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Supplement to: Detection of phosphoglucomutase-3 (PGM3) deficiency by lectin-based flow cytometry

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METHODS

Human subjects

All patients provided informed consent on NIH IRB-approved research protocols designed to study atopy (NCT01164241), hyper-IgE syndromes (NCT00006150), or known or suspected congenital disorders of glycosylation (NCT00369421 and NCT02089789). Clinical histories, review of available outside records, and clinical evaluations were all performed at the Clinical Center of the National Institutes of Health (NIH). Clinical immunologic laboratory tests were performed by the Department of Laboratory Medicine at NIH, Bethesda, MD.

Lectin flow cytometric assays

Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll (Ficoll-Paque PLUS; GE Healthcare) gradient centrifugation. Cells were surface stained with Live/Dead Fixable Blue viability dye (Invitrogen, Carlsbad, Calif), anti-CD19 APC, anti-CD11c v450, anti-CD3 AF700, anti-CD56 PE-Cy7, anti-CD4 BV711, anti-CD8 APC-H7 (BD Biosciences, San Jose, CA), and anti-CD45RO TRPE (Beckman-Coulter, Pasadena, CA). Fluorescein conjugated L-phytohemagglutinin (L-PHA) or concanavalin A (Con A) (Vector Laboratories, Burlingame, CA) were used to stain *N*-glycans. Where indicated, PBMCs were treated with 100 units/μl of glycerol free PNGase F (N-glycosidase F) (New England Biolabs, Ipswich, MA) in 20μL HBSS at 37°C for 1 hour prior to surface staining with antibodies and L-PHA. Cells were washed, fixed with 4% paraformaldehyde and 200,000 events per condition were collected on an LSR Fortessa (BD Biosciences). All events were analyzed using FlowJo software (Treestar, Ashland, OR), and are gated on live single cells as shown in Fig S2.

Nucleotide Sugar Quantification

High performance anion exchange chromatography (HPAEC) was performed on a Dionex ICS-5000 with a CarboPac PA1 column 2x250 and PA1 2x50 guard column (ThermoFisher, Waltham, MA). Isolated PBMCs were washed with PBS, and the resulting pellet was lysed in 75% ethanol (100µl/1x10^6 cells) by cuphorn sonication. After sonication, the suspension was centrifuged, the supernatant was saved and the solvent was removed by Savant SpeedVac (ThermoFisher). The concentrated residue was resuspended in an appropriate volume of 40 mM sodium phosphate buffer and any insoluble material removed by centrifugation.

HPAEC analysis was performed by as described (E5). Briefly, 10 uL of lysate was injected into a 10 uL sample loop and species were separated on the column utilizing a combination of two eluents: A= 1mM NaOH B= 1mM NaOH/1M NaOAc. HPAEC was run with a flow rate of 0.25 mL/min and the following gradient elution was utilized: T_{0min} = 2% B, T_{10min} = 30%B, T_{15min} = 30%B, T_{16min} = 55%B, T_{30min} = 55%B, T_{33min} = 100%B, T_{50min} = 100%B, T_{55min} = 2%B, T_{65min} = 2%B. Standards [UDP-GalNAc and UDP-GlcNAc (Sigma-Alrich, Saint Lous, MO)] were dissolved to 1 mM in 100 mM sodium phosphate buffer and then diluted for standard curves. Samples were compared to standard curves and UDP-HexNAc concentrations defined. Due to a large signal corresponding to ADP in some UV traces, PAD was utilized for quantitation. UV absorbance collected at 260 nm and ED detection done with IntAmp and AgCl electrode using Gold Standard PAD waveform.

