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Human Neutrophils Secrete Bioactive Paucimannosidic Proteins from Azurophilic Granules into Pathogen-Infected Sputum*[S]

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Background: Protein paucimannosylation is considered an important invertebrate- and plant-specific glycoepitope.
Results: Azurophilic granule-specific human neutrophil proteins from pathogen-infected sputum displayed significant core-fucosylated paucimannosylation generated by maturation- and granule-specific β-hexosaminidase A and were preferentially secreted from non-lysosomal origins into sputum upon *P. aeruginosa* stimulation.
Conclusion: Human neutrophils produce, store, and selectively secrete bioactive paucimannosidic proteins.
Significance: This work will aid in understanding the function(s) of human paucimannosylation in glycoimmunology.

Unlike plants and invertebrates, mammals reportedly lack proteins displaying asparagine (*N*)-linked paucimannosylation (mannose$_{1-3}$fucose$_{0-1}$*N*-acetylglucosamine$_2$Asn). Enabled by technology advancements in system-wide biomolecular characterization, we document that protein paucimannosylation is a significant host-derived molecular signature of neutrophil-rich sputum from pathogen-infected human lungs and is negligible in pathogen-free sputum. Five types of paucimannosidic *N*-glycans were carried by compartment-specific and inflammation-associated proteins of the azurophilic granules of human neutrophils including myeloperoxidase (MPO), azurocidin, and neutrophil elastase. The timely expressed human azurophilic granule-resident β-hexosaminidase A displayed the capacity to generate paucimannosidic *N*-glycans by trimming hybrid/complex type *N*-glycan intermediates with relative broad substrate specificity. Paucimannosidic *N*-glycoepitopes showed significant co-localization with β-hexosaminidase A and the azurophilic marker MPO in human neutrophils using immunocytochemistry. Furthermore, promyelocyte stage-specific expression of genes coding for paucimannosidic proteins and biosynthetic enzymes indicated a novel spatio-temporal biosynthetic route in early neutrophil maturation. The absence of bacterial exoglycosidase activities and paucimannosidic *N*-glycans excluded exogenous origins of paucimannosylation. Paucimannosidic proteins from isolated and sputum neutrophils were preferentially secreted upon inoculation with virulent *Pseudomonas aeruginosa*. Finally, paucimannosidic proteins displayed affinities to mannose-binding lectin, suggesting immune-related functions of paucimannosylation in activated human neutrophils. In conclusion, we are the first to document that human neutrophils produce, store and, upon activation, selectively secrete bioactive paucimannosidic proteins into sputum of lungs undergoing pathogen-based inflammation.

Asparagine (*N*)-linked glycosylation adds unprecedented structural and functional heterogeneity to polypeptide chains by the covalent attachment of oligosaccharides (hereafter called glycans) to motif-specific asparagine residues. Although advances in glycobiology and analytical glycoscience continually improve the understanding of protein *N*-glycosylation, the structure-function relationships of most protein glycoforms remain unknown. Dysregulation of protein *N*-glycosylation by the deletion or modulation of its diverse intra- and extracellular functions is a common cause and/or effect of numerous pathologies (1, 2). Our understanding of the conserved mammalian *N*-glycosylation as a structural and functional modulator of proteins is most critically built on the principles of the well described glycoprotein biosynthetic machinery (3). The defined complex and dynamic enzymatic biosynthesis of glycoproteins in the secretory pathway produces three well recognized *N*-glycan classes that are abundantly displayed on mammalian proteins, *i.e.* high mannose, hybrid, and complex type, all of which are based on a common trimannosylated chitobiose core (2).

Contrary to this dogma, several recent glycomics-based studies indicate that a fourth type of protein *N*-glycosylation, referred to as paucimannosylation, with monosaccharide compositions less than or equal to the *N*-glycan trimannosylchitobiose core, *i.e.* mannose(Man)$_{1-3}$fucose(Fuc)$_{0-1}$*N*-acetylglucosamine(GlcNAc)$_2$, is present in mammals. These structures do not correspond to the defined *N*-glycan types nor can their synthesis be described by established mammalian biosynthetic pathways. To date, mammalian protein paucimannosylation has been suggested to be present in (i) human buccal epithelial cells (4), (ii) human colorectal cancer epithelial cells and tissue (5–7), (iii) kidney tissue from mice suffering from systemic

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lupus erythematosus (8), (iv) mouse embryonic neural stem cells (9), and (v) rat brain (10). In addition, we recently indicated the presence of paucimannosylation in pathogen-infected sputum derived from individuals with cystic fibrosis (CF)[2] and upper respiratory tract infection (URTI) (11). Importantly, these observations were all based on molecular profiling of *N*-glycans released from cell/tissue-derived proteins and thus disregarded the protein carrier identities. Consequently, exogenous origin(s) of paucimannosylation could not be ruled out. Mammalian paucimannosylation was supported by immunohistochemistry and immunocytochemistry of selected human and murine tissues and cells using paucimannose-reactive antibodies (12–14). In general, however, mammals including human and mice have usually been reported to lack protein paucimannosylation (15–21). As such, human paucimannosylation remains controversial in the context of our current understanding of mammalian glycobiology.

Outside the vertebrate subphylum, invertebrates such as *Caenorhabditis elegans* (16, 18, 22–24) and *Drosophila melanogaster* (15, 17, 25, 26), plants (27), and other "lower" organisms (28) abundantly produce protein paucimannosylation. Paucimannose synthesis in these species is facilitated by high β-*N*-acetylhexosaminidase activity, allowing partial suppression of the complex *N*-glycan biosynthetic route. Although the exact functions and effector mechanisms still remain elusive, the *N*-glycosylation of the paucimannose-rich *C. elegans* and *D. melanogaster* has been associated with roles in the immune response against bacterial pathogens (19) and the organism lifespan (21).

Here, we present unequivocal evidence that paucimannosylation is also a significant host-derived molecular signature of sputum proteins from pathogen-infected human lungs. Enabled by recent developments in system-wide biomolecular detection, we document that inflammation-associated proteins, localizing to the azurophilic granules of human neutrophils, abundantly display paucimannosylation. In line with their presence in specific micro-environments that are central to inflammation and pathogen infection, we confirm that the timely expressed human azurophilic granule-resident β-hexosaminidase A (Hex A) enzymatically facilitates the generation of protein paucimannosylation by trimming hybrid/complex type *N*-glycan intermediates using a machinery, which is formed during early myeloid maturation, and functionally associate paucimannosidic proteins with roles in innate immunity upon secretion from activated human neutrophils.

EXPERIMENTAL PROCEDURES

Sputum and Bacteria Origin/Handling—Saliva-free whole sputum (>1 ml/donor) was sampled with informed consent from individuals with ($n = 5$) or without ($n = 4$) CF by non-invasive expectoration at Westmead Hospital, Sydney, Australia (see Ref. 11 for donor data). Two of the non-CF individuals were diagnosed with URTI and two were diagnosed with pathogen-free pneumonia or chronic obstructive pulmonary disease. The sputum of the seven pathogen-positive individuals was infected primarily by mucoid/non-mucoid *Pseudomonas aeruginosa*, but also *Aspergillus fumigatus*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* were identified. *P. aeruginosa* laboratory wound (PAO1) and CF sputum (PASS1–4) strains were isolated and cultured (Table 1). Sputum from all donors showed inflammation characteristics ($>1 \times 10^{10}$ polymorphonuclear cells/l sputum). Soluble proteins were isolated from washed sputum plugs (whole sputum) as described (11). In brief, sputum proteins were reduced, and alkylated and intact cells, cellular debris, and insoluble mucins/proteins were removed by centrifugation. The concentration of soluble sputum proteins was measured (Direct Detect, Millipore) and normalized prior to biomolecular characterization.

Origin and Isolation of Human Neutrophils—Resting human neutrophils were isolated to high purity from healthy blood donors as described (29, 30). In brief, the neutrophils were isolated using dextran sedimentation ($1 \times g$), hypotonic lysis of erythrocytes, and centrifugation in a Ficoll-Paque gradient. Resting neutrophils were resuspended in a Krebs-Ringer phosphate buffer and counted. Neutrophils were pelleted ($4{,}000 \times g$, 5 min), and proteins were extracted by cell lysis (6 M urea, 0.1% (w/v) SDS).

Handling of Human Neutrophil-like Cells—Human promyelocytic leukemia cells (HL-60, ATCC CCL-240) were differentiated (5–6 days, 1.3% (v/v) DMSO, Sigma) to meta/band/segmented neutrophil-like cells. HL-60 cells were cultured (RPMI 1640, Gibco, 10% fetal bovine serum, 2 mM L-glutamine, and 50 units/ml penicillin and 50 μg/ml streptomycin) at 37 °C under 5% CO_2. High differentiation efficiencies (>50%) were obtained as assessed by morphology of Wright-Giemsa stained cells (see below). Cells were washed in PBS before use.

LC-MS/MS-based N-Glycome Characterization—N-Glycans were released by 2–5 units of *N*-glycosidase F (PNGase F, *Flavobacterium meningosepticum,* Roche Applied Science)/10 μg of proteins (37 °C, 10–12 h) of protein extracts from sputum, human neutrophils, neutrophil-like cells, and *P. aeruginosa* as described (31) (see supplemental Table 1 for details of sample handling and data acquisition of all LC-MS/MS *N*-glycome experiments). The resulting *N*-glycans (and *N*-glycan reference compounds, Dextra Laboratories) were profiled in their hydroxylated state after porous graphitized carbon (PGC) solid phase extraction desalting (31) using PGC-LC (Hypercarb, Thermo Scientific, 5 μm; 180 μm × 10 cm; pore size: 250 Å) (LC: Ultimate 3000, Dionex) collision-induced dissociation negative ion MS/MS (HCT three-dimensional ion trap, Bruker Daltonics or LTQ-XL, Agilent).

LC-MS/MS-based Proteome and Glycoproteome Profiling—The CF sputum proteome and *N*-glycoproteome were mapped following a crude fractionation on 4–12% SDS-PAGE (fractions 1–5: 65–100, 50–65, 35–50, 20–35, and 5–20 kDa, respectively). The fractions were digested individually in 20 μl of 50 mM NH_4HCO_3 (aq), pH 7.8, using porcine trypsin (Pro-

[2] The abbreviations used are: CF, cystic fibrosis; GM1, Galβ1,3GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glcβ-ceramide; GM2, GalNAcβ1,4(NeuAcα2,3)-Galβ1,4Glcβ-ceramide; GM3, NeuAcα2,3Galβ1,4Glcβ-ceramide; Hex A, β-hexosaminidase A; HCD, higher-energy collisional dissociation; LAMP, lysosome-associated membrane protein; Man, mannose; MBL, mannose-binding lectin, MPO, myeloperoxidase, NE, neutrophil elastase; PGC, porous graphitized carbon; PMA, phorbol myristate acetate; URTI, upper respiratory tract infection; ER, endoplasmic reticulum; DMSO, dimethyl sulfoxide; aq, aqueous.

mega, 50 ng/μl, 8 h, 37 °C). The resulting peptide mixtures were used for proteome and glycoproteome mapping. *N*-Glycopeptides were de-*N*-glycosylated using 3 units of *N*-glycosidase F/fraction (12 h, 37 °C) and desalted prior to proteomics or enriched in their intact form using hydrophilic interaction liquid chromatography solid phase extraction prior to *N*-glycoproteomics as described (32). All fractions were analyzed individually using C_{18} LC-Orbitrap Elite-MS/MS (Thermo Scientific) with higher-energy collision-induced dissociation (HCD) and collision-induced dissociation MS/MS fragmentation as described (10) (see supplemental Tables 2–3 for details of sample handling and data acquisition of all LC-MS/MS proteome and glycoproteome experiments).

LC-MS/MS Data Handling—*N*-Glycans were manually characterized using (i) molecular mass, PGC-LC retention time, and *de novo* MS/MS sequencing, and (ii) MS/MS spectral and PGC-LC retention time matching to reference compounds (Dextra Laboratories). Proteome HCD-MS/MS data were searched separately against UniProt *Homo sapiens* and *P. aeruginosa* PAO1 (Mascot v2.4). *N*-Glycoproteome *m/z* 204.08-filtered HCD-MS/MS spectra were searched against a targeted sputum proteome and *N*-glycome database (Byonic™ v1.2, Protein Metrics) (10). Protein identifications were filtered to 1% false discovery rate. *N*-Glycopeptide identifications (≥4 *b*/*y*-ions in HCD-MS/MS) were validated using collision-induced dissociation-MS/MS (see supplemental Fig. 1 for *N*-glycopeptide annotation). Accurate MS-based relative quantitation of proteins, *N*-glycans, and *N*-glycopeptides was performed (33).

