

Isolating and Culturing of Sputum Macrophages: A Potential Ex Vivo/In Vitro Model

Journal:	<i>Experimental Lung Research</i>
Manuscript ID	UELR-2018-0098.R2
Manuscript Type:	Original Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Bølling, Anette Kocbach ; Norwegian Institute of Public Health,, Domain of Infection Control and Environmental Health Steensen, Tonje Berg; Norwegian Institute of Public Health,, Domain of Infection Control and Environmental Health Alexis, N.E.; University of North Carolina, Chapel Hill, Center for Environmental Medicine, Asthma and Lung Biology Sikkeland, Liv Ingunn Bjoner; University of Oslo, Institute of Clinical Medicine; Oslo University Hospital, Rikshospitalet, Dept. of Respiratory Medicine
Keywords:	induced sputum, airway macrophages, cell culture

SCHOLARONE™
Manuscripts

Isolating and Culturing of Sputum Macrophages: A Potential *Ex Vivo/In Vitro* Model

¹Anette Kocbach Bølling, ¹Tonje Berg Steensen, ²Neil E. Alexis, ³Liv Ingunn Bjoner Sikkeland

¹Domain of Infection Control and Environmental Health, Norwegian Institute of Public Health, Oslo, Norway. ²Center for Environmental Medicine, Asthma and Lung Biology, UNC Chapel Hill, Chapel Hill, North Carolina, USA. ³Department of Respiratory Medicine, Rikshospitalet, Oslo University Hospital AND University of Oslo, Oslo, Norway

Correspondence to:

Liv Ingunn Bjoner Sikkeland,
Department of Respiratory Medicine
Institute of Clinical Medicine, Faculty of Medicine
University of Oslo
PostBox 4950 Nydalen, 0424 Oslo
Norway

l.i.b.sikkeland@medisin.uio.no

Key words: Induced sputum, airway macrophages and cell culture.

Abstract

Purpose

This paper aimed to test whether induced sputum samples acquired from human volunteers could be used to isolate and culture airway macrophages for *in vitro* exposures. This was assessed in terms of the culturing success rate, culture purity, viability and responsiveness of cultured cells.

Materials and Methods

The isolation and culturing procedure was performed over three days. On Day 1, induced sputum samples were obtained, processed and seeded in culture wells. Differential cell counts and viability tests were performed to allow for calculation of viable macrophage numbers and appropriate sample dilution. After a 1 h rest seeded wells were washed to remove non-adherent cells, resulting in macrophage isolation. Then, cells rested overnight (Day 1 – Day 2), before *in vitro* exposure for 2-24 h (Day 2 – Day 3). The criteria for progressing into the culturing procedure was cell viability > 40 % and total cell number > 10⁶. Successful culturing was evaluated based on cell attachment (N=40). Culture purity by differential cell analysis and viability was monitored during culturing (N=4-8). Macrophage responsivity was assessed by measurement of inflammatory cytokine gene expression (N=4) and cytokine levels (N=6) following *in vitro* exposure to lipopolysaccharide (LPS) (2-24 h) and live bacteria (*S. aureus*) (4h).

Results

Overall, 88 % (35/40) of the samples acquired were suitable for isolation, and 80 % (32/40) were successfully progressed through the 2-3 day culturing protocol. Macrophage purity (88 %) and viability (85 %) were adequate. Moreover, cultured macrophages were responsive to *in vitro*

1
2
3 stimulation with LPS and viable *S. aureus* showing positive mRNA responses for TNF α , IL-1 β
4
5 and IL-8 and release of IL-1 β , respectively.
6

7
8 *Conclusion*
9

10 Sputum macrophage isolation by plate adherence and subsequent culturing of sputum
11
12 macrophages was successfully performed and represents a promising *in vitro* model for e
13
14 examination of airway macrophage behavior.
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Introduction

Culturing macrophages from the airways of humans provides an opportunity to examine their phenotypic and functional characteristics in isolation, in order to better understand their role in maintaining an immunologically healthy airway environment. Specifically, one can examine *ex vivo*, biological responses of macrophages both before and following exogenously applied environmental pollutants. Induced sputum is a relatively non-invasive method by which to acquire airway macrophages from the surfaces of the central airways,^[1] a region of the lung where pathophysiologic events often occur. The majority of induced sputum samples contain a sufficient number of viable cells that allow for *ex vivo/in vitro* assays to be performed.^[2] In healthy volunteers, sputum samples comprise mainly macrophages and neutrophils, and to a lesser extent monocytes, eosinophils, lymphocytes and basophils. Therefore, in order to study macrophages in isolation, an optimal enrichment technique is needed to avoid the confounding effects from other cell types such as neutrophils. This is important both when measuring mRNA expression and protein levels. To date, a few isolation methods, including a cell culture method, have been attempted but none have proven optimally effective. Horva'th and co-authors (1998) isolated sputum macrophages for western blotting by culturing the whole sputum sample on plates but had varied success in terms of macrophage adherence and subsequent isolation.^[3] Others, including our group have utilized magnetic bead isolation, cell sorting by flow cytometry

1
2
3 and a culture method, but concluded these methods either have significant limitations for use in
4
5 subsequent *in vitro* assays, or require further refinement.^[4,5]
6
7

8 The purpose of this paper is to describe a fully refined method to culture and isolate macrophages
9
10 from induced sputum samples acquired from human volunteers. Method criteria under
11
12 examination were macrophage viability, macrophage purity, success and survival rate of
13
14 macrophages post-isolation, and post-culture macrophage activation following exogenously
15
16 applied inflammatory stimuli, such as LPS and viable bacteria. We report results from 33 healthy
17
18 subjects who underwent sputum induction for culturing of airway macrophages.
19
20
21
22
23
24

25 **Materials and Methods**

26 ***Experimental Design***

27
28 As shown in Figure 1, the entire experimental protocol occurred over 3 days. On Day 1, sputum
29
30 induction, sample processing, cell seeding and adherence of the cells took approximately 4 hours
31
32 (h) to complete. An overnight rest period was followed by an LPS (1 µg/ml) exposure on Day 2.
33
34 Cells were harvested for mRNA analysis after 2, 5, 8 or 24 h exposure (Day 2 or 3). The culture
35
36 purity and macrophage viability was assessed in the exposure time interval (0-24 h).
37
38
39
40
41

42 ***Subjects***

43
44 Healthy adult volunteers (N=33; ages 20 to 58 years old; 16 female vs. 17 male) underwent
45
46 sputum induction. All volunteers were non-smokers and were free from upper respiratory tract
47
48 infections for a period at least 4 weeks prior to study enrollment. Informed consent was obtained
49
50 from all volunteers and ethical approval was obtained from the Norwegian Regional Committees
51
52 for Medical and Health Research Ethics (REC; 2015/1322). Five of the volunteers provided 2
53
54
55
56
57
58
59
60

1
2
3 samples, while one volunteer provided 3 samples. Thus, the total number of sputum inductions
4
5 preformed was 40.
6
7

8 ***Sputum induction and processing***

9
10 Induced sputum collection was performed according to Alexis et al.^[6] In brief, post-
11
12 bronchodilator spirometry (FEV₁) was measured (Airomir Autohaler, Teva) and safety values (10
13
14 % and 20 % fall from post bronchodilator FEV₁) were determined. Then, hypertonic saline of 3
15
16 % (w/V), 4 %, and 5 % was inhaled via an ultrasonic nebulizer (DeVilbiss, Sunrise Medicals)
17
18 during three 7-minute inhalation periods. Sample expectorates were collected after each
19
20 inhalation period into a sterile specimen cup placed on ice. Care was taken to avoid throat
21
22 scraping to minimize potential squamous epithelial cell contamination. Whole sputum samples
23
24 were dissolved in 0.1 % (w/V) dithiothreitol (DTT)(Sigma, MO, USA) in cold Phosphate
25
26 Buffered Saline (PBS) on a shaker for 10 minutes before dilution to 0.05 % DTT in PBS. The
27
28 sample was filtered through a 50µM nylon mesh filter (Sintab, Sweden) and centrifuged at 340 x
29
30 g for 5 minutes. The cell pellet was resuspended in 1ml complete cell culture medium, consisting
31
32 of RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10 % heat inactivated fetal
33
34 bovine serum (Sigma-Aldrich), 100 U/ml penicillin (Sigma-Aldrich), 100 µg/ml streptomycin
35
36 (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich) and 0.5 mg/ml Gibco™ Amphotericin B
37
38 (Thermo Fisher Scientific). The sputum sample was kept on ice during all stages of the
39
40 processing, except for the shaking with DTT.
41
42
43
44
45
46
47

