Assessment of bacterial exposure on phagocytic capability and surface marker expression of sputum macrophages and neutrophils in COPD patients

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Methods

Sputum processing

Sputum samples were processed to obtain cell pellets for immune cell counting and culture according to a previous method [19]. Briefly, sputum plugs were selected from saliva and put on ice (minimum weight 0.1 g). A four-fold volume of 0.1% DTT was added and the mixture was incubated for 15 min on a roller mixer on ice, vortexed every 5 min, filtered using 48 μm nylon-mesh filter and centrifuged. Cell pellets were resuspended in 1 ml of PBS to perform haemocytometer cell counts, cytospin differential cell counts and re-adjusted to $1x10^6$/ml in Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies, Paisley, UK) which was supplemented with 10% human serum and 1mM pencillin-streptomycin (both Sigma-Aldrich, Poole, UK). Amphotericin B (Life Technologies, Paisley, UK) was added to the media at a concentration of 1% to kill any pre-existing bacteria.

Sputum macrophages and neutrophils were isolated by plating sputum cells in 48 well plates at $0.1x10^6$ macrophages per well and leaving to adhere for 2 hours. Non-adherent cells were removed, counted and plated at $0.1x10^6$ cells per well. Cells were cultured overnight in antibiotic free IMDM with 10% human serum. Adherent cell population was confirmed by differential cell counts as 92% macrophages and non-adherent population were 83% neutrophils (Supplement Figure 1, Supplement Table 1)

Bacterial culture

Bacteria was cultured in supplemented brain heart infusion broth (Sigma-Aldrich, Poole, UK). 500ml of broth was made with distilled water and supplemented with 10μg/ml each of Hemin and β-Nicotinamide dinucleotide (both Sigma-Aldrich, Poole, UK). The broth was aliquotted into 50ml tubes and stored at 4°C.

To generate bacterial stocks, a frozen bead of *Streptococcus pneumonaie* (strain ATCC 700902 chosen as a representative clinical strain Representative strain of the Pneumococcal Molecular

Epidemiology Network (PMEN)) was transferred to 10ml of supplemented brain heart infusion broth (Sigma-Aldrich, Poole, UK) and left overnight at 37°C, 5% CO_2.

The next day, the optical density (OD) was measured using a BMG plate reader. If an OD of 0.4 or above had been achieved, the culture was centrifuged and re-suspended in a) 5ml PBS then aliquoted into 5x1ml aliquots to create short-terms stocks, or b) in a 10ml solution of 90% supplemented brain heart infusion broth with 10% glycerol (Sigma-Aldrich, Poole, UK) to create 1ml glycerol stocks for long term use. A sample of PBS-suspended bacteria was taken for a Miles-Misra assessment to determine how many colony forming units were present in the short-term bacterial stocks.

Bacterial quantification: Miles Misra assessment

Quantification of viable bacteria was determined by Miles-Misra protocol. Eight 10-fold serial dilutions were prepared from a 1ml aliquot of fresh PBS-suspended bacterial stock. 20µl of bacterial stock and 180µl of PBS was used to create the following dilution series: $1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$, $1x\ 10^5$, $1x10^6$, $1x10^7$, $1x10^8$. Chocolate agar plates were split into 8 equal quadrants and 2 drops of each serial dilution were plated on a quadrant. Drops were left to dry then plates inverted and incubated overnight at 37°C, 5% CO_2.

Developed colonies were counted in the quadrant with the highest number of colonies where individual colonies were discernible. The following calculation was performed: number of colonies/number of drops x dilution factor x 100 to give the number of colony forming units per millilitre (CFU/ml). Using the CFU assessment, volume required for a multiplicity of infection (MOI) of 10 was calculated.

Multiplicity of infection (MOI) calculation

In order to keep the bacterial load consistent in experiments, MOI was used. This is the ratio of bacteria per cell (macrophages, neutrophils or total cell counts depending on experiment type). MOI of 10 was used in all experiments.

From the Miles Misra assessment, MOI was determined by calculating CFU/ml and working out how much bacteria were required. Cell numbers were $1x10^4$ so 100,000/ CFU/ml x1000 was used to determine how much bacterial stock to add in microlitres. A Miles Misra of the bacterial MOI was also taken at each experiment to check bacterial numbers.

Gentamycin Protection assay

Sputum cells were counted to determine macrophage numbers..

Cells were plated in 48 well plates (Greiner BioOne, Stonehouse, UK) at a concentration of $0.1x10^6$ macrophages per well and left to adhere for at least 2 hours to isolate macrophages. Cells were then washed to remove non-adherent cells. The wash fraction from all wells was collected and non-adherent cells counted. $0.1x10^6$ non-adherent cells were added to tubes. The assay was performed on both adherent and non-adherent cells in parallel.

Media was changed to antibiotic free IMDM with 10% human serum and all cells were left overnight prior to commencing the assay. For the non-adherent cells, each time a wash or media change was performed thereafter, cells were spun down at 400g for 3 minutes to pellet cells before washing.

In fresh antibiotic-free IMDM. *Streptococcus pneumonaie* (*S. pneumoniae*) underwent opsonisation in assay media (10% human serum) (Supplement Figure 13) and was added at a multiplicity of infection of 10:1 or PBS alone was added to unstimulated controls and incubated for 3 hours at 37°C, 5% CO_2. Cells were then incubated with 20µg/ml gentamycin (Life technologies, Paisley, UK) and 40U Benzyl-pencillin (Sigma-Aldrich, Poole, UK) for 2 hours in fresh media. Following three washes with 1ml PBS, cells were treated with 0.02% saponin solution (Sigma-Aldrich, Poole, UK) and left to lyse for 12 minutes. The solution was then energetically pipetted several times to release intracellular bacteria. Living internalised *S. pneumoniae* was quantified by Miles Misra.

