Title: Intercompartment monocyte modulation relates to TNF dysregulation in neutrophilic asthma

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SUPPLEMENTAL ONLINE INFORMATION

Supplemental methods:

Study inclusion and exclusion criteria

Variable airflow obstruction was defined as airway hyperresponsiveness (PD_{15}<15mL to hypertonic saline or other standard challenge agent), bronchodilator responsiveness (change in post-bronchodilator FEV_1>12% or >200 mL), peak flow variability >12% over at least one week of monitoring, or FEV_1 variability >12% (two values measured in two months of each other). Exclusion criteria were respiratory infection or asthma exacerbation requiring oral corticosteroids, antibiotics or change in maintenance therapy in the last month, current lung cancer or other malignancy, smoking within the last six months, pregnancy or breastfeeding, and respiratory diseases other than asthma. Participants were not excluded if they were receiving long-term macrolide therapy for their asthma.

Sputum induction

Sputum induction (after administration of salbutamol) or sputum challenge and induction (withholding the reliever) were performed by hypertonic saline challenge. Nebulized saline (4.5%) was inhaled for increasing time periods (30 seconds, 1 minute, 2 minutes, and 3 x 4 minutes) from an Ultra-NEB™ ultrasonic nebulizer (DeVilbiss, Model 2000 or similar) up to a maximum of 15.5 minutes or until FEV_1 had fallen by more than 20%. One minute after each saline dose, FEV_1 was measured and participants were asked to expectorate into a sterile container. Sputum was processed by separating mucus from saliva and dispersing the cells with dithiothreitol and phosphate buffered saline (PBS). A total cell count was performed under the light microscope. Cell viability was assessed by trypan blue exclusion. Differential cell counts were performed on cytospins stained with May-Grünwald-Giemsa, counting 400 non-squamous cells.

Collection of whole blood

Venous blood was collected in EDTA anticoagulant collection tubes and an automated full blood count was performed (CELL-DYN Ruby, Abbott). To generate plasma, the collection tubes were centrifuged for 10 minutes at 1,730 x g, 4°C. Aliquots of the supernatant were stored at -80°C until use.

Flow cytometry of sputum and whole blood

Sputum samples were centrifuged (400 x g, 10 minutes, 4°C) and cells were resuspended in flow cytometry buffer (PBS, 2 mM EDTA, 1% fetal calf serum, 0.01% sodium azide). Depending on availability, $1 \times 10^5 – 1.5 \times 10^6$ cells were stained. Human Fc block (BD biosciences) was added, and samples were incubated for 10 minutes on ice. Cells were washed with flow cytometry buffer and centrifuged (400 x g, 7 minutes, 4°C). The cell pellet was resuspended in the antibody cocktail containing 50 µL Brilliant Stain Buffer (BD Biosciences) and antibodies against CD45 (BD Biosciences, clone HI30), CD14 (BD Biosciences, clone M5E2), CD16 (BD Biosciences, clone 3G8), HLA-DR (BD Biosciences, clone G46-6), CD206 (Biolegend, clone MMR). The volumes of the antibodies were adjusted according to the number of sputum cells to be stained. Cells were incubated for 20 minutes on ice in the dark, centrifuged (400 x g, 7 minutes, 4°C) and resuspended in flow cytometry buffer. A viability dye (7-AAD, BD Biosciences) was added. After 10 minutes incubation on ice in the dark, samples were analysed on a flow cytometer (LSRFortessa X-20, BD Biosciences).

Whole blood (100 µL) was added to an antibody cocktail consisting of Brilliant Stain Buffer and the same antibodies used for sputum except the antibody against the macrophage marker CD206. The used antibody concentrations were established through antibody titration. Samples were stained in the dark for 20 minutes at room temperature. Erythrocyte lysis buffer (154 mM NH_4Cl, 10 mM Tris, pH 7.5) was added and incubated for 15 minutes in the dark at room temperature. Cells were centrifuged (500 x g, 5 minutes, 4°C) and washed with flow cytometry buffer, followed by repeat centrifugation. Cells were resuspended in flow cytometry buffer and analysed on the flow cytometer.