FIGURES

Figure E1. Discrete differences in *N*-glycan complexity exist among PGM3 deficient leukocyte populations. (A) MFI of L-PHA in total T cells ($CD3^+$), natural killer cells ($CD56^+$), $CD45RO^-$ and $CD45RO^+$ $CD8^+$ cells, B cells ($CD19^+$), and $CD11c^+$ cells from the same cohort as in Figure 1. Surface markers were used to identify T cells ($CD3^+$), natural killer cells ($CD56^+$), $CD8^+$ $CD45RO^-$ T cells, $CD8^+$ $CD45RO^+$ T cells, B cells ($CD19^+$), and $CD11c^+$ cells. Mann-Whitney test; **P<0.01, ****P<0.0001.

Figure E2. Gating strategy for defining leukocyte subsets. PBMCs were gated by forward and side scatter and cell clusters were excluded by side scatter. All dead cells were excluded using viability dye, and remaining populations were divided into $CD3^+$ and $CD3^-$ groups. Natural killer cells (defined as $CD56^+$), B cells (defined as $CD19^+$), and $CD11c^+$ cells were identified from the $CD3^-$ group. $CD3^+$ T cells were further divided into $CD4^+$ or $CD8^+$ populations, and then into $CD45RO^+$ or $CD45RO^-$ groups.

Figure E3. Total *N*-glycan expression appears preserved in PGM3 deficient leukocyte subsets. (A) Schematic of complex- (top) and hybrid-type (bottom) branching *N*-glycans showing α-mannose residues bound by concanavalin A (Con A) indicated in red dashed boxes. Mean fluorescent intensity (MFI) of Con A staining of PBMCs, total T cells, $CD45RO^-$ CD4 and CD8, $CD45RO^+$ CD4 and CD8, natural killer cells ($CD56^+$), B cells ($CD19^+$), and $CD11c^+$ cells isolated from control individuals (n = 7), as well as atopic dermatitis (AD, n = 3) and PGM3 deficient individuals (n = 3). Mann-Whitney test.