Bacterial phagocytosis

Cells were re-suspended in IMDM antibiotic-free media (Sigma-Aldrich, Poole, UK) at $1x10^6$ per ml and 100ul ($0.1x10^6$ cells) was plated per well of a 96 flat bottomed well plates (Greiner Bio-One, Stonehouse, UK). Five extra wells were included to be used as fluorescence minus one (FMO) controls at each time point.

pHrodo labelled heat killed Non-typeable *Haemophilus influenzae* (strain ATCC 53600 chosen as a representative clinical bacterium, isolated from the sputum of a patient with chronic bronchitis) (pHrodo NTHi, a gift from GSK) was reconstituted in assay buffer (HBSS with 200uM HEPES solution [both Sigma Aldrich, Dorset, UK]) at a concentration of 5mg/ml. 25ul was added to relevant wells including FMO controls and 25ul of assay buffer alone was added to unexposed wells. Cells were left to phagocytose at 37°C, 5% CO_2 for 30 minutes and 3 hours. 30 minutes was chosen for neutrophil phagocytosis, and 3 hours was chosen as an optimal timepoint for macrophages. pHrodo NTHi underwent opsonisation in assay media (10% human serum) (Supplement Figure 13).

After incubation, cells were placed on ice for 5 minutes then energetically pipetted and gently scraped with a pipette tip to release cells from the plate. Harvested cells were collected in labelled polypropylene tubes and washed with 2ml PBS then centrifuged at 400g for 5 minutes.

FcR block (Biolegend, Bristol, UK) was added to cells for 5 minutes at 4°C. Antibodies (BD Biosciences, Oxford and Biolegend, Bristol, UK) to CD45 (APC-Cy7), CD66b (FITC), CD206 (APC), CD16 (PerCP Cy5.5) and CD14 (PE-Cy7) were added for 30 minutes. After staining, cells were washed twice with 2ml FACS buffer (PBS supplemented with 1% bovine serum albumen [Sigma Aldrich, Dorset, UK] and 0.1% sodium azide) then centrifuged at 400g for 5 minutes. Cells were re-suspended in 350ul FACS buffer and vortexed. Samples were acquired immediately after processing using Becton Dickinson Canto II flow cytometer (Becton Dickinson, Berkshire, UK).

Cells were gated as follows (Supplement Figure 2); CD45+ cells were gated on (P1) and from this population, single cells were gated by plotting FSC-H v FSC-A (P2). From P2, when plotted on SSC-A vs FSC-A, viable cell population was seen and debris excluded (P3). P3 was used as a stopping gate. Gating for granulocytes (CD66b FITC), macrophage (CD206 APC). Where possible, $10x10^4$ events were recorded under the stopping gate for each condition, and a minimum of $5x10^4$ events for FMO controls. To calculate percentage of phagocytosing cells, channels of the markers of interest were plotted against PE-A (pHrodo NTHi). The gate for positive bacterial internalisation was set using unexposed controls which contained no pHrodo NHTi bacteria. Flowjo software (Portland, Oregon, USA) was used for further analysis to identify cell populations and levels of phagocytosis. FMO controls were used to check the staining was genuine and not due to autofluorescence.

Lactate dehydrogenase assay

Sputum cell viability was measured by the Pierce lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (Thermo Scientific) assay. Cells were re-suspended in IMDM antibiotic-free media (Sigma-Aldrich, Poole, UK) at $1x10^6$ per ml and 100ul ($0.1x10^6$ cells) and either exposed to pHrodo labelled heat killed Non-typeable *Haemophilus influenzae* or left unexposed for 30 minutes and 3 hours at 37°C, 5% CO_2. 50 µl of supernatant was transferred into a new plate and mixed with reaction buffer. After 30 minutes incubation at room temperature, reactions were stopped by adding Stop Solution. Absorbance at 490 nm and 680 nm was measured using a plate-reading spectrophotometer to determine LDH activity.

In-situ cell death assay

Sputum cell viability was measured by *In Situ* Cell Death Detection Kit, Fluorescein kit (ROCHE, UK). Cells were re-suspended in IMDM antibiotic-free media (Sigma-Aldrich, Poole, UK) at $1x10^6$ per ml and 100ul ($0.1x10^6$ cells) and either exposed to pHrodo labelled heat killed Non-typeable *Haemophilus influenzae* or left unexposed for 30 minutes and 3 hours at 37°C, 5% CO_2. Cytospins were generated and cells were fixed with 4% paraformaldehyde in PBS (Sigma Aldrich) for 1 hour. Slides were rinsed with PBS and permeablised with freshly prepared permeablisation buffer (0.1%Triton X in 0.1% sodium citrate) for 2 minutes on ice. 50 µl of label solution was added to the negative control; and the TUNEL mix was freshly prepared according to manufacturer's instruction and added to the pre-treated cells. Slides were protected from light and incubated for 1 hour at 37^0 C. Slides were mounted in mounting media containing the nuclear stain DAPI and immunofluorescence was detected by fluorescence microscopy. The percentage of TUNEL positive cells was calculated.

Results

Phagocytosis of pHrodo NTHi at 30 minutes and 3 hours

Phagocytosis of pHrodo NTHi was numerically increased in both CD206+ and CD66b+ cells at 3 hours compared to 30 minutes (Supplement Figure 7). This increase over time reached significance for in CD66b+ cells for HS ($p<0.05$).

Macrophage expression of CD206 and CD14 after 30 minutes pHrodo NTHi exposure

Following exposure to pHrodo NTHi the percentage of CD206+$CD14^{high}$ cells were decreased in HNS and HS and CD206+$CD14^{low}$ cells in HS after 30 minutes (Supplement Figure 8A and 8B respectively). Conversely the percentage of CD206- $CD14^{high}$ expressing cells was increased HS and COPD after 30 minutes (Supplement Figure 8C). There was no difference in the percentage of CD206- $CD14^{low}$ after 30 minutes in any group (Supplement Figure 8D). It appears cells move from CD206- $CD14^{high}$ group at 30 minutes to CD206- $CD14^{low}$ at 3 hours.

Phagocytosis of pHrodo NTHi by neutrophil subpopulations at 30 minutes and 3 hours exposure

Phagocytosis of pHrodo NTHi was increased in CD66b+ CD16+ and CD66b+ CD16- cells at 3 hours compared to 30 minutes (Supplement Figure 12). This increase over time reached significance for all groups in CD66b+ CD16+ cells ($p<0.05$).

