a.u., Arbitrary units; CFSE, carboxyfluorescein succinimidyl ester; Tn, naive T cell; Tmem, memory T cell. *P < .05, **P < .01, and ***P < .001.

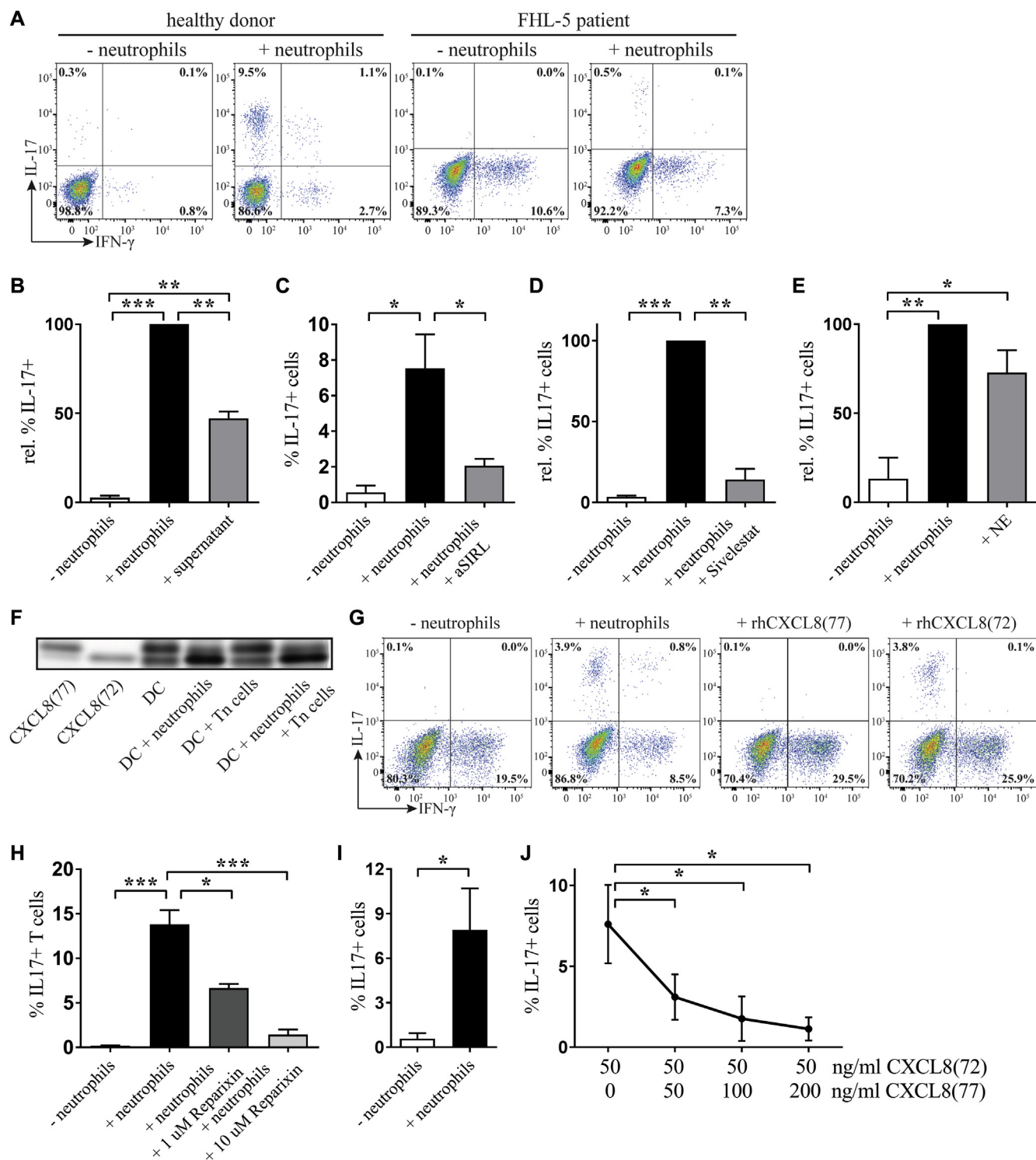


FIG 2. NE processes CXCL-8(77) into CXCL-8(72), which is necessary for human T_H17 cell development. T_H17 cell development from highly purified human $CD4^+CD45RA^+CD45RO^-$ naive T cells upon coculture with *C albicans*-activated DCs with or without neutrophils (healthy donor vs FHL-5 patient) (**A**), or with supernatant from fMLP-activated neutrophils (**B**), or in the presence of SIRL-1-activating antibodies (**C**), Sivelestat, a selective NE inhibitor (**D**), NE (**E**), recombinant CXCL-8(77) or CXCL-8(72) (**G**), or reparixin, a specific inhibitor of CXCR1 and CXCR2 (**H**) (all $n = 3$, mean $\pm$ SEM). **F**, A representative silverstain of CXCL-8 immunoprecipitated from culture supernatants of indicated cocultures. **I**, T_H17 cell development from $CD4^+CXCR1^+$ T cells sorted 4 days after activation with *C albicans*-activated DCs in the presence or absence of neutrophils ($n = 5$, mean $\pm$ SEM). **J**, T_H17 cell development from naive $CD4^+$ T cells after activation with *C albicans*-activated DCs in the presence of different ratios of CXCL-8(72) and CXCL-8(77) ($n = 3$, mean $\pm$ SEM). *aSIRL*, Anti-SIRL-1; *fMLP*, *N*-formyl-methionyl-leucyl-phenylalanine; *Rel*, % IL-17+ cells, relative expression of IL-17+ cells in specific conditions normalized to conditions with neutrophils; *SIRL-1*, signal inhibitory receptor on leukocytes-1; *Tn*, naive T cell; *Tmem*, memory T cell. * $P < .05$, ** $P < .01$, and *** $P < .001$.