Western Blotting of Paucimannosidic Proteins—Paucimannosidic proteins derived from sputum were visualized using paucimannose-recognizing Mannitou IgM (12) (undiluted concentration, 8–10 h) and HRP-conjugated anti-mouse IgM (Life Technologies) (1:3000, 1 h) on SDS-PAGE-separated sputum proteins (10 μg) transferred to nitrocellulose membranes (Bio-Rad). HRP chemiluminescent substrates (Millipore) were used in a ChemiDoc MP system (Bio-Rad).

Substrate Specificity of Hex A—The substrate specificity of human Hex A (Sf 21 baculovirus-derived, R&D Systems, >95% purity and >1.2 nmol/min/μg of 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide activity) was assessed by monitoring the exoglycosidase activity on complex (Dextra Laboratories) and hybrid (chicken ovalbumin, Sigma) *N*-glycans and GM1–3 (Matreya LLC, Pleasant Gap, PA) using positive ion MALDI time-of-flight MS (MicroFlex, Bruker Daltonics) or negative ion PGC-LC-MS/MS (above). Digestion was performed in 100 mM sodium citrate, 250 mM NaCl (aq), pH 4.5 (25:1 substrate/enzyme molar ratio, 0–108 h, 37 °C, gentle agitation). 10:1 substrate/enzyme molar ratios were used after 18 h to drive the reaction further.

Sequence Homology of Hex A—The sequence homology of human Hex A (α/β) to hexosaminidases from paucimannose-rich organisms was assessed using EMBOSS Needle.

Subcellular Localization of Neutrophilic Proteins—The subcellular localizations were established using granule-specific proteome libraries of human neutrophils (34) to classify identified sputum proteins and by immunocytochemistry of DMSO-differentiated, fixed (4% (v/v) formaldehyde), and membrane-permeabilized (0.5% (v/v) Triton X-100 in PBS (aq), 20 min, 25 °C) HL-60 cells plated on 0.02% (v/v) polylysine-coated coverslips using the following antibodies: mouse anti-human myeloperoxidase (MPO) IgG (1:2000), rabbit anti-human Hex A IgG (1:500) (Santa Cruz Biotechnology), the paucimannose-recognizing Mannitou IgM (undiluted concentration) (12), goat anti-mouse IgG Alexa Fluor 546 (1:400), and goat anti-rabbit IgG Alexa Fluor 488 (1:400). Nuclei were DAPI-stained. Images were taken using Olympus FV-1000 and Zeiss LSM510 MetaUV confocal microscopes. Co-localization was evaluated semi-quantitatively via iMaris (Bitplane) using Manders' coefficient of six representative cells (35).

Bacterial Exoglycosidase Activity Assay—Bacterial α-sialidase, β-galactosidase, α-fucosidase, β-hexosaminidase, and α-mannosidase activities were assessed by monitoring the *N*-glycan profiles (above) of bovine fetuin, human IgG, and bovine ribonuclease B (Sigma) with and without inoculation of *P. aeruginosa* PAO1 and PASS1–3 (10^5 bacteria/μg of protein, 12 h, 37 °C).

Temporal Gene Expression of Paucimannosidic Proteins and Enzymes—Maturation stage-specific expression of genes coding for paucimannosidic biosynthetic enzymes and proteins was investigated using a transcriptional profile of terminal granulocytic human neutrophil differentiation following bone marrow and peripheral blood collection from healthy individuals and granulocyte isolation using density gradient centrifugation and immune-magnetic sorting (GEO accession number GSE19556, platform GPL96). The promyelocytic and myelocytic expression levels of genes coding for putative paucimannosidic enzymes, *i.e. HEXA* (ID 215155_at/201765_s_at), *HEXB* (201944_at), *MAN2A2* (219999_at), *MAN2B2* (214703_s_at), and *MANBA* (203778_at), and proteins, *i.e. AZU1* (214575_s_at), *MPO* (203949_at), *CD63* (200663_at), and *LAMP2* (200821_at/203042_at/203041_s_at) were represented as a -fold change relative to levels in mature resting human neutrophils.

Pathogen-induced Paucimannosidic Protein Secretion—Pathogen-induced secretion of paucimannosidic proteins was monitored by inoculating DMSO-differentiated HL-60 cells (1–3 h, 37 °C, $n = 3$) and neutrophil-rich pathogen-free whole sputum (8 h, 37 °C, $n = 4$) with and without *P. aeruginosa* PAO1 and PASS1. *N*-Glycan profiling (above) was performed on acetone-precipitated proteins (80% (v/v), 8 h) secreted into the culture medium (1:100 HL-60:bacteria cell ratio). For sputum neutrophils, 10^5 bacteria/μg of whole sputum was used. Bacteria were estimated based on optical density (assuming A_{600}: $0.2 \sim 7.8 \times 10^7$ CFU/ml). Bacteria and HL-60 co-cultures were performed in serum- and antibiotics-free RPMI 1640 media (37 °C, 5% CO_2, gentle agitation). Cell counts and viabilities were monitored using an electronic cell counter (Bio-Rad) and trypan blue exclusion. Phorbol myristate acetate (PMA) treatment (200 nM, 1–3 h, 37 °C) of HL-60 cells served as activation controls. HL-60 morphology was monitored using Wright-Giemsa-stained smears prepared with a Cytospin centrifuge (Shandon).

MBL Binding Assay—Binding of paucimannosidic proteins derived from pathogen-positive sputum and commercially sourced *N*-glycans (Dextra Laboratories) to agarose-conjugated mannose-binding lectin (MBL) (Thermo Scientific) was assessed in 300 μl of 20 mM $CaCl_2$, 1.25 M NaCl, 10 mM Tris-

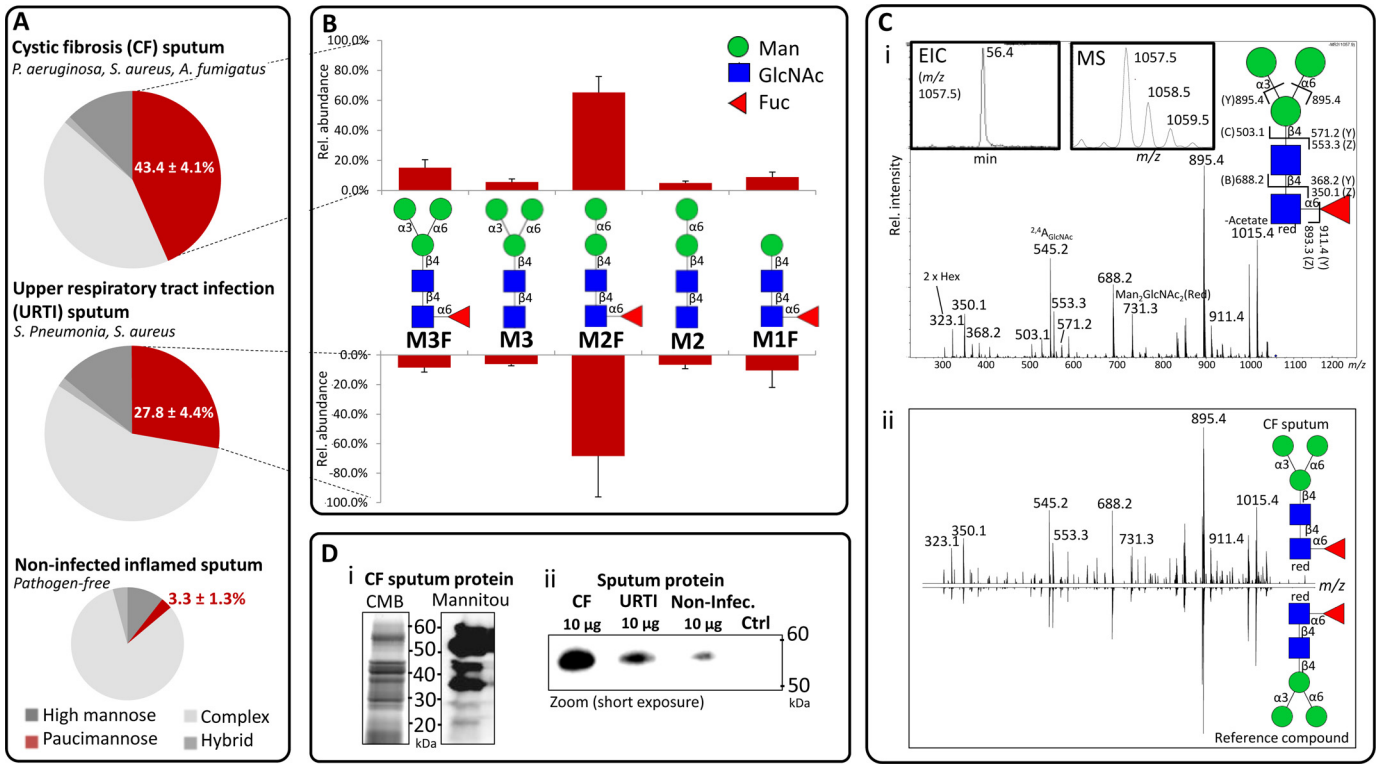


FIGURE 1. **Paucimannosylation is an abundant glycoepitope of proteins derived from pathogen-infected sputum.** *A*, *N*-glycan type distribution of sputum proteins from pathogen-infected individuals with CF (*top*) and URTI (*middle*) and pathogen-free individuals with other inflammatory lung conditions (*bottom*) (*red*: total proportion of paucimannosylation of the entire *N*-glycome) (see Ref. 11 for sputum donor-specific data). *B*, distribution of paucimannosidic *N*-glycans released from proteins derived from pathogen-infected sputum (see *inset* for nomenclature). *Rel. abundance*, relative abundance. *C*, example of structural confirmation of a paucimannosidic *N*-glycan (M3F) using LC-MS/MS for (*panel i*) *de novo* sequencing and (*panel ii*) spectral matching to a well characterized reference compound (see supplemental Table 1); *red* refers to a $NaBH_4$-reduced *N*-glycan reducing end. *EIC*, extracted-ion chromatogram; *Rel. intensity*, relative intensity. *D*, the paucimannose-recognizing Mannitou antibody was highly reactive toward CF sputum proteins (*panel i*). CF and URTI sputum proteins (zoom of 50–60-kDa region, short exposure) were more reactive toward Mannitou relative to proteins derived from pathogen-free sputum (*panel ii*). *CMB*, Coomassie blue; *Non-infec.*, non-infected; *Ctrl*, negative control. For all data: mean ± S.D.

HCl (aq), pH 7.4, in a ratio of 1 nmol of analyte to 100 µl of washed MBL-agarose slurry (12 h, 4 °C, end-over-end rotation). The unbound fractions were collected for separate analysis after centrifugation. MBL-agarose beads were washed twice in 500 µl of binding buffer. Bound analytes were eluted with 300 µl of 2 mM EDTA, 1.25 M NaCl, 10 mM Tris-HCl (aq), pH 7.4 (8 h, 25 °C, end-over-end rotation). Paucimannosidic proteins and *N*-glycans in the MBL unbound and bound fractions were glycoprofiled using PGC-LC-MS/MS after glycan release/clean-up (see explicit description above).

Statistics—The significance of the individual experiments was assessed by one- or two-tailed Student's *t* tests where *p* less than 0.05 was chosen as the minimum acceptable level of confidence to support a rejection of the proposed null hypothesis, *e.g.* difference of two means. In general, tests fulfilling the minimum confidence level of significance were indicated by *; stronger confidence was indicated by ** and ***. The sample number (*n*) is given for the individual experiments. Data points are presented as a mean, and error is presented as S.D. or S.E.

RESULTS

Paucimannosylation, the Fourth Type of Human N-Glycosylation—Among other *N*-glycan alterations, we recently suggested that inflamed pathogen-infected sputum of individuals with CF and URTI displayed paucimannose-rich *N*-glycome signatures (43.4 ± 4.1 and 27.8 ± 4.4%, respectively) relative to sputum from pathogen-free, but neutrophil-positive, lungs of individuals suffering from pneumonia and chronic obstructive pulmonary disease (3.3 ± 1.3%, $p = 2.7–9.7 \times 10^{-3}$) (11) (as summarized in Fig. 1*A*). Mucoid/non-mucoid *P. aeruginosa*, *A. fumigatus*, *S. pneumoniae*, and *S. aureus* were identified in the pathogen-infected paucimannose-rich sputum (Table 1), illustrating a pathogen species-unspecific link to paucimannosylation.