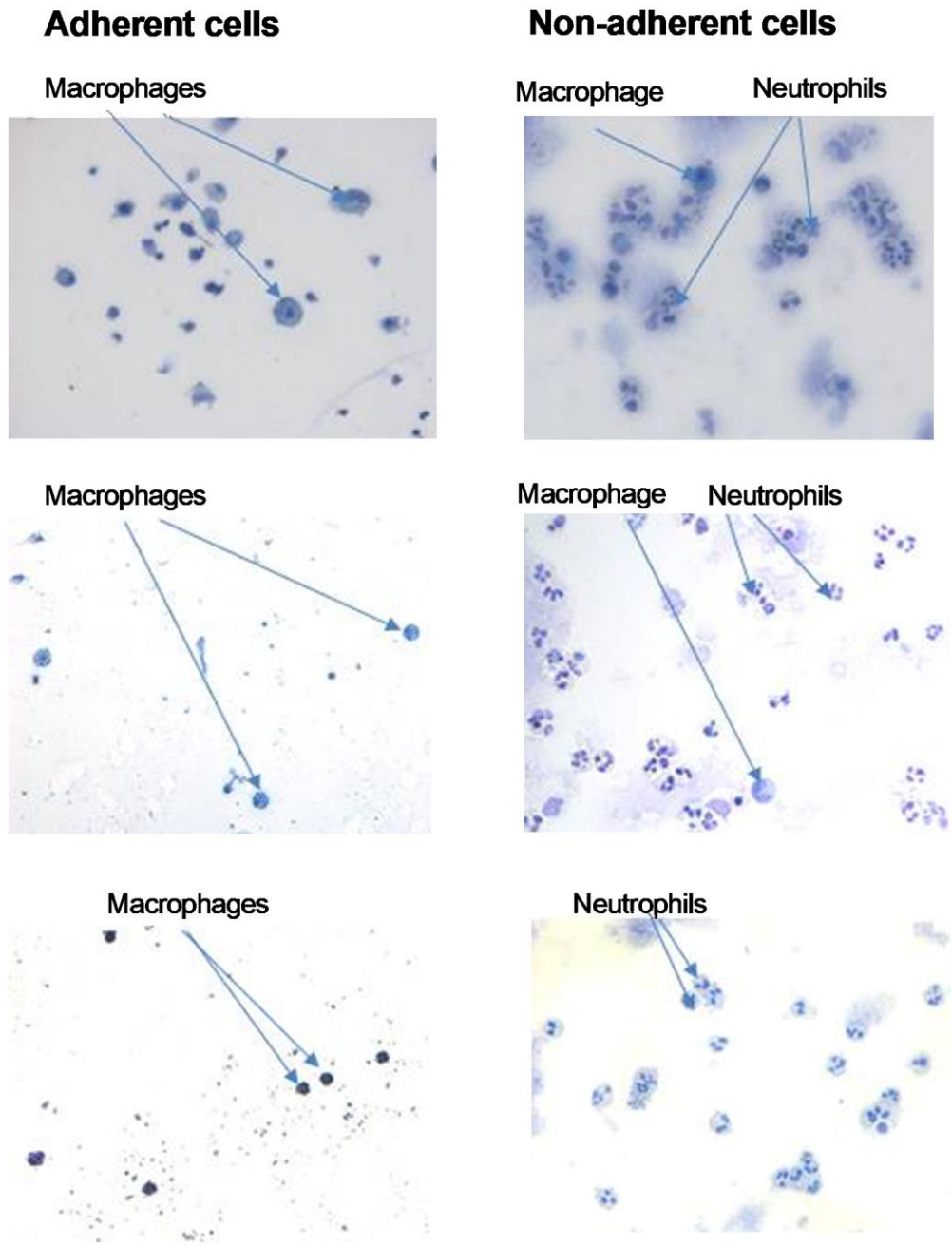
Supplementary Table 1

	Adherent cells DCC %			**Non-adherent cells DCC %**		
Donor	**Macs**	**Neuts**	**Other**	**Macs**	**Neuts**	**Other**
1	91.5	4.0	3.5	11.5	85.5	3
2	93.0	3.0	2.0	6.5	92.75	0.75
3	91.0	4.0	5.0	26.5	70.5	3.0
Mean (SD)	91.83 (1.04)	3.67 (0.58)	3.5 (1.50)	14.83 (10.41)	82.92 (11.35)	2.25 (1.30)