severely decreased percentages of T_H17 cells in their CD4⁺ memory T-cell compartment compared with healthy controls (Fig 1, A). Moreover, DCs activated by heat-killed *C albicans* hyphae were not able to induce the development of T_H17 cells from highly pure healthy human CD4⁺CD45RA⁺CD45RO⁻ naive T cells. The presence of *C albicans*-activated neutrophils (purity > 97%; see Fig E1, A, in this article's Online Repository at www.jacionline.org) was essential to induce T_H17 cells as reflected by cytokine production and expression of T_H17 cell-specific transcription factor *RORC2* and *IL-17A* mRNA (Fig 1, B and C; Fig E1, B). In contrast, the presence of neutrophils did not affect T_H1 cell development (*TBX21* transcript levels, IFN- γ production; Fig E1, C-E), proliferation of candida-specific naive T cells (Fig E1, F), or the *in vitro* expansion of *in vivo*-formed IL-17-producing memory T cells (Fig E1, G). It should be noted that contamination with minute populations of contaminating memory (CD45RA⁻CD45RO⁺) or recently activated (CD45RA⁺CD45RO⁺) T cells during the activation of naive CD4⁺ T cells *in vitro* resulted in a major T_H17 cell population independent of neutrophils (Fig E1, H and I). Neutrophils alone were insufficient to activate naive or memory CD4⁺ T cells (Fig 1, D). In addition to monocyte-derived DCs, human skin-derived Langerhans cells and dermal DCs needed neutrophils to potently induce T_H17 cells (see Fig E2, A, in this article's Online Repository at www.jacionline.org). Also, T_H17 cell formation in response to another fungus (*Aspergillus fumigatus*) and to bacteria was dependent on the presence of neutrophils (Fig E2, B).

Importantly, stimulation of FHL-5 patient-derived naive CD4⁺ T cells, neutrophils, and DCs by *C albicans* failed to result in T_H17 induction, indicating that degranulation of neutrophils is essential for T_H17 induction (Fig 2, A). Indeed, supernatant of *N*-formyl-methionyl-leucyl-phenylalanine-induced degranulated neutrophils from healthy donors supported induction of *C albicans* hyphae-specific T_H17 cells (Fig 2, B). In addition, ligation of signal inhibitory receptor on leukocytes-1, which limits neutrophil extracellular trap (NET) formation,⁶ resulted in a strong inhibition of T_H17 cell induction (Fig 2, C). Because these data suggested the involvement of soluble factors present in neutrophilic granules or attached to NETs, we further investigated the role of neutrophil elastase (NE). Remarkably, selective inhibition of NE by Sivelestat completely abrogated the induction of T_H17 cells (Fig 2, D). Moreover, replacing neutrophils with human NE resulted in similar T_H17 cell development (Fig 2, E). The amount of NE release upon *C albicans*-mediated activation of neutrophils was irrespective of the presence of DCs or T cells (see Fig E3, A, in this article's Online Repository at www.jacionline.org). Blocking of 2 other major neutrophil granule components, that is, cathepsin G or myeloperoxidase, did not affect T_H17 cell development (data not shown).

NE is known to cleave CXCL8 of 77aa (CXCL8(77)) at the N-terminus into the short form of 72aa, CXCL8(72).⁷ *C albicans*-activated DCs produced CXCL8, which was not significantly augmented by neutrophils (Fig E3, B). Yet, the presence of neutrophils resulted in a clear loss of CXCL8(77) and induction of CXCL8(72) (Fig 2, F). Importantly, rhCXCL8(72) but not rhCXCL8(77) strongly promoted T_H17 cell development from naive T cells by *C albicans*-activated DCs without consistently affecting T_H1 cell development (Fig 2, G). Moreover, reparixin (inhibitor of

CXCL8Rs CXCR1 and CXCR2) inhibited T_H17 cell formation (Fig 2, H). While naive T cells lack expression of CXCL8Rs, a subpopulation of T cells expressed CXCR1 after 4 days of activation and more T_H17 cells appeared to develop from CXCR1⁺ T cells than from CXCR1⁻ T cells (Fig E3, C and D). In line with these findings, only recently activated CXCR1⁺ T cells that were primed in the presence of neutrophils (and thus CXCL8(72)) developed into T_H17 cells (Fig 2, J). Finally, CXCL8(77), which binds to the same receptor as CXCL8(72), dose dependently inhibited the induction of T_H17 cells by CXCL8(72) (Fig 2, J), explaining why low levels of CXCL8(72) and abundance of CXCL8(77) produced by DCs in the absence of neutrophils was insufficient to drive T_H17 cell development.

In conclusion, we show that neutrophils are essential for the induction of human T_H17 cells. Upon activation, neutrophils release NE that processes DC-derived CXCL8 into a truncated potent T_H17 cell-inducing form. This novel insight is crucial for the development of new strategies for treatment of both bacterial and fungal infections and chronic inflammatory disorders characterized by the presence of activated neutrophils and/or T_H17 cells that are linked to the pathophysiology of these diseases.⁸ The efficacy of targeting neutrophils is supported in a mouse model for inflammatory bowel disease showing that NE inhibition ameliorated inflammatory bowel disease.⁹ Inhibition of neutrophil activation, inhibition of NE, or blocking of CXCL8 may provide a suitable alternative to prevent the generation of T_H17 cells.