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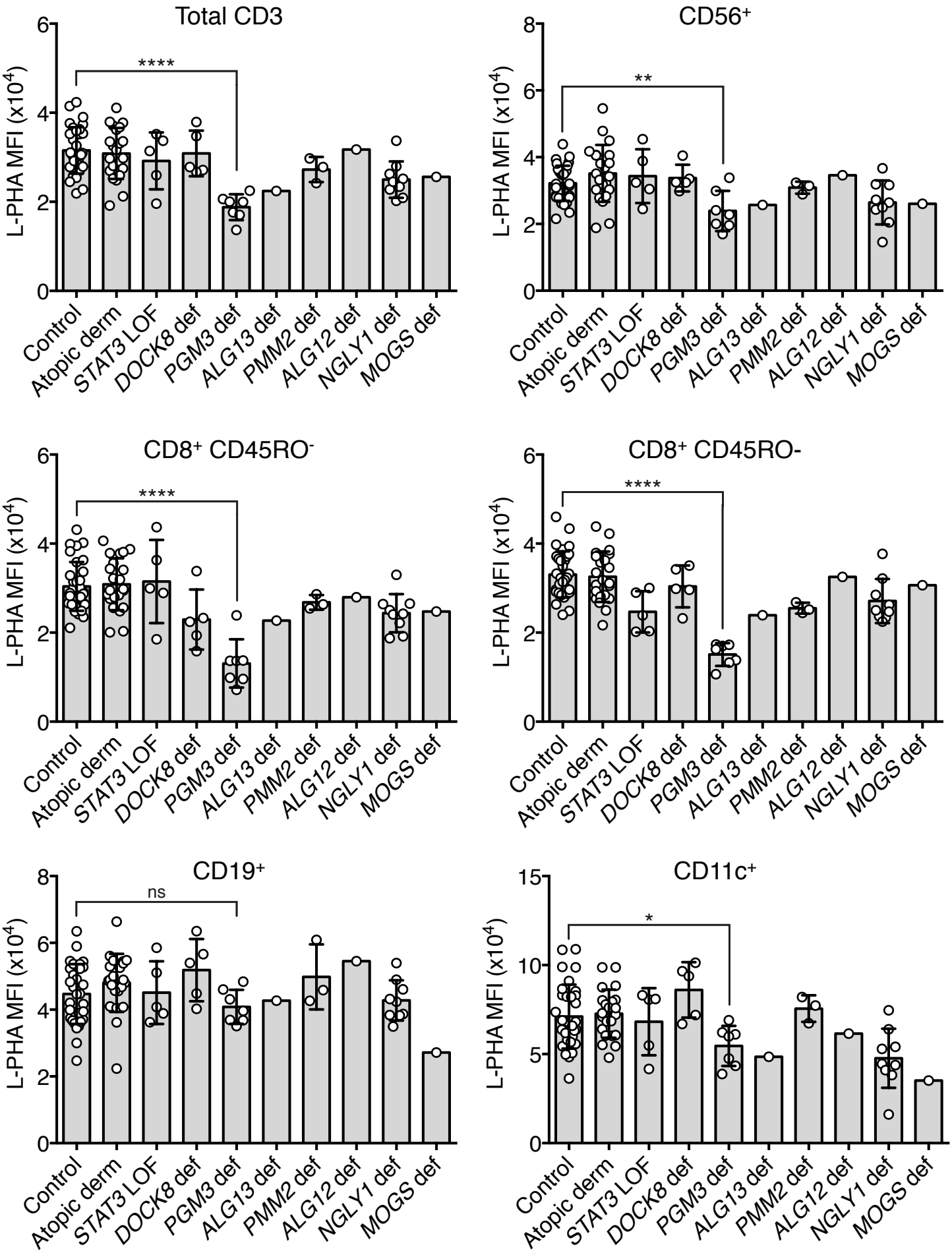
Figure E1.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CD8+ CD45RO-
CD8+ CD45RO-
CD19+
CD11c+
