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Quantification of *O*-GlcNAc Protein Modification in Neutrophils by Flow Cytometry

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Abstract

Observations of intracellular *O*-linked *β*-*N*-acetylglucosamine (*O*-GlcNAc) protein modification are primarily performed by Western blot or immunofluorescence microscopy. The goal of this study was to develop a flow cytometric-based assay for *O*-GlcNAc signaling and thus provide a more quantitative and rapid method to facilitate clinical analyses. Isolated peripheral blood neutrophils were stimulated with fMLF after adherence to glass cover slips. Cells in suspension were treated with either fMLF or PMA. Unstimulated cells served as controls. Neutrophils were fixed with formaldehyde and permeabilized with cold methanol before intracellular *O*-GlcNAc staining. Cells on cover slips were analyzed by fluorescence microscopy, and suspension cell data were acquired by flow cytometry. *O*-GlcNAc protein modification was increased following neutrophil stimulation with either 100 nM fMLF or 10 nM PMA. Increases were detected following either treatment using both flow cytometry and fluorescence microscopy. The time necessary for the completion of staining, data acquisition, and analysis was considerably less using flow cytometry. In addition, flow cytometry allows for the analysis of a substantially greater number of cells. Neutrophil protein modifications by *O*-GlcNAc are rapidly detected using flow cytometry and provide information similar to that observed using fluorescence microscopy. Published 2008 Wiley-Liss, Inc.†

Key terms

neutrophil; *O*-GlcNAc; fMLF; PMA; intracellular protein; flow cytometry; immunofluorescence microscopy

INTRACELLULAR *O*-glycosylation of ser/thr residues with *O*-linked *β*-*N*-acetylglucosamine (*O*-GlcNAc) is now recognized as a novel transmembrane signaling mechanism. The mechanisms and timing of *O*-GlcNAc cycling are akin to phosphorylation and dephosphorylation reactions, which are vastly different from the modification of extracellular glycans (1). Targets of O-GlcNAcylation include ser/thr residues that might otherwise undergo phosphorylation, but instead are covalently modified by *O*-GlcNAc (2). In addition, protein glycosylation with *O*-GlcNAc is distinct from classical pathways in that O-GlcNAcylated proteins are found primarily in the cytoplasm and nucleoplasm, and the glycan is not elongated by further modification (3). GlcNAc signaling begins with the hexosamine biosynthesis pathway (HBP). The HBP, whose signaling role was first recognized by Traxinger and coworkers (4), provides the precursor (UDP-GlcNAc) necessary for *O*-GlcNAc signaling (5). GlcNAc signaling is a crucial regulator of glucose transport. In addition, this posttranslational modification results in the alteration of a number of functions including cell-cycle progression, transcription, tumor growth, nutrient sensing, diabetes, and stress response (2). Recent work has also indicated a role for O-GlcNAcylation in agonist-induced neutrophil motility (6).

Evaluation and identification of *O*-GlcNAc modified proteins are traditionally performed by immunoblot (7) and, more recently, immunofluorescence microscopy (6). These methods are primarily qualitative and may require several days to

complete. A recent review by Hart et al. (3) indicated that the development of sensitive, yet straightforward tools for detection, quantitation, and site localization of *O*-GlcNAc are still needed. In this report, we describe a rapid, semiquantitative approach to evaluate overall changes in *O*-GlcNAc modification using flow cytometry.

Materials and Methods

Sample Preparation and Treatment

Peripheral blood samples for neutrophil isolation by discontinuous density gradient centrifugation were obtained from healthy adult volunteers by venipuncture. Briefly, 6 ml of blood anticoagulated with EDTA were layered over 3.5 ml Histopaque 1077 and 3.5 ml Histopaque 1119 (Sigma-Aldrich; St. Louis, MO) and centrifuged at 300*g* for 45 min at room temperature. Neutrophils were collected from the 1077 and 1119 interface and washed twice with Hank's Balanced Salt Solution (HBSS) containing calcium and magnesium (Invitrogen; Carlsbad, CA). If necessary, contaminating red blood cells were lysed by incubation with 0.2% sodium chloride for 1 min. Isotonicity was restored by adding an equal volume of 1.6% sodium chloride. Isolated neutrophils suspended in HBSS (1×10^6 cells/ml) were either preincubated with 5 μg/ml cytochalasin D (Sigma; St. Louis, MO) before stimulation with *N*-formylmethionyl-leucyl-phenylalanine (fMLF; Sigma) or stimulated with phorbol 12-myristate 13-acetate (PMA; Sigma).

