C5a mediates peripheral blood neutrophil dysfunction in critically ill patients.

Andrew Conway Morris[1]*, Kallirroi Kefala[1]*, Thomas S Wilkinson[1], Kevin Dhaliwal[1], Lesley Farrell[1], Tim Walsh[2], Simon J Mackenzie[2], Hamish Reid[3], Donald J Davidson[1], Chris Haslett[1], Adriano G Rossi[1], Jean-Michel Sallenave[1], A John Simpson[1]

Online Data Supplement

SUPPLEMENTARY METHODS

Patients and volunteers

Patients with suspected VAP fulfilled criteria described in the main paper. Exclusion criteria comprised PaO_2<8kPa on FiO_2>0.7, positive end-expiratory pressure >15cmH_2O, active bronchospasm, myocardial infarction within the last 3 months, unstable arrhythmia, mean arterial pressure <65mmHg on vasopressor therapy, bleeding diathesis (including platelet count <$20x10^9$/litre) and initiation or modification of antibiotics in the preceding 3 days.

All patients had an acute physiology and chronic health evaluation II (APACHE II) score calculated within 24 hours of ICU admission. To allow the influence of important clinical conditions to be studied, patients with suspected VAP were subdivided according to whether they had confirmed VAP (defined as growth of pathogens at $>10^4$ colony forming units (CFU) per ml of BALF), or acute lung injury/adult respiratory distress syndrome (ALI/ARDS) (defined according to internationally recognized criteria (S1)) in the absence of VAP. Remaining patients were considered to be in a 'non-VAP, non ALI/ARDS' group. Among the patients, 17 (24%) had confirmed VAP and 21 (29%) had ARDS/ALI without evidence for co-existent pneumonia.

After recruitment of 40 patients the (anonymized) age and sex of each patient was communicated to a local primary care practice, where staff unconnected with the study randomly identified matching individuals and sent out invitations to participate. The first twenty-one respondents were enrolled. Exclusion criteria comprised hypoxia (SaO_2 <92% on

air), bleeding diathesis, anticoagulant therapy, insulin-dependent diabetes mellitus, arrhythmia, bronchospasm not responding to nebulized β_2 agonist, or clinical evidence of respiratory tract infection. Eligible volunteers provided blood and had fiberoptic bronchoscopy performed. Separate university staff and students provided blood to generate healthy PBNs for *in vitro* experiments.

Bronchoscopy, bronchoalveolar lavage (BAL), and blood collection

Eligible patients had fiberoptic bronchoscopy and BAL performed using a standardized technique by a single experienced operator. Briefly, the bronchoscope was wedged in a subsegment corresponding to the area of radiological involvement. In the case of diffuse radiographic change the bronchoscope was wedged in a subsegment producing visible purulent secretion or (in the absence of purulent secretions) in the posterior segment of the right lower lobe. 20ml of warm sterile saline was instilled and the aspirate (representing a 'bronchiolar' sample) discarded, then 200ml of sterile saline was instilled in aliquots and the aspirate (representing an alveolar sample) retained. Exactly the same procedure was followed for bronchoscopy and BAL in volunteers with the exception that volunteers were conscious (or sedated using midazolam), received lignocaine throat spray, and were not mechanically ventilated. The bronchoscope was passed via the per-oral route in all volunteers, and all procedures were performed by the same operator as described above. Whole blood was collected into 0.38% sodium citrate (final concentration).

Processing of BAL fluid (BALF) and whole blood

One ml of BALF was collected for culture, and the remainder centrifuged at 700g for 10 minutes. The supernatant was immediately frozen at -80^{o}C until further analysis. The cellular

pellet was resuspended in warmed Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen, Paisley, UK) and used for subsequent experiments whilst fresh.

Thirty ml of citrated whole blood was separated into purified neutrophils and serum. Briefly, serum was prepared by adding 1M calcium chloride to plasma. Peripheral blood neutrophils (PBNs) were isolated by sequential dextran sedimentation and Percoll gradient extraction. Preparations invariably yielded PBNs of >95% purity and were used fresh in all cases.