48 The total cell counts were evaluated using a Bürker hemocytometer, combined with Trypan blue
49
50 (0.4 %) (Sigma Aldrich) staining to determine leukocyte viability. Non-viable cells contained
51
52 Trypan blue stain in the interior of the cell. Cytospins for the differential cell counts were
53
54 obtained by adding 10 000 cells at a concentration of 10⁶ cells/ml to the cytospin slide chamber.
55
56
57

1
2
3 The slides were centrifuged for 5 min at 140 x g, and then air dried for 10 minutes. Then the
4
5 slides were stained using Diff-Quik (Medion Diagnostics, Duedingen, Switzerland) and mounting
6
7 with Eukitt quick-hardening mounting medium (Sigma-Aldrich, St. Louis, USA). The staining
8
9 and mounting process took about 15 minutes, allowing for rapid differential cell counts and
10
11 subsequent calculation of macrophage concentrations based on these counts. The cell suspension
12
13 was then diluted to a concentration of 4.0×10^5 macrophages/ml. The initial criteria for
14
15 processing and culturing sputum macrophages were a) cell viability above 40 %, based on the
16
17 viability threshold suggested by the ERS/ATS working group on induced sputum^[7], and b) a total
18
19 cell count greater than 10^6 cells, based on the necessary number of wells for an *in vitro* exposure
20
21 experiment.
22
23
24
25

26 27 ***Culturing of airway macrophages***

28
29 The suspension of sputum cells was seeded in 12 or 48 well plates to yield 3.2×10^5
30
31 macrophages/well (800 μ L) or 10^5 macrophages/well (250 μ L), respectively (Falcon, Corning
32
33 inc., NY, USA). The 12 well plates were used for mRNA analysis since the mRNA yield was too
34
35 low when using 48 well plates. The 48 well plates were applied for monitoring of cells during
36
37 culturing. The cells were incubated for 1h in an atmosphere of 37° C and 5 % CO₂ to allow
38
39 macrophages to attach to the culture wells. Then, the wells were washed twice with PBS and
40
41 detached cells were discarded (supernatant), before fresh medium was added. Figure 2 shows
42
43 sputum cells (on a cytospin) before seeding (a) and following attachment in the culture dish (b).
44
45 Preliminary data (not shown) indicated high levels of pro-inflammatory cytokines post
46
47 macrophage seeding (i.e. at -20 h, Figure 1), so we instilled a rest period of 20 h before *in vitro*
48
49 exposure/stimulation.
50
51
52
53
54

55 56 ***Monitoring of cells during culturing***

1
2
3 At different time points during the culturing, cells were monitored in terms of cell numbers
4 (counting squares), viability (Trypan blue exclusion) and differential cell count (staining with
5 Diff Quik for preparation of cytopins). The number of attached cells were counted in a square
6 drawn onto the bottom of the cell culture plate using an x-y axis grid, and presented as the
7 percentage of the initial cell count (-20 h time point). For viability tests, the attached cells were
8 stained for 1 min with 1:1 diluted Trypan blue (0.4 %) in cell culture medium. This was then
9 replaced with 1:50 diluted Trypan blue to allow for light microscopy assessment, before a
10 minimum of 150 cells were counted (Trypan blue exclusion). In addition, cells in suspension
11 were used to monitor the viability of cells detached during culturing (1:10 dilution of Trypan
12 blue).

13
14
15
16
17
18
19
20
21
22
23
24
25
26
27 Differential cell counts of detached cells post-culturing, was performed at the 0 h time point
28 (Figure 1), using Accutase to detach the cells. In brief, cells were washed with PBS, incubated at
29 37 °C with Accutase for 9 min, with physical manipulation (shaking/knocking of plate) every
30 third minute. Then, the cells were transferred to an Eppendorf tube and an additional rinsing of
31 the well was performed with PBS, before centrifugation. A cytospin was prepared of these
32 detached cells, and stained for differential cell counts as described above.

33 34 35 36 37 38 39 40 41 ***RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)***

42 For mRNA analysis, sputum macrophages were exposed to 1 µg/mL of the toll-like receptor
43 (TLR)-4 agonist lipopolysaccharide (LPS) from *E. coli* strain 026:B6 (Sigma-Aldrich) for 2-24 h.
44 Total RNA was extracted from approximately 300,000 sputum macrophages using QIAGEN
45 RNeasy Mini Kit (VWR International, Oslo, Norway) with the addition of RNase-free DNase
46 according to the manufacturer's instructions. 100 ng of total RNA was reverse transcribed using
47 TaqMan RT reagents (Applied Biosystems, Foster City, CA) in a final volume of 50 µl. Negative
48
49
50
51
52
53
54
55
56
57
58
59
60

controls were performed to ensure that PCR amplification cDNA was not a result of contaminating, genomic DNA. Reverse-transcribed cDNA was amplified by using a TNF α probe/primer mix (Hs00174128_m1, 4331182, Applied Biosystems), or 200 nM primers (Eurogentec) and 100 nM probes (Probelibrary, Exqion, Denmark) for human IL-1 β , IL-6 or IL-8, and 1X TaqMan Universal Master Mix (Applied Biosystems) in a total volume of 25 μ l.

Primers were designed using Exiqon ProbeLibrary Assay Design Center (<http://www.universalprobelibrary.com>), and were constructed as follows: IL-1 β ; left primer AAAGCTTGGTGATGTCTGGTC, right primer GGACATGGAGAACACCACTTG to probe 39, IL-6; left primer GCCCAGCTATGAACTCCTTCT, right primer GAAGGCAGCAGGCAACAC to probe 45, and IL-8; left primer AGACAGCAGAGCACACAAGC, right primer ATGGTTCCTTCCGGTGGT to probe 72. Human PGK (phosphoglyceratekinase 1) (4310885E, Applied Biosystems) served as endogenous control. Real-time PCR was performed using the Applied Biosystems prism 7900 sequence detector following the manufacturer's instructions. Results were expressed as fold induction of mRNA, which was determined by normalizing cytokine threshold cycle values against PGK. These data were further normalized against samples from unstimulated cells at 0 h.

Stimulation with viable bacteria and cytokine analysis

To further test the responsivity of the sputum macrophages after 40-44 h in culture, the cells were exposed to viable bacteria for 4 h at the 20 h time-point. *Staphylococcus aureus* (*S. aureus*) strains Newman was a kind gift from Tadashi Baba, Juntendo University School of Medicine, Tokyo, Japan. Viable *S. aureus* were added to the cell culture at multiplicity of infection (MOI) of 1 and 10, corresponding to ratios of *S. aureus* to sputum macrophages of 1:1 and 10:1, respectively. After 4 h exposure to *S. aureus*, the supernatant was harvested and centrifuged for

1
2
3 10 min at 100 x g to remove cells and for 10 min at 5000 x g to remove bacteria, and stored at -80
4
5 °C until cytokine analysis. The levels of IL-1 β were determined by an enzyme-linked
6
7 immunosorbent assay (ELISA) kits for human IL-1 β from R&D Systems Inc. (MN, USA)
8
9 according to the manufacturer's instructions.
10
11
12
13
14
15

16 **Statistical analysis**

17
18 Statistical comparisons were performed using 2-way ANOVA with Bonferroni post-tests to
19
20 assess effects of LPS stimulation. Additionally, to compare the cytokine gene expression level at
21
22 20 h compared to 0 h, a one sample t-test with hypothetical value 1 (i.e. 0 h control value) was
23
24 used. All analyses were performed in GraphPad Prism Version 5.04. In these analyses $p < 0.05$
25
26 was considered statistically significant, and the data reflected 4 independent experiments from 4
27
28 different subjects.
29
30
31
32
33
34

