Letter to the Editor

Charcot-Leyden crystals promote neutrophilic inflammation in patients with nasal polyposis

To the Editor:

Since their discovery, Charcot-Leyden crystals (CLCs) have been considered merely a degradation product and marker of eosinophilic inflammation.[1] CLCs are composed of galectin-10 (Gal10), a protein produced by eosinophils, basophils, and some T cells that autocrystalizes when eosinophils are intensely activated and undergo cytolysis associated with extrusion of DNA extracellular traps, a process referred to as EETosis.[2,3] CLCs are abundantly present in mucosa and mucus from patients with chronic rhinosinusitis with nasal polyps (CRSwNP). Recent work provided evidence that CLCs, by analogy to other crystals, such as uric acid and cholesterol crystals, can activate the NLR family pyrin domain containing 3 (NLRP3) inflammasome and cause IL-1β–driven inflammation after uptake by human macrophages *in vitro*.[4] In addition, we have shown that CLCs can stimulate innate and adaptive immunity and act as a type 2 adjuvant, promoting key features of asthma in an NLRP3-independent manner in a mouse model.[2]

To date, direct effector functions of CLCs on human airway samples have not been reported. In this study we sought to investigate whether and how CLCs activated patient-derived nasal polyp tissues, isolated airway epithelial cells, and peripheral blood leukocytes. Details of the materials and methods used in this study are shown in the Methods section in this article's Online Repository at www.jacionline.org.

The process of eosinophil extracellular trap cell death (EETosis) preferentially occurs at epithelial barrier defects in the mucosa and mucus of patients with CRSwNP.[2,5] Recently, EETosis was shown to be at the basis of CLC formation.[2,3] Inspection of tissue from patients with CRSwNP with a type 2 and IL-5–high profile showed that CLCs were often lining the epithelial layer in zones with denuded or abnormal epithelium.[2,3] In line with these observations, we found that CLCs correlated negatively ($P < .001$, $R = -0.8792$, n = 12; Fig 1, *A*) with the relative percentage of normal pseudostratified epithelium in polyp tissue from patients with CRSwNP.

To address the question of whether CLCs could directly cause epithelial damage, we incubated nasal epithelial cell monolayers with CLCs grown from recombinant Gal10 and a soluble crystallization-resistant galectin-10 mutein (Gal10mut) carrying a Tyr69Glu point mutation.[2] A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed a small but significant decrease in the metabolic activity of primary epithelial cells after 24 hours of incubation with CLCs ($P < .05$, n = 6; Fig 1, *B*). However, passive leakage of lactate dehydrogenase (LDH) was not detected in the supernatant, suggesting that CLCs interacted with epithelial cells and that cell injury was minimal after 24 hours.

In mouse models lung administration of CLCs results in influx of neutrophils into the airway lumen.[2,4] In line with these observations, we measured a significantly increased migration ($P < .05$; Fig 1, *C*) of neutrophils to CLC-stimulated epithelial cells using a modified Boyden chamber assay, demonstrating that CLCs stimulate epithelial cells to cause neutrophil recruitment.

Next, we investigated whether CLCs induced chemokine or cytokine responses that could activate neutrophils or other inflammatory cells. Nasal polyp tissue fragments that were stimulated with 100 μg/mL recombinant CLCs and soluble Gal10mut (see Fig E1 in this article's Online Repository at www.jacionline.org) released significantly increased amounts of IL-1β ($P < .05$; see Fig E1, *A*), IL-1α ($P < .05$; see Fig E1, *B*), TNF-α ($P < .05$; see Fig E1, *C*), IL-6 ($P < .05$; see Fig E1, *D*), and IL-8 ($P < .05$; see Fig E1, *E*) when measured 24 hours later and compared with soluble Gal10mut and normal saline control values. The observed cytokine release in the supernatant was dependent on the CLC concentration that was applied (see Fig E2 this article's Online Repository at www.jacionline.org). Concentrations of IL-5, IFN-γ, and IL-17 were unaltered after stimulation with either CLCs or Gal10mut compared with control values (see Fig E3 this article's Online Repository at www.jacionline.org). In addition, we found that treatment of primary epithelial monolayers with recombinant CLCs and Gal10mut (Fig 1) for 24 hours resulted in a significant increase in IL-1α ($P < .05$; Fig 1, *E*), TNF-α ($P < .05$; Fig 1, *F*), GM-CSF ($P < .01$; Fig 1, *G*), and IL-6 ($P < .05$; Fig 1, *H*) levels and a nonsignificant increase in IL-1β (Fig 1, *A*) and IL-8 (Fig 1, *I*) levels. Levels of secreted thymic stromal lymphopoietin, IL-33, and IL-25 were less than the detection limit for all conditions (data not shown).