Herein, we undertake a thorough investigation of this indication of human protein paucimannosylation by performing in-depth spatio-temporal analyses of the structure, function, and biosynthesis of paucimannosidic proteins in neutrophil-rich sputum from pathogen-infected individuals, isolated blood-derived human neutrophils, and neutrophil-like cells (HL-60). The detailed structures and distribution of five chromatographically pure paucimannosidic *N*-glycans (M1F, M2, M2F, M3, and M3F) were determined in pathogen-infected sputum (Fig. 1*B*). M2F (Manα1,6Manβ1,4GlcNAcβ1,4(Fucα1,6)GlcNAc) was consistently the most abundant paucimannosidic *N*-glycan; the corresponding α1,3-isomer of M2F (and M2) was absent. The detailed *N*-glycan characterization was facilitated by *de novo* MS/MS sequencing and by spectral and PGC-LC retention time matching to paucimannosidic reference compounds (Fig. 1*C*, see supplemental Table 1 for supporting *N*-glycome data). Although M0F *per se* does not fall under our definition of pauci-

TABLE 1
Overview of isolated and cultured *P. aeruginosa* strains used in the study

P. aeruginosa strain	Tissue origin	Patient origin[a]	Strain origin	Type	Biofilm formation[b]	Genome known
PAO1	Wound	NA[c]	Laboratory	Non-mucoid	Yes	Yes
PASS1	Sputum	CF5	Clinical	Mucoid	Yes	Yes[d]
PASS2	Sputum	CF1	Clinical	Mucoid	No	Yes[d]
PASS3	Sputum	CF2	Clinical	Non-mucoid	Yes	Yes[d]
PASS4	Sputum	CF4	Clinical	Mucoid/Non-mucoid	Yes	Yes[d]

[a] See Ref. 11 for de-identified sputum donor information.
[b] Determined on a flow cell system on LB and minimal media with glucose (data not shown).
[c] NA, not applicable.
[d] Recently genome-sequenced and functionally characterized by Prof. Ian Paulson, Macquarie University, Sydney, Australia (unpublished).

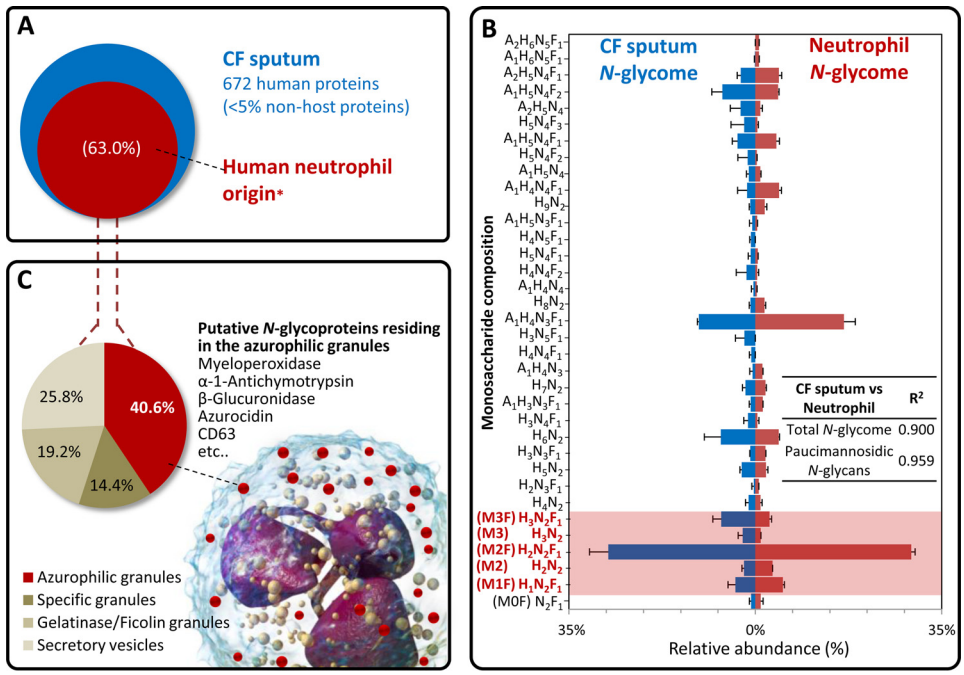


FIGURE 2. **Pathogen-infected sputum and human neutrophils share biomolecular characteristics.** *A*, similar proteomes from CF sputum (*blue*) and human neutrophils (*red*) (*, see Ref. 34). *B*, near identical *N*-glycomes of proteins derived from CF sputum (*blue*) and blood-derived resting human neutrophils (*red*) (mean ± S.D.) (see *inset* for correlation, R^2); *A*, NeuAc; *H*, Hex; *N*, GlcNAc; *F*, fucose. *C*, the granular distribution of predicted *N*-glycoproteins identified in CF sputum using neutrophilic granule libraries (34) shows that the putative glycoproteins (when not considering their type of *N*-glycosylation) localize to all four main compartments of the human neutrophil, but preferentially to the azurophilic granules (*red*) (see supplemental Tables 1 and 2 for more). Graphics modified and used with permission from Blausen Medical (Blausen Gallery 2014).

mannosylation, the presence of this paucimannosidic-related structure was confirmed in the CF sputum. Trace levels of the truncated M1, M0, (Fucα1,6)GlcNAc, or single GlcNAc residues may have been present below the detection limit or not released efficiently by *N*-glycosidase F. In support of these observations, Western blotting using the paucimannose-reactive Mannitou antibody showed high reactivity to CF and URTI sputum proteins (Fig. 1*D*).

Possible exogenous bacterial origins of the abundant paucimannosylation in sputum were ruled out by the absence of paucimannosidic *N*-glycan signatures of proteomes obtained from isolated and cultured laboratory wound (PAO1) and CF (PASS1–2) *P. aeruginosa* strains. In addition, no significant α-sialidase, β-galactosidase, β-hexosaminidase, α-fucosidase, and α-mannosidase activities were detected in any of the investigated *P. aeruginosa* strains using a series of digestion assays with well characterized glycoproteins displaying a spectrum of glycoepitopes and LC-MS/MS *N*-glycan profiling, thus confirming that sputum paucimannosylation does not result from exogenous *P. aeruginosa* exoglycosidase activities.

Granule-specific Paucimannosylation of Human Neutrophil Proteins—LC-MS/MS-based proteome mapping of CF sputum revealed the protein characteristics of significant leukocytes, *e.g.* abundance of MPO, neutrophil elastase (NE), eosinophil peroxidase, lactoferrin, catalase, and aminopeptidase N (see supplemental Table 2 for supporting proteome data). The neutrophil-specific proteome (as predicted from a relatively unique transcriptional profile of human neutrophils among other immune cell types (36)) was significantly represented in the CF sputum proteome (63% of 672 human proteins) (34) (Fig. 2*A*), which was supported by morphology-based identification by microscopy of sputum neutrophils (data not shown). Exogenous bacterial proteins were negligible in the CF sputum proteome, *i.e. P. aeruginosa* proteins constituted <2% of the sputum proteome. Further evidence supporting strong neutrophilic molecular signatures in sputum was obtained by the near identical *N*-glycomes of CF sputum proteins and blood-derived

TABLE 2
Human paucimannosidic proteins and their relative glycoform distribution identified in pathogen-infected sputum using glycoproteomics

Protein	UniProt	Asn site	Σ_{pauci} (%)	Paucimannosidic glycans (%)				
				M3F	M3	M2F	M2	M1F
MPO	P05164	323	63.0	13.5	3.9	39.9	5.6	0.2
		355	0.6		0.6			
		483	69.5	67.3		2.2		
β-Glucuronidase	P08236	272	86.1	73.0		11.1	2.1	
LAMP2	P13473	58	100			100		
		356	100			100		
NE	P08246	88	100				100	
LAMAN	O00754	692	100		100			
Azurocidin	P20160	171	100			100		
CEACAM6	P40199	197	100		13.0		87.0	
CD63	P08962	130	100	100				
CREG1	O75629	160	67.0		7.5		59.5	
α-1-Antichymotrypsin	P01011	186	100		100			
		271	100				100	
Aspartylglucosaminidase	P20933	38	54.1		20.2		34.0	
γ-Glutamyl hydrolase	Q92820	203	73.6		20.2		53.3	
NGAL[a]	P80188	85	0.2	0.1	0.1			
Phospholipase B-like 1	Q6P4A8	71	10.2		10.2			
		366	44.0		44.0			
α-1-Antitrypsin	P01009	271	100		100			
Ig α-2 chain C	P01877	205	1.6	1.6				
Ig μ chain C	P01871	46	7.7	7.7				
UPF0762 protein C6orf58	Q6P5S2	69	3.1		3.1			

[a] NGAL, neutrophil gelatinase-associated lipocalin.

human neutrophil proteins ($R^2 = 0.90$); in particular, strong correlation of the paucimannosidic *N*-glycans ($R^2 = 0.96$) was observed (Fig. 2*B*).

Utilizing sequon-based (N*X*(T/S), $X \neq$ P) prediction of *N*-glycosylation and published granule proteome libraries of human neutrophils (34), the putative *N*-glycoproteins of CF sputum were shown to localize to all four main subcellular granular compartments of the human neutrophil, *i.e.* azurophil, specific, gelatinase/ficolin granules, and secretory vesicles. However, significant proportions of the *N*-glycoproteome (~40%) resided in the azurophilic granule (Fig. 2*C*).

Unequivocal evidence for human protein paucimannosylation in pathogen-infected sputum and its granule specificity was generated by system-wide mapping of intact glycopeptides using our recently developed glycoproteomics technology (10). Site-specific paucimannosylation was identified on 18 abundant proteins (23 *N*-sites, 35 unique *N*-glycopeptides) of the total of 30 human *N*-glycoproteins identified in CF sputum (36 *N*-sites, 115 unique *N*-glycopeptides), quantitatively covering ~20% of the CF sputum proteome (Table 2, see also supplemental Table 3 and supplemental Fig. 1 for supporting glycoproteome data). By overlaying these data onto granule proteome libraries of human neutrophils (34), paucimannosylation was found to be highly enriched in the azurophilic granules ($p = 2.3 \times 10^{-4}$) relative to the other three main compartments of the neutrophil (Fig. 3, *A* and E). High mannose and complex type *N*-glycoproteins displaying β-galactosylation, Lewis type fucosylation, and α-sialylation localized predominantly to the other granules (Fig. 3, *B*–*E*). These observations were supported by partial co-localization of the azurophilic marker MPO and paucimannosidic epitopes in DMSO-differentiated human neutrophil-like cells using immunocytochemistry (data not shown).

Spatio-temporal Paucimannose Generation by Human β-Hex A—The biosynthetic mechanisms of human paucimannosylation were investigated. We have previously shown that *N*-glycan processing and the solvent accessibility of *N*-glycosylation sites on maturely folded proteins are closely correlated (37). The identified paucimannosidic *N*-glycosylation sites on sputum proteins were found to be significantly more accessible than the spatially hidden high mannose sites ($p = 8.0 \times 10^{-4}$) showing solvent accessibilities similar to the highly processed complex sites (Fig. 4*A*), indicating that paucimannosylation results from significant exoglycosidase processing of the solvent-exposed *N*-glycan structures. No specific sequence recognition motifs for the paucimannosidic *N*-glycosylation sites were evident as assessed by a frequency plot.

In paucimannose-rich invertebrates, hexosaminidases are required for the removal of the β1,2-GlcNAc residue on the 3′-mannose arm to form paucimannosidic structures (28). The α and β subunits of the heterodimeric human Hex A showed high sequence similarities to hexosaminidases of such organisms, in particular to *C. elegans* Hex A (53.8 and 52.9%) and *Arabidopsis thaliana* Hex1–3 (49.1 and 47.9%) (Fig. 4*B*). *In vitro* incubation of human Hex A with β1,2-GlcNAc-terminating *N*-glycan substrates, at physiologically realistic conditions, *i.e.* enzyme/substrate ratio, temperature, and organelle-like pH, generated M3F, albeit at low enzymatic rates (Fig. 4*C*). Furthermore, β1,2-GlcNAc-terminating afucosylated complex and hybrid type *N*-glycans and β1,4-GalNAc-terminating GM2 glycolipids were found to be acceptable Hex A substrates (Fig. 4*D*). Hex A showed no activity on β1,2-GlcNAc-terminating *N*-glycan substrates when proximal antennas carried β1,4-galactosylation or β1,4-bisecting GlcNAcylation (Fig. 4*E*).

