Online supplement:

Phosphoinositide 3 kinase inhibition restores neutrophil migratory accuracy in the elderly: towards targeted treatments for immunesenescence.

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Methods

Surface receptor expression

Surface expression of chemo-attractant receptors (CXCR1[CXCL8 receptor], CXCR2 [CXCL8 and CXCL1 receptor], FPR1[fMLP receptor], C5aR[C5a Receptor] and BLT1[LTB4 receptor]) were measured on freshly isolated peripheral blood neutrophils. $2x10^6$ neutrophils were re-suspended in 1% PBS/BSA (Sigma-Aldrich) and incubated with relevant FITC, PE, APC and APCcy7-labelled antibodies. Receptor expression was determined by flow cytometry ($CyAN_{ADP}$, Beckman Coulter) counting 10,000 events in duplicate to reduce variability. Receptor anti-human antibodies included; mouse CXCR1 2µg/ml (clone#:42075); mouse CXCR2 3µg/ml (clone#:48311); mouse FPR1 3µg/ml (clone#:350418); mouse BLT1 3µg/ml (clone #:203/14F11); mouse C5aR1 2µg/ml (clone#:347214); (all R&D Systems). Isotype control antibodies included Mouse IgG2a 2µg/ml (clone#:DAK-GO5) and mouse IgG1 3µg/ml (clone#:DAK-GO1), from Dako Cytomation.

Sputum collection

Previously healthy patients, with no significant medical history admitted to hospital with a lower respiratory tract infection with a sputum culture positive for *Streptococcus pneumoniae* (n=5) were recruited to provide a sputum sample within 24 hours of admission. Sputum was collected over four hours from waking with mouth washing procedures prior to collection to reduce saliva contamination[1E]. The sputum sample was divided into 2 aliquots, the first aliquot was used for quantitative culture[2E]. The median bacterial load was calculated from each individual patients results.

The second aliquot was centrifuged to form a sol phase, used to determine mediator concentrations[3E]. All patient sol phases were pooled prior to measurement of mediators.

All sputum mediators were assessed by commercially available ELISA (CXCL8, GRO□, C5a) or parameter assays (LTB4); all R&D Systems, UK. Plasma and serum samples were used to measure systemic TNFα□ and IL-1β concentrations using high sensitivity commercially available ELISA assays (R&D Systems, UK). ELISA and parameter assays were performed in triplicate, and the average reading given (expressed as the median with interquartile range).

PI3K expression

PI3K expression was assessed by Western blotting, with neutrophils incubated with CXCL8 (100nM; R&D Systems, UK) for 0, 1, 2 or 5 minutes. Neutrophils were then pelleted (1500xg, 4°C, 2 minutes) and re-suspended in lysis buffer (20mM 3-[N-Morpholino] propanesulfonic acid (MOPS), 50mM NaF, 50nM β-glycerophosphate, 10mM Na_3VO_4 (Sodium Orthovanadate), 1% Triton X-100 (Sigma-Aldrich, UK), 1mM 4-(2-Aminoethyl)

benzensulfonyl fluoride hydrochloride (AEBSF, Sigma Aldrich), 1mM Dithiothreitol (DTT, [Sigma Aldrich]) and protease inhibitor cocktail [Sigma Aldrich]). Lysates were then mixed 1:1 with 2x SDS-sample buffer (4% SDS, 0.1M Dithiothreitol, 20% Glycerol, 0.0625M Tris-HCL [pH 6.8], 0.004% bromophenol blue). Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis(SDS-PAGE) and transferred onto a 0.45 micron PVDF membrane (Geneflow Ltd, Fradley, UK). Blots were processed in duplicate and probed with relevant primary and horseradish peroxidase conjugated secondary antibodies. Proteins were visualised using enhanced chemiluminescence (EZ-ECL, Geneflow Ltd). Primary antibodies used were rabbit anti-human phospho-PI3K p85 (Tyr485)/p55 (Tyr199) (Cell Signalling Technology) and mouse anti-human β-actin (Sigma Aldrich).

References for online supplement

E1. Sapey E, Bayley D, Ahmad A, Newbold P, Snell N, Stockley RA**.** Interrelationships between inflammatory markers in stable COPD patients with bronchitis; the intra and inter patient variability. Thorax. 2008;63:493 - 9.