Because CLCs triggered epithelial cells to recruit neutrophils and produce neutrophil-activating cytokines, such as GM-CSF, TNF-α, and IL-1, that could potentially prime neutrophil effector function, we measured the effects of CLCs on neutrophil function. An important aspect of neutrophil effector function is extrusion of DNA in the form of neutrophil extracellular traps (NETs) that are rich in DNA covered with citrullinated histones.[6] Peripheral blood neutrophils were first primed with GM-CSF and subsequently stimulated with CLCs or soluble Gal10mut. Stimulation with CLCs evoked NET formation, which resulted in a significantly increased percentage of neutrophils undergoing NETosis ($P < .05$; Fig 2, *A-C*) based on DNA staining of the neutrophils on coverslips. In line with these observations, the amount of extracellular DNA release measured was significantly greater when neutrophils were exposed to CLCs compared with vehicle ($P < .0001$; Fig 2, *D*) or soluble Gal10mut ($P < .001$; Fig 2, *D*).

Neutrophils were stained for citrullinated H3 (citH3; Fig 2, *E*) and counted to further verify that the observed DNA was the result of induced neutrophil extracellular trap cell death (NETosis). The number of $citH3^+$ neutrophils was significantly increased after stimulation with CLCs ($P < .05$; Fig 2, *F*). Remarkably, no additive effect of CLCs on NETosis was observed when costimulated with phorbol 12-myristate 12-acetate (data not shown). The effect of CLCs on eosinophil EETosis was also studied, and although stimulation with CLCs after priming showed some induction of eosinophil extracellular traps (EETs) on coverslips, this effect was not significant and was not further confirmed by using DNA quantification in the supernatant (data not shown).

Collectively, these data show that CLCs cause epithelial cells to recruit neutrophils and produce cytokines that prime neutrophil function. Subsequently, CLCs steer neutrophils to undergo