Partial co-localization of human Hex A and paucimannosidic glycoepitopes as evaluated by immunocytochemistry of DMSO-differentiated human neutrophil-like cells supported the involvement of Hex A in paucimannose production (Fig. 4*F*). Azurophilic granule residence of human Hex A was indicated by moderate/strong co-localization of Hex A with the azurophilic marker MPO (Fig. 4*G*). This was supported by proteomics-based identifications of α and β subunits of Hex A in isolated azurophilic granules of human neutrophils (34, 38).

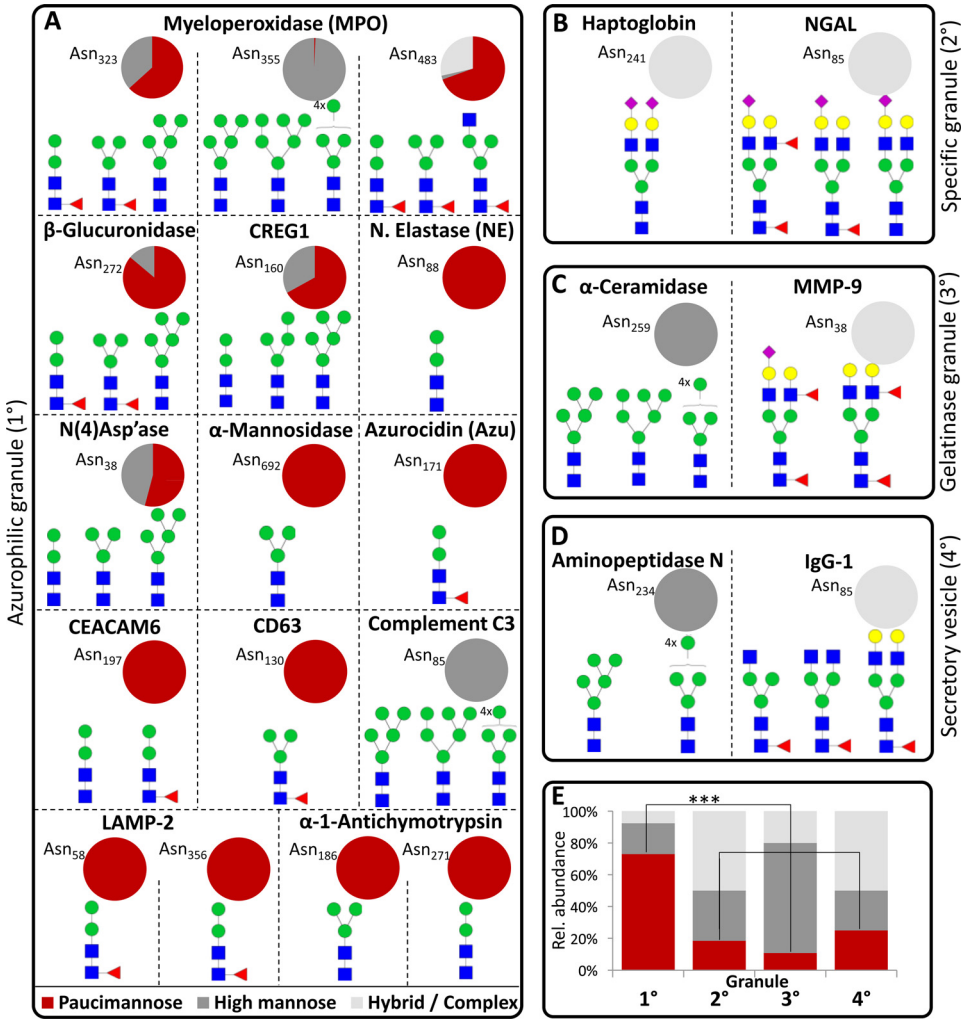
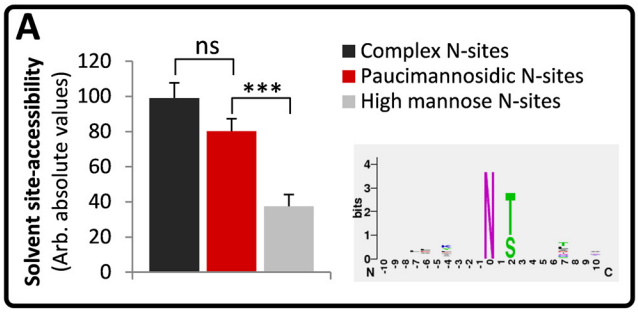


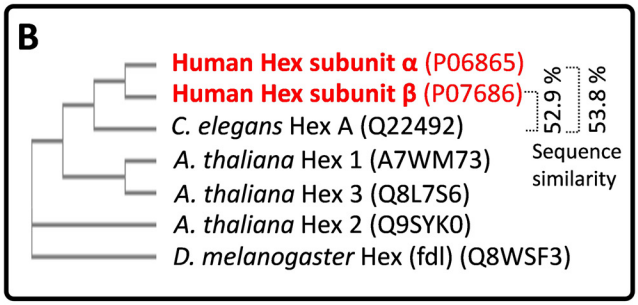
FIGURE 3. **Paucimannosylation is carried by proteins localizing to the azurophilic granules of human neutrophils.** *A–D*, the granular classification (34) of the characterized site-specific glycoprofiles of the CF sputum glycoproteome confirms that azurophilic proteins are the main carriers of paucimannosylation (*A*), whereas proteins localizing to the specific granules (*B*), gelatinase/ficolin granules (*C*), and secretory vesicles (*D*) display predominantly hybrid and complex *N*-glycosylation. Proteins carrying the under-processed high mannose type *N*-glycosylation localize to all compartments (see supplemental Table 3 for more information). *NGAL*, neutrophil gelatinase-associated lipocalin. *E*, the granule *N*-glycan type distribution as derived from the *N*-glycoproteomics data confirms that paucimannosylation is highly enriched in the azurophilic granules (primary, 1°) relative to other neutrophilic compartment (secondary-quaternary granules/vesicle, 2–4°) (***, $p < 0.001$). *Rel. abundance*, relative abundance.

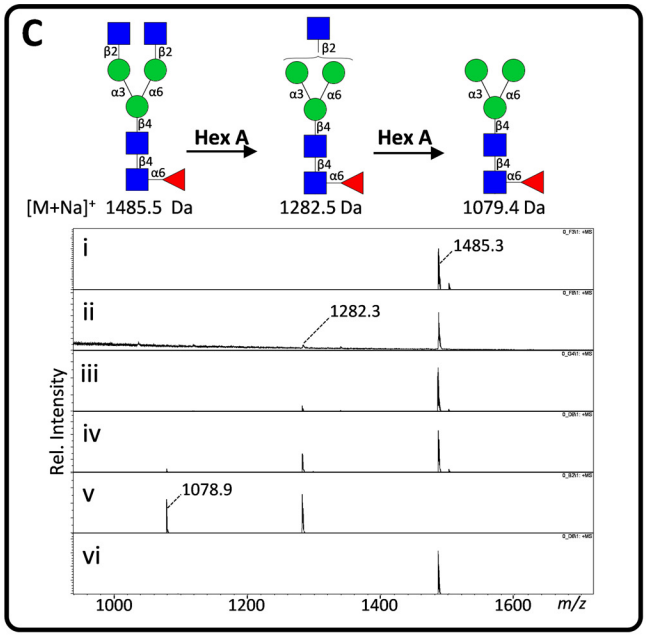
High expression of the genes coding for the putative paucimannosidic enzymes, *e.g. HEXA* and *HEXB* (coding for β-hexosaminidase subunit α and β, respectively, which together hydrolyze β-GlcNAc- and α-GalNAc-terminating glycoconjugates), and for paucimannosidic proteins, *e.g. MPO* and *AZU1* (coding for MPO and azurocidin, respectively) in promyelocytes, relative to levels in myelocytes and mature (resting) neutrophils, indicated assembly of the synthetic machinery for paucimannosylation (but not necessarily the complete biosynthetic generation of paucimannosidic proteins), early in the bone marrow maturation (Fig. 4*H*). M3(F) truncation to M2(F), M1(F), and M0(F) may be facilitated by human α- and β-mannosidases previously identified in the azurophilic granules of human neutrophils (34, 38); promyelocytic stage-specific expression of the corresponding mannosidase genes, *i.e. MAN2A2/MAN2B2* (coding for α-mannosidases, which hydrolyze terminal α1,3/6-linked mannosides) and *MANBA* (coding for β-mannosidase, which hydrolyzes terminal β-linked mannosides), was indeed observed. Taken together, we propose a new granule- and maturation-specific assembly of the biosynthetic machinery for human protein paucimannosylation in the azurophilic granules during early myeloid maturation of neutrophil precursors in the bone marrow (Fig. 4*I*).

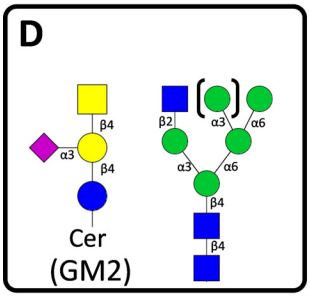
Pathogen-induced Secretion of Paucimannosidic Proteins—Pathogen-induced secretion of paucimannosidic proteins was observed *in vitro* from DMSO-differentiated neutrophil-like HL-60 cells (Fig. 5*A*, *top*). *P. aeruginosa* PAO1 and PASS1 induced a time-dependent (1 h *versus* 3 h, $p = 1.8 \times 10^{-3}$) release of paucimannosidic proteins, preferentially M2F glycoforms (Fig. 5*A*, *bottom*), into the culture medium. Upon *P. aeruginosa* inoculation, the total levels of paucimannosylation were consistently above the unchallenged secretion levels ($p = 5.4 \times 10^{-3}$ to 5.0×10^{-5}); less elevation of the paucimannosylation levels was observed when the neutrophil-like HL-60 cells were activated with PMA ($p < 0.05$, 1 h *versus* 3 h). The unchanged morphology and cell counts of PAO1-challenged (1 h) neutrophils and PMA-activated neutrophils relative to the resting level suggested active secretion of paucimanno-

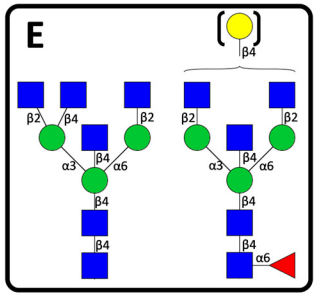
sidic proteins from viable cells by degranulation mechanisms. However, reduced cell numbers and viability after prolonged bacteria inoculation (2–3 h), in particular with the virulent CF-derived PASS1 strain, indicated that paucimannosidic proteins may, in part, be released into the culture media upon cell death under these conditions. Interestingly, only PASS1 inoculation induced significant secretion (but not M2F-specific secretion) of paucimannosidic proteins into neutrophil-rich pathogen-free sputum (31.9% ± 8.4%) relative to the unchallenged counterpart (9.4% ± 2.7%, $p = 2.2 \times 10^{-2}$, $n = 4$) (Fig. 5*B*).