Supplemental figure legends:

Figure S1. Gating strategy for sputum cells and overlay of identified cell populations in a final dot plot. Identification of viable monocytes ($CD45^{+}$, SSC^{med}, HLA-DR^{+}, $CD14^{+/++}$, $CD206^{-/medium}$, $CD16^{-/+}$, 7-AAD^{-}, green), viable macrophages ($CD45^{+}$, $SSC^{medium/high}$, HLA-DR^{+}, $CD14^{+}$, $CD206^{high}$, $CD16^{+}$, 7-AAD^{medium}, orange), viable neutrophils ($CD45^{+}$, SSC^{medium}, HLA-$DR^{-/low}$, $CD14^{-}$, $CD206^{-}$, $CD16^{++}$, 7-AAD^{-}, purple) and viable eosinophils ($CD45^{+}$, SSC^{low}, HLA-$DR^{-/low}$, $CD14^{-}$, $CD206^{-}$, $CD16^{-}$, 7-AAD^{-}, red). SSC, side scatter; FSC, forward scatter; Macs, macrophages; Monos, monocytes; Eos, eosinophils; CM, classical monocytes; IM, intermediate monocytes; NCM, non-classical monocytes.

Figure S2. Gating strategy for blood cells and overlay of identified cell populations in a final dot plot. Identification of monocytes ($CD45^{+}$, $SSC^{low/medium}$, HLA-DR^{+}, $CD14^{+/++}$, $CD16^{-/+}$, green), neutrophils ($CD45^{+}$, SSC^{high}, HLA-DR^{-}, $CD14^{-}$, $CD16^{++}$, purple) and eosinophils ($CD45^{+}$, SSC^{high}, HLA-DR^{-}, $CD14^{-}$, $CD16^{-}$, red). FSC, forward scatter; SSC, side scatter; Eos, eosinophils; CM, classical monocytes; IM, intermediate monocytes; NCM, non-classical monocytes.

Figure S3. Gating of classical (CM), intermediate (IM) and non-classical monocytes (NCM) in blood (left) and sputum (right). Monocyte subsets were selected based on CD14 and CD16 expression. Events are displayed as dot plots (pseudocolor; a, b) and contour plot (c, d).

Figure S4. Comparison of a) mTNFα, b) mTNFR1 and c) TNFR2 expression on blood (red) and sputum (green) immune cells in non-asthma controls (n=8). Bars and whiskers indicate the median with interquartile range. The Wilcoxon matched-pair signed rank test was used for statistical comparison. Ns, not significant; * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Figure S5. Differential TNF marker expression on monocyte subsets. a) mTNFα, b) mTNFR1, c) TNFR2 expression on sputum monocyte subsets. d) mTNFα, e) mTNFR1, f) mTNFR2 expression on blood monocyte subsets (n=45). The Kruskal-Wallis test was used for statistical comparison. P-values

of the overall comparison are indicated in the graphs. CM, classical monocytes; IM, intermediate monocytes; NCM, non-classical monocytes; ns, not significant; ** $p < 0.01$, **** $p < 0.0001$.