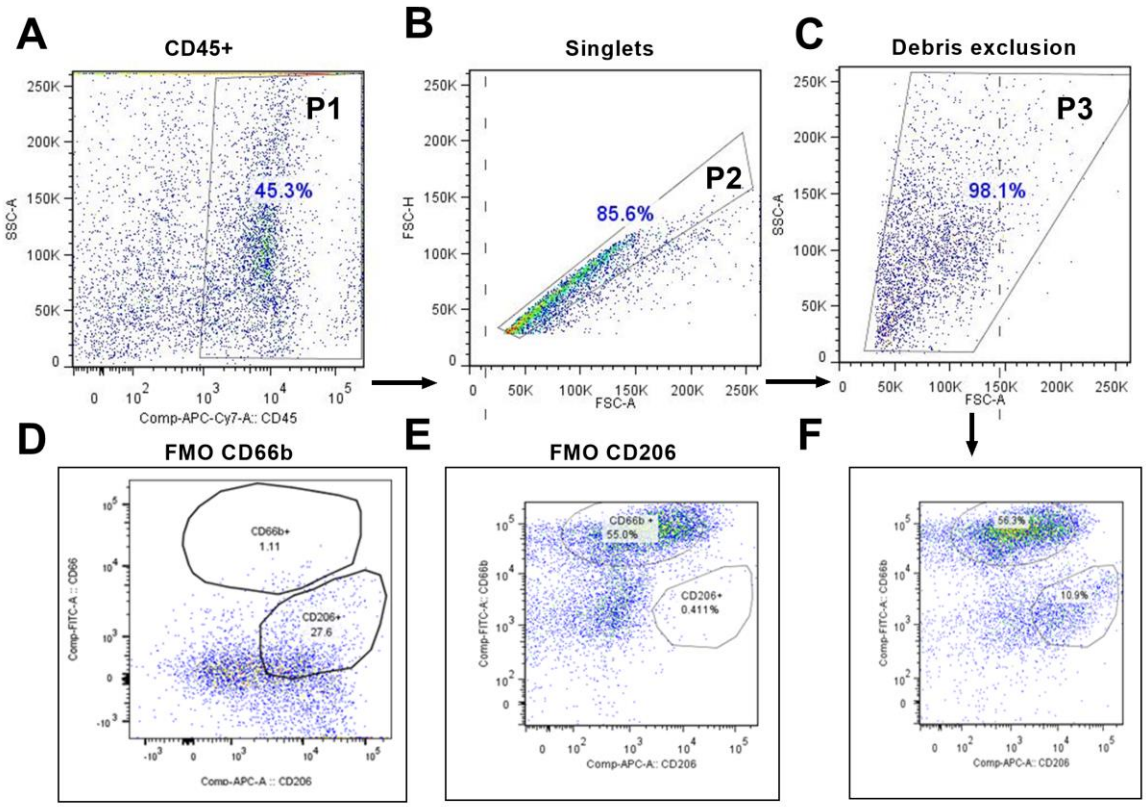
Supplementary Table 1

Differential cell counts from sputum samples obtained from COPD patients (n=3). Adherent and non-adherent cell counts post separation.

Supplement Figure 1

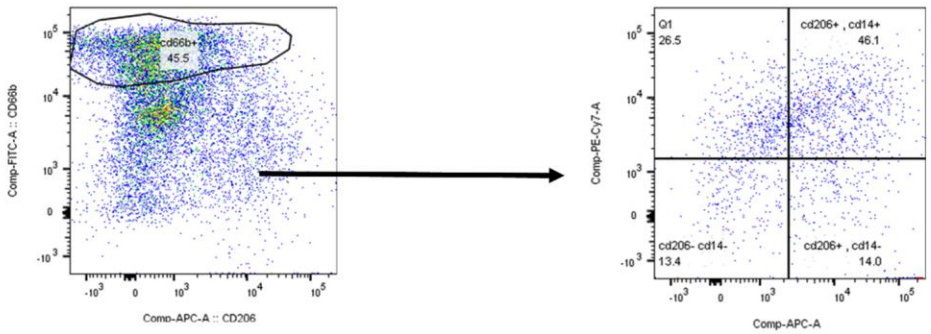


Supplement Figure 2



Supplement Figure 3

A



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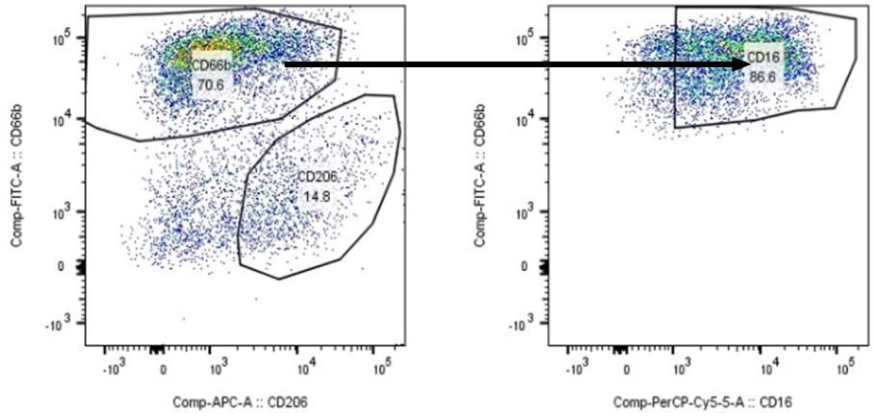

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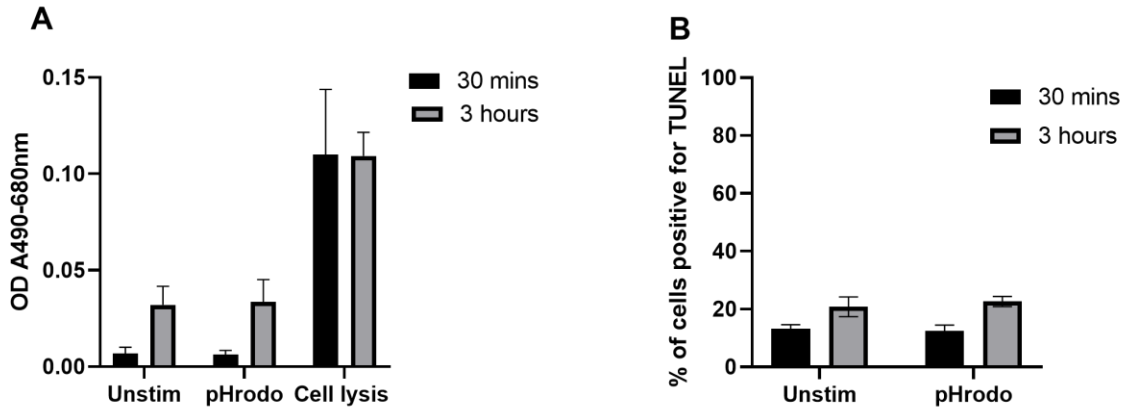

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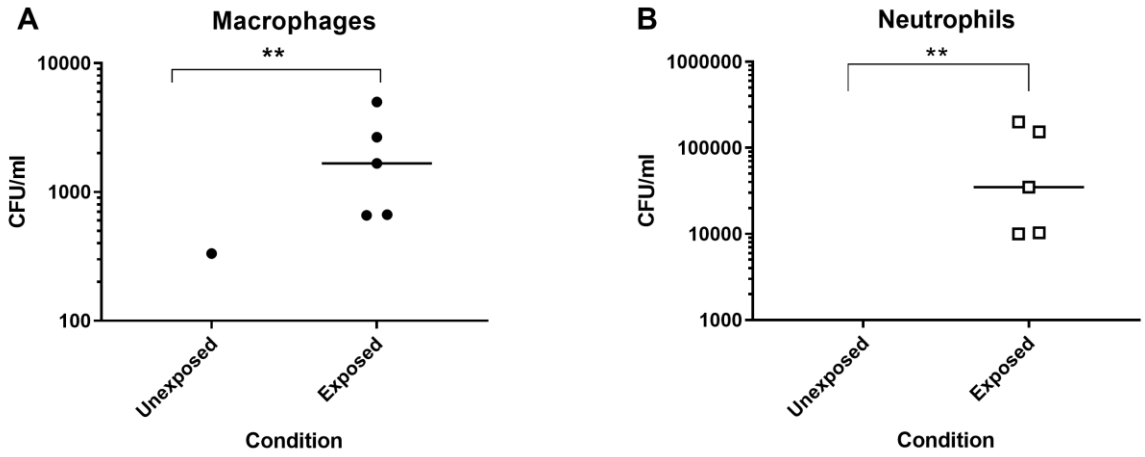