Immunostaining

For fluorescent microscopy. Neutrophils (7.5×10^4 cells) in HBSS with and without cytochalasin D were loaded onto glass cover slips and incubated at 37°C, 5% CO_2 for 30 min before stimulation. Adhered neutrophils were stimulated with fMLF for 2 min in the presence of cytochalasin D or for 60 min with PMA alone. *O*-GlcNAc staining was performed as described by Kneass and Marchase (6) with minor modification. Briefly, stimulated cells were fixed with 3% formaldehyde (Tousimis; Rockville, MD) in phosphate-buffered saline (formaldehyde/PBS) for 20 min at room temperature. Cells were permeabilized with cold methanol for 2 min, rinsed with PBS, and blocked with Image-iT FX (Invitrogen) for 30 min at room temperature. After blocking with 1% casein/PBS containing 2% Tween-20 for 30 min at room temperature, the cells were incubated for 1 h at 37°C with a 1:250 dilution of anti-*O*-GlcNAc antibody (monoclonal antibody clone CTD110.6; Covance; Berkeley, CA) in 1% casein/PBS/2%Tween-20. Cover slips were washed three times with PBS before additional blocking for 10 min with 10% normal goat serum in PBS. This was followed by incubation at room temperature for 45 min in 1:250 dilution of Alexa Fluor 488-conjugated goat anti-mouse IgM (Invitrogen). Finally, cover slips were washed three times with PBS and mounted on glass slides with ProLong Gold antifade reagent containing DAPI (Invitrogen). Mounted coverslips were cured overnight before image acquisition.

For flow cytometry. Neutrophils (1×10^6 cells) in HBSS containing cytochalasin D were incubated in 12 mm × 75 mm polysterene tubes for 20 min at 37°C, 5% CO_2 before stimulation with fMLF. PMA stimulation for 60 min at 37°C, 5% CO_2 occurred in the absence of cytochalasin D. Following treatment, cells were fixed with 3% formaldehyde/PBS for 10 min at 37°C. Cells were pelleted by centrifugation at 300*g* for 5 min, permeabilized for 20 min on ice with cold 90% methanol (Sigma), and washed once with cold 1% casein/PBS (Blocker Casein; Pierce; Rockford, IL). Permeabilized cells were incubated 30 min at 4°C with a 1:50 dilution of anti-*O*-GlcNAc antibody in 1% casein/PBS (100 μl final volume). To demonstrate specificity of *O*-GlcNAc antibody immunoreactivity, 500 mM GlcNAc was preincubated with the monoclonal antibody for 5 min at 4°C before addition to permeabilized cells. Incubation was terminated by adding 500 μl of 1% casein/PBS, pelleted by centrifugation, and washed with additional 1% casein/PBS. This was followed by incubation at 4° C for 15 min in 100 μl of a 1:1,000 dilution of Alexa Fluor 488-conjugated goat anti-mouse IgM (Invitrogen) in PBS containing 10% normal goat serum (Sigma). Incubation was again terminated by adding 500 μl of 1% casein/PBS followed by washes with 1% casein/PBS and PBS, respectively. Final cell pellets were suspended in 1% paraformaldehyde/PBS for flow cytometric acquisition of 10,000 events with a FACSCalibur flow cytometer (BD Biosciences; San Jose, CA).

Immunofluorescence Microscopy

Neutrophils were viewed and images acquired with a Nikon Eclipse TE2000 Quantum inverted fluorescence microscope (Nikon Instruments, Melville, NY) and an Andor Technologies iXon model DV8 16-bit electron multiplying CCD camera cooled to −90°C (Andor Technologies, South Windsor, CT). A 96320 HYQ filter module (Nikon) was used for *O*-GlcNAc. A second filter set containing a D355HT15 exciter, 390DCLP dichroic, and 405DF43 emitter was used for DAPI imaging. The intensity of *O*-GlcNAc fluorescence labeling was quantified with MetaMorph image analysis software (Molecular Devices, Danville, PA).