We thank M. L. Kapsenberg for critical scientific discussions and reviewing the manuscript. We thank R. Mebius, E. Eldering, and T. Geijtenbeek for critical reading of the manuscript. We thank R. Pouw for preparing anti-mouse sepharose, G. van Mierlo for help with measuring elastase, and S. Zaat for using the laboratory to grow microorganisms.

Yuri Souwer, PhD^{a,*}

Tom Groot Kormelink, PhD^{b,*}

Esther W. Taanman-Kueter, BSc^b

Femke J. Muller, BSc^a

Toni M. M. van Capel, BSc^b

Domonkos V. Varga, MSc^a

Yotam E. Bar-Ephraim, PhD^c

Marcel B. M. Teunissen, PhD^c

S. Marieke van Ham, PhD^d

Taco W. Kuijpers, MD, PhD^{e,f}

Diana Wouters, PhD^d

Linde Meyaard, PhD^g

Esther C. de Jong, PhD^b

From ^athe Department of Cell Biology & Histology, ^bthe Department of Experimental Immunology, and ^cthe Department of Dermatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; ^dthe Department of Immunopathology, Sanquin Blood Supply and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; ^eEmma Children's Hospital, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; ^fthe Department of Blood Cell Research, Sanquin Blood Supply and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; and ^gthe Laboratory of Translational Immunology, Department of Immunology, University Medical Center Utrecht, Utrecht, The Netherlands. E-mail: e.c.dejong@amc.nl.

*These authors contributed equally to this work.

This work was supported by grants from the Landsteiner Foundation for Blood Research (LSBR, grant no. 0816), the Dutch Technology Foundation STW (grant no. 10725), and Netherlands Organization for Scientific Research (NWO) Vici (grant no. 91815608).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

REFERENCES

1. Weaver CT, Elson CO, Fouser LA, Kolls JK. The Th17 pathway and inflammatory diseases of the intestines, lungs, and skin. *Annu Rev Pathol* 2013;8:477-512.
2. Puel A, Cypowyj S, Bustamante J, Wright JF, Liu L, Lim HK, et al. Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science* 2011;332:65-8.
3. van Beelen AJ, Zelinkova Z, Taanman-Kueter EW, Muller FJ, Hommes DW, Zaat SA, et al. Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells. *Immunity* 2007;27:660-9.
4. Breedveld A, Groot Kormelink T, van Egmond M, de Jong EC. Granulocytes as modulators of dendritic cell function. *J Leukoc Biol* 2017;102:1003-16.
5. Kamenyeva O, Boularan C, Kabat J, Cheung GY, Cicala C, Yeh AJ, et al. Neutrophil recruitment to lymph nodes limits local humoral response to *Staphylococcus aureus*. *PLoS Pathog* 2015;11:e1004827.
6. van Avondt K, van der Linden M, Naccache PH, Egan DA, Meynard L. Signal inhibitory receptor on leukocytes-1 limits the formation of neutrophil extracellular traps, but preserves intracellular bacterial killing. *J Immunol* 2016;196:3686-94.
7. Padrines M, Wolf M, Walz A, Baggiolini M. Interleukin-8 processing by neutrophil elastase, cathepsin G and proteinase-3. *FEBS Lett* 1994;352:231-5.
8. Bardeel BW, Kenny EF, Sollberger G, Zychlinsky A. The balancing act of neutrophils. *Cell Host Microbe* 2014;15:526-36.
9. Morohoshi Y, Matsuoka K, Chinen H, Kamada N, Sato T, Hisamatsu T, et al. Inhibition of neutrophil elastase prevents the development of murine dextran sulfate sodium-induced colitis. *J Gastroenterol* 2006;41:318-24.

Available online January 31, 2018.
<http://dx.doi.org/10.1016/j.jaci.2018.01.003>

Characterization of drug-neutralizing antibodies in patients with Fabry disease during infusion



To the Editor:

Enzyme replacement therapy (ERT) leads to infusion-associated reactions (IARs) as well as the formation of neutralizing antidrug antibodies (ADA) in male patients with Fabry disease (FD).¹ Recently, we demonstrated that ADA are also associated with increased left ventricular mass and progressive loss of renal function.² Furthermore, we also showed that immunosuppression due to transplantation lead to a decrease of ADA titers in affected patients.³ However, due to the lack of standardized assays, the impact of ADAs on disease progression is still controversially discussed and the isotype of antibodies is yet unknown.¹

In the current study, we aimed to identify the IgG isotype mediating ERT inhibition in FD patients and to establish an improved inhibition assay with minimized background signal by application of isolated IgGs. Consequently, we used this improved assay to determine the rescued galactosidase A (GLA) activity of the infused enzyme overcoming neutralizing ADAs. This approach may help optimizing therapy strategies by individually tailored infusion dosages.

To demonstrate a direct effect of IgGs on ERT inhibition in patients with FD and to overcome protocol-dependent disadvantages limiting the application of currently used inhibition assays,¹ we modified the existing protocols for inhibition measurements first described by Linthorst et al⁴ by using purified total IgG antibodies from 24 patients with definite serum-mediated inhibition status instead of raw serum samples. The clinical characteristics of the patients are described in Table E1 in this article's Online Repository at www.jacionline.org.