Phagocytosis assays

Core protocol

The core protocol is outlined in the main paper but is reproduced in detail here for completeness. Zymosan particles (Sigma, Gillingham, UK), derived from *Saccharomyces cerevisiae*, were pre-incubated with 50% autologous serum for one hour at 37^{o}C. PBNs were adhered to tissue culture plates at 500,000 per well in IMDM containing 10% autologous serum at 37^{o}C and 5%CO_2. After 1 hour cells were washed and incubated in IMDM containing zymosan particles (0.02mg/ml) and 1% autologous serum for one hour under the same conditions. PBNs were washed, air dried, fixed with methanol and stained with Reastain Diff-Quik (Reagena, Toivala Finland). Phagocytosis was quantified using light microscopy to determine the proportion of PBNs containing 2 or more zymosan particles. Counts were performed on four randomly selected microscope fields per duplicate well.

Variations of this core protocol were performed to explore mechanisms underlying defective phagocytosis. Candidate abnormalities were considered to be: deficient opsonization, a serum inhibitor(s) preventing ligation of phagocytic receptors, generation of an inflammatory

mediator capable of down-regulating phagocytosis, or deficient expression/activation of phagocytic receptors. The latter mechanism is considered under *Flow Cytometry* below.

Opsonization

Zymosan was opsonized in serum from patients known to exhibit poor PBN phagocytosis (<40%, see Figure 1 in main manuscript) or matched volunteers (known to exhibit efficient PBN phagocytosis) for one hour at 37^{o}C. PBNs from healthy volunteers were then exposed to opsonized zymosan at 0.02mg/ml in IMDM with 1% patient or healthy volunteer serum, incubated for 1 hour at 37^{o}C and then washed, stained and counted as per the core protocol above. To demonstrate the need for opsonization for efficient phagocytosis in this assay, similar experiements were conducted in which zymosan was incubated with heat-inactivated serum (60^{o}C for 60 minutes) or with PBS before being exposed to the PBNs.

Serum inhibitors

Healthy volunteer PBNs were incubated with serum from patients known to exhibit poor PBN phagocytosis (<40%) or from matched volunteers (known to exhibit efficient PBN phagocytosis) at 37^{o}C for one hour. The PBNs were adhered as per the core protocol above, then exposed to zymosan opsonized with either patient serum or healthy volunteer serum for 1 hour at 37^{o}C. Washing, staining and counting were as per the core protocol.

Inhibition of phagocytosis by inflammatory mediators

Healthy volunteer PBNs in IMDM containing 1% autologous serum were incubated with recombinant C5a (Sigma, Gillingham, UK) or medium alone for one hour at 37^{o}C. Zymosan particles opsonized in autologous serum were added at 0.02mg/ml and incubated for a further hour. Washing, staining and counting were as per the core protocol.

In a variation, healthy volunteer PBNs were incubated with either 1μg/ml of S5/1 (Abcam, Cambridge, UK) - an antibody known to block CD88 - or with 1μg/ml pre-immune murine IgG1 (Invitrogen, Paisley, UK) for 30 minutes at 37^{o}C, prior to addition of C5a (or control) as above.

In a separate variation, 0.3nM (5ng/ml) recombinant human GM-CSF (Affinity Bioreagents, Golden,CO, USA) or medium was added for an hour at 37^{o}C after the addition of C5a (or control) and before the application of zymosan.

Pseudomonas killing assay

The ability of healthy volunteer neutrophils, pre-incubated in various media, to kill *Pseudomonas aeruginosa* was assayed in the following manner. *P. aeruginosa* strain PA01 was grown for 12 hours in Luria-Bertani (LB) broth at 37^{o}C, before being subcultured into fresh LB Broth at 1:200 dilution and grown for a further 3 hours so as to enter into early logarithmic phase. An aliquot of this early log culture was taken and diluted to an optical density of 0.1 at 595nm in IMDM. This was further diluted by a factor of 1:1000 in IMDM, with the resultant bacterial suspension being used at this concentration. Duplicates of this suspension were diluted $1x10^{2}$ and $1x10^{4}$ and plated on LB Agar for overnight incubation to determine baseline colony counts. Simultaneously $1x10^{6}$ neutrophils suspended in IMDM were incubated with various treatments including BALF supernatant from patients, BALF supernatants from healthy volunteers, C5a at 100nM or vehicle control for one hour at 37^{o}C. Following this 1ml of the *P. aeruginosa* suspension was added and the resultant mixture incubated for a further thirty minutes. Alongside, *P. aeruginosa* was incubated without neutrophils as a positive control. Following this, the neutrophils were lysed with triton 0.1% (Sigma, Gillingham, UK) and the suspension diluted $1x10^{2}$ and $1x10^{4}$ and plated on LB Agar for overnight incubation to determine colony counts.