Flow Cytometry

O-GlcNAc intensity data were analyzed with CellQuest Pro software (version 5.2.1; BD Biosciences). Neutrophils were gated on size and granularity characteristics. Data were visualized using density dot plots of forward scatter versus side (90° light) scattering properties. Intracellular abundance of *O*-GlcNAc was recorded as the mean fluorescence intensity (MFI) of all gated neutrophils as observed using histogram plots.

Statistical Analysis

Nonparametric paired *t* test (Wilcoxon signed ranks test) was used to evaluate differences due to fMLF or PMA treatments (SPSS 14.0; SPSS; Chicago, IL). Differences were considered significant when $P < 0.05$.

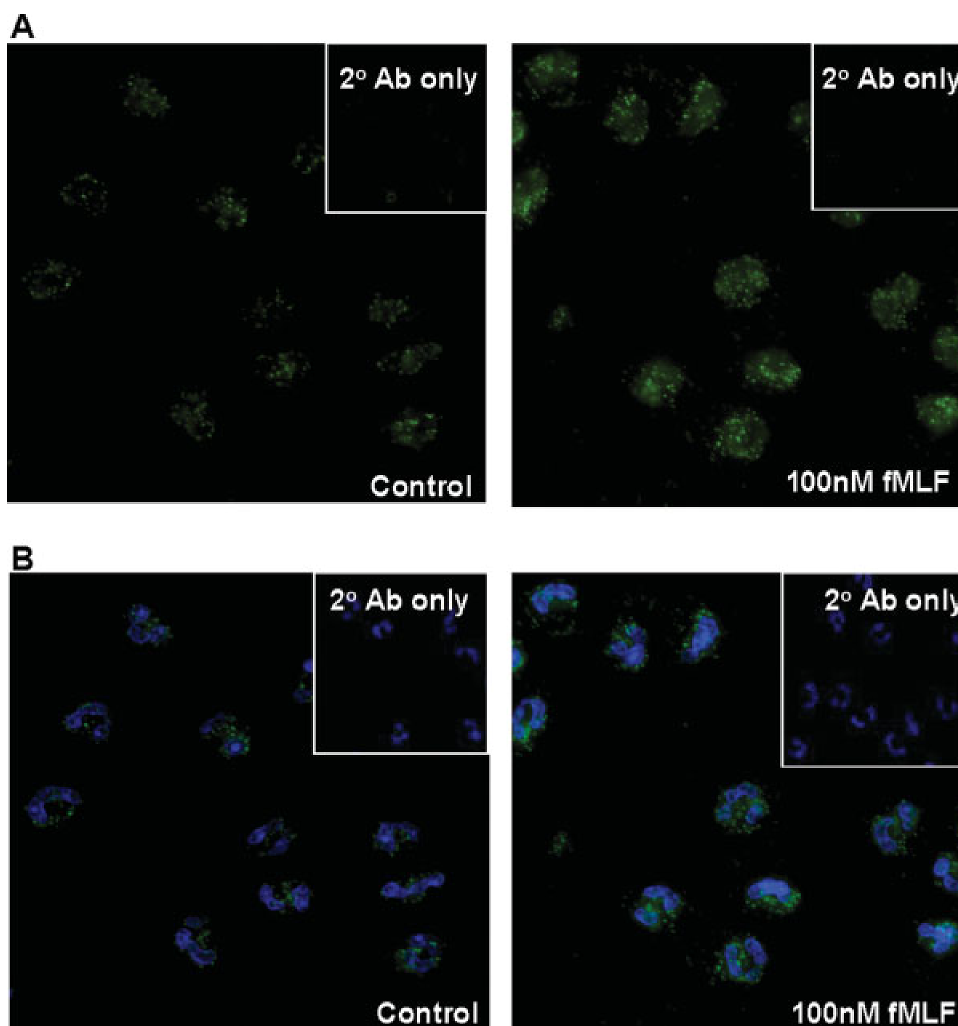

Figure 1. Analysis of *O*-GlcNAc levels by immunofluorescence microscopy. Neutrophils adhered to coverslips demonstrated brighter *O*-GlcNAc fluorescence after stimulation with 100 nM fMLF for 2 min relative to unstimulated (control) cells (Panel **A**). Proteins modified by *O*-GlcNAc appear to be primarily cytoplasmic relative to DAPI-stained nuclei shown in the image overlays of Panel **B**.