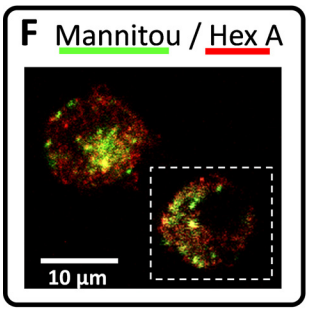


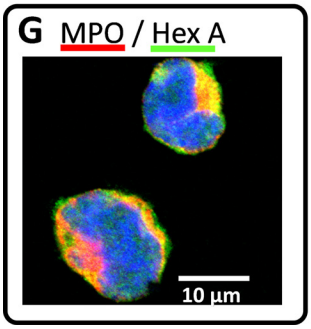


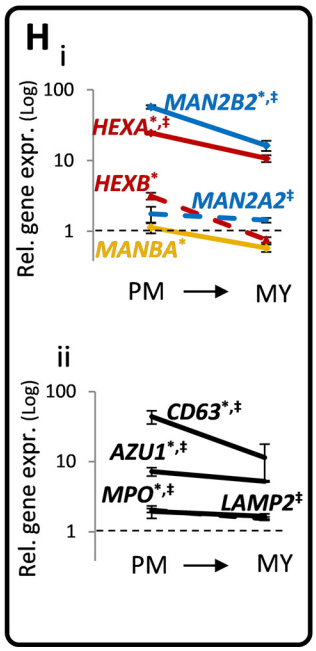


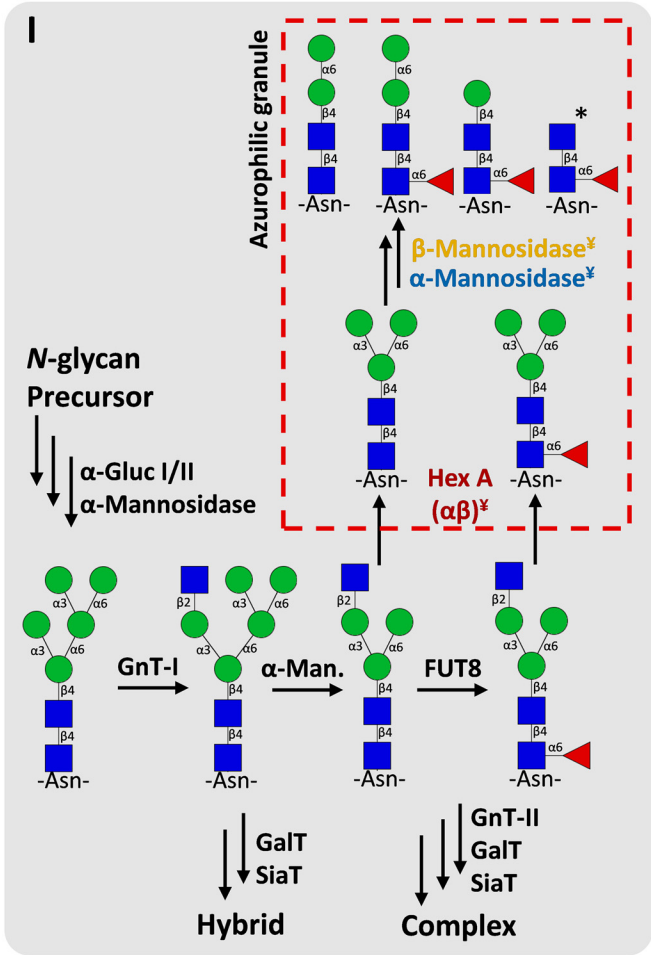












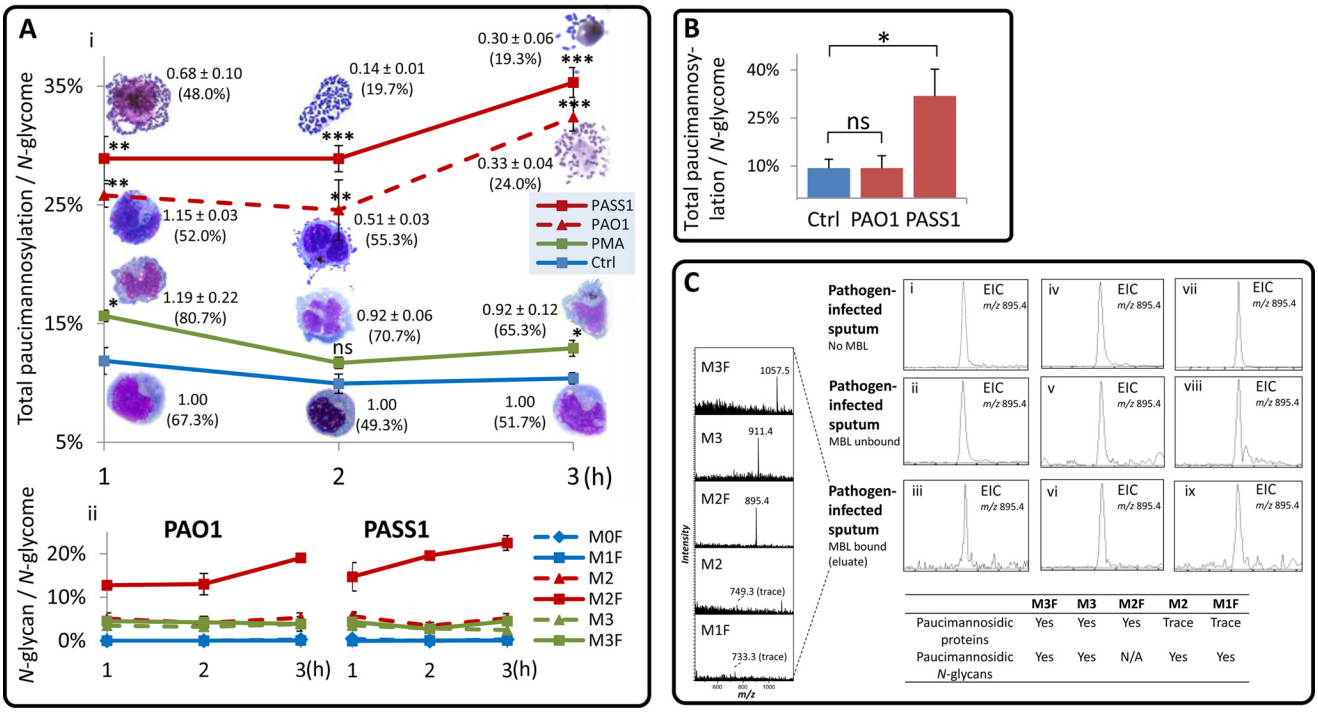


FIGURE 5. **Multiple functions of paucimannosidic proteins in neutrophil biology.** *A*, inoculation and incubation of laboratory wound (PAO1, *broken red line*) and virulent CF (PASS1, *red line*) *P. aeruginosa* strains and PMA activation (*green line*) of DMSO-differentiated HL-60 cells showing induced secretion of paucimannosidic proteins as measured by the degree of total paucimannosylation of the entire *N*-glycome in the culture medium relative to unchallenged DMSO-differentiated HL-60 cells (control, *blue line*) (*top panel*). Representative morphologies of Wright-stained neutrophils, total cell counts (normalized to control), and cell viabilities (in %) are presented for the individual time points. M2F-specific secretion in response to the bacterial inoculation was observed (*bottom panel*) ($n = 3$ for all time points and conditions). Although M0F *per se* does not fall under our definition of paucimannosylation, this paucimannosidic-related structure was included because it was observed in the HL-60 *N*-glycome. *ns*, not significant. *B*, PASS1-specific induction of paucimannosidic protein secretion in inflamed (neutrophil-rich) pathogen-free sputum ($n = 4$ for all conditions). *C*, all five paucimannosidic glycoepitopes displayed on sputum proteins have affinities to MBL (M2 and M1F were observed at low abundance in sputum) (*left spectra*). The abundant M2F (extracted ion chromatogram of *m/z* 895.4) found in CF sputum (*top panels*, *i*, *iv*, and *vii*) was present in the MBL unbound (*middle panels*, *ii*, *v*, and *viii*) and bound (eluted) fractions (*bottom panels*, *iii*, *vi*, and *ix*). *Lower*, table providing overview of MBL affinities for paucimannosidic proteins derived from CF sputum and commercially sourced paucimannosidic *N*-glycans (Dextra Laboratories) (M2F was not available). *EIC*, extracted-ion chromatogram; *N/A*, not applicable. For all *A* and *B panels*: mean ± S.E., *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Binding of Paucimannosidic Proteins to Mannose Receptors—Potential involvement of protein paucimannosylation in complement activation was assessed by evaluating the binding capacity of paucimannosidic glycoepitopes to MBL. All paucimannosidic glycoforms carried by the CF sputum proteins showed affinities to MBL (Fig. 5*C*). Isolated paucimannosidic *N*-glycans from commercial sources showed similar binding behavior. However, the significant presence of paucimannosidic glycoforms in the MBL unbound fractions indicated low binding affinities or MBL saturation under the assayed conditions. High mannose-containing bovine ribonuclease B did not bind to MBL, whereas free high mannose *N*-glycans showed significant affinities (data not shown).

DISCUSSION

Augmenting established glycobiology (15–21), we here, for the first time, demonstrate that humans also produce bioactive paucimannosidic proteins similar to lower organisms such as nematodes, insects, and plants (15, 16, 26, 28) (see Fig. 6 for overview of our findings). However, in contrast to lower organ-

FIGURE 4. **Human Hex A facilitates paucimannosidic N-glycan generation.** *A*, glycosylation sites carrying paucimannosidic *N*-glycans, which are not sequon-constrained beyond the conventional N*X*(S/T) glycosylation motifs (*shaded*, *inset*), are significantly more accessible than the spatially hidden high mannose *N*-glycans (***, $p < 0.001$, mean ± S.E.) when compared with published data (37), implying sterical access of these glycoepitopes to exoglycosidase processing. *ns*, not significant. *B*, subunit α and β of human Hex A show high sequence homology to hexosaminidases from paucimannose-rich organisms. *C*, human Hex A can generate M3F *in vitro* from a complex *N*-glycan intermediate as monitored by MALDI-MS following (i) 0 h (no treatment), (ii) 6 h, (iii) 18 h, (iv) 90 h, (v) 108 h of Hex A incubation and (vi) 108 h without enzyme (control). *Rel. intensity*, relative intensity. *D* and *E*, Hex A also acts on hybrid *N*-glycan intermediates and ganglioside GM2, but shows no activity toward β1,2-GlcNAc-containing structures displaying bisecting β1,4-GlcNAc or β1,4-galactose residues on proximal antennae. *Cer*, ceramide. *F*, partial co-localization (*yellow*) of paucimannosidic epitopes as detected by the paucimannose-reactive Mannitou (*green*) and human Hex A (*red*) antibodies in DMSO-differentiated HL-60 cells supports the involvement of Hex A in paucimannose generation (*inset* is a neutrophil-like cell from a separate field with the same scale and exposure). *G*, moderate/strong co-localization (*yellow*) of the human azurophilic marker MPO (*red*) and Hex A (*green*) in differentiated neutrophil-like HL-60 (multi-lobular nuclei in blue). *H*, temporal gene expression data (GSE19556) of enzymes putatively related to human paucimannosylation including *HEXA* (*red*), *HEXB* (*broken red*), *MAN2A2* (*blue*), *MAN2B2* (*broken blue*), and *MANBA* (*green*) (*panel i*) and paucimannosidic proteins including *AZU1*, *MPO* (*broken line*), *CD63*, and *LAMP2* (*panel ii*) in promyelocytes (*PM*) relative to myelocytes (*MY*) and mature (resting) neutrophils (normalized to 1, indicated by *broken line*). Mean ± S.D., $p < 0.05$ (*, PM *versus* MY; ‡, PM *versus* resting neutrophil). *Rel. gene expr.*, relative gene expression. *I*, proposed biosynthetic route of protein paucimannosylation in azurophilic granules of maturing human promyelocytes. The enzymes putatively responsible for the generation of paucimannosidic proteins are presented in the same color scheme as in *panel H*. ¥, paucimannose biosynthetic enzymes previously identified in the azurophilic granules of human neutrophils (34, 38). *, although M0F *per se* does not fall under our definition of paucimannosidic type structures, it is included here because this paucimannosidic-related structure was observed in pathogen-infected sputum.

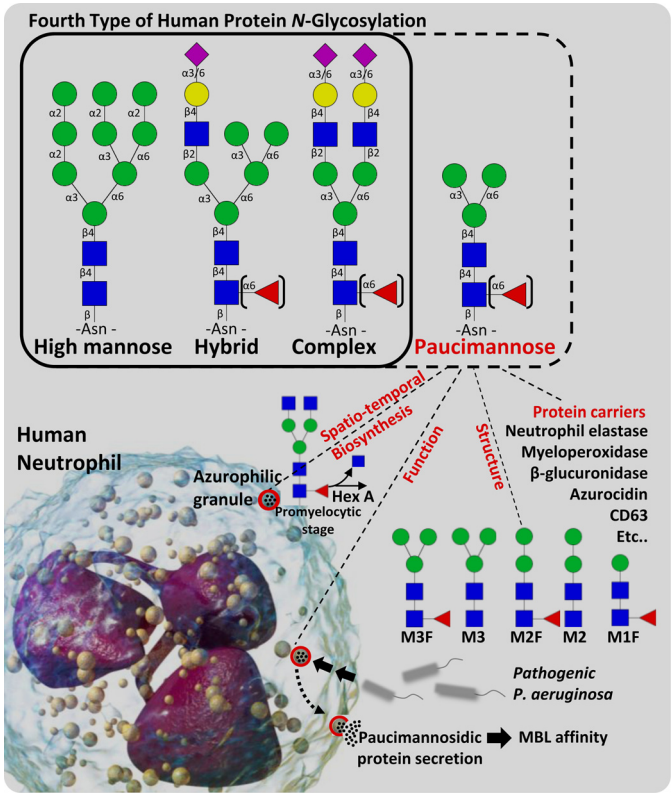


FIGURE 6. **Structure, biosynthesis, and function of paucimannosylation as a fourth type of protein *N*-glycosylation in human neutrophils.** Shown is a schematic overview illustrating in a simplified form the main findings of this work in terms of structure (*N*-glycan species and protein carriers), temporal, and spatial aspects of the biosynthetic route, as well as the functions of protein paucimannosylation in the human neutrophil. As such, protein paucimannosylation constitutes a new and fourth type of human *N*-glycosylation in addition to the established high mannose, hybrid, and complex type *N*-glycans. Graphics modified and used with permission from Blausen Medical (Blausen Gallery 2014).

isms where paucimannosylation is generated in the classical ER-Golgi secretory pathway and constitute a ubiquitous class of structures in the *N*-glycome repertoire, humans appear to use a non-classical ER-Golgi-granule pathway to produce paucimannosidic proteins. Irrespective of this diverted (organelle-specific) and time-demanding hydrolytic route, we argue that the abundance of paucimannosylation in infected sputum and the presentation of these structures on intact and functionally bioactive proteins support their classification as a fourth *N*-glycan type (in addition to high mannose, hybrid, and complex type) in neutrophil-rich environments central to inflammation and infection.