Superoxide anion production

Neutrophil superoxide anion production was assayed by a cytochrome C reduction assay, using a stimulus of 100nM platelet activitating factor (PAF, Calbiochem, Nottingham, UK) and 100nM N-formyl-methionyl-leucyl-phenylalanine (fMLP, Sigma, Gillingham, UK).

Transmigration assay

PBNs from healthy volunteers were added to polystyrene inserts (Corning Costar, Lowell, MA, USA) containing pores of 3μm diameter, at 100,000 cells per insert. The inserts were in turn placed in 24-well plates containing a) IMDM and fMLP (as positive control for migration, final concentration 100nM), b) IMDM alone (as negative control), c) BALF supernatant from patients or d) BALF supernatant from volunteers. Plates were incubated at 37°C for one hour. The upper surface of each insert was gently scraped to remove adherent neutrophils then inserts were stained with Diff-Quik and fixed in ethanol. Transmigration was quantified by counting the number of neutrophils on the lower surface of the insert, counting ten randomly selected fields per insert.

Flow cytometry

Samples comprised freshly prepared PBNs incubated in IMDM, centrifuged at 300g for 5 minutes at 4^{o}C then resuspended in PBS containing 4% murine serum (with the exception of cells stained for CD88 which were resuspended in 4% caprine serum). Primary antibodies consisted of

- murine monocloncal anti-CD11b, -CD32 (Fcγ receptor II), and -CD64 (Fcγ receptor I) (all Invitrogen Paisley, UK) labelled with phycoerythrin (PE), fluorescein-5-isothiocyanate (FITC) and PE respectively;

- murine antibody against the activation epitope of CD11b, CBRM1/5 (eBioscience, San Diego,CA, USA), labelled with FITC; and
- murine monoclonal anti-CD88 P12/1 (Abcam,Cambridge, UK), unlabelled.

In the case of anti-CD88, a secondary goat-anti mouse antibody conjugated with alexa-647 (Invitrogen, Paisley, UK) was applied.

In parallel, appropriate isotype control antibodies were applied. Cells were washed and fixed with 10% formalin and analyzed by flow cytometry (FACSCalibur, BD Bioscience, Oxford, UK) within 18 hours. Expression was quantified as geometric mean fluorescence.

Stimulation of cells with opsonized zymosan was performed to assess the activation of CD11b. Neutrophils at 500,000/ml were exposed to zymosan opsonized in autologous serum at 0.02mg/ml. Cells were then stained with the activation epitope specific antibody CBRM1/5 as per the protocol above.

SUPPLEMENTARY RESULTS

All patients recruited to the study had clinically suspected VAP. On quantitative cultures of lavage however, only 17 (24%) had VAP confirmed by growth of organisms at $>10^4$ CFU per ml of BALF. 22 (29%) had ARDS/ALI by consensus criteria without evidence of pneumonia, with the remaining 33 falling into a 'non-VAP/non-ARDS' group. The demographic and clinical details of these three sub-groups are shown in Table S1. Other than the patients with ARDS/ALI having significantly higher numbers of neutrophils in BALF and being ventilated for 2-3 days fewer than those in the VAP or 'non-VAP/non-ARDS', there were no significant demographic or clinical differences.

Phagocytosis by patients

Figure S1 shows the rates of phagocytosis by the PBNs from each sub-group. No significant differences were found when comparing these groups.