L-PHA MFI (x10^4)
****
**
ns
*
Control
Atopic derm
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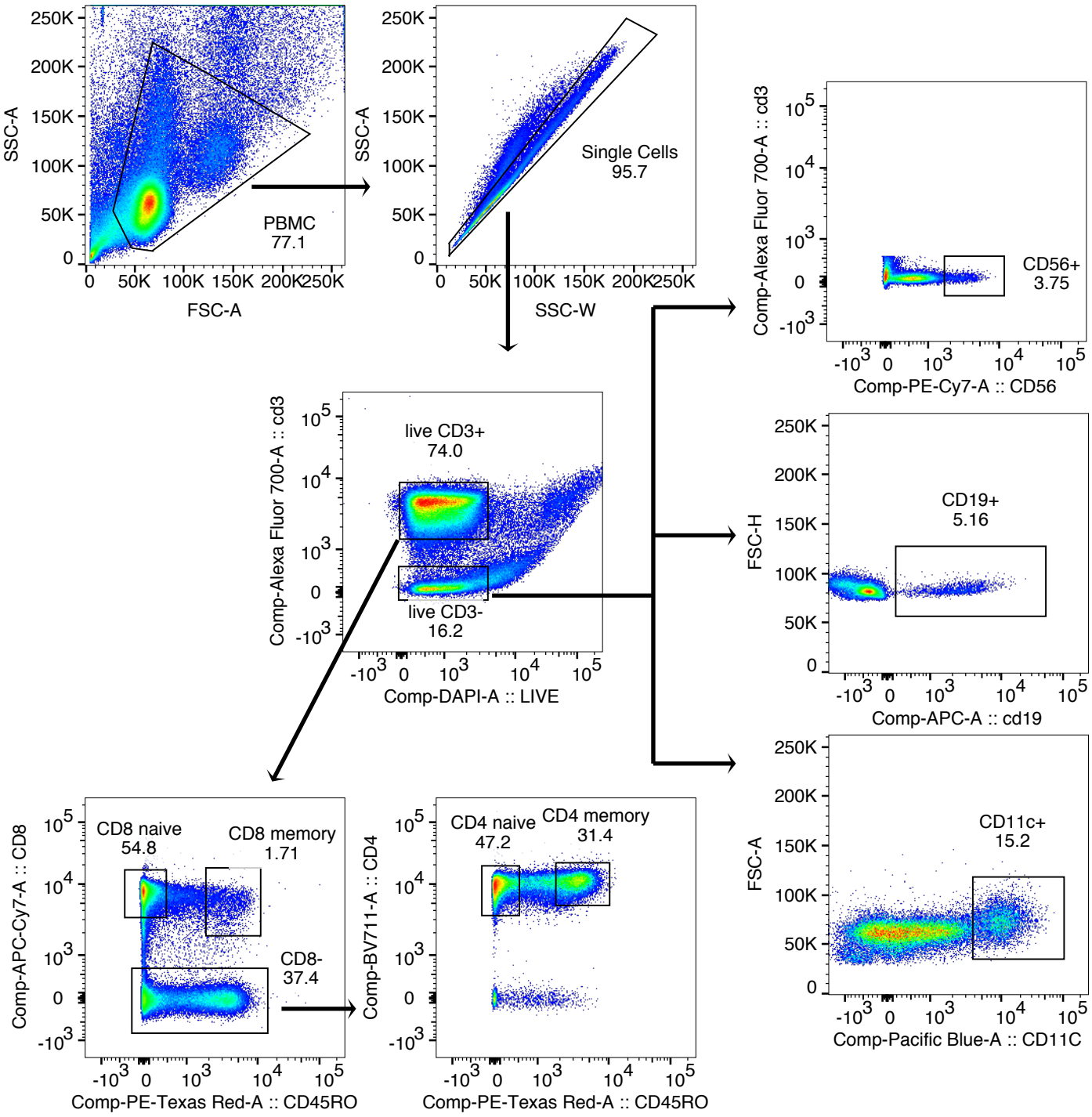
PGM3 def
ALG13 def
PMM2 def
ALG12 def
NGLY1 def
MOGS def