E2 Pye A, Stockley RA, Hill SL**.** Simple method for quantifying viable bacterial numbers in sputum. J Clin Path. 1995;48:719 - 24.

E3. Woolhouse IS, Bayley D.L., Stockley RA. Effect of sputum processing with dithiothreitol on the detection of inflammatory mediators in chronic bronchitis and bronchiectasis. Thorax. 2002;57(8):667 - 71.

Online table E1. Neutrophils from older healthy subjects migrate with preserved chemokinesis but reduced chemotaxis and accuracy towards GRO□, LTB4 and C5a

	Chemokinesis um/min (SD)	Chemotaxis um/min (SD)	Chemotactic Index cs (SD)
RPMI (negative control)			
Age < 35	1.9 (0.3)	0.02 (0.1)	0.01 (0.01)
Age > 65	2.0 (0.5)	0.01 (0.2)	0.02 (0.01)
GRO□□(10nM)			
Age < 35	4.2 (0.4)	0.7 (0.1)	0.14 (0.03)
Age > 65	4.4 (0.7)	0.08 (0.2) *	-0.02 (0.04)*
LTB4 (10nM)			
Age < 35	4.2 (0.3)	1.4 (0.7)	0.22 (0.04)
Age > 65	3.9 (0.4)	0.2 (0.3)*	0.06 (0.01)*
C5a (1 nM)			
Age < 35	3.9 (0.6)	1.3 (0.4)	0.19 (0.04)
Age > 65	4.1 (0.8)	0.4 (0.3)*	0.01 (0.03)*

Legend.
Neutrophils from healthy controls migrated in shallow gradients of negative carrier control (RPMI) or towards single mediators (as shown) and migratory parameters observed. Measurements were taken from 10 randomly selected cells from each individual, with 10 subjects in each group. The average results for each subject were calculated, and an overall average was used for comparisons between groups (data in parenthesis are the standard deviation). Chemokinesis (random speed of movement in any direction) and Chemotaxis (speed of movement towards the chemoattractant) were measured in micrometres per minute. Chemotactic Index is a measure of the cell's directional orientation, calculated by the cosine of the angle between the cell's direction and the orientation of the chemoattractant gradient. This measure is expressed in a comparative scale ranging from -1 to 1. * = significant difference in migratory parameter from comparable results for neutrophils from younger subjects ($p<0.05$).

Online table E2. Characteristics of pooled sputum used in migratory assays

	Median mediator/ bacteria concentration. (Interquartile range)
CXCL8 (nM)	60.8 nM (59.4 – 64.1)
GRO□ (nM)	1.4 nM (1.1 – 1.9)
LTB4 (nM)	16.3 nM (12.2 – 18.9)
C5a (nM)	3.3 nM (2.0 – 3.7)
***Streptococcus pneumoniae* (cfu/ml)**	7.2×10^7 cfu/ml (6.3×10^6 – 8.9×10^8)

Legend.
Each median mediator concentration is formed from pooled sputum from n=5 patients admitted with a lower respiratory tract infection secondary to *streptococcus pneumoniae*. The pooled sputum sol phase sample was assessed in triplicate with the median result (and interquartile range) provided. Pooled sample concentrations were used to guide single mediatory concentrations in migratory assays to ensure physiological values were included. Bacterial load was assessed using standard quantitative culture techniques(E2). Each sample was cultured and then median bacterial load was calculated.

Online table E3. LTB4 and C5a receptor expression on the surface of circulating neutrophils isolated from healthy young and older subjects

	Age < 35	Age > 65
C5aR MFI (range)	112 (41 – 144)	138 (101 – 254)
BLT1 MFI (range)	103 (32 – 160)	149 (76 – 289)

Legend.
Receptors are expressed as mean median fluorescence intensity (MFI) for each young and old individual with the range in parenthesis. MFI was calculated by subtracting the MFI for neutrophils incubated with anti-human BLT1 (LTB4 receptor) and C5aR for each subject from the MFI for neutrophils from the same subject incubated with isotype matched irrelevant FITC/PE labelled $IgG_{2A.}$ All data sets follow normal distribution (Kolmogorov-Smirnov test). There were no differences between groups**.**