35 **Results**

36 ***Sputum sample characteristics pre-culturing (N=35)***

37
38 Based on the initial criteria (mean viability > 40 %, total cell number > 10⁶), 35 of 40 sputum
39
40 samples from healthy donors were suitable for culturing. Overview of sample characteristics of
41
42 sputum samples (N=35) are given in Table 1 and Figure 3. The mean (\pm standard deviation (SD))
43
44 number of total viable leukocytes in these samples was 5.5 (\pm 5.3) million. With a mean
45
46 macrophage percentage of 68 (\pm 16) %, ranging from 26-93 %, this resulted in 0.5 to 14.1 million
47
48 macrophages for culturing. The mean neutrophil percentage was 31 (\pm 17) %, while the
49
50 percentages of eosinophils and leukocytes were negligible (Table 1). Note that of these 35
51
52
53
54
55
56
57
58
59
60

1
2
3 samples, 7 originated from participants that were invited to provide a sample a second or third
4
5 time as their samples exhibited high cell numbers.
6
7
8
9

10 In three of the 35 samples there was not sufficient adherence of the macrophages to the cell
11 culture well during the initial 1 h rest. These samples displayed relatively low macrophage
12 percentages ($< 60\%$), combined with a sub-optimal viability ($< 60\%$), resulting in poor
13 conditions for cellular attachment in the cell culture wells. For the remaining 32 samples, the
14 culturing was successful (Figure 3). Exclusion of the 7 repeated visits resulted in 25 out of 32
15 samples with successful culturing, and a success rate for culturing of sputum macrophages of 78
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Purity of culture and cell survival (N=3-8)

During sputum processing, macrophages were the dominating cell type ($58 \pm 7\%$) followed by neutrophils ($23 \pm 10\%$) and squamous epithelial cells ($19 \pm 9\%$) (Figure 4a). After 20 h in culture, differential cell counts of detached viable cells showed increased macrophage purity, with mean macrophage percentages of $88 (\pm 11)\%$, with squamous epithelial cells as the only other identified cell type ($12 \pm 11\%$) (Figure 4b). To evaluate whether the squamous cell percentages were reduced during the seeding and culturing procedure, the percentages of squamous cells relative to macrophages should be considered. During sputum processing these squamous cell percentages were $27 (\pm 17)\%$, while they were reduced to $14 (\pm 8)\%$ after 20h in culture, resulting in an average reduction of $47 (\pm 21)\%$.

1
2
3 The number of attached cells decreased for increasing incubation (Figure 5a), with a
4 meanpercentage of attached cells of 70 (\pm 15) % for the 20 h time point which corresponds to
5 0.7x10⁵ cells per well in 48 well plates. However, the mean viability of the attached cells
6
7 remained relatively constant, around 85 %, throughout the incubation period, and at the 20h time
8 point the viability of the attached cells was 88 (\pm 8) % (n=8) (Figure 5b). The viability for the
9 detached macrophages (supernatant) was 83 (\pm 8) % (n=4).

20 ***LPS stimulation and gene expression analysis (N=4)***

21
22 Macrophage activation was assessed by cytokine gene expression. The TNF α gene expression
23 was 50 fold higher at the -20 h compared to the 0 h time point ($p < 0.05$), whereas the expression
24 levels of IL-1 β , IL-6 and IL-8 were relatively unchanged during the same period (Figure 6).

25
26 Thus, a 20 h rest of the macrophages post-culture, but pre LPS stimulation resulted in a low basal
27 TNF α expression at the time of LPS exposure (0 h). The cells responded to LPS stimulation with
28 significantly increased gene expression levels of TNF α at 2 and 5 h, IL-1 β at 8 h and IL-6 at 2, 5
29 and 8 h. These data confirm that the cultured sputum macrophages are capable of responding to
30 inflammatory stimuli during an acute period of time but not chronic (24 h).

41 ***Stimulation with viable bacteria and protein analysis (N=6)***

42
43 The cells responded to exposure to viable *S. aureus* at multiplicity of infection of 1 and 10 after
44 4h exposure at the 20h time-point. Both *S. aureus* concentrations resulted in significantly
45 increased release of IL-1 β from the cultured sputum macrophages ($p < 0.05$) (Figure 7).

Discussion

Pulmonary macrophages play an important role in the first line of defense against inhaled particulates and pathogens, and also help orchestrate the immune response and contribute to resolution of inflammation.^[8,9] Sputum samples represent secretions from the surfaces of the central airways and are comprised of a mixture of cell populations.^[1] The predominant cell type present in sputum samples in healthy individuals are macrophages, with neutrophils occupying relatively lesser percentages, followed by monocytes and lymphocytes. Due to their location, sputum macrophages have the potential to provide valuable information concerning homeostatic as well as pathological mechanisms. However, a method to isolate and then culture these cells for subsequent *ex vivo/in vitro* experiments has not been optimized. The present study demonstrates successful isolation and culturing of sputum macrophages in healthy volunteers for up to 48 h post sample collection. We report that 88 % (35/40) of the samples acquired were suitable for isolation, and of these 80 % (32/40) were successfully progressed through the 2-3 day culturing protocol. Indeed, the isolated macrophages were viable and responsive to *in vitro* stimulation with LPS and viable *S. aureus* showing significant mRNA TNF α responses and release of IL-1 β , respectively. This confirmed that the macrophages successfully survived the isolation, adherence and culture conditions applied in this study.

For sputum samples, two processing methods are commonly applied to the raw sample; plug selection or processing of the whole sputum sample.^[7] Although these two methods have been reported to provide comparable results with some endpoints,^[7,10] plug selection seems to be the commonly preferred method in sputum processing today. However, in the current study we used the whole sputum processing method as it was advantageous in terms of reduced processing times and generating increased recovery of total numbers of immune cells, two factors critical in

1
2
3 optimizing the isolation and culture conditions of sputum macrophages Advantages of the plug
4 selection method include reduced squamous cell contamination, decreased amounts of saliva, and
5 increased cell viability.^[7,10] As important as these factors are for differential cell count and
6 protein analysis, they were not significant effect modifiers with our culture approach. Here, the
7 squamous cell numbers were reduced in the initial seeding step, dead cells were eliminated due to
8 lack of adherence, and saliva was removed during the centrifugation step in the initial processing.
9

10 We implemented a 20 h rest period for the sputum macrophages before the *in vitro* stimulation
11 exposures were introduced. This was based on preliminary data, and also supported by
12 bronchoalveolar lavage (BAL) data where alveolar macrophages exhibited a marked pro-
13 inflammatory transcriptional profile that declined after a resting period of 24 to 48 h.^[11] In this
14 study, we observed that gene expression analysis of TNF α decreased dramatically during the pre-
15 *in vitro* exposure rest period (-20 h to 0 h). Likewise, in alveolar macrophages from BAL,
16 transcriptional responsiveness to inflammatory stimuli (LPS and poly IC) was restored after a
17 resting period^[11]. This highlights the importance of including a rest or deactivation period prior to
18 culturing sputum macrophages for *in vitro* stimulation. The enhanced expression of pro-
19 inflammatory cytokines immediately after culturing could possibly reflect a cellular response to
20 the culturing and attachment on plastic or cellular activation during the isolation procedure^[5,11].
21 Alternatively, removal from an anti-inflammatory environment in the airways and from the
22 ‘environmental breaks’ present there, such as prostaglandin E2 (PGE2) and transforming growth
23 factor β (TGF β), could also explain the transient increase in expression of pro-inflammatory
24 cytokines^[11-14].
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51

52 We observed that the number of attached macrophages decreased during the 48 hours in culture,
53 but the mean viability was relatively constant (85 %) for both attached and detached
54
55
56
57
58
59
60