In comparison to serum-mediated inhibition assays with samples from ERT-naïve patients, advanced inhibition assays

(AIAs) with increasing amounts of purified total IgGs from ERT-naïve FD patients (n = 12) and samples from inhibition-negative patients under ERT (n = 9) demonstrated no significant changes for rescued GLA activities and no significant background signal ($P = .6869$ and $P = .3139$) (see Fig E1, A-D and Fig E2 in this article's Online Repository at www.jacionline.org). By contrast, purified total IgGs from inhibition-positive patients revealed a concentration-dependent significantly decreased GLA activity ($P = .0036$) (see Fig E1, C and D). AIAs also demonstrated different inhibitory capacity of neutralizing antibodies per microgram total IgG in patients (Fig E1, E).

To determine the isotype of inhibitory IgG antibodies, we measured IgG titers by ELISA before ERT (naïve) and during ERT (43 ± 19 months) in 8 patients with known inhibition status, demonstrating stable antibody titers in ERT inhibition-negative (ERT^{-}) patients, while ERT inhibition-positive (ERT^{+}) patients presented with up to 18-fold increased IgG₄ titers (2.6- to 18.9-fold) (see Fig E3, A in this article's Online Repository at www.jacionline.org), which was confirmed by representative Western blots (see Fig E4 in this article's Online Repository at www.jacionline.org). Between-group analysis confirmed the IgG₄ increase (ERT^{-} : 1.22 ± 0.03 vs ERT^{+} : 9.80 ± 3.40 ; $P = .0452$). To demonstrate a direct effect of IgG₄ antibodies on ERT inhibition, we performed an antibody-based GLA enzymatic activity rescue (GEAR) experiment, demonstrating that the rescued GLA activity decreased significantly in patients with specific anti-GLA IgG₄ isotype antibodies by $-18.7 \pm 2.3\%$ ($P < .001$), while rescued GLA activity in patients without anti-GLA antibodies remained unchanged ($P = .4330$) (Fig E3, B). A different impact of agalsidase- α , or - β on IARs or ADA formation was not observed. Of note, analysis of lyso-Gb3 levels as a marker of disease burden and therapy response revealed decreased levels in patients without measurable ADAs after infusion (each n = 4; 36.9 ± 13.0 vs 90.6 ± 29.9 ng/mL; $P = .0093$).

The general dose-response discussion has raised the question, whether increased total infused ERT amounts per infusion might overcome antibody titers. To analyze the impact of ERT on antibody titers during infusion, total IgGs directly before (pre) and after (post) infusions were purified and ERT inhibition per microgram total IgG was determined from 8 ERT inhibition-positive patients. Individual profiles demonstrated differences in ERT inhibition directly after infusions (Fig 1, A-H). Of note, we detected a significant correlation between increasing amounts of ERT and reduced ERT inhibition after infusion (Spearman $r = -0.8128$, $P = .0058$) (Fig 1, I). Consequently, we monitored ADA titers in patient 16 receiving different amounts of agalsidase- β within 2 weeks (Fig 2). During these 2 independent infusion settings, repetitive serum samples (opposite arm, every 30 minutes) were drawn. Interestingly, while receiving 35 mg only a slight decrease of ADA titers was observed (Fig 2, A) ($P = .0881$), and ADAs were still present at the end of infusion. By contrast, 70 mg agalsidase- β caused a significant decrease of ADA titers ($P = .0013$) (Fig 2, B).

By using a modified IgG-based inhibition assay, we characterized neutralizing ADAs in male patients with FD. Our main findings are (1) neutralizing ADAs against recombinant GLA are of the IgG₄ isotype, (2) the modified assay features minimal background signal and allows improved FD patient classification, and most importantly, (3) the total amount of infused recombinant GLA per infusion correlates with the

METHODS

Cell isolation

Human PBMCs were isolated from fresh, heparinized (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) blood from healthy donors. In a selected set of experiments, peripheral blood of patients with diverse neutrophil function disorders was used. The following patients were included: an ELANE patient with congenital neutropenia, a patient with a defect in CARD9, the signaling molecule downstream of the curdlan-recognizing Dectin-1, and 2 patients with familial hemophagocytic lymphohistiocytosis (FHL-5), in which neutrophils do not degranulate.^{E1-E4} Isolation was performed by density gradient centrifugation on Lymphoprep ($d = 1.077 \pm 0.001$ g/mL; Axis-Shield, Oslo, Norway). PBMCs were subsequently separated into monocyte and peripheral blood lymphocyte (PBL) fractions by density gradient centrifugation on percoll (GE Healthcare, Hoevelaken, The Netherlands). For this procedure, 3 different percoll solutions were used (34%, 47.5%, and 60%, all in Iscove's Modified Dulbecco's Medium [IMDM; Lonza, Verviers, Belgium] supplemented with 1% heat-inactivated [HI]-FCS [Hyclone; Thermo Fischer Scientific Inc, Waltham, Mass] and gentamycin [86 µg/mL, Duchefa Biochemie B.V., Haarlem, The Netherlands]). Upon harvesting, the monocyte fraction was washed with IMDM supplemented with 1% HI-FCS and gentamycin. PBLs were simultaneously harvested and washed with HBSS supplemented with 2% HI-FCS and gentamycin. CD4⁺ T cells were isolated from the PBL fraction using a CD4⁺ T-cell isolation kit II (Miltenyi Biotec Inc, Leiden, The Netherlands) and CD4⁺CD45RA⁺ naive cells were subsequently separated from the CD4⁺CD45RO⁺ memory cells by positive selection of memory T cells using CD45RO-Phycoerythrin antibody (DAKO, Heverlee, Belgium) and magnetic-labeled anti-PE beads (Miltenyi Biotec). Purity of the obtained naive T cells was always more than 99%, as analyzed by fluorescence-activated cell sorting.