The discovery of this alternate type of human *N*-glycosylation, as exemplified in pathogen-infected sputum, was enabled by recent analytical developments in system-wide characterization of protein glycosylation (10, 31). Protein paucimannosylation was found to be abundant in sputum from inflamed pathogen-infected lungs irrespective of lung disease/condition, infecting microorganism, gender, age, and antibiotic treatment. Pathogen-free sputum, although derived from neutrophil-rich lungs (39), displayed negligible amounts of protein paucimannosylation. This implies that human protein paucimannosylation is neither genotype-specific, microbe-specific, nor disease-specific, but rather a general molecular feature common to inflamed micro-environments of hosts undergoing pathogenic attack.

Five human paucimannosidic *N*-glycans were found to be carried by 18 abundant human sputum proteins that localized specifically to the azurophilic granules of the multi-compartmentalized human neutrophil (40). Neutrophilia is well established in CF and other respiratory conditions including URTI, featuring high proportion (>95%), counts (>10^7 cells/g of sputum), and viability (>70%) of neutrophils in sputum (41). Strong support of paucimannosidic subcellular-specific localization in azurophilic granules and the proposed association between sputum protein paucimannosylation and neutrophil activation by pathogens comes from the observation that purified neutrophil proteins, including human MPO (42, 43), proteinase 3 (PR3) (44), azurocidin (45), Hex B (46), and bovine α-mannosidase (47), which localize to azurophilic granules (34), were previously shown to carry monosaccharide compositions corresponding to paucimannosidic *N*-glycans. Our glycoproteomics data indicate that sputum glycoproteins localizing to the specific and gelatinase granules and secretory vesicles in human neutrophils carried preferentially complex and high mannose *N*-glycans, suggesting a compartment-specific production and storage of paucimannosidic proteins in azurophilic granules. Highly similar *N*-glycosylation profiles of pathogen-infected sputum and neutrophil proteins, including the paucimannosidic profiles, further supported the neutrophilic origin of paucimannosylation and are congruent with studies indicating mammalian paucimannosylation in cancer and systemic lupus erythematosus (5, 7, 8, 12), which are neutrophil-rich pathologies. The neutrophil *N*-glycosylation profile in our study resembles a previously reported human neutrophil *N*-glycan profile (48) in which the low mass paucimannosidic structures however were not reported. The biosynthetically intriguing mono-antennary sialo-*N*-glycans ($NeuAc_1Gal_1$ Man_3-$GlcNAc_3Fuc_{0-1}$) were abundant in the neutrophil and CF sputum *N*-glycomes, but did not appear to be directly related to the azurophilic granule-specific paucimannosylation based on their attachment to proteins localizing to other neutrophil granules (supplemental Fig. 1).

The fact that paucimannosidic *N*-glycans are carried by highly solvent-accessible sites on sputum proteins suggests that they are derived from extensive exoglycosidase processing (37). The high solvent accessibilities also explain the prevalence (>85%) of the accessibility-dependent α1,6-(core) fucosylation on the paucimannosidic *N*-glycans. High core fucosylation, in turn, implies that the paucimannosidic biosynthetic route involves *N*-glycan intermediates displaying terminal β1,2-GlcNAcylation, a substrate requirement for fucosyltransferase 8 (49). This implies again that paucimannose generation follows the initial synthesis of *cis*-Golgi-localized fucosylated hybrid/complex glycan intermediates.

Hexosaminidases are highly expressed in paucimannose-rich organisms including *C. elegans*, *D. melanogaster*, and plants (21). We observed high sequence homology of the α and β subunits of the heterodimeric human Hex A to these hexosaminidases in line with a previous study (20). In addition, the specific identification of human Hex A α/β subunits in azurophilic granules of neutrophils (34, 38) and our immunocyto-

chemistry data showing partial co-localization of both human Hex A and paucimannosidic glycoepitopes with an azurophilic granule marker (MPO) in differentiated neutrophil-like HL-60 cells together support the Hex A-driven compartment-specific paucimannosylation pathway proposed herein. The capacity of Hex A to generate paucimannosidic *N*-glycans *in vitro* from biosynthetic intermediates at realistic physiological conditions, albeit at low enzymatic rates, also confirms this relationship. The similarities of primary (66.5% sequence similarity) (data not shown) and higher structural levels of α and β subunits of Hex A (50, 51) suggest that the homodimeric Hex B ($\beta\beta$) and Hex S ($\alpha\alpha$) isoenzymes may also be able to catalyze paucimannosylation. The relative broad substrate specificity of hexosaminidases to both β-GlcNAc-terminating and α-GalNAc-terminating glycoconjugates (52), together with the low activity observed here, may evolutionarily be more beneficial than high enzyme activity considering the prolonged storage of Hex A and glycoprotein substrates in the azurophilic granules: from the compartment assembly early in the neutrophil maturation in the bone marrow (40) over blood circulation averaging 5 days (53), to transmigration and mobilization via degranulation mechanisms at the inflammatory site. The relative efficiency of Hex A-driven paucimannosylation of membrane, *e.g.* lysosome-associated membrane protein 2 (LAMP2) and soluble *e.g.* NE proteins and any co-factor requirements for optimal paucimannosidic protein generation as reported for GM2 ganglioside degradation by Hex A (54), await further investigation.

Assembly of the paucimannose generating azurophilic granules and its molecular components early during myeloid maturation in the bone marrow was supported by the temporal gene expression of paucimannosidic proteins and putative paucimannose biosynthetic enzymes in promyelocytes in excellent agreement with previous studies (55, 56). Gene set enrichment analysis (57) revealed a high gene expression of paucimannosidic proteins in isolated blood leukocytes from *S. pneumoniae*-infected (but not *S. aureus*-, *Escherichia coli*-, and influenza A virus-infected) individuals (9-fold enrichment, $p = 3.4 \times 10^{-3}$) relative to healthy individuals, suggesting that the assembly of the paucimannose biosynthetic machinery in neutrophil precursors can be shifted from the bone marrow to the blood circulation via infection-dependent "left shifts."

The "targeted-by-timing biosynthesis" hypothesis mechanistically explaining the formation of the granule-specific proteomes in neutrophils (34, 40, 58) is congruent with our observation of compartment-specific *N*-glycosylation; the majority of all glycoproteins trafficking through the *N*-glycosylation machinery at the promyelocytic stage of the neutrophil development are directed to the azurophilic granules by vesicles budding from the *cis*-Golgi without reaching the late *N*-glycan maturation stage, *e.g.* β-galactosylation and α-sialylation in the *trans*-Golgi network (59). As expected, proteins localizing to the specific (and other) granules, which are synthesized exclusively in the myelocyte and more mature stages of the neutrophil development by vesicles budding off from the *trans*-Golgi network (58, 59), displayed complex type *N*-glycosylation. This subcellular-specific *N*-glycosylation is further supported by the absence of human α-sialidases and β-galactosidases in azurophilic granules (34, 38), which suggest that Hex A does not function in concert with other outer-arm exoglycosidases and may explain why the sterically protected GlcNAc-terminating *N*-glycans are unacceptable substrates for human Hex A. This fascinating feature of compartment-specific *N*-glycosylation is not unique to neutrophils (60).

The further trimming of the paucimannosidic glycoforms by human α-mannosidases appears to be linkage-specific as shown by the specific generation of the α1,6-linked mannose-terminating M2F and M2 isomers (see supplemental Table 1). This agrees well with the exclusive presence of the α1,6-mannose isomer of M2F and M2 previously reported on neutrophil-derived human proteins (45) and the preferential hydrolysis of α1,3-linked mannosides by human α-mannosidase (61). Human β-mannosidase may be responsible for yielding amannosylated di- (M0) or tri- (M0F) saccharides. The detection of α- and β-mannosidases in isolated azurophilic granules (34, 38) and the promyelocyte-specific expression of genes coding for the corresponding mannosidases support their association with protein paucimannosylation. By overlaying our observations on the existing map of the mammalian *N*-glycosylation machinery (3), we propose a new spatio-temporal restricted biosynthetic route enabling paucimannosylation of human neutrophil proteins (Fig. 4*I*).

Azurophilic granules of human neutrophils share some functional and molecular commonalities with the glycoprotein-degrading lysosome, yet the two compartments are separate entities as displayed by their unique proteomes as well as by the mobile characteristics of azurophilic granules as secretory components (59, 62). In addition, bidirectional lysosomal glycoprotein degradation is facilitated by a suite of exoglycosidases and proteases, leaving the released and partially degraded *N*-glycans without the reducing-end β-GlcNAc and α1,6-fucose residues (61), contrary to the paucimannosidic *N*-glycans and indeed the *N*-glycoproteins identified in this study. Together this indicates that protein paucimannosylation in pathogen-infected sputum is of non-lysosomal origin. The absence of significant co-localization of paucimannosidic glycoepitopes and several established lysosomal and ER/Golgi markers in other paucimannose-positive human cells further supported a non-lysosomal/ER/Golgi-based paucimannose synthesis.[3] As such, we argue that protein paucimannosylation in the azurophilic compartment of the human neutrophil should not be perceived as a degradation product aimed to salvage monosaccharides and amino acids, but rather as a cellular mechanism to generate an arsenal of releasable biomolecules displaying unique glycoepitopes and activities.

Sorting of proteins to storage granules is not unique to neutrophils, but seems to occur by mechanisms common to all cells (59). Intrigued by the temporal and compartment-specific biosynthetic route for paucimannosylation in neutrophils proposed here, we are investigating whether paucimannosylation is unique to neutrophils or common across cell types. We have recently reported that cultured human colon and breast cancer epithelial cells produce paucimannosidic epitopes (6, 60), albeit in lower quantities (typically less than 15–20% of the total

[3] A. C. Dahmen, M. T. Fergen, C. Laurini, B. Schmitz, I. Loke, M. Thaysen-Andersen, and S. Diestel, submitted for publication.

N-glycome), supporting a possible oncofetal antigen potential of paucimannosylation (12) and the possible molecular and functional similarity of neutrophils and epithelial cells (58). However, the lack of azurophilic granules in epithelial cells implies that production, storage, and secretion of paucimannosidic proteins may be facilitated by other mechanisms in these systems.

Containing an ensemble of bioactive molecules, including antimicrobial peptides and proteases, the azurophilic granule is the microbicidal compartment of the neutrophil (62, 63). Thus, the azurophil-specific localization of protein paucimannosylation in neutrophils is potentially of high biological significance. It has been established that azurophilic granules are mobilized as the last compartment upon phagocytosis (40, 62, 63), emptying their soluble content into the phagolysosome and the extracellular environment to combat invading pathogens (40). The virulence-specific release of paucimannosidic proteins into sputum upon *P. aeruginosa* stimulation indicates an infection-dependent mobilization of azurophilic granules, aspects we are currently investigating by bacterial genome sequencing and proteomics. The importance of granule mobilization for innate immunity is well illustrated in the Chediak-Higashi syndrome where immobile azurophilic granules reduce the host response to pathogens (64). The induced secretion of paucimannosidic proteins from neutrophils presented here indicates that, as reported (40, 62), granules fuse not only with the phagolysosome, but also with the plasma membrane upon activation.