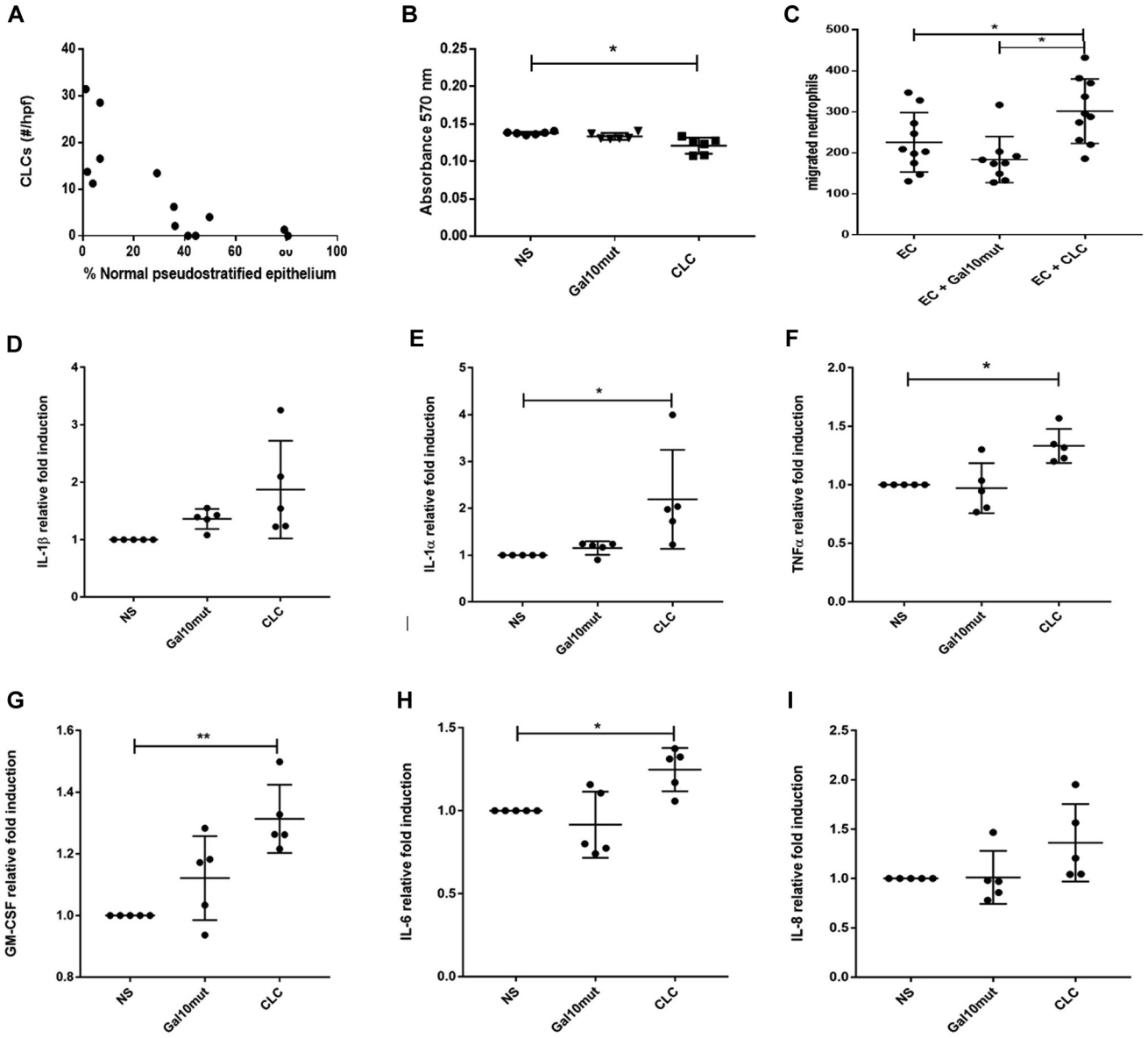

FIG 1. Effects of CLCs on primary nasal epithelial cells. **A,** Scatter plot of the number of CLCs per high-power field as a function of percentage of normal pseudostratified epithelium. **B,** MTT assay after stimulation of epithelial monolayers for 24 hours with vehicle (normal saline *[NS]*), soluble Gal10mut *(Gal)*, or CLCs. **C,** Boyden chamber assay using peripheral neutrophils toward epithelial cells treated for 24 hours with vehicle (epithelial cells *[EC]*), Gal10mut *(EC + Gal10mut)*, or CLCs *(EC + CLC)*. **D-I,** Released IL-1β (Fig 1, *D*), IL-1α (Fig 1, *E*), TNF-α (Fig 1, *F*), GM-CSF (Fig 1, *G*), IL-6 (Fig 1, *H*), and IL-8 (Fig 1, *I*) levels after 24 hours of stimulation with vehicle (normal saline *[NS]*), 100 μg/mL soluble Gal10mut *(Gal10mut)*, or 100 μg/mL CLCs. Data are normalized as fold induction with respect to cells treated with vehicle. $*P < .05$ and $**P < .01$.

NETosis, which might serve as a hallmark of intense activation. In response to CLCs, epithelial cells from nasal polyp tissue, as well as isolated nasal epithelial cells, produced GM-CSF, a known priming stimulus for neutrophils. Strikingly, other cytokines that prime neutrophils for crystal-induced NETosis, such as IL-1α and TNF-α, were also found to be produced by polyp tissue and epithelium under these conditions.[7] The precise mechanisms by which CLCs trigger NETosis require further study. Potentially, the size of the crystals and inability of the crystals to be phagocytized by neutrophils might be involved in turning on NETosis through reactive oxygen species–dependent translocation of neutrophil elastase to the nucleus, a mechanism proposed for sensing of large extracellular pathogens.[8] The observed interindividual variation in response indicates that the role of CLCs in the pathophysiology of CRSwNP might be valid in only a specific subtype of CRSwNP. No information is currently available on how the applied CLC concentration in the *in vitro* experiments relates to the concentration in the patient, and therefore patient-intrinsic dose-response sensitivity might also emerge in the future.

