

Expression of Muscarinic Receptors by Human Macrophages

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Abstract

Macrophages increase and are highly activated in chronic obstructive pulmonary disease (COPD). Muscarinic receptor antagonists inhibit acetylcholine stimulated-release of neutrophilic chemoattractants suggesting that acetylcholine may regulate macrophage responses. Therefore, expression and function of components of the non-neuronal cholinergic system in monocyte-macrophage cells was investigated.

RNA was isolated from monocytes, monocyte-derived macrophages (MDM), lung and alveolar macrophages from non-smokers, smokers and COPD patients and expression of the high-affinity choline transporter, choline acetyltransferase, vesicular acetylcholine transporter and muscarinic receptors (M_1 - M_5) ascertained using real-time PCR. M_2 and M_3 receptor expression was confirmed using immunocytochemistry. Release of IL-8, IL-6 and LTB_4 were measured by ELISA or EIA.

All monocyte-macrophage cells expressed mRNA for components of the non-neuronal cholinergic system. Lung macrophages expressed significantly more M_1 mRNA compared with monocytes, and both lung macrophages and alveolar macrophages expressed the highest levels of M_3 mRNA. Expression of M_2 and M_3 protein was confirmed in MDM and lung macrophages. Carbachol-stimulated release of LTB_4 from lung macrophages (buffer: 222.3 ± 75.1 vs. carbachol: 1118 ± 622.4 pg/ml, $n=15$, $p<0.05$) but not IL-6 or IL-8. LTB_4 release was attenuated by the M_3 antagonist, 4-DAMP (EC_{50} : 5.2 ± 2.2 nM, $n=9$).

Stimulation of macrophage M_3 receptors promotes release of LTB_4 suggesting anti-muscarinic agents may be anti-inflammatory.

Background

Macrophages are the predominant inflammatory cell found in the lung. Their role is primarily to remove any inhaled particles and pathogens and maintain sterility of the respiratory tract. However, in lung diseases such as chronic obstructive pulmonary disease (COPD), macrophage numbers increase by more than ten-fold and are highly activated producing increased levels of inflammatory mediators [1]. At present, pharmacotherapy for COPD is largely symptomatic with no treatments capable of decreasing the underlying inflammatory response and improving lung function [2]. Long acting muscarinic antagonists such as tiotropium bromide have been shown to have efficacy in patients with COPD [3] although whether this drug could also act to control the inflammatory components of the disease or act upon the small airways where COPD is manifest is unclear. Recently, tiotropium bromide has been shown to suppress chemotactic activity released by macrophages following stimulation with acetylcholine (ACh) [4]. This suggests that macrophage mediated inflammation may, in part, be regulated by components of the non-neuronal cholinergic system.

Classically, ACh is synthesised in nerve terminals and is released to regulate many activities including regulation of airway contraction and dilation of vessels [5,6]. More recently, essential components of the non-neuronal cholinergic system, including the high-affinity choline transporter (CHT1), ACh, choline acetyltransferase (ChAT), and muscarinic and nicotinic ACh-receptors are expressed by a number of non-neuronal cells including peripheral blood mononuclear cells (PBMC) and lymphocytes [7,8]. Many of these cells, not only release ACh upon stimulation but also can be activated by ACh [9-11]. Bovine alveolar macrophages release neutrophil, monocyte and eosinophil chemotactic factors after stimulation with ACh [12] and it has been suggested that human alveolar macrophages release leukotriene (LT)_{B₄} in

response to ACh stimulation [4]. Cells obtained from induced sputum have been also been shown to express muscarinic M_2 and M_3 receptors and that following stimulation with ACh increased the release of LTB_4 in cells from COPD patients but not cells from non-smokers or smokers [13]. Taken together, these data suggest that non-neuronal ACh might be involved in the pathophysiology of COPD by stimulating the release of inflammatory mediators from macrophages.

This study examined the expression of components of the non-neuronal cholinergic system including CHT1, ChAT and vesicular acetylcholine transporter (VAChT) in cells of the monocyte-macrophage lineage together with expression of muscarinic receptors (M_{1-5}). The putative roles of these receptors on macrophages were then evaluated using functional assays.