The strong neutrophilic association with the paucimannosidic proteins observed in sputum from pathogen-infected inflamed lungs prompted us to investigate possible functional aspects of human paucimannosylation, *i.e.* lectin-based recognition by the immune system via mannose-receptor interactions. Mannose-terminating glycoconjugates are infrequent in the extracellular environment of healthy human cells/tissues (60), but such determinants from internal membranes or granules may be exposed under specific cellular conditions, *e.g.* immature ER-resident glycoepitopes were shown to be exposed in apoptotic cells serving as "eat me" signals for macrophage-based clearance (65). Exposure of α- and β-mannose determinants on solvent-accessible glycosylation sites such as those presented by paucimannosidic proteins may be a unique feature to enable molecular and cellular communication of activated neutrophils via mannose receptors in the micro-environment by mechanisms of active secretion (degranulation) of paucimannosidic proteins or by release upon cell death (39). The abundant paucimannosidic determinants found in infected sputum may also arise from the release of granular contents into neutrophil extracellular traps (NETs) via the activation and NETosis of polymorphonuclear cells (66). We have demonstrated binding of paucimannosidic proteins and *N*-glycans to MBL; however, the exact roles of paucimannosylation in the downstream complement activation clearly need further investigation. In addition, paucimannosidic binding to other lectins including the macrophage mannose receptors (CD206 and CD280) and dendritic C type lectins (*e.g.* CD209, CD299, and CD303) may provide avenues for the neutrophils to communicate with other immune cells (67). Opportunistic pathogens may also recognize exposed mannosidic determinants as an avenue for host adherence, *e.g. E. coli* fimbrial FimH adhesin shows high affinity for high mannose type as well as paucimannosidic *N*-glycans (68, 69).

In addition, bacteriostatic and bactericidal effects of the paucimannose-rich neutrophilic proteins NE, azurocidin, and cathepsin G have previously been reported (70). We are currently investigating the functional role of the carbohydrate moieties on these human paucimannosidic proteins in the context of bacterial killing and growth inhibition.

Finally, we speculate that paucimannosylation may carry out modulatory roles in the generation and/or recognition of anti-neutrophil cytoplasmic autoantibodies by masking or presenting immunogenic epitopes on the anti-neutrophil cytoplasmic autoantibody-typic and paucimannose-rich LAMP2, PR3, MPO, and NE (71). As such, it becomes clear that paucimannosylation may be linked to multiple diverse functional roles in the micro-environments where paucimannosidic proteins appear to be enriched following neutrophil activation, *i.e.* in phagolysosomes (39, 62), microvesicles/ectosomes (72) and neutrophil extracellular traps (73).

In conclusion, we document that human neutrophils produce, store, and selectively secrete bioactive paucimannosidic proteins into sputum of lungs undergoing pathogen-based inflammation and infection. We show that the azurophilic granules of neutrophils are the biosynthetic "venues" and that human hexosaminidases are the enzymatic "facilitators" of this fourth type of human protein *N*-glycosylation. In line with established neutrophil biology, we propose that paucimannosidic proteins are targeted to the azurophilic granule "by timing" rather than by selective sorting in the early bone marrow maturation of developing myeloid cells. The rather narrow temporal and spatial nature of paucimannosidic proteins in micro-environments surrounding inflammation, and in response to pathogen infection, suggests specialized biomolecular immune functions and may explain how protein paucimannosylation to date has remained under the radar in human glycobiology.

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Note Added in Proof—Fig. 4F did not indicate that the images of the neutrophil-like HL-60 cells were obtained from separate fields in the version of this article that was published on February 2, 2015 as a Paper in Press. This error has been corrected.

REFERENCES

1. Freeze, H. H. (2013) Understanding human glycosylation disorders: biochemistry leads the charge. *J. Biol. Chem.* **288,** 6936 – 6945
2. Moremen, K. W., Tiemeyer, M., and Nairn, A. V. (2012) Vertebrate protein glycosylation: diversity, synthesis and function. *Nat. Rev. Mol. Cell*

Biol. **13,** 448–462
3. Aebi, M. (2013) *N*-linked protein glycosylation in the ER. *Biochim. Biophys. Acta* **1833,** 2430–2437
4. Everest-Dass, A. V., Jin, D., Thaysen-Andersen, M., Nevalainen, H., Kolarich, D., and Packer, N. H. (2012) Comparative structural analysis of the glycosylation of salivary and buccal cell proteins: innate protection against infection by *Candida albicans*. *Glycobiology* **22,** 1465–1479
5. Balog, C. I., Stavenhagen, K., Fung, W. L., Koeleman, C. A., McDonnell, L. A., Verhoeven, A., Mesker, W. E., Tollenaar, R. A., Deelder, A. M., and Wuhrer, M. (2012) *N*-Glycosylation of colorectal cancer tissues: a liquid chromatography and mass spectrometry-based investigation. *Mol. Cell. Proteomics* **11,** 571–585
6. Sethi, M. K., Thaysen-Andersen, M., Smith, J. T., Baker, M. S., Packer, N. H., Hancock, W. S., and Fanayan, S. (2014) Comparative *N*-glycan profiling of colorectal cancer cell lines reveals unique bisecting GlcNAc and α-2,3-linked sialic acid determinants are associated with membrane proteins of the more metastatic/aggressive cell lines. *J. Proteome Res.* **13,** 277–288
7. Joosten, C. E., Cohen, L. S., Ritter, G., Batt, C. A., and Shuler, M. L. (2004) Glycosylation profiles of the human colorectal cancer A33 antigen naturally expressed in the human colorectal cancer cell line SW1222 and expressed as recombinant protein in different insect cell lines. *Biotechnol. Prog.* **20,** 1273–1279
8. Hashii, N., Kawasaki, N., Itoh, S., Nakajima, Y., Kawanishi, T., and Yamaguchi, T. (2009) Alteration of *N*-glycosylation in the kidney in a mouse model of systemic lupus erythematosus: relative quantification of *N*-glycans using an isotope-tagging method. *Immunology* **126,** 336–345
9. Yagi, H., Saito, T., Yanagisawa, M., Yu, R. K., and Kato, K. (2012) Lewis X-carrying *N*-glycans regulate the proliferation of mouse embryonic neural stem cells via the Notch signaling pathway. *J. Biol. Chem.* **287,** 24356–24364
10. Parker, B. L., Thaysen-Andersen, M., Solis, N., Scott, N. E., Larsen, M. R., Graham, M. E., Packer, N. H., and Cordwell, S. J. (2013) Site-specific glycan-peptide analysis for determination of *N*-glycoproteome heterogeneity. *J. Proteome Res.* **12,** 5791–5800
11. Venkatakrishnan, V., Thaysen-Andersen, M., Chen, S. C., Nevalainen, H., and Packer, N. H. (2015) Cystic fibrosis and bacterial colonization define the sputum *N*-glycosylation phenotype. *Glycobiology* **25,** 88–100
12. Zipser, B., Bello-DeOcampo, D., Diestel, S., Tai, M. H., and Schmitz, B. (2012) Mannitou monoclonal antibody uniquely recognizes paucimannose, a marker for human cancer, stemness, and inflammation. *J. Carbohydr. Chem.* **31,** 504–518
13. van Remoortere, A., Bank, C. M., Nyame, A. K., Cummings, R. D., Deelder, A. M., and van Die, I. (2003) *Schistosoma mansoni*-infected mice produce antibodies that cross-react with plant, insect, and mammalian glycoproteins and recognize the truncated biantennary *N*-glycan Man3GlcNAc2-R. *Glycobiology* **13,** 217–225
14. Kaprio, T., Satomaa, T., Heiskanen, A., Hokke, C. H., Deelder, A. M., Mustonen, H., Hagström, J., Carpen, O., Saarinen, J., and Haglund, C. (2015) *N*-Glycomic profiling as a tool to separate rectal adenomas from carcinomas. *Mol. Cell. Proteomics* **14,** 277–288
15. Sarkar, M., Leventis, P. A., Silvescu, C. I., Reinhold, V. N., Schachter, H., and Boulianne, G. L. (2006) Null mutations in *Drosophila N*-acetylglucosaminyltransferase I produce defects in locomotion and a reduced life span. *J. Biol. Chem.* **281,** 12776–12785
16. Schachter, H. (2009) Paucimannose *N*-glycans in *Caenorhabditis elegans* and *Drosophila melanogaster*. *Carbohydr. Res.* **344,** 1391–1396
17. Altmann, F., Staudacher, E., Wilson, I. B., and März, L. (1999) Insect cells as hosts for the expression of recombinant glycoproteins. *Glycoconj. J.* **16,** 109–123
18. Zhang, W., Cao, P., Chen, S., Spence, A. M., Zhu, S., Staudacher, E., and Schachter, H. (2003) Synthesis of paucimannose *N*-glycans by *Caenorhabditis elegans* requires prior actions of UDP-*N*-acetyl-D-glucosamine:α-3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase I, α3,6-mannosidase II and a specific membrane-bound β-*N*-acetylglucosaminidase. *Biochem. J.* **372,** 53–64
19. Shi, H., Tan, J., and Schachter, H. (2006) *N*-Glycans are involved in the response of *Caenorhabditis elegans* to bacterial pathogens. *Methods Enzymol.* **417,** 359–389
20. Gutternigg, M., Kretschmer-Lubich, D., Paschinger, K., Rendić, D., Hader, J., Geier, P., Ranftl, R., Jantsch, V., Lochnit, G., and Wilson, I. B. (2007) Biosynthesis of truncated *N*-linked oligosaccharides results from non-orthologous hexosaminidase-mediated mechanisms in nematodes, plants, and insects. *J. Biol. Chem.* **282,** 27825–27840
21. Schachter, H., and Boulianne, G. (2011) Life is sweet! A novel role for *N*-glycans in *Drosophila* lifespan. *Fly* **5,** 18–24
22. Altmann, F., Fabini, G., Ahorn, H., and Wilson, I. B. (2001) Genetic model organisms in the study of *N*-glycans. *Biochimie* **83,** 703–712
23. Cipollo, J. F., Costello, C. E., and Hirschberg, C. B. (2002) The fine structure of *Caenorhabditis elegans N*-glycans. *J. Biol. Chem.* **277,** 49143–49157
24. Haslam, S. M., Gems, D., Morris, H. R., and Dell, A. (2002) The glycomes of *Caenorhabditis elegans* and other model organisms. *Biochem. Soc. Symp.*117–134
25. Altmann, F., Schwihla, H., Staudacher, E., Glössl, J., and März, L. (1995) Insect cells contain an unusual, membrane-bound β-*N*-acetylglucosaminidase probably involved in the processing of protein *N*-glycans. *J. Biol. Chem.* **270,** 17344–17349
26. Léonard, R., Rendic, D., Rabouille, C., Wilson, I. B., Préat, T., and Altmann, F. (2006) The *Drosophila fused lobes* gene encodes an *N*-acetylglucosaminidase involved in *N*-glycan processing. *J. Biol. Chem.* **281,** 4867–4875
27. Dam, S., Thaysen-Andersen, M., Stenkjær, E., Lorentzen, A., Roepstorff, P., Packer, N. H., and Stougaard, J. (2013) Combined *N*-glycome and n-glycoproteome analysis of the *Lotus japonicus* seed globulin fraction shows conservation of protein structure and glycosylation in legumes. *J. Proteome Res.* **12,** 3383–3392
28. Schiller, B., Hykollari, A., Yan, S., Paschinger, K., and Wilson, I. B. (2012) Complicated *N*-linked glycans in simple organisms. *Biol. Chem.* **393,** 661–673
29. Jin, C., Ekwall, A. K., Bylund, J., Björkman, L., Estrella, R. P., Whitelock, J. M., Eisler, T., Bokarewa, M., and Karlsson, N. G. (2012) Human synovial lubricin expresses sialyl Lewis x determinant and has L-selectin ligand activity. *J. Biol. Chem.* **287,** 35922–35933
30. Bøyum, A., Løvhaug, D., Tresland, L., and Nordlie, E. M. (1991) Separation of leucocytes: improved cell purity by fine adjustments of gradient medium density and osmolality. *Scand. J. Immunol.* **34,** 697–712
31. Jensen, P. H., Karlsson, N. G., Kolarich, D., and Packer, N. H. (2012) Structural analysis of *N*- and *O*-glycans released from glycoproteins. *Nat. Protoc.* **7,** 1299–1310
32. Mysling, S., Palmisano, G., Højrup, P., and Thaysen-Andersen, M. (2010) Utilizing ion-pairing hydrophilic interaction chromatography solid phase extraction for efficient glycopeptide enrichment in glycoproteomics. *Anal. Chem.* **82,** 5598–5609
33. Leymarie, N., Griffin, P. J., Jonscher, K., Kolarich, D., Orlando, R., McComb, M., Zaia, J., Aguilan, J., Alley, W. R., Altmann, F., Ball, L. E., Basumallick, L., Bazemore-Walker, C. R., Behnken, H., Blank, M. A., Brown, K. J., Bunz, S. C., Cairo, C. W., Cipollo, J. F., Daneshfar, R., Desaire, H., Drake, R. R., Go, E. P., Goldman, R., Gruber, C., Halim, A., Hathout, Y., Hensbergen, P. J., Horn, D. M., Hurum, D., Jabs, W., Larson, G., Ly, M., Mann, B. F., Marx, K., Mechref, Y., Meyer, B., Möginger, U., Neusüβ, C., Nilsson, J., Novotny, M. V., Nyalwidhe, J. O., Packer, N. H., Pompach, P., Reiz, B., Resemann, A., Rohrer, J. S., Ruthenbeck, A., Sanda, M., Schulz, J. M., Schweiger-Hufnagel, U., Sihlbom, C., Song, E., Staples, G. O., Suckau, D., Tang, H., Thaysen-Andersen, M., Viner, R. I., An, Y., Valmu, L., Wada, Y., Watson, M., Windwarder, M., Whittal, R., Wuhrer, M., Zhu, Y., and Zou, C. (2013) Interlaboratory study on differential analysis of protein glycosylation by mass spectrometry: the ABRF Glycoprotein Research Multi-Institutional Study 2012. *Mol. Cell. Proteomics* **12,** 2935–2951
34. Rørvig, S., Østergaard, O., Heegaard, N. H., and Borregaard, N. (2013) Proteome profiling of human neutrophil granule subsets, secretory vesicles, and cell membrane: correlation with transcriptome profiling of neutrophil precursors. *J. Leukoc. Biol.* **94,** 711–721
35. Zinchuk, V., Wu, Y., and Grossenbacher-Zinchuk, O. (2013) Bridging the gap between qualitative and quantitative colocalization results in fluorescence microscopy studies. *Sci. Rep.* **3,** 1365
36. Abbas, A. R., Baldwin, D., Ma, Y., Ouyang, W., Gurney, A., Martin, F.,