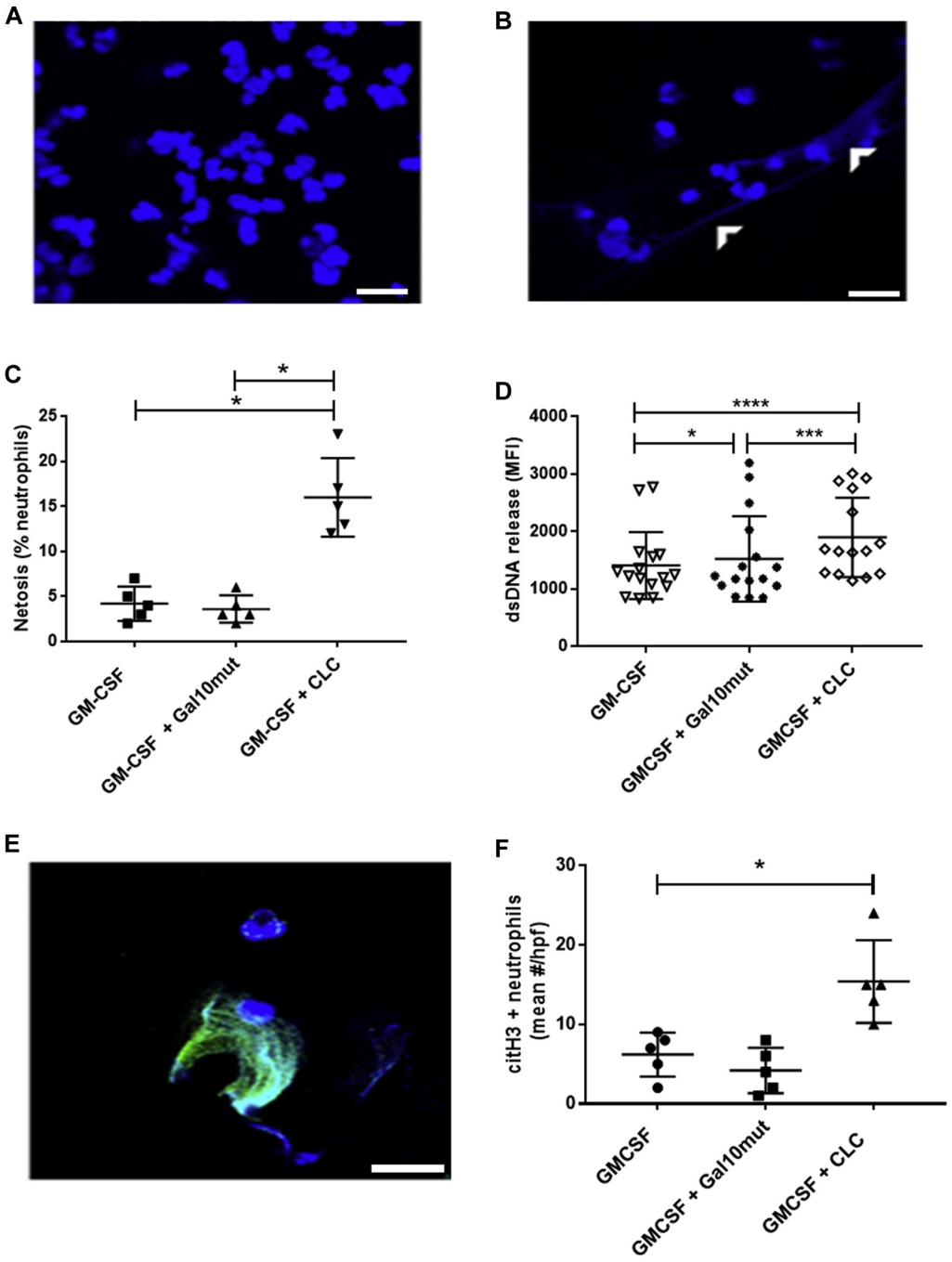

FIG 2. CLCs cause NETosis. **A** and **B,** Immunofluorescent image for DNA (blue) of stimulated neutrophils with Gal10mut (Fig 2, *A*) or CLCs (Fig 2, *B*), indicating formation of NETs *(arrowheads)* after treatment with CLCs (Fig 2, *B*). *Scale bar* = 25 μm. **C,** Relative number of neutrophils involved in NET formation after priming with GM-CSF and followed by stimulation with vehicle *(GM-CSF)*, Gal10mut *(GM-CSF + Gal10mut)*, and CLCs *(GM-CSF + CLC)*. **D,** Released DNA in supernatants of neutrophils stimulated with vehicle *(GM-CSF)*, Gal10mut *(GM-CSF + Gal10mut)*, and CLCs *(GM-CSF + CLC)*. Values are presented as mean fluorescence intensity *(MFI)*. **E,** Immunofluorescent image for citH3 (green) and DNA (blue) of peripheral blood neutrophils after stimulation with CLCs. *Scale bar* = 25 μm. **F,** Quantification of the number of citH3$^+$ neutrophils after stimulation.