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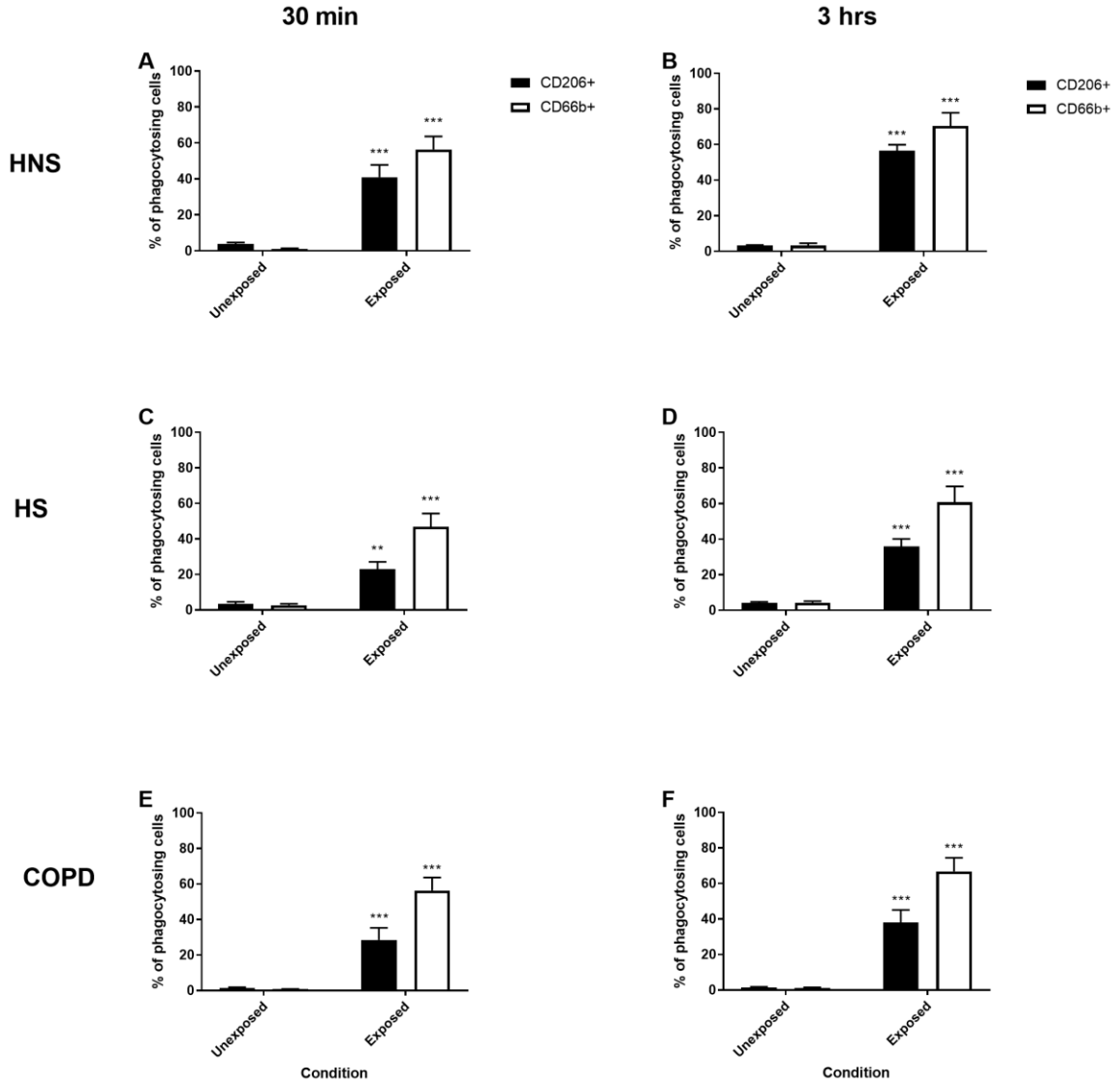