Methods

Subject Selection

Subjects were recruited from clinics at the Royal Brompton Hospital NHS Trust, from staff of the Royal Brompton Hospital and National Heart & Lung Institute, or volunteers known to the clinical research group of the Asthma Laboratory, National Heart & Lung Institute. Alveolar macrophages from BAL fluid of non-smokers, smokers and patients with COPD were obtained from consenting patients at Heatherwood Hospital, Ascot, Wexham Park Hospital, Slough. Smokers had a smoking history of at least 10 pack years and COPD patients were stable and fulfilled the American Thoracic Society criteria [14]. All studies were approved by the Ethics Committee of Royal Brompton and Harefield NHS Trust and National Heart and Lung Institute Ethics Committee, the East Berkshire Local Research Ethics Committee or St Mary's NHS Trust Ethics Committee. All subjects gave informed written consent.

Preparation of monocytes

Monocytes were isolated from peripheral blood, centrifuged on discontinuous Percoll gradients, and purified either by adherence to tissue culture plastic [15] or by negative immunoselection using a MACS monocyte isolation kit (Miltenyi Biotec; Bisley, UK) and magnetic depletion columns according to the manufacturer's instructions. Cells were cultured in complete media (RPMI-1640 media supplemented with 10% (v/v) foetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin and 2mM L-glutamine) in a 6-well plate and were then resuspended in lysis buffer and stored at -70°C.

Preparation of Monocyte-Derived Macrophages

After separation of PBMC, cells were resuspended at 2×10^6 cells/ml in complete media. The cells were seeded onto 48 well plates, or chamber slides and incubated for 2 h at 37°C in a humidified incubator with 5% (v/v) CO₂. After incubation the supernatant was removed and replaced with complete media supplemented with 2ng/ml GM-CSF. The cells were differentiated in culture for 12 d towards a macrophage phenotype.

Preparation of Lung-Derived Macrophages

Lung-derived macrophages were isolated from lung tissue as previously described [16]. Briefly, lung tissue from patients undergoing surgical resection for carcinoma was lavaged by injection of RPMI-1640 containing 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5µg/ml amphotericin and 2mM L-glutamine. The cells were washed and resuspended in 2ml of PBS and separated by centrifugation (25 min; 18°C; 1100 x g) using Percoll density gradient [65%/35%/25% (v/v)]. Macrophage-enriched fraction was collected at the 25% and 35% Percoll interface. The cells were washed in HBSS and resuspended in RPMI-1640 medium supplemented with 10% (v/v) foetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5µg/ml amphotericin and 2mM L-glutamine resuspended at 1×10^6 cells/ml and seeded into 24 well plates at 5×10^5 cells/well, 48 well plates at 3.2×10^5 cells/well or chamber slides at 4×10^5 cells/well. After 2 h of incubation in at 37°C in a humidified incubator with 5% (v/v) CO₂, the non-adherent cells were removed and fresh medium was added. The adherent purified macrophages were incubated overnight and the medium was

changed the next day before beginning the experiment. Macrophage purity was confirmed by anti-CD68 staining as described previously [17].

Preparation of Alveolar Macrophages

Bronchoscopy and processing were performed according to the guidelines of the European Respiratory Society (ERS) task force [18] and alveolar macrophages isolated as described previously [19].

Preparation of Sputum Cells

Sputum was induced by inhalation of hypertonic saline and processed with 0.05% (w/v) dithiothreitol [20]. After centrifugation, the cell pellet was resuspended with HBSS and cytopins prepared.

Real-time PCR

Total RNA was extracted from cells using an RNeasy RNA extraction kits (Qiagen, Crawley, UK) and isolated RNA was quantified using the Ribogreen quantification Assay (Molecular Probes; Leiden, Netherlands). RNA was reverse transcribed using an Taqman reverse transcriptase (RT) mastermix (Taqman RT buffer, MgCl₂ 5.5mM, dNTPs 500μM, random hexamers 2.5μM, RNase Inhibitor 0.4U/μl and RT enzyme 1.25U/μl) (Applied Biosystems; Foster City, U.S.A.) according to the manufacturer's instructions. Reverse transcription products were amplified by PCR. cDNA (5μl) was added to 20μl of a solution containing Universal master mix, water, sense and antisense primers and 6-carboxy-tetramethyl-rhodamine (FAM) labelled probe. Primers and probes were designed by Applied Biosystems. An ABI Prism 7500Sequence Detection System was used for thermal cycling, which consisted of an initial activation step of 50°C for 2 min and 95°C for 10 min, followed by 45 cycles

of 95°C for 15 s and 60°C for 1 min. Each analysis included a standard curve (1.25-20 ng) consisting of cDNA synthesised from a panel of 5 control human RNAs (human RNA control panel, Becton Dickinson). Samples were analysed in duplicate and levels of expression for each specific gene calculated by extrapolating from the standard curve. For each test gene, endogenous control (HPRT) levels were also analysed on the same plate, calculated using extrapolation of standard curve values.