1
2
3 macrophages, the latter present in the supernatant. Thus, the detaching of macrophages from the
4 cell culture wells was not due to cell death, but mediated through other mechanisms. The applied
5 isolation method, using cellular attachment to the culture wells, resulted in relatively high cell
6 purity (88 % macrophages). Culturing in serum-free medium has been reported to promote
7 adhesion of macrophages.^[15] However, the impact of culturing in serum-free medium was not
8 tested in the present study. Macrophage purity was assessed using smaller sized 48 well plates,
9 which limited the possibility of applying vigorous washing that would have otherwise enhanced
10 macrophage purity, although washing could also have resulted in decreased numbers of attached
11 macrophages.
12
13
14
15
16
17
18
19
20
21
22
23

24 Our initial criteria for progressing isolated macrophages to the culturing procedure, was cell
25 viability > 40 % and total cell number > 10⁶. The viability threshold was based on the study from
26 ERS/ATS working group on induced sputum,^[7] while the total cell number was based on the
27 necessary number of wells for an *in vitro* exposure experiment. Of the 35 sputum samples
28 appropriate for adherence isolation, three exhibited a lack of macrophage adherence to the cell
29 culture wells. These samples exhibited viability of < 60 % combined with macrophage
30 proportions of < 60 %, reflecting samples dominated by non-macrophage non-adhering cells, e.g.
31 dead cells, squamous cells and neutrophils, most likely leading to an environment sub-optimal for
32 macrophage adherence. Thus, extra attention should be paid to samples with a combination of
33 cell viability < 60 % and macrophage proportions < 60 %, as these exhibit a low success rate for
34 attachment after the wash step on Day 1. Overall, our experiences from the series of experiments
35 suggest that the initial criteria of viability > 40 %, total cell number > 10⁶ were adequate and can
36 be expanded with a criteria of macrophage numbers > 50 %.
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 Exposure to the toll-like receptor (TLR)-4 agonist LPS (1 $\mu\text{g}/\text{ml}$) resulted in significantly
4 increased expression of TNF α , IL-1 β and IL-6 after 2-5, 8 and 2-8 h respectively. Moreover, the
5
6
7 4h exposure to viable *S. aureus* at the 20h time-point significantly increased the release of IL-1 β .
8
9
10 Together these data demonstrate that these cultured sputum macrophages are responsive to
11
12 inflammatory stimuli during the entire 24h *in vitro* exposure period. Previous work by our group
13
14 has shown that cultured sputum macrophages can display different cytokine patterns depending
15
16 on the stimulating agent. For example LPS, Pam₃Cys (TLR2 agonist), isoproterenol and the pro-
17
18 inflammatory cytokine IL-1 β , all induced differential responses demonstrating that cultured
19
20 sputum macrophages have recognition specificity in terms of pro-inflammatory stimuli.^[4]
21
22
23

24
25 Culturing of sputum macrophages constitutes an *ex vivo/in vitro* human primary macrophage
26
27 model allowing for *in vitro* exposure to various inhaled environmental agents like particulate
28
29 matter, fungi, environmental chemicals etc., commonly studied in other macrophage models.^[16,17]
30
31 However, one must exercise caution in equating this new model with those currently in use. For
32
33 example, animal and human monocyte-derived macrophage (MDM) models are commonly used
34
35 to study *in vitro* exposure to various inhaled environmental agents,^[18-21] despite noted differences
36
37 between MDMs and primary airway macrophages.^[10,22-24] Furthermore, our group has shown
38
39 functional differences between sputum macrophages and BAL macrophages from the same
40
41 individual while other studies report that BAL macrophages possess different properties than
42
43 MDMs.^[6,7,10] Therefore, cultured sputum macrophages may represent an *ex vivo/in vitro* model
44
45 that only applies to immune cells derived from the surfaces of the central airways. Future studies
46
47 will be needed however, to confirm whether sputum macrophage models are similar or different
48
49 than other *in vitro* macrophage models that use MDMs, differentiated cell lines or macrophages
50
51 acquired from BAL.
52
53
54
55
56
57
58
59
60

1
2
3 Although the isolation of sputum macrophages by plate adherence resulted in a viable and
4 responsive culture of primary human airway macrophages, the method has some challenges and
5 limitations. First, there are some practical challenges related to the experiment planning. More
6 specifically, the number of wells for *in vitro* experiments resulting from a sputum induction was
7 unpredictable and varied from 0 to >100. This was due to large variations in the yield for viable
8 macrophages ($0.5 - 14.1 \times 10^6$) and a success rate for culturing below 100%, since around 20%
9 of the healthy volunteers provided samples that were not suitable for culturing. Second, the
10 obtained cultures contained some squamous cell contamination (approximately 10%) and the
11 impact of this contamination on the macrophage responses is not characterized. Finally, there is
12 limited knowledge regarding the impact of the isolation and culturing on the phenotype of the
13 sputum macrophages, which limits the generalizability of the data resulting from these cultures.
14 Tomlinson et al. 2012 encountered similar challenges during culturing of BAL macrophages, and
15 suggested a comparison of cultured macrophages and *in situ* macrophages in tissue samples to
16 assess the impact of culturing^[11].
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35

36 Induced sputum is a non-invasive method with minimal discomfort for the recruited human
37 volunteers. The described method for isolation and culturing of macrophages from induced
38 sputum samples requires approximately 4 hours work, and was demonstrated to result in airway
39 macrophage cultures of adequate purity and viability. Moreover, successful post-culture
40 activation of the airway macrophages by the TLR agonist LPS and viable *S. aureus* bacteria was
41 demonstrated. Thus, isolation by plate adherence and subsequent culturing of sputum
42 macrophages represents a promising *ex vivo/in vitro* model in which to examine airway
43 macrophage behavior.
44
45
46
47
48
49
50
51
52
53

54 Acknowledgments

1
2
3 This work was supported by the Norwegian research council under Grant number 228129 and the
4
5 Working Environmental Fund, Confederation of Norwegian Enterprise. Dr. Håkon Valen Rukke
6
7 is greatly acknowledged for supervision during planning and conduction of the experiments with
8
9 the viable bacteria, which were performed at the Nordic institute of Dental Materials (NIOM).
10
11
12
13
14

15 **Declaration of interest statement**

16
17 The authors declare no conflict of interest.
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

References

1. Alexis NE, Hu SC, Zeman K, Alter T, Bennett WD. Induced sputum derives from the central airways: confirmation using a radiolabeled aerosol bolus delivery technique. *American journal of respiratory and critical care medicine*. 2001;164(10 Pt 1):1964-1970.
2. Pavord ID, Pizzichini MM, Pizzichini E, Hargreave FE. The use of induced sputum to investigate airway inflammation. *Thorax*. 1997;52(6):498-501.
3. Horváth I, Donnelly LE, Kiss A, Paredi P, Kharitonov SA, Barnes PJ. Raised levels of exhaled carbon monoxide are associated with an increased expression of heme oxygenase-1 in airway macrophages in asthma: a new marker of oxidative stress. *Thorax*. 1998;53(8):668-672.
4. Sikkeland LI, Dahl CP, Ueland T, et al. Increased levels of inflammatory cytokines and endothelin-1 in alveolar macrophages from patients with chronic heart failure. *PloS one*. 2012;7(5):e36815.
5. Sikkeland LI, Kongerud J, Stangeland AM, Haug T, Alexis NE. Macrophage enrichment from induced sputum. *Thorax*. 2007;62(6):558-559.
6. Alexis N, Soukup J, Ghio A, Becker S. Sputum phagocytes from healthy individuals are functional and activated: a flow cytometric comparison with cells in bronchoalveolar lavage and peripheral blood. *Clinical immunology (Orlando, Fla)*. 2000;97(1):21-32.
7. Hamid Q, Kelly MM, Linden M, et al. Methods of sputum processing for cell counts, immunocytochemistry and in situ hybridisation. *European Respiratory Journal*. 2002;20(37 suppl):19s.
8. Grabiec AM, Hussell T. The role of airway macrophages in apoptotic cell clearance following acute and chronic lung inflammation. *Seminars in immunopathology*. 2016;38(4):409-423.
9. Morales-Nebreda L, Misharin AV, Perlman H, Budinger GR. The heterogeneity of lung macrophages in the susceptibility to disease. *European respiratory review : an official journal of the European Respiratory Society*. 2015;24(137):505-509.
10. Spanevello A, BeghÉ B, Bianchi A, et al. Comparison of Two Methods of Processing Induced Sputum: Selected versus Entire Sputum. *American journal of respiratory and critical care medicine*. 1998;157(2):665-668.
11. Tomlinson GS, Booth H, Petit SJ, et al. Adherent human alveolar macrophages exhibit a transient pro-inflammatory profile that confounds responses to innate immune stimulation. *PloS one*. 2012;7(6):e40348.
12. Gardai SJ, Xiao YQ, Dickinson M, et al. By binding SIRPalpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. *Cell*. 2003;115(1):13-23.
13. Roth MD, Golub SH. Human pulmonary macrophages utilize prostaglandins and transforming growth factor beta 1 to suppress lymphocyte activation. *Journal of leukocyte biology*. 1993;53(4):366-371.
14. Takayama K, Garcia-Cardena G, Sukhova GK, Comander J, Gimbrone MA, Jr., Libby P. Prostaglandin E2 suppresses chemokine production in human macrophages through the EP4 receptor. *The Journal of biological chemistry*. 2002;277(46):44147-44154.
15. Williams MR, Cauvi DM, Rivera I, Hawisher D, De Maio A. Changes in macrophage function modulated by the lipid environment. *Innate immunity*. 2016;22(3):141-151.
16. Hansen JF, Bendtzen K, Boas M, et al. Influence of phthalates on cytokine production in monocytes and macrophages: a systematic review of experimental trials. *PloS one*. 2015;10(3):e0120083.