Highly stable CLCs that remain after EETosis can thus act as effectors in patients with CRSwNP, sustaining chronic neutrophilic inflammation and eventually resulting in NETosis. We suggest that neutrophils driven by large numbers of CLCs found in the mucosa and mucus of patients with CRSwNP contribute to the persistence of severe airway disease and might render the inflammation nonresponsive to current therapeutic possibilities. Appropriate studies will be required to substantiate this hypothesis.

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E.G and K.V. were supported by a postdoctoral research fellowship from the FWO (FWO/PDO/108 and FWO-12A5517N). I.H. was supported by a predoctoral grant from Ghent University (BOF/DOC2017005501). C.B. and E.G. were supported by grants from FWO Flanders (1515516N) and the Interuniversity Attraction Poles Grant P7/30. B.N.L. is supported by an ERC Advanced grant. This work was supported by Fonds Wetenschappelijk Onderzoek Vlaanderen (FWO Flanders) under EOS project no. GOG2318N and grant FWO/OPR2017004401 (to B.N.L.), and by a Concerted Research Action (GOA) grant from Ghent University (to B.N.L. and S.N.S.), and the VIB. E.P. was supported by a grant from the Swedish Research Council (2014-6852).

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

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https://doi.org/10.1016/j.jaci.2019.08.027

METHODS

Patient characterization

The study was approved by the local ethics committee and the regulatory authorities of Belgium. Written informed consent was obtained from all subjects before enrollment in the study. Nasal polyp tissue from patients undergoing endoscopic sinus surgery for CRSwNP were collected. Only patients with CRSwNP with a pronounced type 2 inflammation and tissue IL-5 levels of greater than 60 pg/mL were included in the study. Tissues were either used immediately, snap-frozen, and/or embedded in paraffin. Peripheral blood neutrophils were collected from whole blood of healthy volunteers. Exclusion criteria were pregnancy, lactation, or receipt of intranasal, oral, and/or intramuscular corticosteroids within the 4 weeks before surgery.

Cytokine and protein measurements

Snap-frozen tissues were weighed, homogenized, and centrifuged, as described previously.[E1] Samples were assayed for IL-5, IL-4, IL-6, IL-8, IL-1β, IL-17, IFN-γ, GM-CSF, and TNF-α by using commercially available Fluorokine kits from R&D Systems (Minneapolis, Minn) and measured on a Bio-Plex 200 Array Reader (Bio-Rad Laboratories, Hercules, Calif).

Immunofluorescent staining

Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. After rehydration of tissue slides (5 μm) and blocking, the slides were incubated with a polyclonal goat anti-human Gal10 antibody (1:200; Abcam, Cambridge, Mass), followed by a fluorescein isothiocyanate-conjugated secondary antibody (1:400). The slides were mounted with Vectashield containing 4′-6-diamidino-2-phenylindole dihydrochloride (Vector Laboratories, Burlingame, Calif) and analyzed with a confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany). For each patient and each piece of tissue, 5 fields were selected in the studied regions, and the number of CLCs was counted.

For coverslip staining, a similar procedure was used, except for the paraffin-embedding and rehydration steps. A rabbit anti-human histone H3 (citrulline R2 + R8 + R17; 1:200; Abcam) was used as a primary antibody, followed by a fluorescein isothiocyanante–labeled donkey anti-rabbit antibody (1:400; Life Technologies, Grand Island, NY).

Stimulation of tissue fragments and epithelial monolayers

Recombinant Gal10mut, a crystallization-deficient Gal10 carrying a Tyr69-Glu point mutation, and CLCs were obtained, as described previously.[E2] Fresh tissue (20 mg per stimulation) was stimulated with 10, 50, and 100 μg/mL Gal10mut or CLCs in RPMI (Life Technologies) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies) and 0.1% BSA (Sigma, St Louis, Mo). After 24 hours, the supernatant was collected by means of centrifugation and stored at −20°C until further measurement.

Epithelial cells from patients with CRSwNP were collected and cultured, as described previously.[E3] At passage 3, the cells were seeded in 24-well plates at 50,000 cells/well. After 48 hours, cells were stimulated with 100 μg/mL Gal10mut, CLCs, or PBS in complete Bronchial Epithelial Growth Medium (Lonza, Basel, Switzerland). After 24 hours of stimulation, the supernatant was collected and stored at −20°C until further measurement. Cells were washed twice with PBS and used for PCR or Western blotting, as described elsewhere in this article.