Immunocytochemistry of M₂ and M₃ receptors

Slides were immersed for 10 min with 4% (wt/vol) paraformaldehyde in PBS (pH 7.4). After washing with PBS, the slides were incubated with either rabbit anti-human M₂ polyclonal antibody (diluted 1:500) or rabbit anti-human M₃ polyclonal antibody (diluted 1:200) (Life Span Biosciences; Seattle, US) or rabbit IgG antibody control (Dako; Ely, UK) in PBS containing 10% (v/v) normal human serum (NHS) for 1 h at room temperature. After washing with PBS, the slides were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (Molecular Probes; Leiden, Netherlands) (diluted 1:1000) in PBS (pH 7.4) containing 10% (v/v) NHS for 1 h at room temperature. The slides were washed with PBS and then incubated with diaminidino phenylindole (DAPI) at 5µM in HBSS for 3 min. After washing the slides were mounted with 50% (v/v) PBS: 50% (v/v) glycerol. The slides were examined using a Leica TCS 4D confocal microscope (Leica Microsystems; Milton Keynes, UK) equipped with argon, krypton, and ultraviolet lasers.

FACS analysis of M₂ and M₃ receptors

Lung macrophages (10⁶ cells/ml) were permeabilised by the addition of ice cold methanol. Cells were then incubated in the absence or presence of either anti-rabbit

IgG, anti-M₂ receptor or anti-M₃ receptor antibodies for 1h at 4°C. The cells were washed with PBS and then resuspended in PBS containing 1% (vol/vol) BSA. All tubes were then incubated with the secondary antibody (goat anti-rabbit IgG) conjugated with PEcy5.5 for 30 min. Cells were washed with PBS and resuspended in FACS fix solution and samples analysed using a BD FACS Canto II flow cytometer and analysed using FACS Diva software (BD Biosciences, Oxford, UK). Data are expressed as the percentage of macrophages expressing the receptor of interest and as the ratio of the median fluorescence intensity (MFI) relative to the isotype control.

Measurement of IL-8 and IL-6 using ELISA

IL-8 and IL-6 were measured in the supernatants from MDM and tissue-derived macrophage incubated with ACh or carbachol using enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, UK). Lower limit of detection of this assay was 16pg/ml for both assays.

Measurement of LTB₄

Release of LTB₄ into cell culture media was measured using a commercially available kit from GE Healthcare (Buckinghamshire, UK) according to the manufacturer's instructions.

Measurement of ACh release

Release of ACh into the cell culture media was measured using a commercially available kit from Invitrogen Ltd., (Paisley, Scotland, UK), according to the manufacturer's instructions. Lower limit of detection of this assay is 1.5µM.

Cell viability assays

Cell viability was determined colourimetrically by measuring the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, MTT, to formazan by

mitochondrial dehydrogenases, as described previously [21]. None of the cell treatments altered cell viability.

Statistical Analysis

GraphPad Prism (GraphPad Software Inc., San Diego, California) was used to perform all statistical tests. When the data were analysed non-parametric distribution was assumed therefore the Wilcoxon matched paired test or Kruskal-Wallis test was used initially with Dunns' post test for ANOVA analysis. Results were considered significant when $p < 0.05$.

Results

Expression of components of the ACh synthesis pathway

In order to examine whether cells of the monocyte-macrophage have the capacity to synthesise ACh, the expression of components of the ACh synthesis pathway, CHT1, ChAT and VACHT were examined by real-time PCR. All three components were expressed by monocytes, MDM, lung macrophages and alveolar macrophages (Table 1). There was no difference in the level of expression of any of the components of the ACh synthesis pathway in any of the cells examined with the exception of significantly reduced expression of ChAT in lung macrophages (Table 1). Low levels of ACh were released and could be measured from lung macrophages ($6\pm 2\mu\text{M}$, $n=4$), however these levels were near the limit of detection of the assay.