Supplemental figures

Figure S1

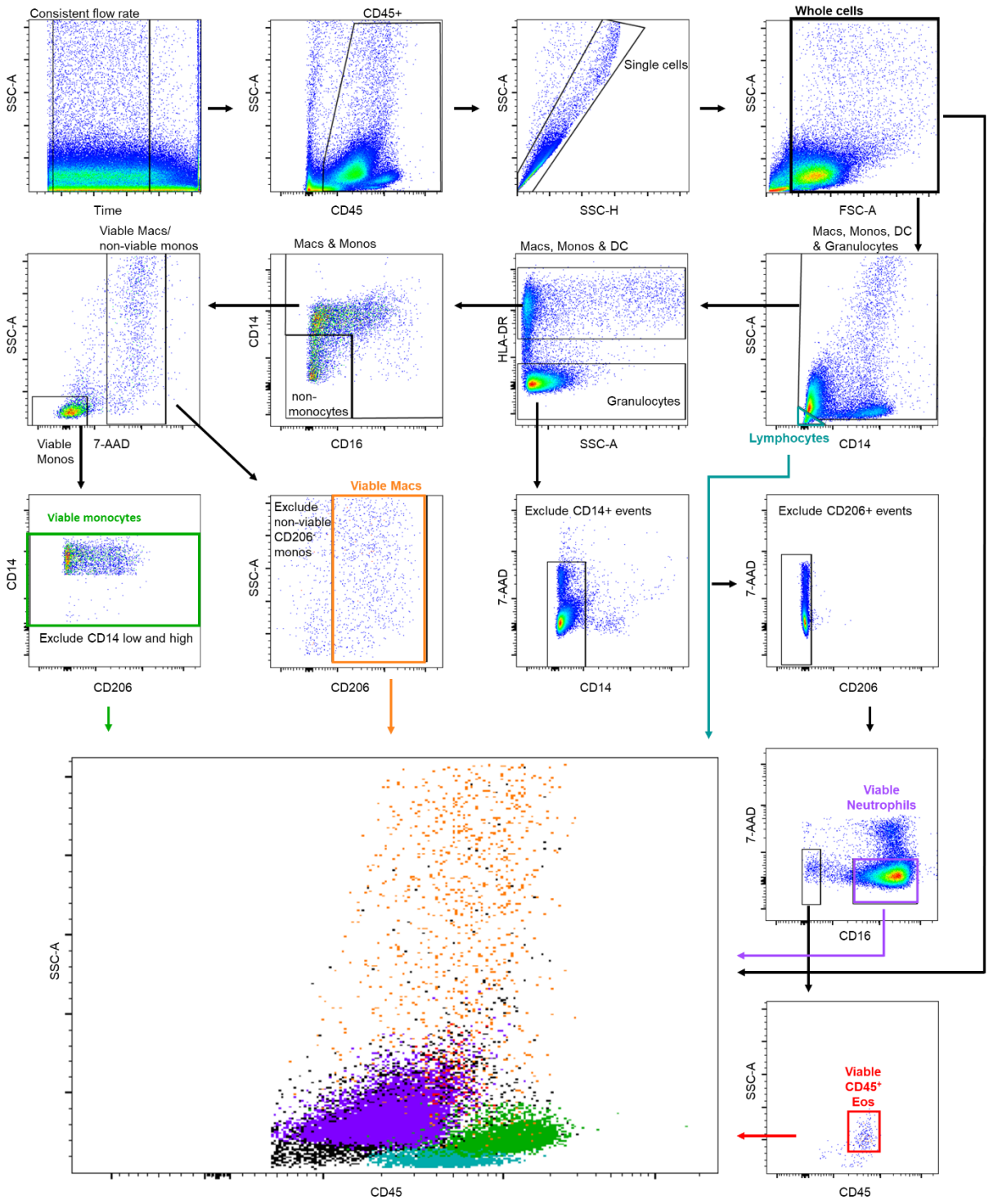


Figure S2

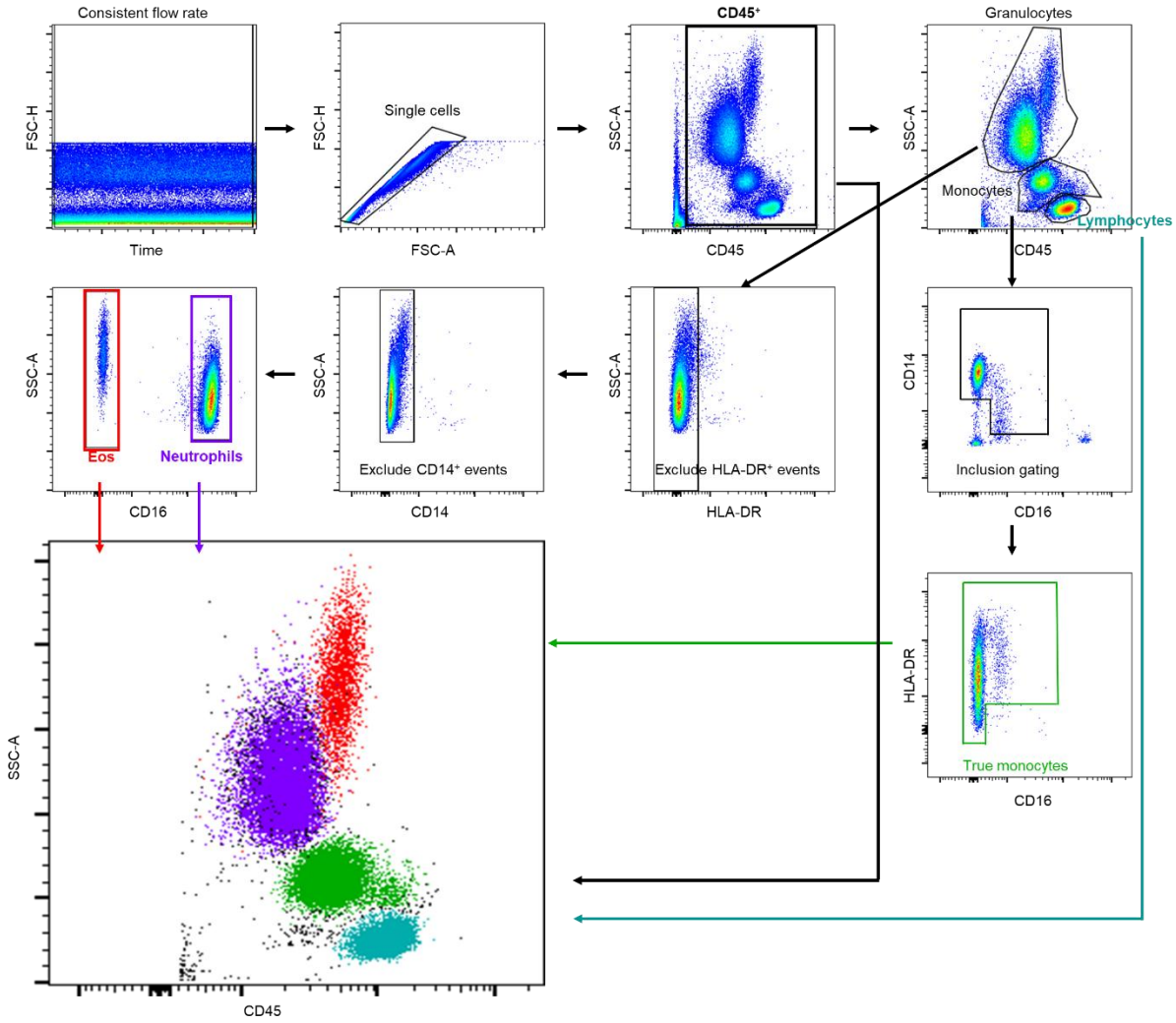

Consistent flow rate
FSC-H
Time
Single cells
FSC-H
FSC-A
CD45⁺
SSC-A
CD45
Granulocytes
SSC-A
Monocytes
Lymphocytes
CD45
CD14
Inclusion gating
CD16
HLA-DR
True monocytes
CD16
SSC-A
Exclude HLA-DR⁺ events
HLA-DR
SSC-A
Exclude CD14⁺ events
CD14
SSC-A
Eos
Neutrophils
CD16
SSC-A
CD45