Phagocytosis by lavage cells from the three sub groups is shown in Figure S2. Again there were no significant differences between the groups.

In-vitro opsonization experiments

Opsonizing zymosan particles in serum from matched volunteers or patients did not alter their phagocytosis by healthy donor neutrophils (Figure S3 Panel A). To validate this assay for opsonization, zymosan particles were incubated with heat inactivated serum then exposed to healthy PBNs; the expected reduction in phagocytosis was observed (Figure S3 Panel B), demonstrating that a heat-labile factor was required for optimal phagocytosis (Panel B). Phagocytosis of non-opsonized zymosan showed no significant difference when compared with phagocytosis of zymosan exposed to heat-inactivated serum (compare the middle and right hand columns in Figure S3 Panel B).

cAMP-mediated inhibition of phagocytosis

Isoproterenol, an agonist for β_1 and β_2 adrenoceptors, is known to inhibit phagocytosis by PBNs via generation of cAMP (29). Figure S4 Panel A shows the effects of isoproterenol on phagocytosis by healthy volunteer PBNs, and demonstrates that this effect can be blocked by pre-incubation with the adenylate cyclase inhibitor SQ22536. In marked contrast, the effects of C5a and fMLP could not be prevented using this inhibitor (Figure S4 panels B and C respectively).

CD11b expression by patient and healthy volunteer neutrophils

Neutrophils from patients showed increased expression of the integrin and phagocytic receptor CD11b relative to matched volunteers (Figure S5 panel A).

CD11b activation by opsonized zymosan

Unstimulated cells from both patients and matched volunteers demonstrated low levels of CD11b activation (Figure S5 Panel B left hand columns). On application of opsonized zymosan, both groups showed activation of CD11b, however there were no significant differences between patients and healthy volunteers (Figure S5 Panel B right hand columns)

Supplementary References

S1. Bernard GR, Artigas A, Brigham KL, Carlet J, Falke K, Hudson L, Lamy M, Legall JR, Morris A, Spragg R. The American-European Consensus Conference on ARDS. Definitions, mechanisms, relevant outcomes, and clinical trial coordination. *Am J Respir Crit Care Med* 1994;149:818-824.

SUPPLEMENTARY FIGURE LEGENDS

Figure E1. Phagocytosis by PBNs of patients by constituent groups.

Data presented as medians, interquartile ranges (box) and range (whiskers), p=0.162 by Kruskal-Wallis test. Data are derived from 68 patients (16 VAP, 19 ARDS, 33 non-VAP/non-ARDS; in the 4 remaining patients insufficient PBNs adhered to tissue culture wells to allow the assay to proceed).

Figure E2. Phagocytosis by lavage cells of patients by constituent groups.

Data presented as medians, inter-quartile ranges (box) and range (whiskers), p=0.279 by Kruskal-Wallis test. Data are derived from 33 patients (7 VAP, 12 ARDS, 14 non-VAP/non-ARDS i.e., all subjects for whom sufficient numbers of phagocytes were available and adhered to tissue culture plates).

Figure E3. The effects of opsonizing zymosan particles in various sera on phagocytosis by healthy neutrophils.

Panel A-phagocytosis by healthy donor PBNs of zymosan opsonized in serum from healthy volunteers and patients with poor phagocytosis (<40%), p= 0.63 by Mann-Whitney U test. n=8 individual PBN donors.

Panel B- phagocytosis by healthy donor PBNs of zymosan treated with serum – from healthy volunteers (HV); heat-inactivated serum from healthy volunteers (HVHT); and PBS (no opsonin), $p<0.0001$ by Kruskal-Wallis, $^{**}p<0.01$, $^{***}p<0.001$ by Dunn's post-hoc test. n=8 individual PBN donors.

Figure E4. C5a- and fMLP-driven impairment of phagocytosis is not mediated by cAMP.

Panel A - phagocytosis by healthy donor PBNs exposed to the beta-adrenoceptor agonist isoproterenol with or without pre-incubation with the adenylate cyclase inhibitor SQ22536, $p=0.006$ by Kruskal-Wallis, $*p<0.05$ by Dunn's post-hoc test (n=5 individual PBN donors).