- 1
2
3 17. Lawal AO. Diesel Exhaust Particles and the Induction of Macrophage Activation and Dysfunction. *Inflammation*. 2018;41(1):356-363.
- 4
5 18. Berntsen HF, Bolling AK, Bjorklund CG, et al. Decreased macrophage phagocytic function due to
6 xenobiotic exposures in vitro, difference in sensitivity between various macrophage models.
7 *Food and chemical toxicology : an international journal published for the British Industrial
8 Biological Research Association*. 2018;112:86-96.
- 9
10 19. Davies JQ, Gordon S. Isolation and Culture of Human Macrophages. In: Vol 290.2004:105-116.
- 11 20. Karlsson H, Lindbom J, Ghafouri B, et al. Wear particles from studded tires and granite pavement
12 induce pro-inflammatory alterations in human monocyte-derived macrophages: a proteomic
13 study. *Chemical research in toxicology*. 2011;24(1):45-53.
- 14 21. Overton NLD, Brakhage AA, Thywissen A, Denning DW, Bowyer P. Mutations in EEA1 are
15 associated with allergic bronchopulmonary aspergillosis and affect phagocytosis of *Aspergillus
16 fumigatus* by human macrophages. *PloS one*. 2018;13(3):e0185706.
- 17 22. Clift MJ, Endes C, Vanhecke D, et al. A comparative study of different in vitro lung cell culture
18 systems to assess the most beneficial tool for screening the potential adverse effects of carbon
19 nanotubes. *Toxicological sciences : an official journal of the Society of Toxicology*.
20 2014;137(1):55-64.
- 21 23. Daigneault M, Preston JA, Marriott HM, Whyte MK, Dockrell DH. The identification of markers of
22 macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages.
23 *PloS one*. 2010;5(1):e8668.
- 24 24. Misharin AV, Morales-Nebreda L, Reyfman PA, et al. Monocyte-derived alveolar macrophages
25 drive lung fibrosis and persist in the lung over the life span. *The Journal of experimental
26 medicine*. 2017;214(8):2387-2404.
- 27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

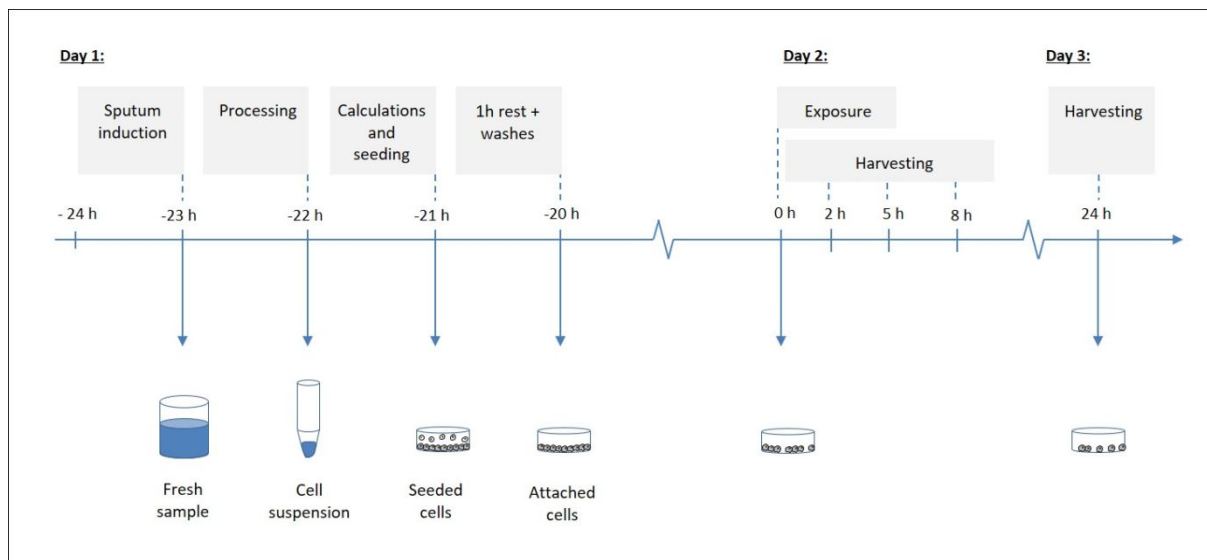
Table 1: Overview of pre-culture sample characteristics of 35 sputum samples that passed the initial criteria for culturing.

The viability originates from the Trypan blue cell count, while the macrophage, neutrophil, eosinophil and lymphocyte percentages were obtained from the differential cell counts performed during sputum processing (pre-culturing).

	Mean \pm SD	Median (min – max)
Sputum weight (g)	5.7 \pm 2.7	5.3 (1.7 – 12.7)
Leukocyte viability (%)	67.7 \pm 11.8	67.0 (44.0 – 97.0)
Total viable leukocytes (10^6)	5.5 \pm 5.3	4.2 (0.5 – 29.0)
Macrophages (%)	68.4 \pm 16.3	72.0 (26.0 – 93.0)
Neutrophils (%)	30.7 \pm 16.8	28.0 (6.0 – 74.0)
Eosinophils (%)	0.1 \pm 0.4	0 (0 – 1.6)
Lymphocytes (%)	0.7 \pm 1.1	0 (0 – 4)
Total viable macrophages (10^6)	3.5 \pm 2.9	2.7 (0.5 – 14.1)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

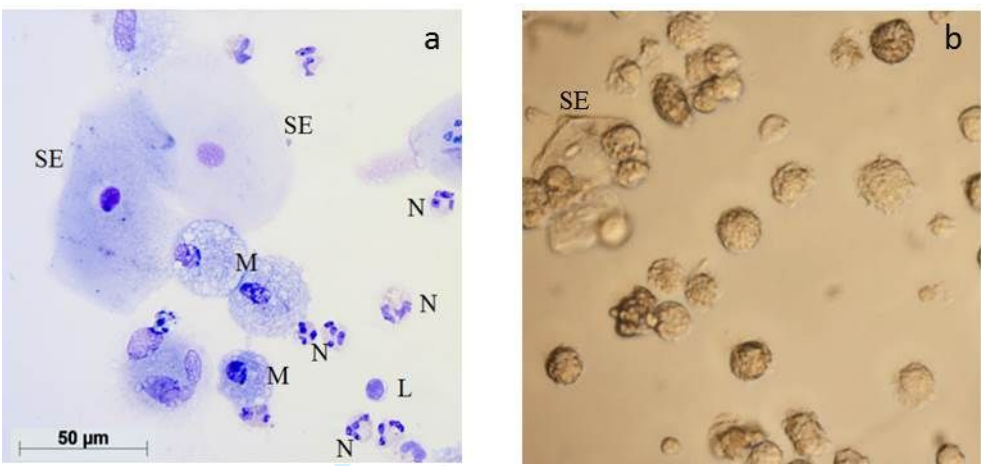
Figure 1:



Pre-Review Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 2:



Peer Review Only

Figure 3:

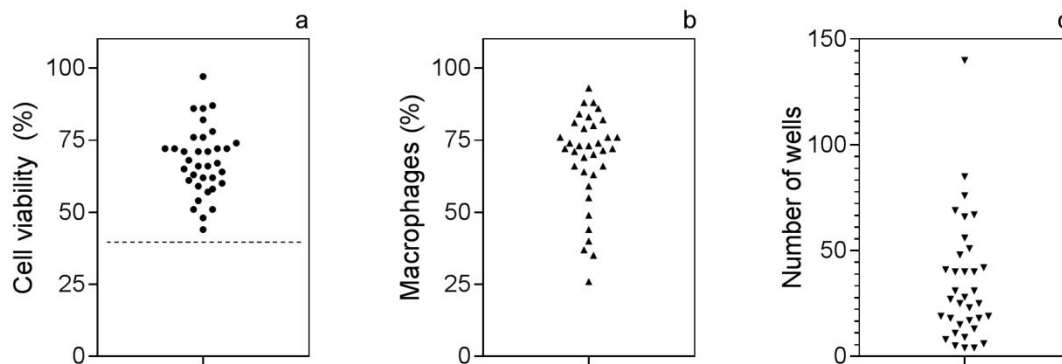
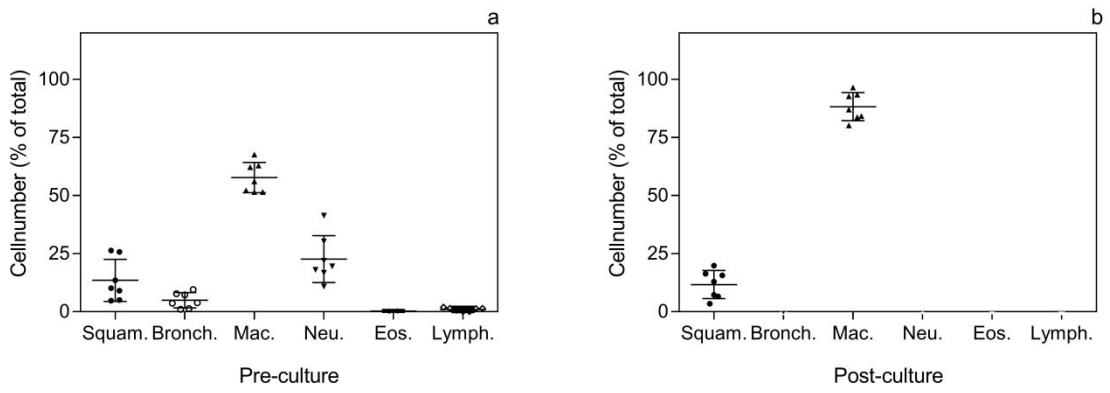
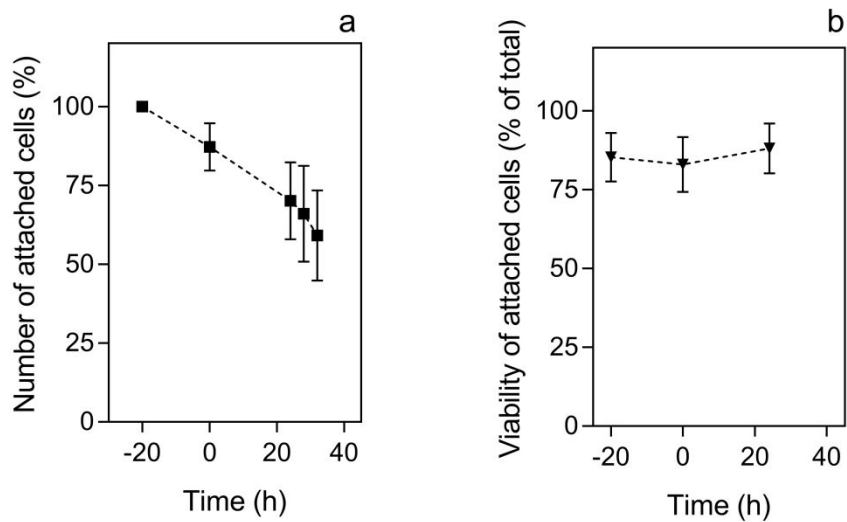


Figure 4:



Peer Review Only

Figure 5:



Review Only

Figure 6:

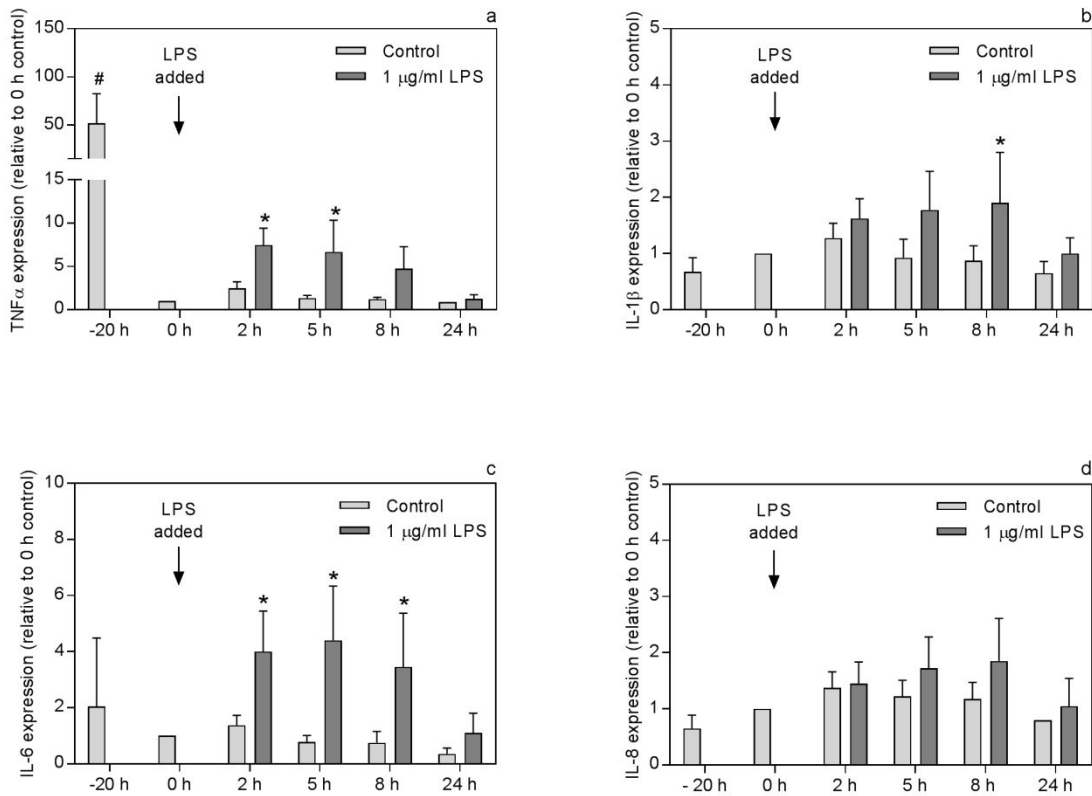


Figure 7:

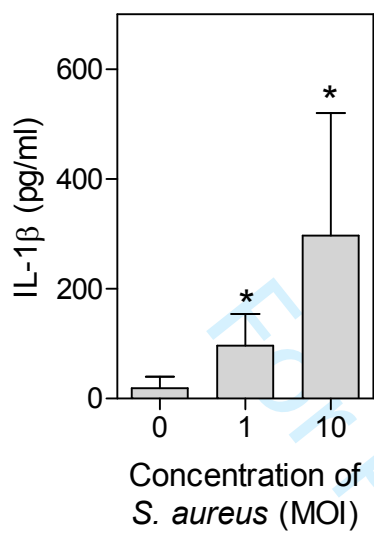


Figure legends

Figure 1: Schematic illustration of culturing of sputum macrophages. The figure shows the timeline of the processing and culturing of sputum cells, illustrating the approximate 4 h duration of sputum induction, the sample processing and seeding of cells (including the 1 h rest allowing for macrophage attachment), the 20 h over-night rest (Day 1 – Day 2) and the 24 h exposure period (e.g. LPS (1 ug/ml)) (Day 2 – Day 3). Cells were examined at 2, 5, 8 and 24 h post LPS exposure.