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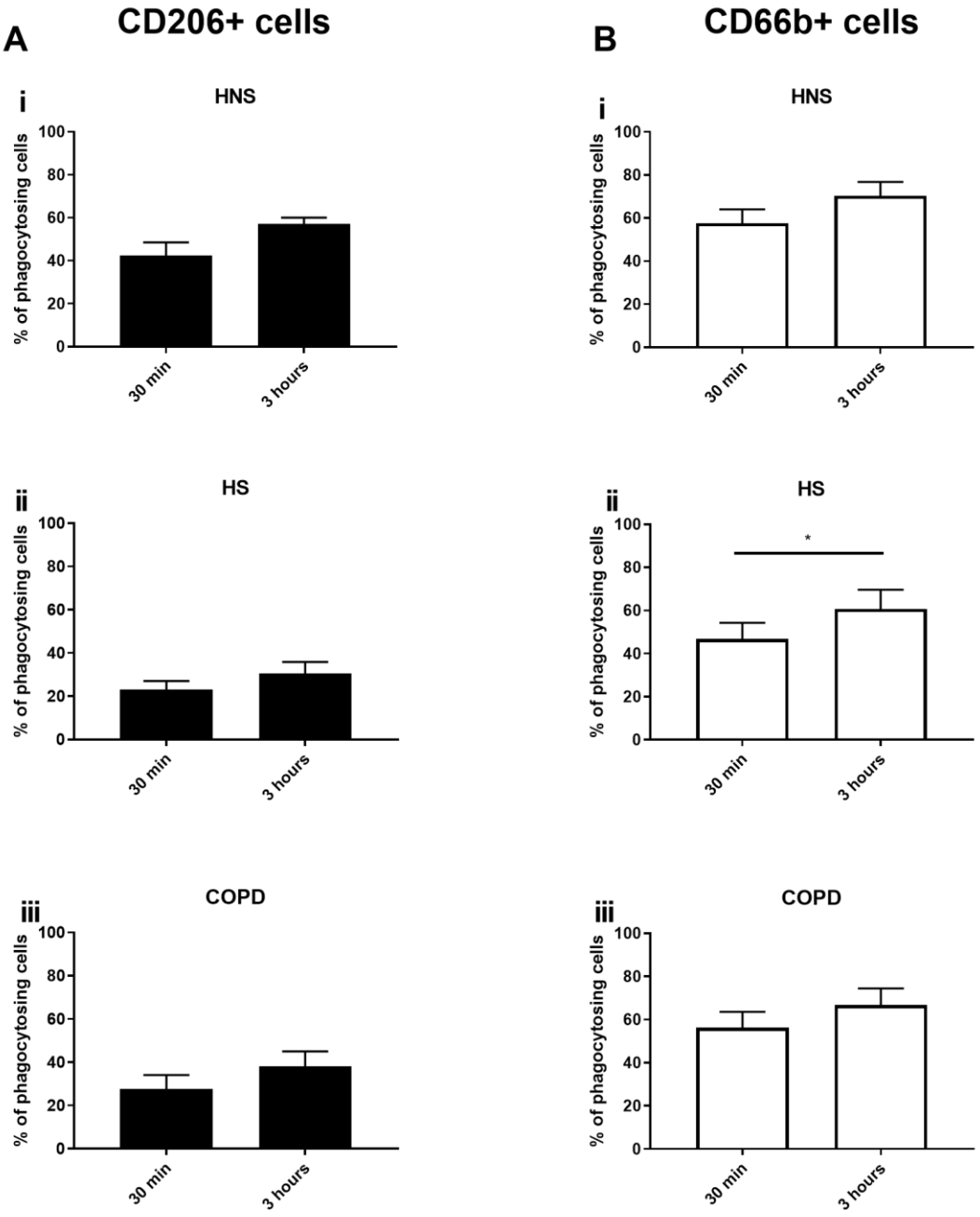
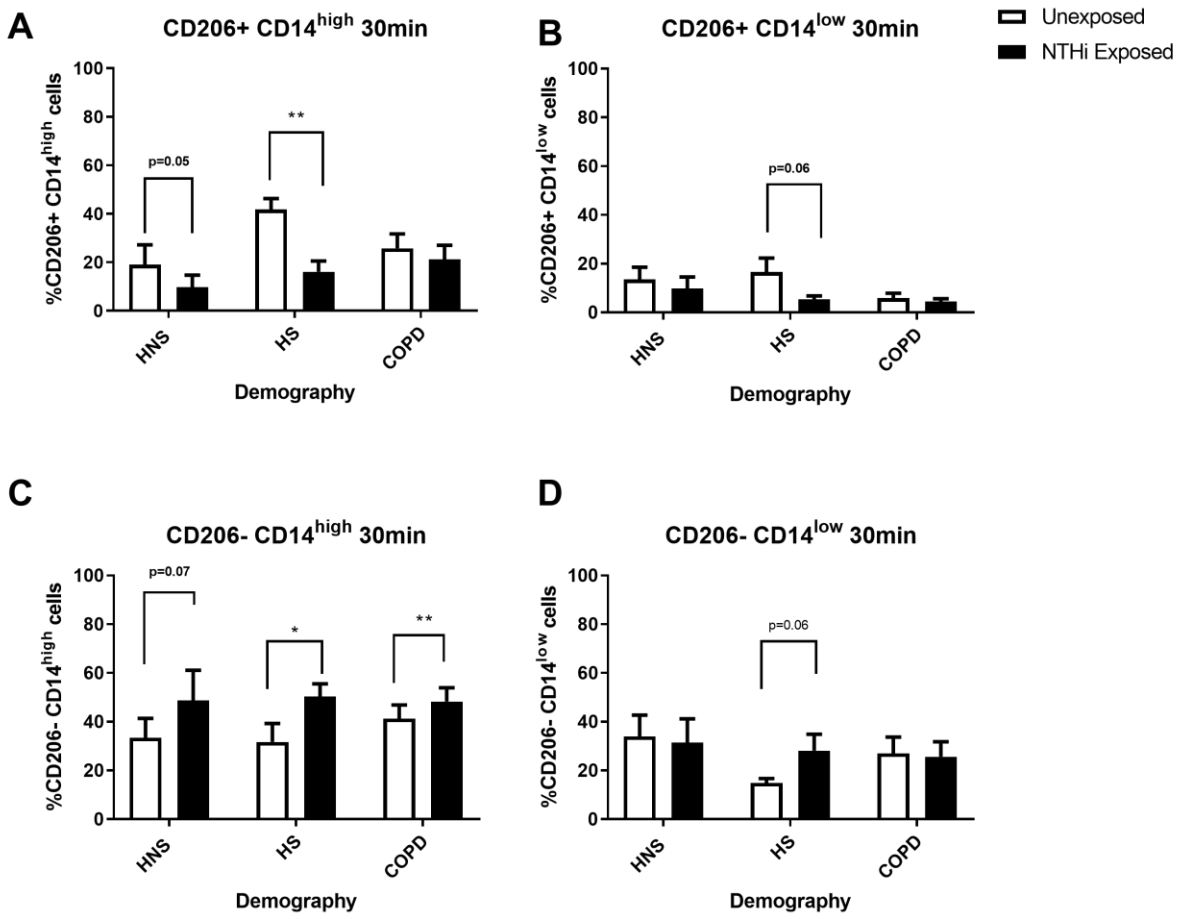
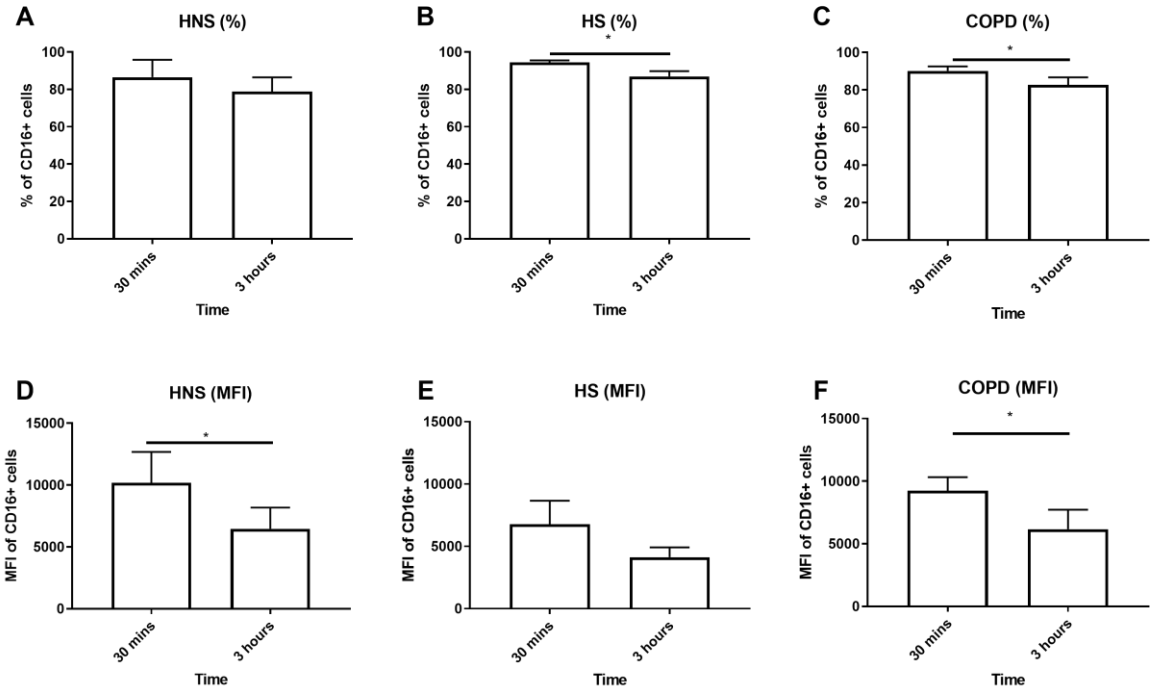