Panel B - phagocytosis by healthy donor PBNs exposed to 100nM C5a with or without pre-incubation with SQ22536, $p=0.003$ by Kruskal-Wallis, $*p<0.05$, $**p<0.01$ by Dunn's post-hoc test (n=5 individual PBN donors).

Panel C- phagocytosis by healthy donor PBNs exposed to 100nM fMLP with or without pre-incubation with SQ22536, $p=0.003$ by Kruskal-Wallis, $*p<0.05$, $**p<0.01$ by Dunn's post-hoc test (n=5 individual PBN donors).

Figure E5. CD11b and CD11b activation status of PBNs from patients and matched volunteers

Panel A-CD11b expression (as determined by flow cytometry) on the surface of PBNs from patients and matched volunteers. ** p=0.006 by t-test. (n=32 study participants)

Panel B- CD11b activation (as determined by flow cytometry using activation epitope-specific antibody CBRM1/5) on PBNs from patients and matched volunteers. US=unstimulated, opZ=exposed to opsonized zymosan. $p<0.0001$ by ANOVA, ***$p<0.001$ NS $p>0.05$ by Bonferoni's post-hoc test. (n=32 study participants)

Table E1. Demographic and clinical data relating to sub-groups of patients.

*by ANOVA, † by Chi-squared, ‡ by Kruskal-Wallis.

	VAP sub-group(n=17)	ARDS/ALI Subgroup (n=21)	Non-VAP/Non-ARDS subgroup (n=34)	P value
Mean Age (range)	58 (32-83)	55 (26-80)	59 (25-87)	0.61*
%male	76%	48%	71%	0.73†
Mean (95% CI) APACHE II score	23 (20-26)	20 (18-22)	22 (20-25)	0.27*
Median (IQR) days of ventilation before enrollment	8 (6-9)	6 (4-8)	9 (7-14)	0.007‡
ICU mortality	35%	43%	32%	0.73†
% with surgical diagnosis on admission	70%	48%	47%	0.24†
% with ≥ 1 co-morbidity	53%	57%	53%	0.95†
% receiving immunosuppressant drugs (including corticosteroids)	12%	19%	6%	0.31†
Median (IQR) Serum C3a des-arg (ng/ml)	585 (495-913)	710 (370-972)	613 (326-826)	0.75‡
Median (IQR) Serum C5a des-arg (ng/ml)	113 (62-170)	121 (60-186)	75 (40-130)	0.32‡
Median (IQR) serum GM-CSF (pg/ml)	2 (0-4)	3(2-4)	2 (0-4)	0.65‡
Median (IQR) BALF C3a (ng/ml)	11 (3-230)	8 (3-348)	20 (4-104)	0.97‡
Median (IQR) BALF C5a (ng/ml)	3 (1-39)	3 (2-32)	8 (0-35)	0.84‡
Median (IQR) BALF GM-CSF (pg/ml)	2 (0-14)	1 (0-6)	3 (1-12)	0.65‡
Median (IQR) BALF Neutrophils (10^5/ml)	2.8 (0.57-21)	9.4 (2.7-19)	0.72 (0.03-12)	0.0031‡
Median (IQR) BALF Macrophages (10^5/ml)	2.3 (0.4-3.9)	1.8 (0.89-3.6)	1.6 (0.1-3.9)	0.8‡