Figure 2: Light microscopy images of cells before and after culturing. a) A Diff Quik stained cytospin slide was prepared from the cell suspension prior to culturing of the cells. b) Macrophages attached to the surface of the cell culture well after 20 h of culturing; one squamous epithelial cell is indicated. Abbreviations: M = macrophage, N = neutrophil, SE = squamous epithelial cell, L = lymphocyte.

Figure 3: Distribution of characteristics for sputum samples for pre-culturing. Dot plots of individual values for a) leukocyte cell viability b) percentage of macrophages and c) the macrophage yield displayed as the number of wells that could be seeded on 48 well plates from the obtained macrophages (N=35).

Figure 4: Differential cell counts pre- and post-culturing. The graphs show the percentages of squamous (Squam.) and bronchial (Bronch.) epithelial cells, macrophages (Mac.), neutrophils (Neu.), eosinophils (Eos.) and lymphocytes (Lymph.) from the same individuals (N=7) a) pre-culturing (during processing of the sputum samples) and b) at the 0 h time point (Figure 1) for cells detached using Accutase. Absence of symbol reflects absence of cell type in sample.

1
2
3 **Figure 5: Number and viability of attached cells.** a) Number and b) viability of attached cells
4 for increasing incubation times, presented as mean \pm SD (N=3-8 independent experiments). In a)
5
6 for increasing incubation times, presented as mean \pm SD (N=3-8 independent experiments). In a)
7
8 100% of attached cells corresponds to 10^5 cells/well in 48 well plates at -20h, while mean \pm SD
9
10 number of attached cells at 24 h and 32 h corresponds to $0.7 \times 10^5 \pm 0.1 \times 10^5$ and $0.6 \times$
11
12 $10^5 \pm 0.1 \times 10^5$ cells/well, respectively.
13
14

15 **Figure 6: Cytokine gene expression levels.** The expression of a) TNF α , b) IL-1 β , c) IL-6 and d)
16
17 IL-8 in cultured airway macrophages were determined at -20, 0, 2, 5, 8 and 24 h time points. At
18
19 the 0 h time point, the cells were stimulated with 1 μ g/ml LPS. Data are displayed as fold change
20
21 from the control value at 0 h for each experiment (mean \pm SD). *p < 0.05 stimulated versus
22
23 unstimulated (Control) cells (2-way ANOVA, with Bonferroni post-tests performed on data from
24
25 2 -- 24 h only, N = 4 independent experiments) and # p < 0.05 -20 h control vs 0 h control (One
26
27 sample t-test; hypothetical value 1 (i.e. 0 h control value)).
28
29
30
31

32 **Figure 7: Exposure to viable bacteria.** Macrophages were exposed to live bacteria (*S. aureus*)
33
34 at a MOI of 1 or 10 for 4h at the 20h time-point. The resulting IL-1 β release was measured at the
35
36 24h time-point. * refers to significant difference from control (repeated measures one-way
37
38 ANOVA, log-transformed data, Dunnett's post-test, N = 6).
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

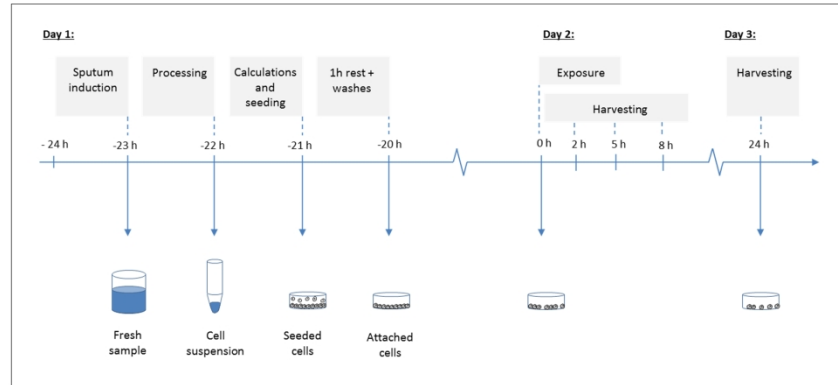


Figure 1: Schematic illustration of culturing of sputum macrophages. The figure shows the timeline of the processing and culturing of sputum cells, illustrating the approximate 4 h duration of sputum induction, the sample processing and seeding of cells (including the 1 h rest allowing for macrophage attachment), the 20 h over-night rest (Day 1 – Day 2) and the 24 h exposure period (e.g. LPS (1 $\mu\text{g}/\text{ml}$)) (Day 2 – Day 3). Cells were examined at 2, 5, 8 and 24 h post LPS exposure.

297x209mm (300 x 300 DPI)

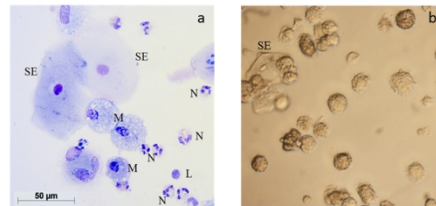


Figure 2: Light microscopy images of cells before and after culturing. a) A Diff Quik stained cytospin slide was prepared from the cell suspension prior to culturing of the cells. b) Macrophages attached to the surface of the cell culture well after 20 h of culturing; one squamous epithelial cell is indicated. Abbreviations: M = macrophage, N = neutrophil, SE = squamous epithelial cell, L = lymphocyte.

297x209mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

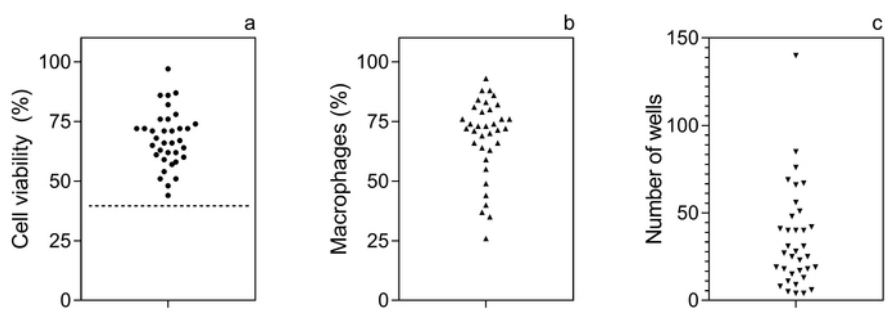


Figure 3
65x25mm (300 x 300 DPI)

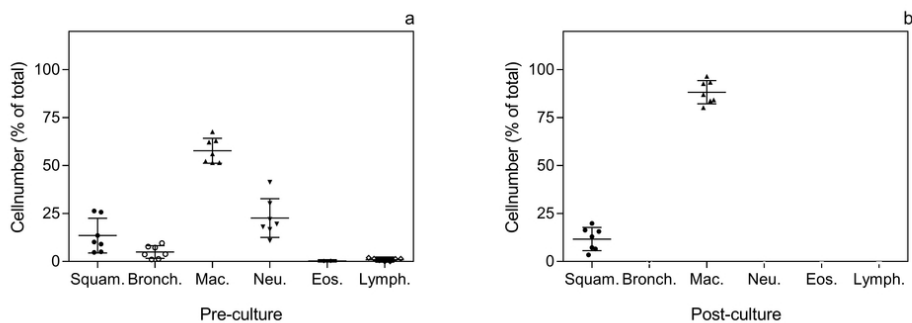


Figure 4: Differential cell counts pre- and post-culturing. The graphs show the percentages of squamous (Squam.) and bronchial (Bronch.) epithelial cells, macrophages (Mac.), neutrophils (Neu.), eosinophils (Eos.) and lymphocytes (Lymph.) from the same individuals (N=7) a) pre-culturing (during processing of the sputum samples) and b) at the 0 h time point (Figure 1) for cells detached using Accutase. Absence of symbol reflects absence of cell type in sample.