Results

Visualization of neutrophil intracellular *O*-GlcNAc abundance was performed with immunofluorescence microscopy (Fig. 1). The staining procedure typically required 3–4 h. Image processing occurred on subsequent days with 1–2 h necessary for image capture whereas 2–3 h was necessary for image analysis. O-GlcNAcylated proteins appeared to be primarily cytoplasmic (Fig. 1B). As previously observed by Kneass and Marchase (6), the average integrated intensity of *O*-GlcNAc fluorescence (60–100 cells per treatment per sample) was greater following fMLF stimulation (Fig. 1 and Table 1). PMA-induced increases in *O*-GlcNAc were also observed using fluorescence microscopy. Unlike observations with fMLF, differences due to PMA stimulation were not significant (Table 1).

A flow cytometric assay for intracellular *O*-GlcNAc fluorescence was developed to offer a more rapid, semiquantitative method of analysis. Time to completion for this procedure included 2–3 h for staining, 0.5–1 h for acquisition, and an additional 0.5–1 h for analysis. Neutrophils isolated by discontinuous gradient centrifugation were always greater than 92% enriched as determined by gating on forward versus side scatter density dot plots (Fig. 2A). Gated neutrophils were assessed for *O*-GlcNAc abundance using histogram plots. A variety of dilutions ranging from 1:50 to 1:1,000 were tested for the anti-*O*-GlcNAc primary antibody whereas Alexa Fluor 488-conjugated IgM secondary antibody was titrated from 1:250 to 1:4,000 (data not shown). The combination of 1:50 primary antibody and 1:1,000 secondary antibody provided the best signal with the least amount of background (Fig. 2B, gray-open histogram versus gray-filled histogram). Formaldehyde levels were also tested at 0.1, 1, and 3% to optimize cellular fixation before permeabilization and staining. Forward and side scatter characteristics were maintained with both 1% and 3% formaldehyde; 3% formaldehyde preserved the greatest mean fluorescence intensity above background in untreated cells (data not shown). Finally, *O*-GlcNAc specificity was demonstrated by competitively blocking antibody binding with free GlcNAc (Fig. 2B).

The effects of fMLF and PMA on intracellular *O*-GlcNAc were assessed using the optimized flow cytometric procedure. As observed using fluorescence microscopy, neutrophil

Table 1. *O*-GlcNAc in isolated PMN: microscopy versus flow cytometry

	ASSAY LENGTH[b]	NO. OF CELLS ANALYZED[c]	INTENSITY VALUE[a] UNSTIMULATED	INTENSITY VALUE[a] STIMULATED	*P*-VALUE[d]
Microscopy					
± 100 nM fMLF	6–10 h	60–100	191,207 ± 26,139	252,221 ± 37,364	0.043
± 10 nM PMA	6–10 h	60–100	232,786 ± 29,504	309,411 ± 83,693	0.080
Flow cytometry					
± 100 nM fMLF	3–5 h	9000–10,000	8.01 ± 0.522	13.08 ± 0.919	0.001
±10 nM PMA	3–5 h	9000–10,000	7.75 ± 0.774	25.72 ± 2.104	0.008

[a] Mean ± standard error of integrated intensity values for microscopy (n = 5) or mean fluorescence intensity values for flow cytometry (±fMLF n = 13; ± PMA n = 9).
[b] Length of staining, data acquisition, and analysis portions of assay; does not include time for cell isolation and treatment. Microscopy assay length is accumulative for the time spent over ~ 2 days.
[c] Number of cells analyzed for each treatment (0 vs. 100 nM fMLF or 0 vs. 10 nM PMA) for each biological replicate.
[d] Wilcoxon signed ranks test.