Figure E1

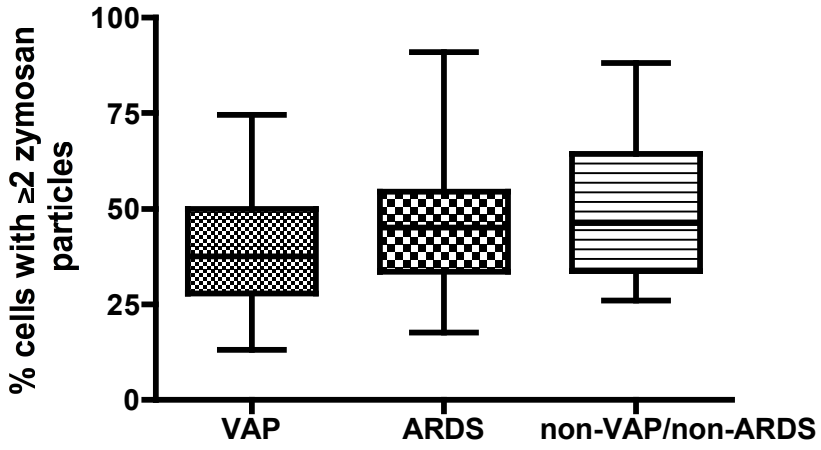
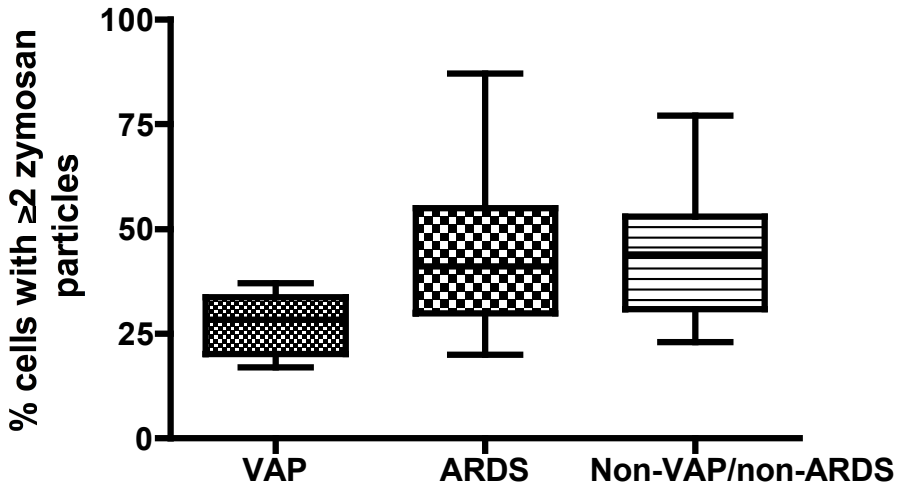

% cells with ≥2 zymosan particles
100
75
50
25
0
VAP
ARDS
non-VAP/non-ARDS

Figure E2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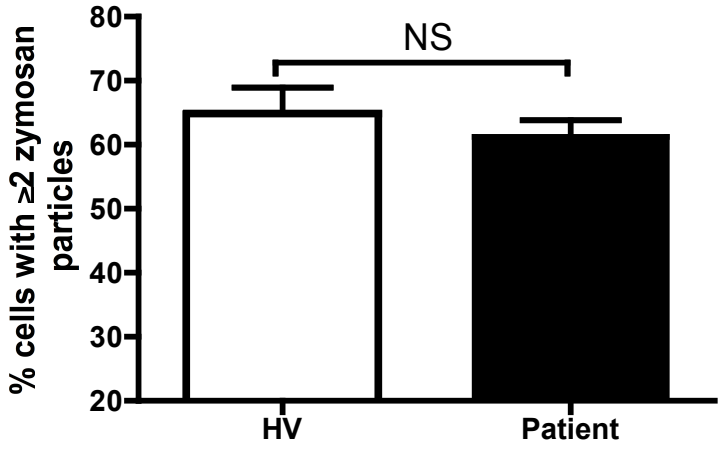
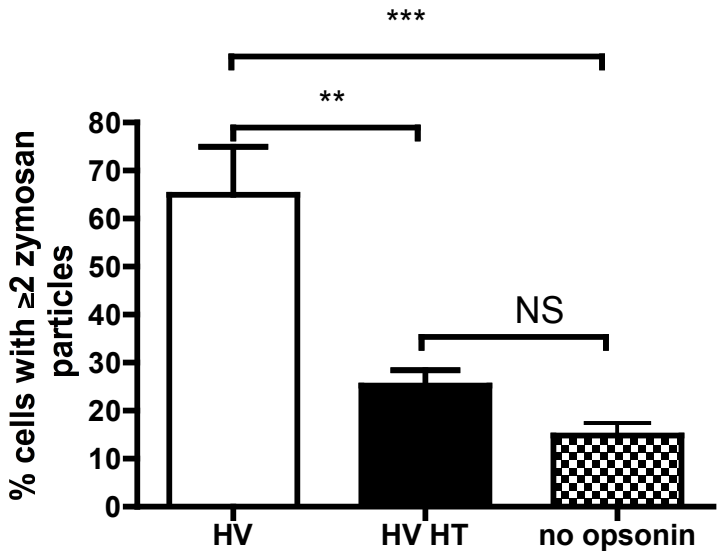
75
50
25
0
VAP
ARDS
Non-VAP/non-ARDS