Online table E4. Non-selective PI3K inhibition improves migratory accuracy of neutrophils from older subjects, while decreasing migratory speed and accuracy of neutrophils from younger adults

	Chemokinesis (um/min)		Chemotaxis (um/min)		Chemotactic Index (cs)	
	Age<35	Age>65	Age<35	Age>65	Age<35	Age>65
Carrier control						
RPMI	2.2 (0.2)	1.9 (0.4)	0.4 (0.2)	0.2 (0.1)	0.02 (0.01)	0.08 (0.03)
CXCL8	4.9 (0.3)*	4.7 (0.5)*	2.0 (0.2)*	1.1 (0.2)*	0.4 (0.02)*	0.2 (0.01)*
LY294002						
RPMI	2.4 (0.2)	2.5 (0.3)	0.3 (0.4)	0.8 (0.5)	0.08 (0.01)	0.1 (0.04)
CXCL8	3.3 (0.4)*§	4.1 (0.3)*	0.8 (0.3)§	1.7 (0.2)*§	0.1 (0.03)§	0.3 (0.02)*§

Legend.
Neutrophils from healthy subjects (aged < 35 or > 65 years of age) migrated in shallow gradients of negative carrier control or towards CXCL8 following incubation with the reversible non-selective PI3K inhibitor, LY2094002 (1uM). Measurements were taken from 10 randomly selected cells from each individual, with 10 subjects in each group. The average results for each subject were calculated, and an overall average was used for comparisons between groups (data in parenthesis are the standard deviation). Chemokinesis, Chemotaxis and Chemotactic Index are defined as described in table E1. * = significant difference in migratory parameter from RPMI data ($p<0.05$), § = significant difference in migratory parameter in LY294002 treated cells compared with cells incubated with carrier control ($p<0.05$).

Online table E5. p38 MAPkinase inhibition does not effect chemokinesis or chemotaxis of neutrophils from older subjects.

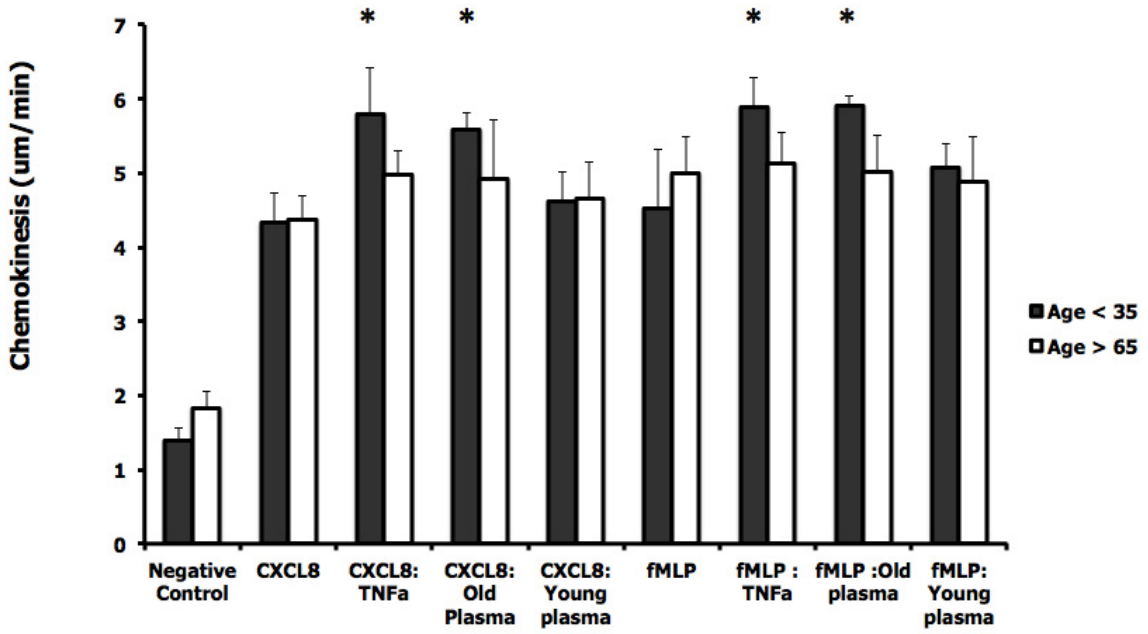
	Chemokinesis (um/min)	Chemotaxis (um/min)
Carrier control		
RPMI	2.1 (0.3)	0.2 (0.1)
CXCL8	3.6 (0.5)*	1.0 (0.3)*
SCIO469		
RPMI	2.2 (0.2)	0.1 (0.1)
CXCL8	3.7 (0.3)	0.9 (0.2)*
VX745		
RPMI	1.9 (0.3)	0.2 (0.2)
CXCL8	3.1 (0.4)*	0.8 (0.4)*