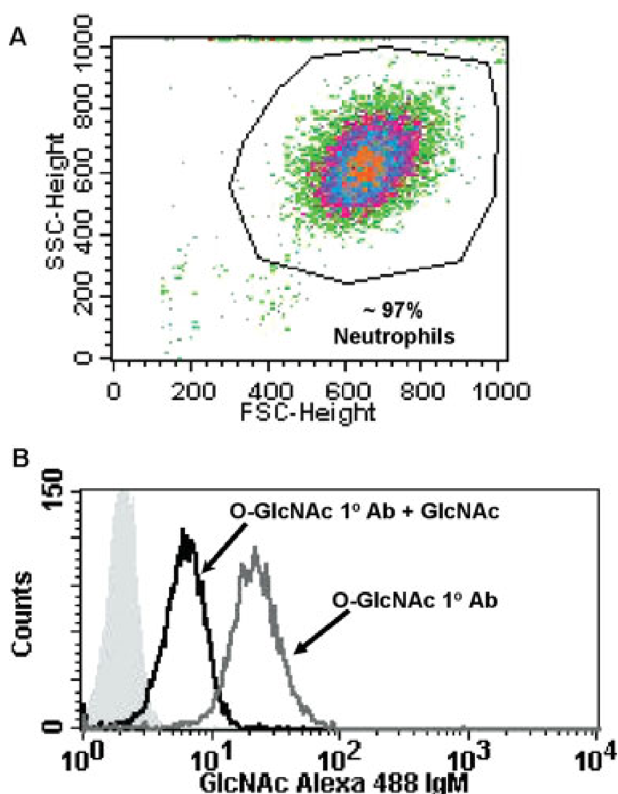

Figure 2. Cell preparations were always at least 92% neutrophils with some as high as 97–98% as is shown in the representative density dot plot (Panel **A**). Neutrophils gated on forward and side scatter were analyzed for *O*-GlcNAc fluorescence using histogram plots. Panel **B** is a representative histogram overlay. The gray-filled histogram represents background staining observed when cells were incubated with Alexa Fluor 488-conjugated IgM only. The gray-open histogram was created by cells incubated first with an *O*-GlcNAc monoclonal antibody (clone CTD110.6) followed by incubation with Alexa Fluor 488-conjugated IgM. To demonstrate antibody-binding specificity, the *O*-GlcNAc monoclonal antibody was incubated with free GlcNAc for 5 min at 4°C before addition to permeabilized neutrophils. The treatment reduced the level of cellular fluorescence as is demonstrated by the black-open histogram. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

stimulation with 100 nM fMLF increased *O*-GlcNAc within 2 min of treatment (Fig. 3A). Unlike previous observations with fluorescence microscopy (6), increases in intracellular *O*-GlcNAc were maintained for at least 2 h of in vitro treatment (Fig. 3B). Incubation with a known *O*-GlcNAcase inhibitor [2 h, 100 μM PUGNAc (*O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino *N*-phenyl carbamate)] also increased the mean fluorescence intensity of neutrophil *O*-GlcNAc and thus, supporting the use of flow cytometry for measurements of O-GlcNAcylation (data not shown). PMA-induced increases in neutrophil *O*-GlcNAc were readily observed at 60 min of stimulation (Fig. 4A). In contrast to fMLF treatment, 10 nM PMA-induced increases in *O*-GlcNAc were time responsive. Increased levels were observed as early as 15 min and the maximum maintained at 45 and 60 min with only slight decreases at 120 min relative to the earlier time point (data not shown). Individual variability is observed in most biological processes and O-GlcNAcylation is no different. Before stimulation, *O*-GlcNAc levels were comparatively similar in neutrophils from healthy adults (Fig. 3C, n = 13 and Fig. 4B, n = 9). The response to fMLF stimulation varied with differences in mean fluorescence intensity between treated and untreated neutrophils ranging from 1.20 to 8.06 across all individuals tested. PMA stimulation induced a greater level of O-GlcNAcylation than fMLF. Intensity differences ranged from 12.47 to 19.50 between mean fluorescence intensity of stimulated versus no treatment with one individual showing a difference of 29.68 (all values are arbitrary units). Even with the observed variation in response, the overall mean in treated cells was significantly greater than that of untreated neutrophils regardless of treatment (Table 1).