Figure E3

A


% cells with ≥2 zymosan particles
80
70
60
50
40
30
20
NS
HV
Patient

B


***
**
% cells with ≥2 zymosan particles
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70
60
50
40
30
20
10
0
NS
HV
HV HT
no opsonin

Figure E4

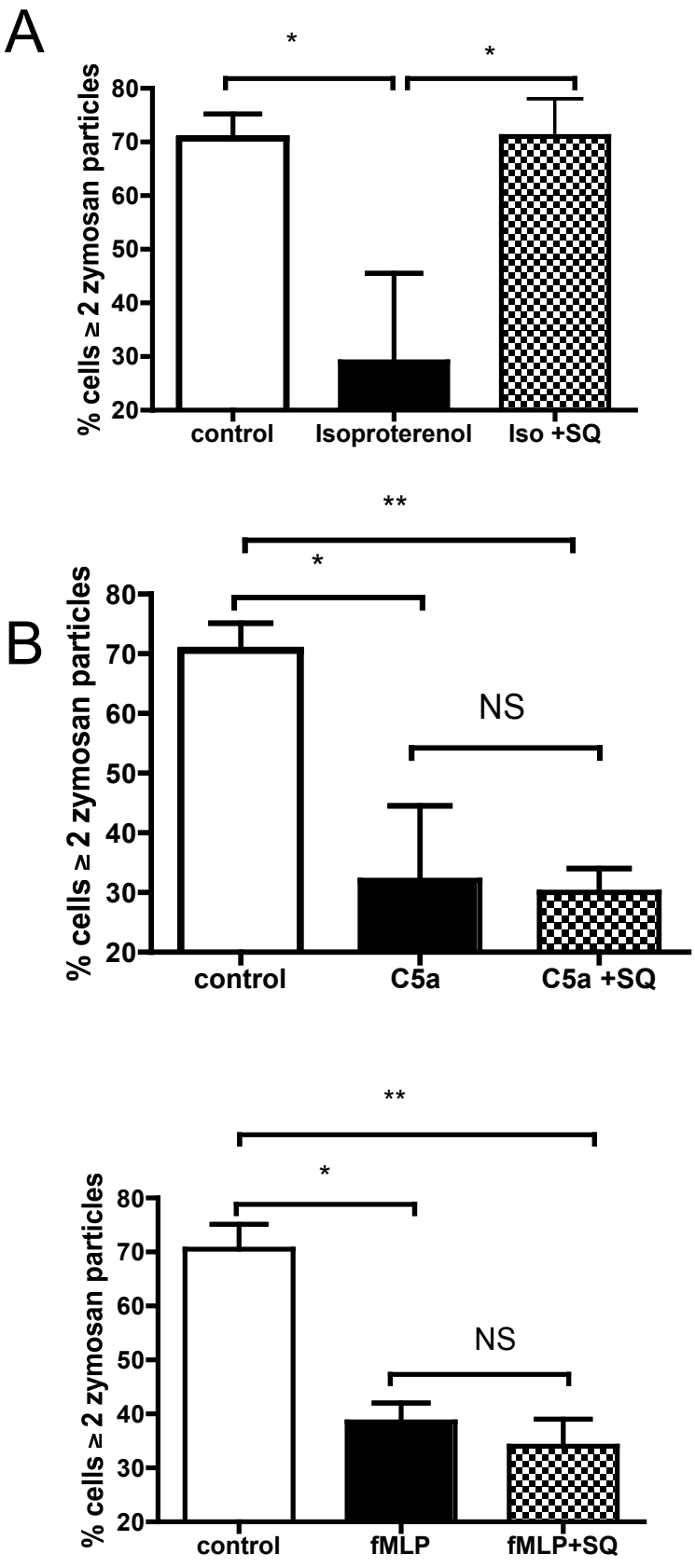
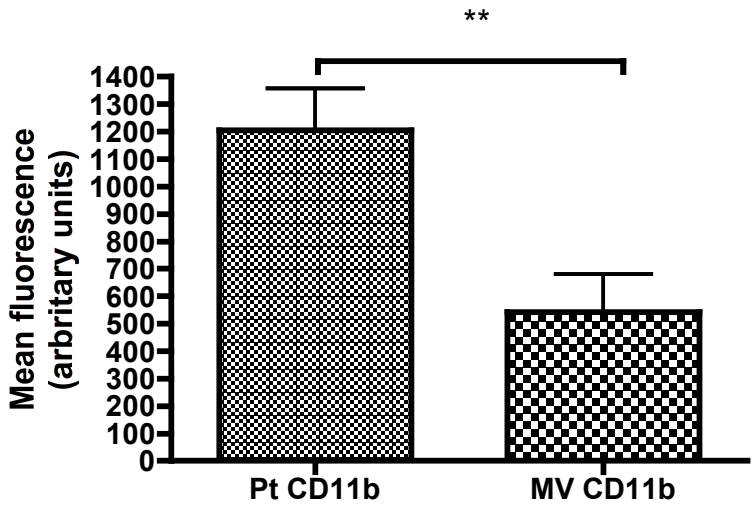