Supplement Figure 8



Supplement Figure 9



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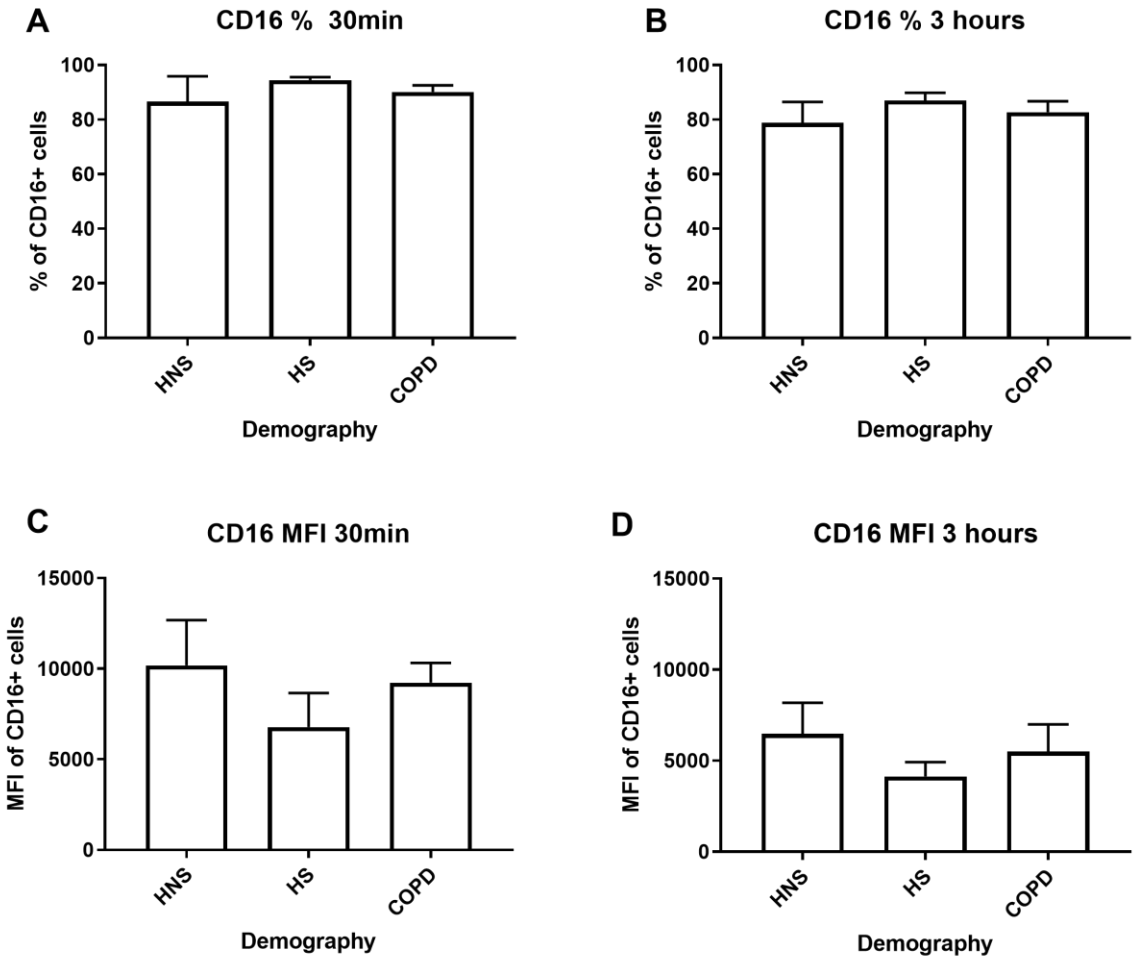