Figure S3

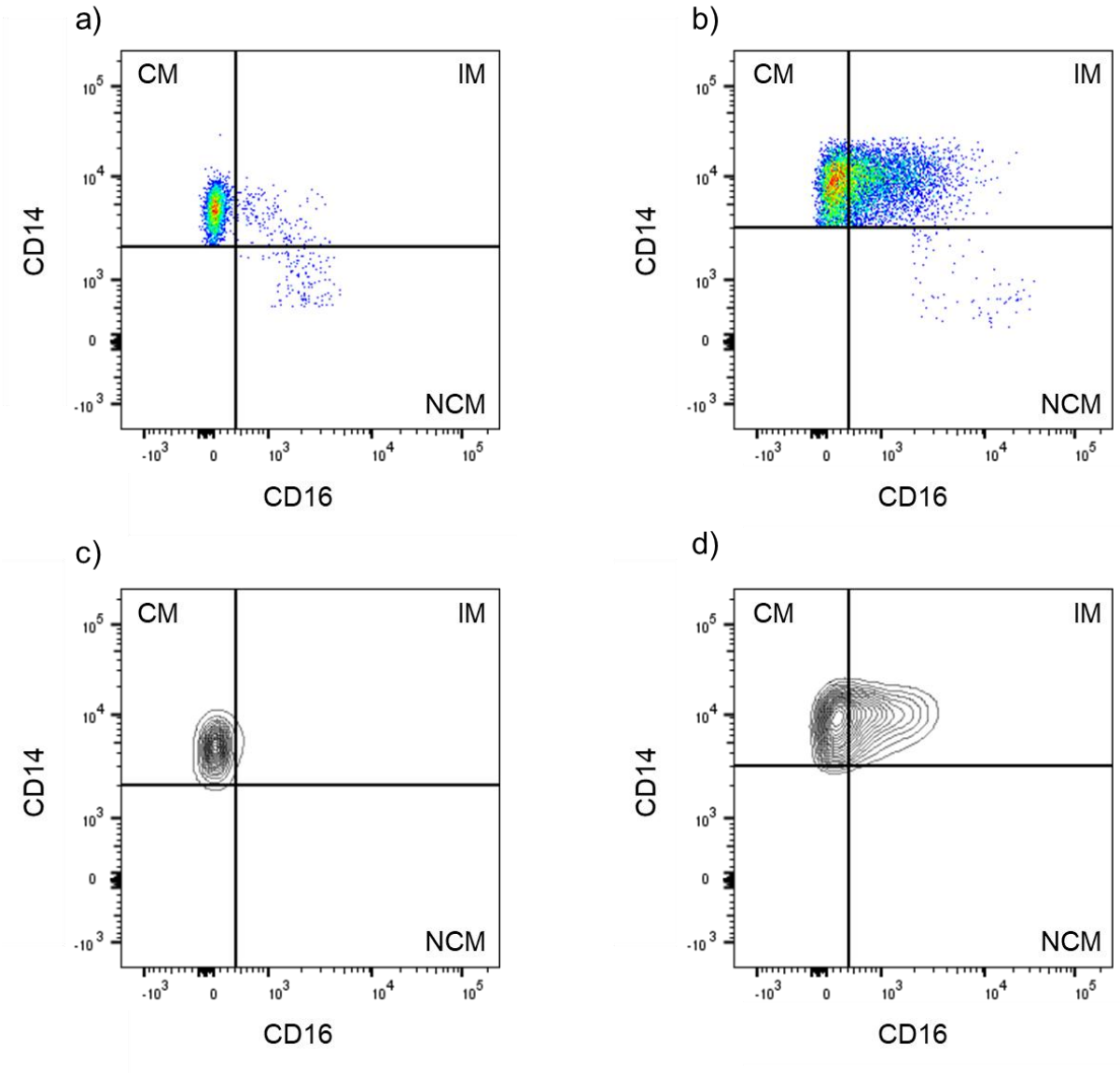


Figure S4

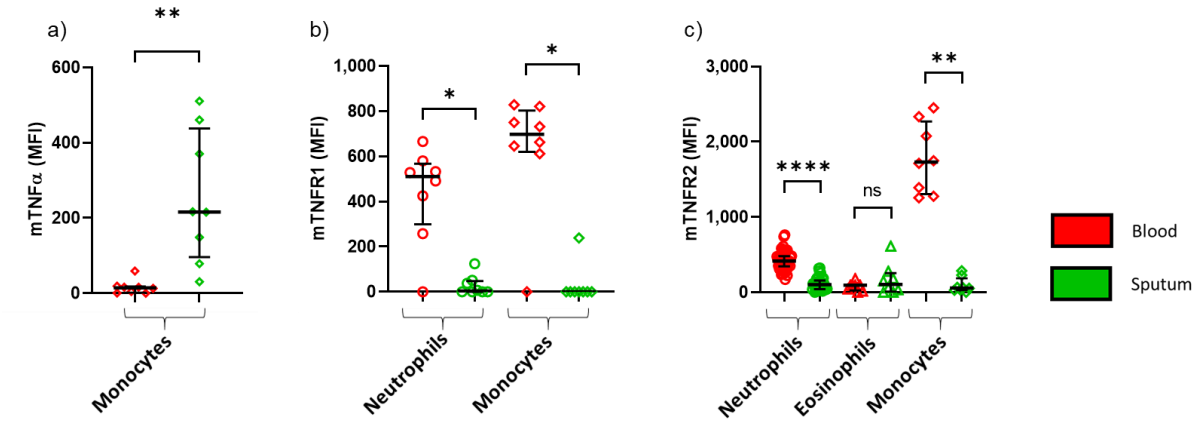
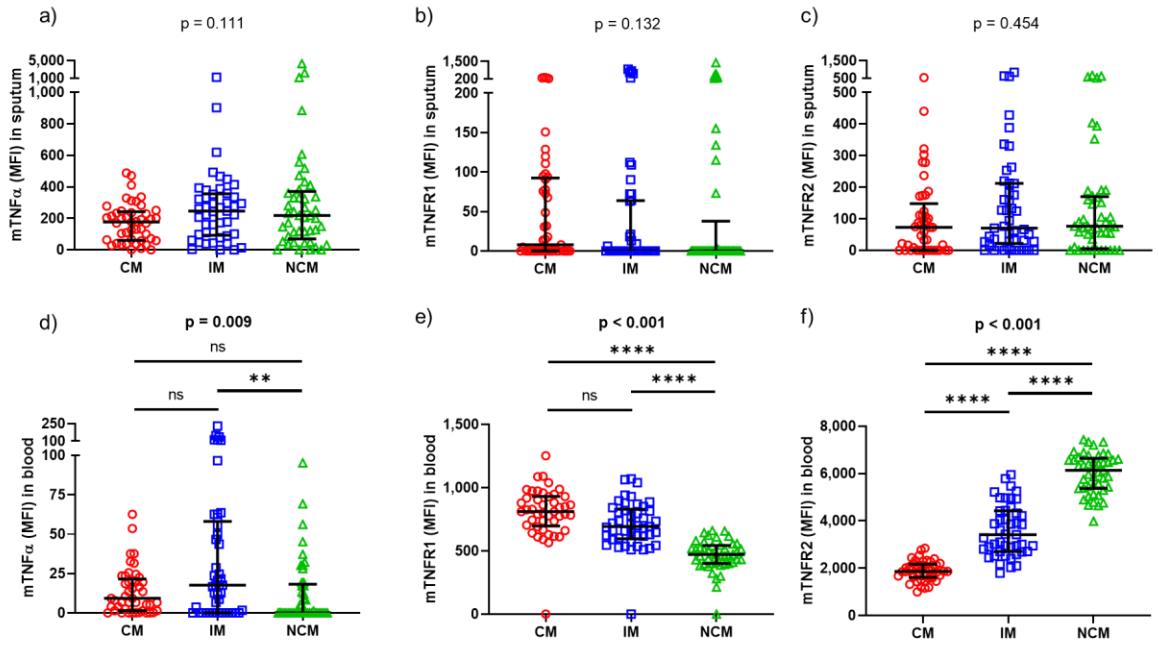

a)
b)
c)
mTNFα (MFI)
mTNFR1 (MFI)
mTNFR2 (MFI)
**
*
*
*
**
****
ns
Monocytes
Neutrophils
Monocytes
Neutrophils
Eosinophils
Monocytes
Blood
Sputum

Figure S5


a)
p = 0.111
mTNFα (MFI) in sputum
5,000
1,000
1,000
800
600
400
200
0
CM
IM
NCM
b)
p = 0.132
mTNFR1 (MFI) in sputum
1,500
200
200
150
100
50
0
CM
IM
NCM
c)
p = 0.454
mTNFR2 (MFI) in sputum
1,500
500
500
400
300
200
100
0
CM
IM
NCM
d)
p = 0.009
ns
**
ns
mTNFα (MFI) in blood
250
100
100
75
50
25
0
CM
IM
NCM
e)
p < 0.001
****
****
ns
mTNFR1 (MFI) in blood
1,500
1,000
500
0
CM
IM
NCM
f)
p < 0.001
****
****
****
mTNFR2 (MFI) in blood
8,000
6,000
4,000
2,000
0
CM
IM
NCM