Fong, S., van Lookeren Campagne, M., Godowski, P., Williams, P. M., Chan, A. C., and Clark, H. F. (2005) Immune response *in silico* (IRIS): immune-specific genes identified from a compendium of microarray expression data. *Genes Immun.* **6,** 319–331
37. Thaysen-Andersen, M., and Packer, N. H. (2012) Site-specific glycoproteomics confirms that protein structure dictates formation of *N*-glycan type, core fucosylation and branching. *Glycobiology* **22,** 1440–1452
38. Lominadze, G., Powell, D. W., Luerman, G. C., Link, A. J., Ward, R. A., and McLeish, K. R. (2005) Proteomic analysis of human neutrophil granules. *Mol. Cell. Proteomics* **4,** 1503–1521
39. Nauseef, W. M., and Borregaard, N. (2014) Neutrophils at work. *Nat. Immunol.* **15,** 602–611
40. Borregaard, N. (2010) Neutrophils, from marrow to microbes. *Immunity* **33,** 657–670
41. Jayaram, L., Labiris, N. R., Efthimiadis, A., Valchos-Mayer, H., Hargreave, F. E., and Freitag, A. P. (2007) The efficiency of sputum cell counts in cystic fibrosis. *Can Respir. J.* **14,** 99–103
42. Ravnsborg, T., Houen, G., and Højrup, P. (2010) The glycosylation of myeloperoxidase. *Biochim. Biophys. Acta* **1804,** 2046–2053
43. Van Antwerpen, P., Slomianny, M. C., Boudjeltia, K. Z., Delporte, C., Faid, V., Calay, D., Rousseau, A., Moguilevsky, N., Raes, M., Vanhamme, L., Furtmüller, P. G., Obinger, C., Vanhaeverbeek, M., Nève, J., and Michalski, J. C. (2010) Glycosylation pattern of mature dimeric leukocyte and recombinant monomeric myeloperoxidase: glycosylation is required for optimal enzymatic activity. *J. Biol. Chem.* **285,** 16351–16359
44. Zoega, M., Ravnsborg, T., Højrup, P., Houen, G., and Schou, C. (2012) Proteinase 3 carries small unusual carbohydrates and associates with α-defensins. *J. Proteomics* **75,** 1472–1485
45. Olczak, M., and Watorek, W. (2002) Structural analysis of *N*-glycans from human neutrophil azurocidin. *Biochem. Biophys. Res. Commun.* **293,** 213–219
46. Schuette, C. G., Weisgerber, J., and Sandhoff, K. (2001) Complete analysis of the glycosylation and disulfide bond pattern of human β-hexosaminidase B by MALDI-MS. *Glycobiology* **11,** 549–556
47. Faid, V., Evjen, G., Tollersrud, O. K., Michalski, J. C., and Morelle, W. (2006) Site-specific glycosylation analysis of the bovine lysosomal α-mannosidase. *Glycobiology* **16,** 440–461
48. Babu, P., North, S. J., Jang-Lee, J., Chalabi, S., Mackerness, K., Stowell, S. R., Cummings, R. D., Rankin, S., Dell, A., and Haslam, S. M. (2009) Structural characterisation of neutrophil glycans by ultra sensitive mass spectrometric glycomics methodology. *Glycoconj J.* **26,** 975–986
49. Paschinger, K., Staudacher, E., Stemmer, U., Fabini, G., and Wilson, I. B. (2005) Fucosyltransferase substrate specificity and the order of fucosylation in invertebrates. *Glycobiology* **15,** 463–474
50. Lemieux, M. J., Mark, B. L., Cherney, M. M., Withers, S. G., Mahuran, D. J., and James, M. N. (2006) Crystallographic structure of human β-hexosaminidase A: interpretation of Tay-Sachs mutations and loss of G_{M2} ganglioside hydrolysis. *J. Mol. Biol.* **359,** 913–929
51. Mark, B. L., Mahuran, D. J., Cherney, M. M., Zhao, D., Knapp, S., and James, M. N. (2003) Crystal structure of human β-hexosaminidase B: understanding the molecular basis of Sandhoff and Tay-Sachs disease. *J. Mol. Biol.* **327,** 1093–1109
52. Hepbildikler, S. T., Sandhoff, R., Kolzer, M., Proia, R. L., and Sandhoff, K. (2002) Physiological substrates for human lysosomal β-hexosaminidase S. *J. Biol. Chem.* **277,** 2562–2572
53. Pillay, J., den Braber, I., Vrisekoop, N., Kwast, L. M., de Boer, R. J., Borghans, J. A., Tesselaar, K., and Koenderman, L. (2010) *In vivo* labeling with ^2H_2O reveals a human neutrophil lifespan of 5.4 days. *Blood* **116,** 625–627
54. Meier, E. M., Schwarzmann, G., Fürst, W., and Sandhoff, K. (1991) The human G_{M2} activator protein. A substrate specific cofactor of β-hexosaminidase A. *J. Biol. Chem.* **266,** 1879–1887
55. Martinelli, S., Urosevic, M., Daryadel, A., Oberholzer, P. A., Baumann, C., Fey, M. F., Dummer, R., Simon, H. U., and Yousefi, S. (2004) Induction of genes mediating interferon-dependent extracellular trap formation during neutrophil differentiation. *J. Biol. Chem.* **279,** 44123–44132
56. Theilgaard-Mönch, K., Jacobsen, L. C., Borup, R., Rasmussen, T., Bjerregaard, M. D., Nielsen, F. C., Cowland, J. B., and Borregaard, N. (2005) The transcriptional program of terminal granulocytic differentiation. *Blood* **105,** 1785–1796
57. Ramilo, O., Allman, W., Chung, W., Mejias, A., Ardura, M., Glaser, C., Wittkowski, K. M., Piqueras, B., Banchereau, J., Palucka, A. K., and Chaussabel, D. (2007) Gene expression patterns in blood leukocytes discriminate patients with acute infections. *Blood* **109,** 2066–2077
58. Borregaard, N., Sørensen, O. E., and Theilgaard-Mönch, K. (2007) Neutrophil granules: a library of innate immunity proteins. *Trends Immunol.* **28,** 340–345
59. Borregaard, N., and Cowland, J. B. (1997) Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* **89,** 3503–3521
60. Lee, L. Y., Lin, C. H., Fanayan, S., Packer, N. H., and Thaysen-Andersen, M. (2014) Differential site accessibility mechanistically explains subcellular-specific *N*-glycosylation determinants. *Front. Immunol.* **5,** 404
61. Winchester, B. (2005) Lysosomal metabolism of glycoproteins. *Glycobiology* **15,** 1R–15R
62. Witko-Sarsat, V., Rieu, P., Descamps-Latscha, B., Lesavre, P., and Halbwachs-Mecarelli, L. (2000) Neutrophils: molecules, functions and pathophysiological aspects. *Lab. Invest.* **80,** 617–653
63. Lacy, P. (2006) Mechanisms of degranulation in neutrophils. *Allergy Asthma Clin. Immunol* **2,** 98–108
64. Kjeldsen, L., Calafat, J., and Borregaard, N. (1998) Giant granules of neutrophils in Chediak-Higashi syndrome are derived from azurophil granules but not from specific and gelatinase granules. *J. Leukoc. Biol.* **64,** 72–77
65. Bilyy, R. O., Shkandina, T., Tomin, A., Muñoz, L. E., Franz, S., Antonyuk, V., Kit, Y. Y., Zirngibl, M., Fürnrohr, B. G., Janko, C., Lauber, K., Schiller, M., Schett, G., Stoika, R. S., and Herrmann, M. (2012) Macrophages discriminate glycosylation patterns of apoptotic cell-derived microparticles. *J. Biol. Chem.* **287,** 496–503
66. Schauer, C., Janko, C., Munoz, L. E., Zhao, Y., Kienhöfer, D., Frey, B., Lell, M., Manger, B., Rech, J., Naschberger, E., Holmdahl, R., Krenn, V., Harrer, T., Jeremic, I., Bilyy, R., Schett, G., Hoffmann, M., and Herrmann, M. (2014) Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines. *Nat. Med.* **20,** 511–517
67. Galli, S. J., Borregaard, N., and Wynn, T. A. (2011) Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. *Nat. Immunol.* **12,** 1035–1044
68. Taganna, J., de Boer, A. R., Wuhrer, M., and Bouckaert, J. (2011) Glycosylation changes as important factors for the susceptibility to urinary tract infection. *Biochem. Soc. Trans.* **39,** 349–354
69. Bouckaert, J., Mackenzie, J., de Paz, J. L., Chipwaza, B., Choudhury, D., Zavialov, A., Mannerstedt, K., Anderson, J., Piérard, D., Wyns, L., Seeberger, P. H., Oscarson, S., De Greve, H., and Knight, S. D. (2006) The affinity of the FimH fimbrial adhesin is receptor-driven and quasi-independent of *Escherichia coli* pathotypes. *Mol. Microbiol.* **61,** 1556–1568
70. Miyasaki, K. T., Bodeau, A. L., and Flemmig, T. F. (1991) Differential killing of *Actinobacillus actinomycetemcomitans* and *Capnocytophaga* spp. by human neutrophil granule components. *Infect. Immun.* **59,** 3760–3767
71. Kain, R., Exner, M., Brandes, R., Ziebermayr, R., Cunningham, D., Alderson, C. A., Davidovits, A., Raab, I., Jahn, R., Ashour, O., Spitzauer, S., Sunder-Plassmann, G., Fukuda, M., Klemm, P., Rees, A. J., and Kerjaschki, D. (2008) Molecular mimicry in pauci-immune focal necrotizing glomerulonephritis. *Nat. Med.* **14,** 1088–1096
72. Timár, C. I., Lorincz, A. M., Csépányi-Kömi, R., Vályi-Nagy, A., Nagy, G., Buzás, E. I., Iványi, Z., Kittel, A., Powell, D. W., McLeish, K. R., and Ligeti, E. (2013) Antibacterial effect of microvesicles released from human neutrophilic granulocytes. *Blood* **121,** 510–518
73. Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y., and Zychlinsky, A. (2004) Neutrophil extracellular traps kill bacteria. *Science* **303,** 1532–1535