Cytotoxicity measurements

Epithelial cells were stimulated, as described previously. After 24 hours, the cells were washed twice with PBS and incubated with complete BEGM containing 5 mg/mL MTT (Sigma-Aldrich) for 4 hours. Then cells were washed twice and subsequently lysed in dimethyl sulfoxide (Sigma). Lysate absorbance was measured at 570 nm.

For the LDH assay (Sigma), the cells were stimulated, as described previously. After 24 hours, the supernatant was analyzed for LDH activity by using an LDH activity kit (Sigma), according to the manufacturer's instructions.

Boyden chamber assay

Three days before the experiment, primary epithelial cells, isolated and cultured as described previously, were seeded at 50,000 cells/well in BEGM medium (Lonza) in the basolateral compartment. Twenty-four hours before the assay, the cells were stimulated with 100 μg/mL Gal10mut, CLCs, or PBS (vehicle).

For the migration assay, blood neutrophils were collected after whole-blood Ficoll-Paque centrifugation from healthy donors, followed by red blood cell lysis. Subsequently, neutrophils were primed for 20 minutes with 100 ng/mL GM-CSF (PeproTech, Rocky Hill, NJ) and then allowed to migrate through 5-μm pore size poly-(vinylpyrrolidone)-free polycarbonate filters (VWR International, Radnor, Pa) for 90 minutes at 37°C to the lower compartment. After the migration assay, migrated cells were collected and subjected to May-Grünwald-Giemsa staining and evaluated for the number of migrated neutrophils.

NET quantification

Blood-derived neutrophils were seeded at a density of 250,000 cells per coverslip (diameter, 13 mm) in X-VIVO medium (Lonza). Subsequently, cells were primed with 100 ng/mL GM-CSF for 20 minutes and subsequently stimulated with vehicle (PBS), 100 μg/mL Gal10mut, or CLCs for 20 minutes. After stimulation, cells were fixed in 10% formalin and stored at 4°C in PBS until further processing. For quantification of NETs based on DNA, coverslips were mounted with Vectashield containing 4′-6-diamidino-2-phenylindole dihydrochloride (Vector Laboratories). For quantification of $citH3^+$ neutrophils, coverslips were subjected to an immunofluorescent stain, as described previously.

Neutrophils were primed and stimulated with CLCs, as described above, to quantify the amount of released DNA. After stimulation, cells were treated for 10 minutes with DNAse (Worthington Biochemical, Lakewood, NJ), the reaction was stopped by adding EDTA (Life Technologies), and the supernatant was collected. The supernatant was measured with the QuantiGene PicoGreen Kit (Life Technologies), according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 7 software (GraphPad Software, La Jolla, Calif). For between-group comparisons, the Mann-Whitney *U* test was used. For multiple-group comparisons of unrelated samples, a Kruskal Wallis-test was used, followed by a Dunn multiple comparison test. For related samples, data were analyzed with a Friedman test, followed by a Dunn multiple comparison test. Correlations were determined with a Pearson correlation or a Spearman rho correlation test. *P* values of less than or equal to .05 were considered statistically significant.