A
*
*
% cells ≥ 2 zymosan particles
80
70
60
50
40
30
20
control
Isoproterenol
Iso +SQ
B
**
*
NS
% cells ≥ 2 zymosan particles
control
C5a
C5a +SQ
C
**
*
NS
% cells ≥ 2 zymosan particles
control
fMLP
fMLP+SQ

Figure E5

A


**
Mean fluorescence (arbritary units)
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
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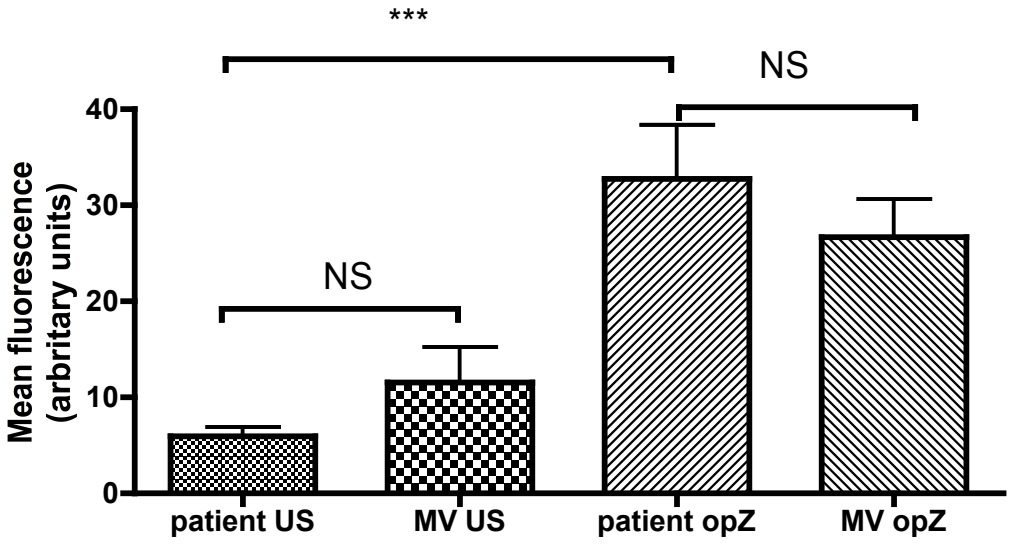
Pt CD11b
MV CD11b

B


***
NS
NS
Mean fluorescence (arbritary units)
40
30
20
10
0
patient US
MV US
patient opZ
MV opZ