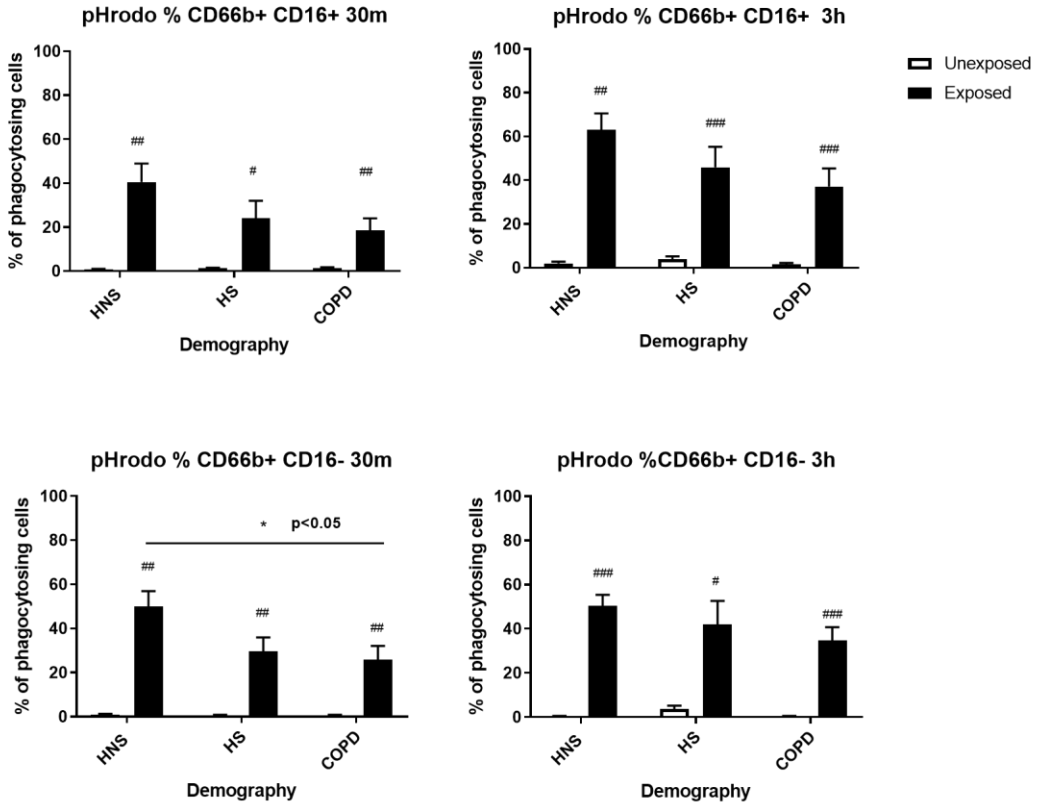
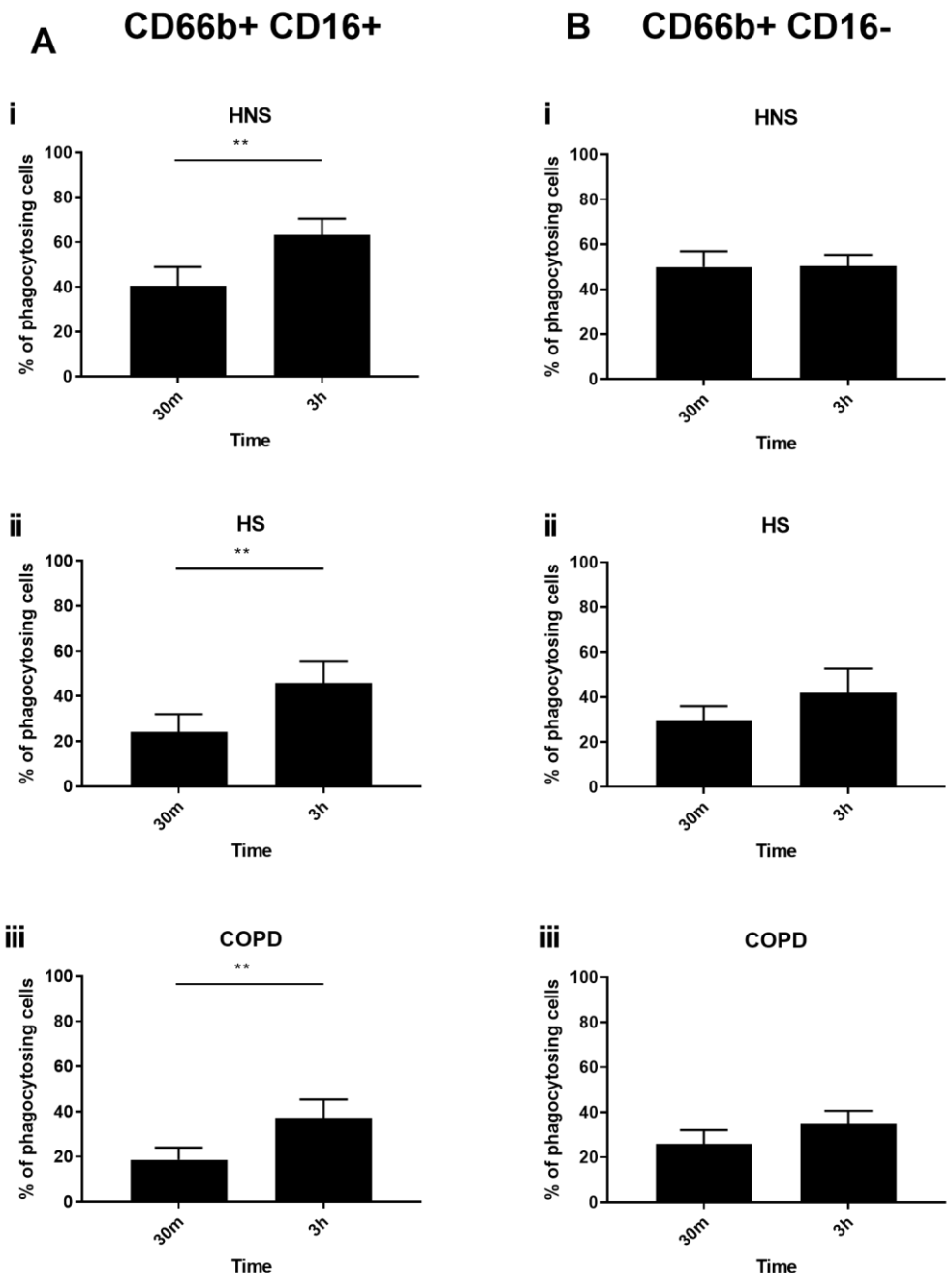


Supplement Figure 11



Supplement Figure 12



Supplement Figure 13