Discussion

Quantitative methods have been developed to analyze many transmembrane signals, such as calcium signals, yet the tools for quantitative analysis of *O*-GlcNAc signaling in cells have not been thoroughly explored (3). Studies of *O*-GlcNAc modification generally use SDS-PAGE/Western blotting or fluorescence microscopy tools. Western-blot assays provide

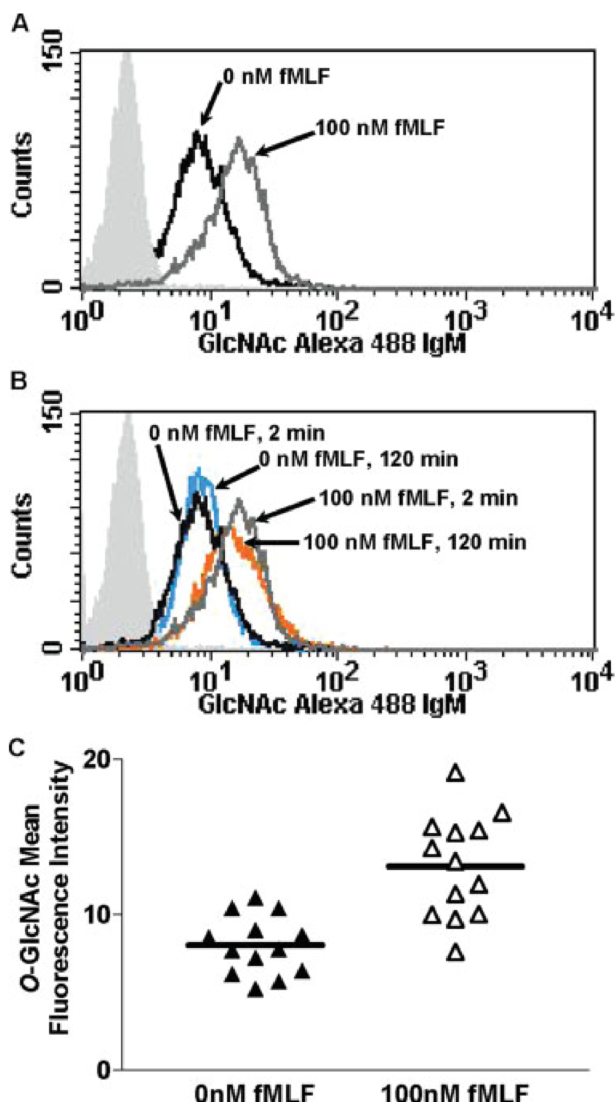

Figure 3. Increased neutrophil *O*-GlcNAc protein modification due to fMLF stimulation can be observed using flow cytometry. The representative histogram in Panel **A** shows a right shift in the histogram peak due to treatment with 100 nM fMLF (gray-open histogram versus control cells, black-open histogram). The gray-filled histogram represents background staining with Alexa Fluor 488-conjugated IgM only. Neutrophils were also incubated in the presence or absence of fMLF for up to 2 h but minimal differences were observed relative to the much shorter, 2 min treatment (Panel **B**). The summary plot in Panel **C** shows that there is variation among individuals ($n = 13$) in the level of *O*-GlcNAc protein modification but the overall mean of control versus cells treated for 2 min with fMLF was significantly different ($P < 0.01$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

qualitative information regarding *O*-GlcNAc modifications such as whether *O*-GlcNAc is added or removed from proteins of particular sizes. Immunofluorescence microscopy is also primarily qualitative but provides the added benefit of visualizing the subcellular localization of modified proteins. Our results suggest that similar observations of general *O*-GlcNAc protein modification can be observed using flow cytometry (Table 1, Figs. 2–4).