Monocyte-derived DCs were generated by plating 0.4×10^6 freshly isolated monocytes in 1 mL in a 24-well plate in IMDM/1% HI-FCS/gentamycin. The plate was incubated at 37°C, 5% CO₂ for 30 to 45 minutes and nonadherent cells were removed by washing with IMDM/1% HI-FCS/gentamycin and monocytes were cultured in IMDM/5% FCS/gentamycin with 500 U/mL recombinant human (rh) GM-CSF (Schering-Plough B.V., Brussels, Belgium) and 10 U/mL rhIL-4 (Miltenyi Biotec). At day 3, medium was refreshed as follows: 500 µL medium was discarded and the wells were replenished with 500 µL IMDM/5% FCS/gentamycin containing 1000 U/mL rhGM-CSF and 20 U/mL rhIL-4. Immature monocyte-derived DCs were harvested on day 6 or 7 for further experiments.

Epidermal and dermal cell suspensions were obtained from residual skin obtained from plastic surgery. Overnight incubation at 4°C in 0.2% dispase II (Boehringer Mannheim, Mannheim, Germany) enabled separation of epidermis and dermis, whereafter epidermal sheets were incubated in 0.25% trypsin solution (5 minutes at 37°C; Invitrogen Life Technologies, Breda, The Netherlands) and dermal sheets in IMDM containing 0.2% collagenase D (Boehringer Mannheim), 40 U/mL DNase I (Boehringer Mannheim), and 2% FCS for 2 hours at 37°C to yield single cells. After labeling with fluorescent-conjugated mAbs (BD Biosciences, San Jose, Calif), highly pure (>95%) CD1a⁺HLA-DR⁺ Langerhans cells and CD3⁺CD14⁺HLA-DR⁺CD11c⁺CD45⁺ dermal DCs were isolated by FACS (BD Biosciences). Medical Ethical Committee approval number was METC 10/309.

Neutrophils were isolated from the erythrocyte pellet obtained after density centrifugation on Lymphoprep. The erythrocyte pellet was transferred to a new 50-mL tube (Greiner Bio-One) and erythrocytes were lysed in ice-cold erythrocyte lysis buffer (containing 0.155 mol NH₄Cl [Sigma-Aldrich], 1 mM KHCO₃ [Merck KGaA, Darmstadt, Germany], and 80 µM EDTA [Merck KGaA], dissolved in sterile water, pH 7.3) for 15 minutes on ice. After centrifugation, the pellet was lysed once more for 5 minutes on ice and subsequently washed with PBS. Neutrophils were then resuspended in IMDM supplemented with 5% HI-FCS (Lonza, Verviers, Belgium) and gentamycin and kept on ice until use. Purity of the neutrophils was always more than 97%, as analyzed by flow cytometry.

Microorganisms

C. albicans cultures (clinical isolate) were maintained at the Department of Experimental Microbiology, Academic Medical Center, Amsterdam. Yeast

particles were resuspended in IMDM supplemented with 10% HI-FCS w/o gentamycin and plated on flat-bottom 96-well plates. (Pseudo-) Hyphae were formed by incubating for 4 hours at 37°C, 5% CO₂, followed by heat-killing for 2 hours at 70°C. *Haemophilus influenza* strains ATCC9007 and ATCC608124 and *Staphylococcus aureus* (clinical isolate) were from the Department of Experimental Microbiology. *Aspergillus fumigatus* was a kind gift from T. Geijtenbeek, AMC.

Cocultures

Unless stated otherwise, cell cultures were done in IMDM supplemented with 5% HI-FCS and gentamycin at 37°C, 5% CO₂. Initial culturing was done in 96-well plates (Costar, Corning Inc, Corning, NY) with 50,000 DCs and T cells, 100,000 neutrophils, and 10,000 heat-killed *C. albicans* hyphae per well. Dependent on the growth of the culture, cells were transferred after 4 to 5 days of culturing to 24-well plates (Costar) and cultures were refreshed every 2 days with rhIL-2 (20 U/mL, Novartis AG, Basel, Switzerland) containing medium. FACS sorting of CXCL8R-positive cells was performed on day 4 and sorted cells were cultured in medium alone as described above. For coculture experiments the following reagents, enzymes, and cytokines were used: rhCXCL8 (77 and 72, both from Miltenyi Biotec; 200, 100, and 50 ng/mL), reparixin (MedChem Express, Monmouth Junction, NJ, 1 and 10 µM), and human NE (Sigma-Aldrich, Zwijndrecht, The Netherlands, 0.2 U/mL). anti-Signal inhibitory receptor on leukocytes-1 antibody was used as previously described.^{E6,E7}

For degranulation, neutrophils were incubated in medium at 37°C in a shaking water bath when adding the (priming) agent cytochalasin B (5 µg/mL, Sigma-Aldrich, 5 minutes) and subsequent stimulation with *N*-formyl-methionyl-leucyl-phenylalanine (1 µM, Sigma-Aldrich, 15 minutes). Hereafter, supernatants were gently centrifuged to get rid of cells and cell debris and supernatants were immediately used in experiments.

Quantitative PCR

RNA was extracted NucleoSpin RNA Isolation Kit (Machery-Nagel, Düren, Germany) and first-strand cDNA (MBI Fermentas) transcribed according to manufacturer's instructions. Gene expression of RORC2 (forward 5'-CCTGA CAGAGATAGAGACC-3', reverse 5'-GTTCCACATCTCCACATG-3'), IL-17A (forward 5'-CTTGAATCTCCACCGCAAT-3', reverse 5'-CGCAG GACCAGATCTCTTG-3'), and T-bet (forward 5'-CCTACCAGAATGCCGA GATTACTC-3', reverse 5'-GATGCTGGTGTCAACAGATGTGTAC-3') was measured as described previously.^{E8} Results were normalized to UBC (forward 5'-GTACCCTGTCTGACTACAACATC-3', reverse 5'-GTGATGGTCTTGC CAGTGAG-3'), β2M (forward 5'-AAGATTCAGGTTTACTCACGTC-3', reverse 5'-TGATGCTGCTTACATGTCTCG-3'), and glyceraldehyde 3-phosphate dehydrogenase (forward 5'-GAAGGTGAAGGTCCGAGTC-3', reverse 5'-GAAGATGGTGATGGGATTTC-3').