Figure E2.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PBMC
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Comp-PE-Cy7-A :: CD56
CD56+
3.75
Comp-DAPI-A :: LIVE
live CD3+
74.0
live CD3-
16.2
FSC-H
Comp-APC-A :: cd19
CD19+
5.16
Comp-APC-Cy7-A :: CD8
Comp-PE-Texas Red-A :: CD45RO
CD8 naive
54.8
CD8 memory
1.71
CD8-
37.4
Comp-BV711-A :: CD4
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CD11c+
15.2

Figure E3.

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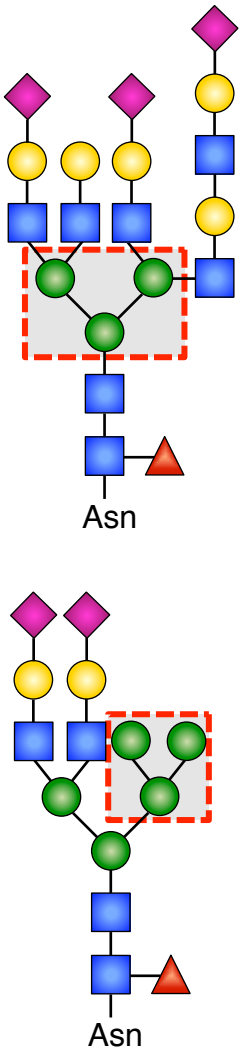
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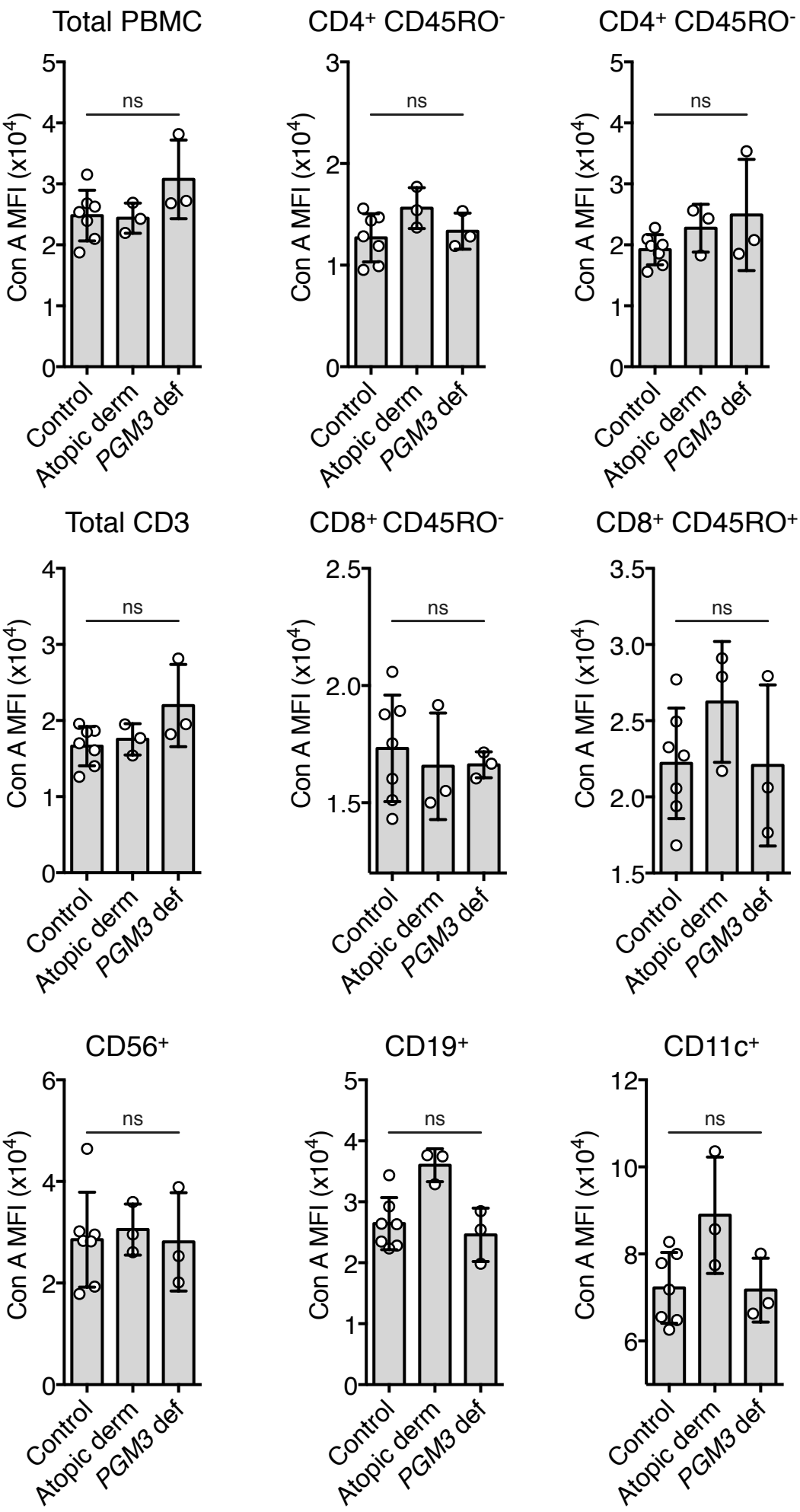
Total PBMC
CD4+ CD45RO-
CD4+ CD45RO-
Total CD3
CD8+ CD45RO-
CD8+ CD45RO+
CD56+
CD19+
CD11c+
Con A MFI (x10^4)
ns
Control
Atopic derm
PGM3 def