Flow cytometric analysis of *O*-GlcNAc protein modification can be used to detect increases (and potentially decreases) of this signal in leukocytes. A major benefit of this approach is the amount of time needed to perform the assay. Western blot and immunofluorescence microscopy require ~2 days before differences can be observed. Overall, changes in *O*-GlcNAc protein modification can be detected in a matter of hours with the flow cytometric assay described. Our observations of increased *O*-GlcNAc modification in neutrophils stimulated 2 min with fMLF (Figs. 1 and 3) are in agreement with the changes detected by Kneass and Marchase (6) using immunofluorescence microscopy. This was not the case for longer fMLF treatments in that we observed very little decrease in *O*-GlcNAc levels over 2 h of treatment. Previous reports indicated that the increase was short lived and that *O*-GlcNAc modification began to decrease within 10 min of stimulation (6). This difference may result from the use of cytochalasin D. Cytochalasins have been shown to increase neutrophil adhesion (8) and superoxide production in response

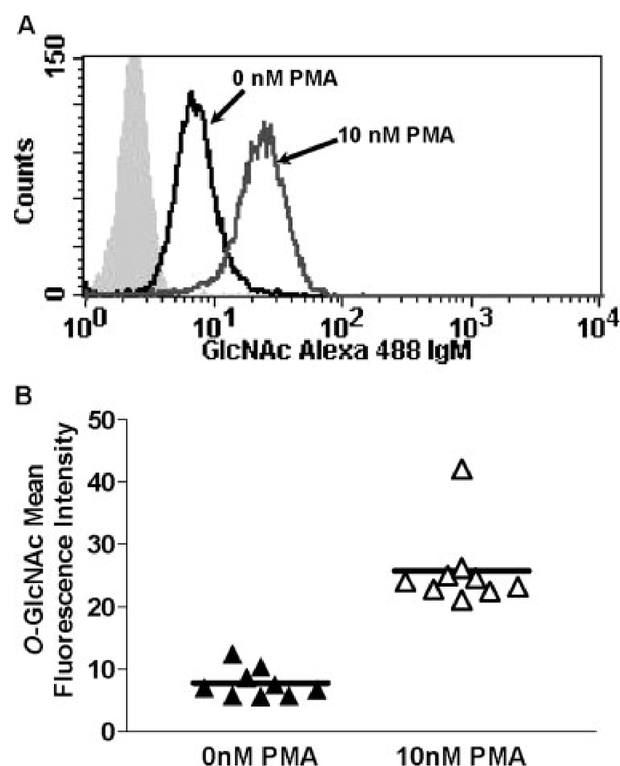

Figure 4. PMA stimulation increases neutrophil *O*-GlcNAc protein modification. The representative histogram in Panel **A** shows a right shift in the histogram peak due to treatment with 10 nM PMA for 60 min (gray-open histogram versus control cells, black-open histogram). The gray-filled histogram represents background staining with Alexa Fluor 488-conjugated IgM only. The summary plot in Panel **B** shows that the PMA-induced increase in *O*-GlcNAc was more consistent across individuals ($n = 9$) relative to that observed with fMLF. Observation is significant at $P < 0.01$.

to ligand stimulation, such as fMLF (9,10). In this study, cytochalasin D was selected over other cytochalasins, because it does not alter cellular glucose transport (11), the major energy source of activated neutrophils. Cytochalasin D may also account for the more robust *O*-GlcNAc signaling response observed in this study as physiological responses to fMLF are much more robust with cytochalasin D treatment. PMA, a more potent neutrophil activator, stimulated even greater levels of O-GlcNAcylation than fMLF treatment regardless of the presence or absence of cytochalasin D. Increases in *O*-GlcNAc were not as rapid with this stimulus but were maintained for over 1 h of treatment. Thus, the sustained *O*-GlcNAc signaling observed increases the reliability of signal detection in comparison with the very transient responses observed previously.

Our findings indicate that flow cytometric analysis of *O*-GlcNAc levels provides a rapid and semiquantitative method that can be used as an alternative or to confirm observations obtained with more qualitative procedures. The described method uses a commercially available IgM monoclonal antibody commonly used in microscopy and immunoblot analysis of *O*-GlcNAcylated proteins. Although this antibody detects modification of nuclear and cytolasmic proteins, the flow cytometric analysis depicted will primarily detect cytoplasmic proteins due to limited nuclear permeability of the antibody isotype. In conclusion, this approach permits the rapid analysis of *O*-GlcNAc modification of cytoplasmic proteins, including those participating in GlcNAc signaling, within neutrophils from multiple donors such as patients in clinical studies.

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