CXCL8 determination

CXCL8 secretion in 48-hour supernatant of cocultures from DCs and neutrophils was measured by ELISA from Invitrogen Life Technologies, according to the manufacturer's instructions.

For detection on gel, CXCL8 was immunoprecipitated from these supernatants with an αCXCL8 antibody (clone 893A6G8, Invitrogen Life Technologies) and αmouse IgG-kappa light chain-coupled sepharose (clone RM19, Sanquin Blood Supply, Amsterdam, The Netherlands). Precipitates were run on a 12% PAGE (Novex; Invitrogen, Breda, The Netherlands) and protein was visualized by silverstaining (SilverQuest, Invitrogen Life Technologies) according to the manufacturer's instructions.

Elastase ELISA

Released elastase in supernatants was measured using a sandwich ELISA. Briefly, a microtiter plate coated with 1.5 µg/mL polyclonal rabbit IgG directed against human elastase (Sanquin Blood Supply) was incubated with supernatants diluted in PBS/0.1% Tween20/0.2% gelatin (PTG) for 1-hour

shaking at room temperature (RT). After subsequent washing with PBS/0.02% Tween20, 1 $\mu\text{g/mL}$ biotinylated rabbit anti-human elastase diluted in PTG was added and the plates were incubated for 1-hour shaking at RT. Thereafter, the plates were washed with PBS/0.02% Tween20 and streptavidin-horseradish peroxidase (Amersham Life Science, Buckinghamshire, UK) diluted 1:1000 in PTG was added and incubated for 30 minutes at RT. Finally, after washing, the plates were developed with 3,3',5,5'-tetramethylbenzidine and conversion was stopped by adding 2 mol H_2SO_4 . The absorbance at 450/540 nm was measured using a Titertek Multiscan (Flow Laboratories, Meckenheim, Germany). Human NE purified from sputum (Elastin Products Co, Owensville, Mo) in PTG was used as a standard.

Flow cytometry

For the determination of cytokine production by T cells, cells were restimulated in round-bottom 96-well plate (Costar) by incubation for 5 hours at 37°C, 5% CO_2 , in IMDM, supplemented with 5% HI-HS and PMA (100 ng/mL), ionomycin (1 $\mu\text{g/mL}$), and brefeldin A (10 $\mu\text{g/mL}$) obtained from Sigma-Aldrich. Cells were fixated in 3.7% formaldehyde (Sigma-Aldrich) in PBS and subsequently permeabilized in 0.5% w/v saponine in PBA (PBS-0.5% w/v BSA-0.05% v/v azide).

The following antibodies were used: $\alpha\text{IFN}\gamma$ -FITC, αCD45RA -FITC, αCD4 -APC (all BD Biosciences), αCD45RO -PE (DAKO), αCD66b -FITC (Sanquin Blood Supply), $\alpha\text{IL-17A}$ -Alexafluor647 (eBioscience, San Diego, Calif), and $\alpha\text{IL-17A}$ -Efluor660 (BioLegend, San Diego, Calif). $\alpha\text{CD4-BB515}$, $\alpha\text{CD16-APC-Cy7}$, and $\alpha\text{Fc}\epsilon\text{RIa}$ -FITC were from BD Biosciences and $\alpha\text{Siglec8-PE}$ and CXCR1-APC from BioLegend. FACSsorting of *ex vivo* skin DCs was performed with $\alpha\text{CD1a-APC}$, $\alpha\text{CD3-PE}$, $\alpha\text{CD14-FITC}$, $\alpha\text{HLA-DR-PerCP}$, $\alpha\text{CD11c-PE-Cy7}$, and $\alpha\text{CD45-Pacific Blue}$ (all BD Biosciences). A total of 10,000 cells were acquired in the live gate on a FACSCanto (BD Biosciences) and further analyzed using FlowJo software (Tree Star Inc, Ashland, Ore).

Statistics

Statistical analysis was performed using Graphpad Prism (Graphpad Software, La Jolla, Calif). Data were analyzed by a *t* test or the Wilcoxon signed rank test to compare groups. Data were presented as mean $\pm$ SEM. *P* values of .05 or less were considered significant.