Legend.
Neutrophils from healthy old subjects (> 65 years of age) migrated in shallow gradients of carrier control or towards CXCL8 following incubation with SCIO469 or VX745. Measurements were taken from 10 randomly selected cells from each individual, with 10 subjects in each group. The average results for each subject were calculated, and an overall average was used for comparisons between

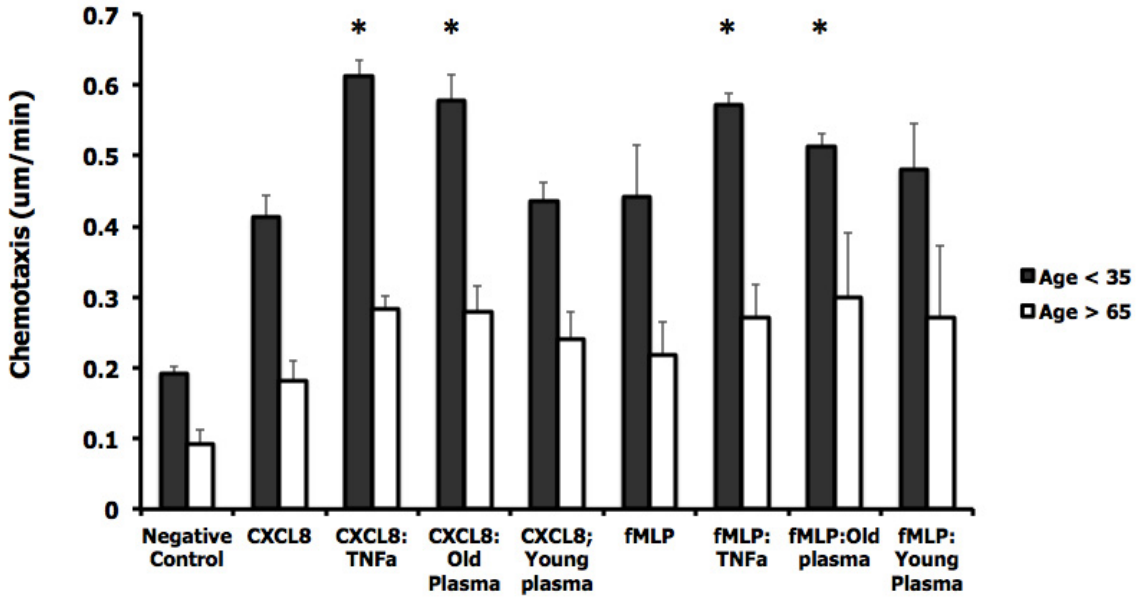
groups (data in parenthesis are the standard deviation). Chemokinesis and Chemotaxis are defined as described in table E1. * = significant difference in migratory parameter from RPMI data ($p<0.05$), § = significant difference in migratory parameter in treated cells compared with cells incubated with carrier control ($p<0.05$).

Online supplement figure E1. Exposing neutrophils to inflammatory signals does not reproduce the "old" migratory phenotype

1A



1B



Legend

Neutrophils from healthy subjects (aged < 35 or > 65 years of age) (n=10 each group) migrated towards CXCL8 (100nM) or fMLP (100nM) following incubation with carrier control, TNFα (1pM) or pooled plasma from healthy older or younger donors for 45 minutes. Measurements were taken from 10 randomly selected cells from each individual. The average results for each subject were calculated, and an overall average was used for comparisons between groups. Bars represent the mean migratory parameter with standard deviation shown as the error line. Chemokinesis and Chemotaxis are calculated as described in table E1. * = significant difference in migratory parameter from carrier control data ($p<0.05$).