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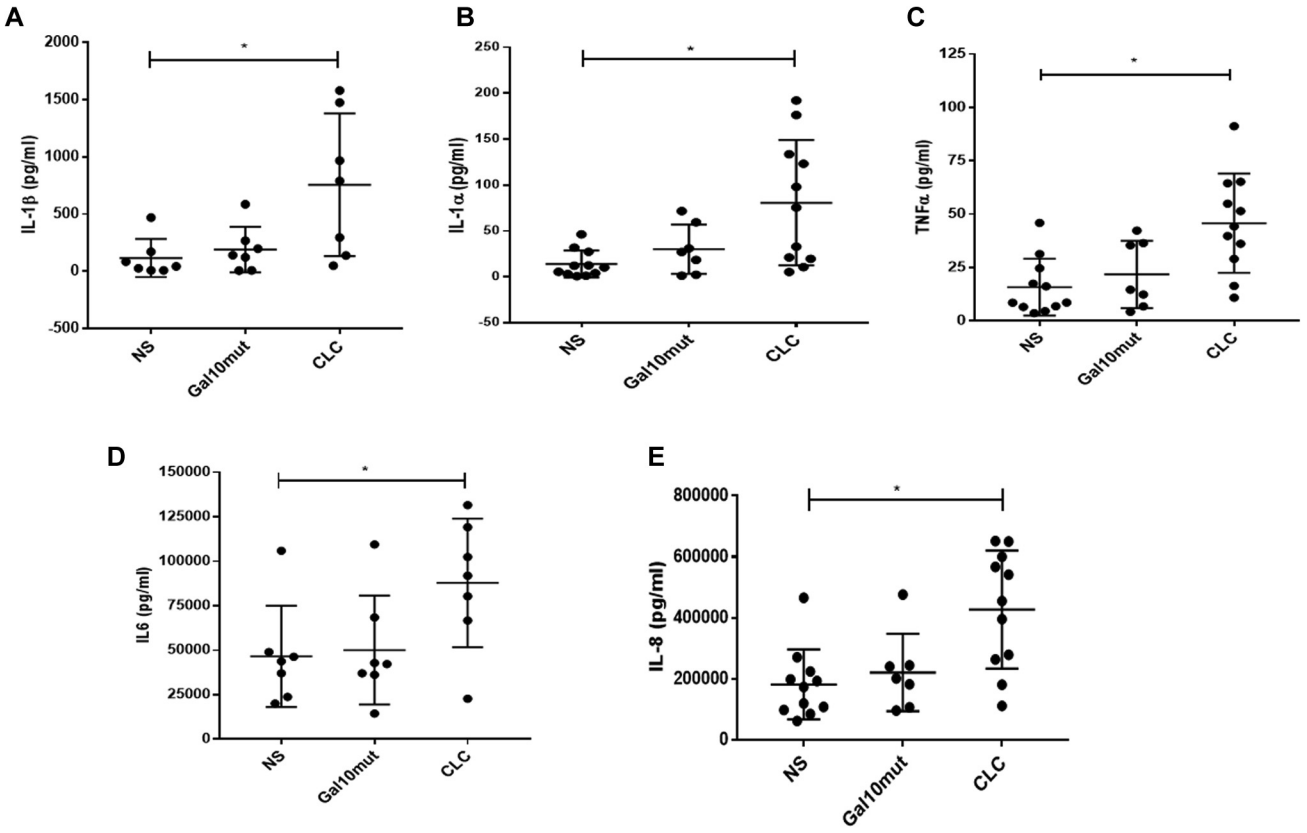

FIG E1. CLCs evoke a proinflammatory response in nasal polyp tissue. **A,** Released IL-1β levels after 24 hours of stimulation with vehicle (normal saline *[NS]*), 100 μg/mL soluble Gal10mut *(Gal10mut)*, or 100 μg/mL CLCs. **B,** Released IL-1α levels after 24 hours of stimulation with vehicle *(NS)*, 100 μg/mL soluble Gal10mut *(Gal10mut)*, or 100 μg/mL CLCs. **C,** Released TNF-α levels after 24 hours of stimulation with vehicle *(NS)*, 100 μg/mL soluble Gal10mut, or 100 μg/mL CLCs. **D,** Released IL-6 levels after 24 hours of stimulation with vehicle *(NS)*, 100 μg/mL soluble Gal10mut *(Gal10mut)*, or 100 μg/mL CLCs. **E,** Released IL-8 levels after 24 hours of stimulation with vehicle *(NS)*, 100 μg/mL soluble Gal10mut *(Gal)*, or 100 μg/mL CLCs. $*P < .05$.