REFERENCES

- E1. van de Vosse E, Verhard EM, Tool AJ, de Visser AW, Kuijpers TW, Hiemstra PS, et al. Severe congenital neutropenia in a multigenerational family with a novel neutrophil elastase (ELANE) mutation. *Ann Hematol* 2011;90:151-8.
- E2. Zhao XW, Gazendam RP, Drewniak A, van HM, Tool AT, van Hamme JL, et al. Defects in neutrophil granule mobilization and bactericidal activity in familial hemophagocytic lymphohistiocytosis type 5 (FHL-5) syndrome caused by STXBP2/Munc18-2 mutations. *Blood* 2013;122:109-11.
- E3. Drewniak A, Gazendam RP, Tool AT, van HM, Jansen MH, van Hamme JL, et al. Invasive fungal infection and impaired neutrophil killing in human CARD9 deficiency. *Blood* 2013;121:2385-92.
- E4. Strasser D, Neumann K, Bergmann H, Marakalala MJ, Guler R, Rojowska A, et al. Syk kinase-coupled C-type lectin receptors engage protein kinase C-sigma to elicit Card9 adaptor-mediated innate immunity. *Immunity* 2012;36:32-42.
- E5. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 2007;8:942-9.
- E6. Steevens TA, Van AK, Westerlaken GH, Stalpers F, Walk J, Bont L, et al. Signal inhibitory receptor on leukocytes-1 (SIRL-1) negatively regulates the oxidative burst in human phagocytes. *Eur J Immunol* 2013;43:1297-308.
- E7. Van Avondt K, van der Linden M, Naccache PH, Egan DA, Meyaard L. Signal inhibitory receptor on leukocytes-1 limits the formation of neutrophil extracellular traps, but preserves intracellular bacterial killing. *J Immunol* 2016;196:3686-94.
- E8. De Wit J, Souwer Y, van Beelen AJ, de Groot R, Muller FJ, Klaasse Bos H, et al. CD5 costimulation induces stable Th17 development by promoting IL-23R expression and sustained STAT3 activation. *Blood* 2011;118:6107-14.