85x31mm (300 x 300 DPI)

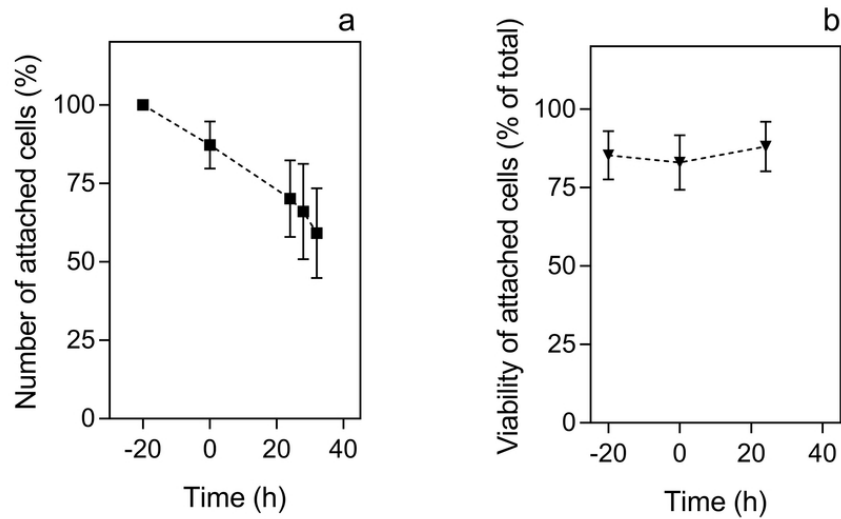


Figure 5: Number and viability of attached cells. a) Number and b) viability of attached cells for increasing incubation times, presented as mean \pm SD (N=3-8 independent experiments). In a) 100% of attached cells corresponds to 10^5 cells/well in 48 well plates at -20h, while mean \pm SD number of attached cells at 24 and 32h correspond to $0.7 \times 10^5 \pm 0.1 \times 10^5$ and $0.6 \times 10^5 \pm 0.1 \times 10^5$ cells/well, respectively.

80x48mm (300 x 300 DPI)

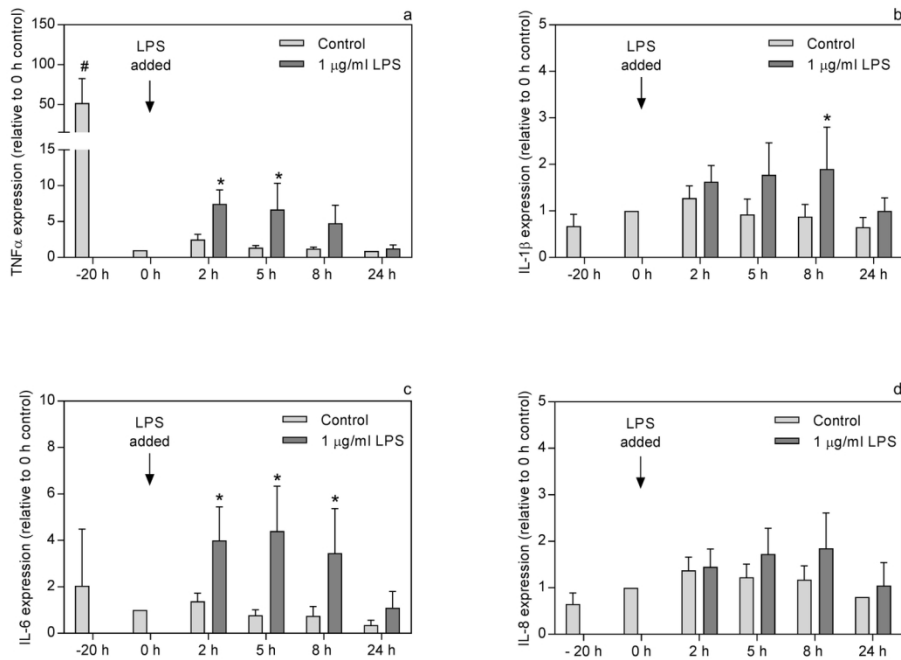


Figure 6: Cytokine gene expression levels. The expression of a) TNF α , b) IL-1 β , c) IL-6 and d) IL-8 in cultured airway macrophages were determined at -20, 0, 2, 5, 8 and 24 h time points. At the 0 h time point, the cells were stimulated with 1 μ g/ml LPS. Data are displayed as fold change from the control value at 0 h for each experiment (mean \pm SD). *p < 0.05 stimulated versus unstimulated (Control) cells (2-way ANOVA, with Bonferroni post-tests performed on data from 2 -- 24 h only, N = 4 independent experiments) and # p < 0.05 -20 h control vs 0 h control (One sample t-test; hypothetical value 1 (i.e. 0 h control value).

139x100mm (300 x 300 DPI)

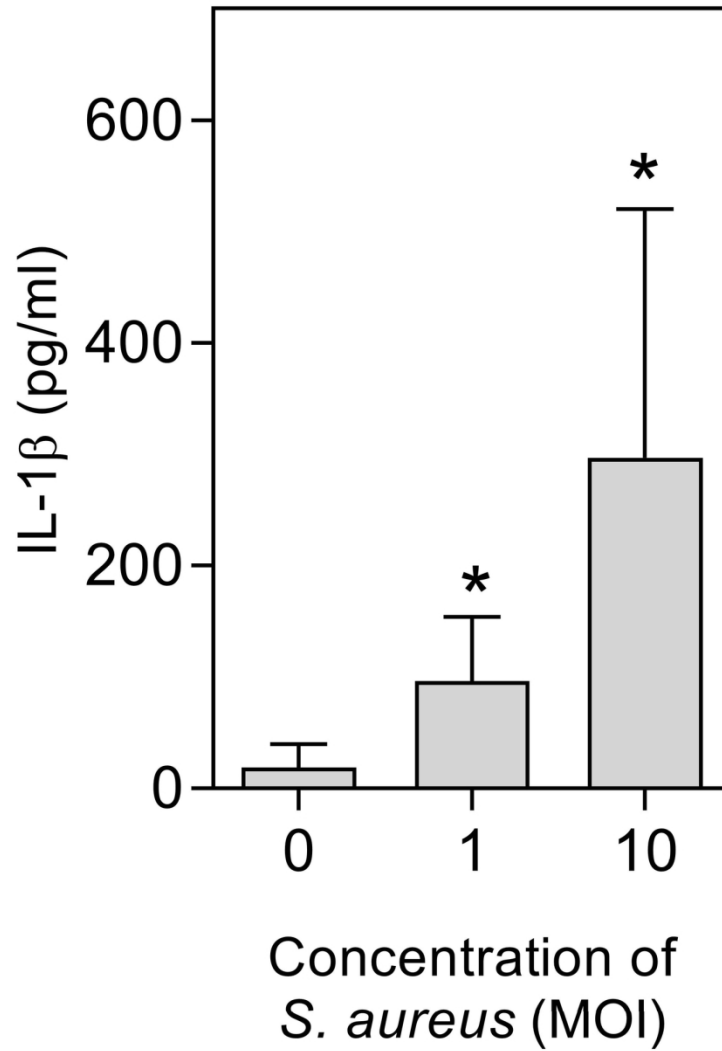


Figure 7: Exposure to viable bacteria. Macrophages were exposed to live bacteria (*S. aureus*) at a MOI of 1 or 10 for 4h at the 20h time-point. The resulting IL-1 β release was measured at the 24h time-point. * refers to significant difference from control (repeated measures one-way ANOVA, log-transformed data, Dunnett's post-test, N = 6).

156x193mm (300 x 300 DPI)