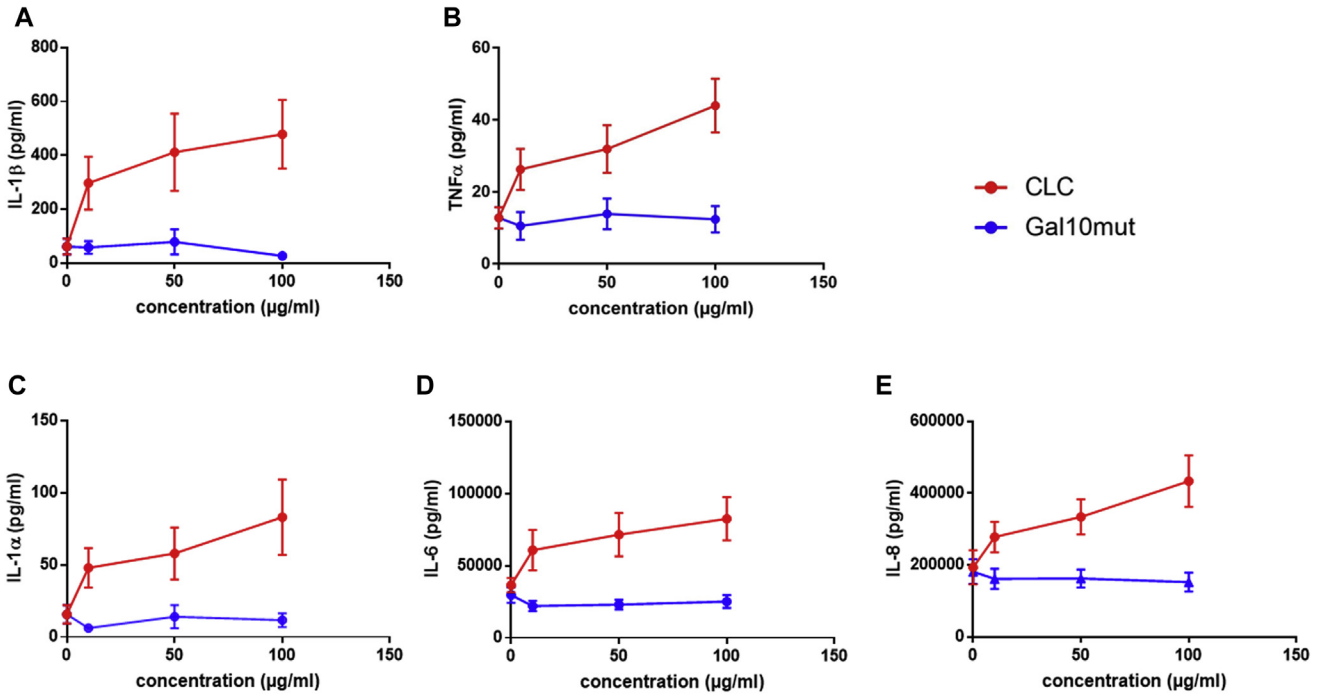

FIG E2. Dose-response curve of the release of cytokines in the function of Gal10mut and CLCs in nasal polyp tissue fragments. Data are presented as means ± SEMs.

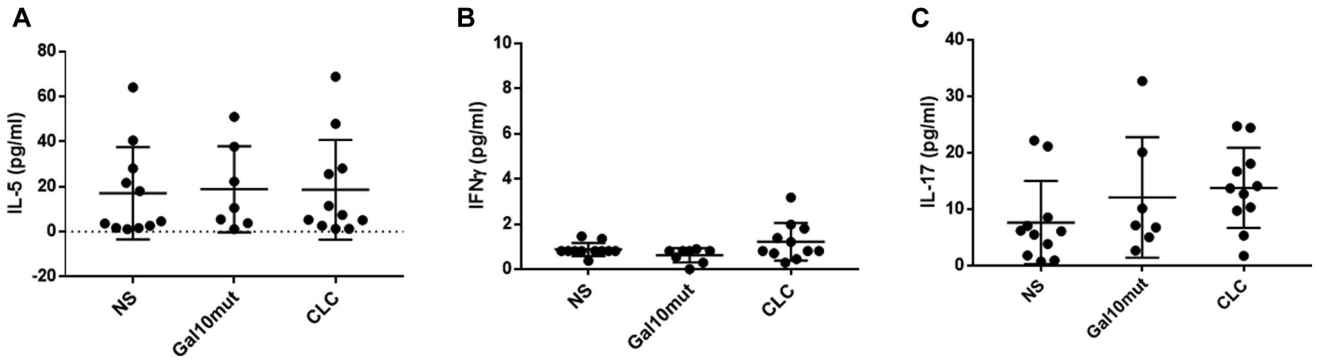

FIG E3. Cytokine release in nasal polyp tissue after stimulation. **A,** Released IL-5 levels after 24 hours of stimulation with vehicle (normal saline *[NS]*), 100 μg/mL soluble Gal10mut *(Gal10mut)*, or 100 μg/mL CLCs. **B,** Released IFN-γ levels after 24 hours of stimulation with vehicle *(NS)*, 100 μg/mL soluble Gal10mut *(Gal10mut)*, or 100 μg/mL CLCs. **C,** Released IL-17 levels after 24 hours of stimulation with vehicle *(NS)*, 100 μg/mL soluble Gal10mut, or 100 μg/mL CLCs.

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Highly stable Charcot-Leyden crystals in nasal polyp tissue are deposited by eosinophils undergoing EETosis and actively promote an inflammatory response that sustains chronic neutrophilic inflammation.