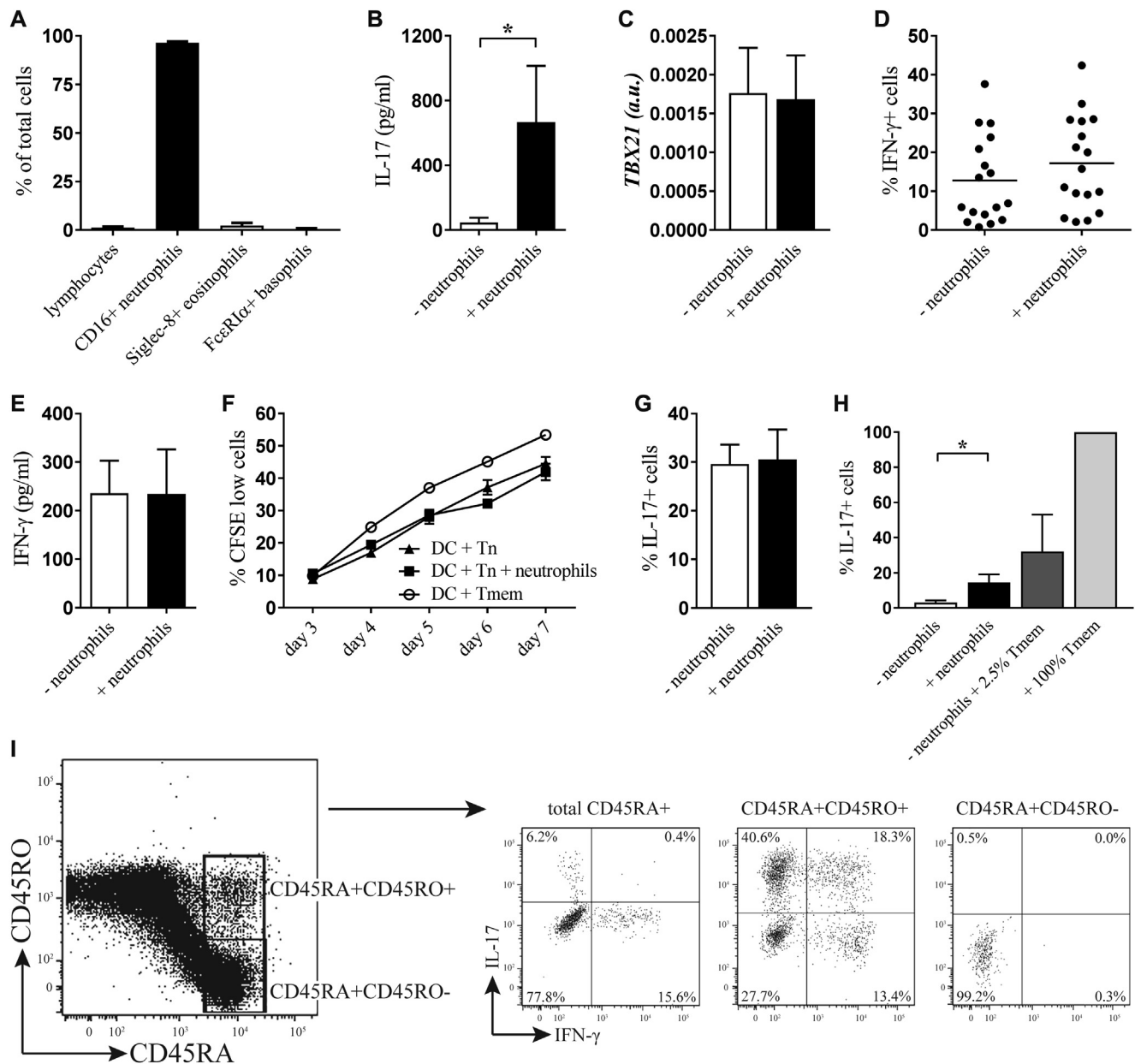


FIG E1. Neutrophils are required for human T_H17 cell development from naive T cells. **A**, Purity of isolated neutrophils ($n = 6$, mean $\pm$ SEM). **B–E**, Naive T cells were primed by *C. albicans*-activated DCs in the absence or presence of neutrophils and restimulated after a resting phase. Shown are IL-17 release (Fig E1, **B**, $n = 7$, mean $\pm$ SEM), *TBX21* (encoding T-bet) mRNA expression in antigen-specific T cells sorted on day 13 (Fig E1, **C**, $n = 9$, mean $\pm$ SEM), percentage of IFN- γ -producing T cells (Fig E1, **D**), and IFN- γ release (Fig E1, **E**, $n = 6$, mean $\pm$ SEM). **F**, The presence of neutrophils did not potentiate or inhibit the proliferation (measured by CFSE dilution) of T cells. **G**, DC-mediated activation of T_H17 cells from *C. albicans*-specific memory T cells in the absence or presence of neutrophils ($n = 6$, mean $\pm$ SEM). **H** and **I**, Influence of the presence of memory and recently activated T cells on the percentage of T_H17 cells upon restimulation ($n = 4$, mean $\pm$ SEM). In Fig E1, **H**, Tn cells and/or Tmem cells were stimulated by *C. albicans*-activated DCs. In Fig E1, **I**, T cells were stimulated with anti-CD3/anti-CD28-coated beads in the presence of IL-1 β and IL-6, combined with neutralizing anti-IFN- γ and anti-IL-4.^{ES} CFSE, Carboxyfluorescein succinimidyl ester; Tn, naive T cell; Tmem, memory T cell. * $P < .05$.

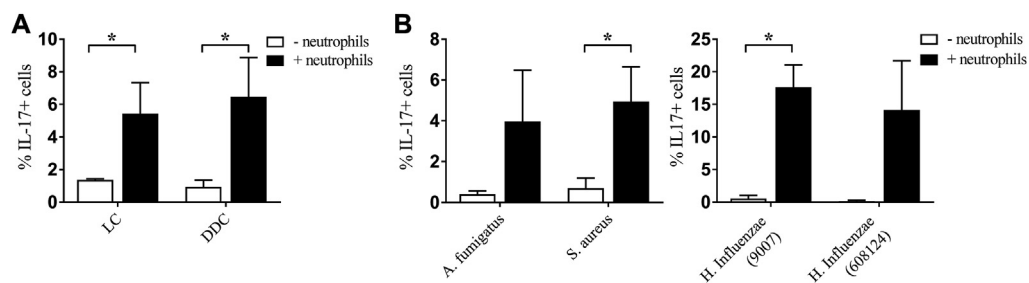


FIG E2. The critical role for neutrophils in T_H17 cell development is independent of DC subset and is not restricted to *C. albicans*. **A**, T_H17 cell induction from purified human $CD4^+CD45RA^+CD45RO^-$ naive T cells by human skin-derived Langerhans cells (LCs) and dermal DCs (DDCs) in the presence or absence of neutrophils ($n = 3$, mean $\pm$ SEM). **B**, DC-mediated induction of yeast and bacteria-specific T_H17 cells from naive T cells ($n = 3$, mean $\pm$ SEM). * $P < .05$.

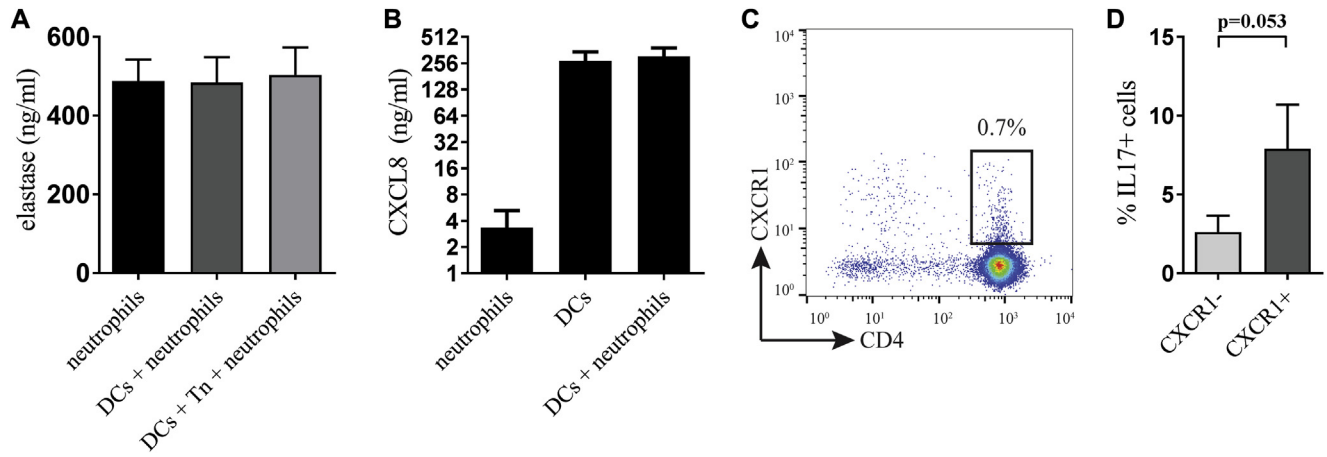


FIG E3. Essential roles for NE and CXCL8 in inducing T_H17 cell formation. **A**, NE concentrations in culture supernatants collected 24 hours after activation with *C. albicans* of indicated cocultures (n = 5, mean ± SEM). **B**, Production of IL-8 by *C. albicans*-activated neutrophils, DCs, and cocultures of both (n = 3, mean ± SEM). **C**, Gating strategy for sorting CXCR1⁺ T cells 4 days after starting a coculture of *C. albicans*-activated DCs and naive T cells, with or without neutrophils. **D**, T_H17 cell development from CXCR1⁻ and CXCR1⁺ T cells sorted as indicated in Fig E3, C, both derived from cocultures containing neutrophils (n = 5, mean ± SEM).