Muscarinic Receptor Expression Analysed by Real-Time PCR

Having ascertained that cells of the monocyte-macrophage lineage express mRNA for proteins to drive synthesis of ACh, we next determined whether these cells could respond to this mediator. To this end, we examined the level of mRNA expression of M_{1-5} muscarinic receptors in cells of the monocyte-macrophage lineage. Expression of the muscarinic receptors M_{1-5} was detected on all cell types (Fig 1). The expression of M_1 receptor mRNA in lung-derived macrophages was significantly greater than that on monocytes and MDM (monocytes: 0.14 (0.06, 1.6)%, $n=24$, MDM: 0.16 (0.03, 0.8)%, $n=52$, lung-derived macrophages: 1.22 (0.5, 4.8)%, $n=27$, AM: 0.65 (0.2, 3.5)%, $n=16$) (Fig 1a). Expression of M_3 receptor mRNA in lung-derived macrophages and alveolar macrophages was significantly greater than that in monocytes and MDM (monocytes: 2.1 (0.9, 4.7)%, $n=15$, MDM: 0.06 (0, 0.3)%,

n=46, lung-derived macrophages: 51.4 (17, 179.4)%, n=22, alveolar macrophages: 42.1, (32, 90)%, n=13) (Fig 1c).

M₂ and M₃ receptor expression estimated by Immunohistochemistry

In order to confirm the expression data obtained using Taqman analysis regarding the expression of muscarinic receptors in cells of the monocyte/macrophage lineage, we performed immunocytochemistry. Due to the poor quality and availability of antibodies against human muscarinic receptors, this study was limited to expression of M₂ and M₃ receptors. M₂ and M₃ receptor expression was evaluated on MDM and lung-derived macrophages (Fig 2) with the M₂ receptor predominant on the cell membrane. Expression of the M₃ receptor on MDM appeared to be both membrane-associated and cytosolic (Fig 2). In lung-derived macrophages expression of M₂ and M₃ receptors was also detected on the cell membrane (Fig 2a). The immunocytochemistry was validated using FACS (Fig 2b), where M₂ expression was observed on 50.8±17.3% of lung macrophages with an MFI of 5.4±1.2, n=5 and M₃ expression was observed on 66.5±17.3% of lung macrophages with an MFI of 6.9±1.3, n=5. Expression was also determined in cells obtained from BAL and sputum samples from non-smokers, smokers and patients with COPD. Cells obtained from BAL from all three groups expressed similar levels of both M₂ and M₃ receptor (Fig. 3), with expression associated with the macrophages. Similarly, macrophages obtained from induced sputum expressed similar levels of the M₂ receptor (Fig 4) with less expression of the M₃ receptor (Fig 4).

Function of muscarinic receptors on MDM and lung-derived macrophages

Having determined the expression of muscarinic M₂ and M₃ receptors on the surface of cells of the macrophage lineage the function of these receptors was then investigated. Neither MDM, nor lung macrophages stimulated with the stable analogue of ACh, carbachol (100µM), for up to 24h released measurable level of either IL-8 or IL-6 (data not shown). Similarly, MDM exposed to 100µM carbachol did not lead to the release of LTB₄ (Fig 5a) (Buffer: 278.9±84.9 vs. carbachol: 230.4 ± 84.8 pg/ml, n=6). In contrast, carbachol stimulated LTB₄ release from lung-derived macrophages (Fig. 5b) (Buffer: 222.3±75.0 vs. carbachol: 1118 ± 622.4 pg/ml, n=15). In order to investigate the mechanism of carbachol-stimulated LTB₄ release from lung macrophages, cells were pre-treated with the muscarinic receptor antagonists 4-DAMP or AF-DX116 prior to stimulation with carbachol. The release of LTB₄ from carbachol-stimulated lung macrophages was inhibited in a concentration-dependent manner by 4-DAMP (Fig. 6a). Maximal inhibition (58.9±6.6%, n=9) occurred at 30nM with an EC₅₀ value of 5.2±2.2 nM. The effect of pre-treating these cells with AF-DX116 and gallamine were less effective with maximal inhibition of 42±15.1%, and 36.4±15.6%, n=5 respectively.

Discussion

This study demonstrated that mRNA for components of the ACh synthesis pathway were expressed by both monocytes and macrophages. Human mononuclear cells have been reported to contain ACh [22] and ChAT is expressed by rat monocytes [23] and human alveolar macrophages [24], however we have now demonstrated expression of CHT1 and VACHT in cells of the monocyte-macrophage lineage suggesting that these cells are capable of ACh synthesis and release of ACh from lung macrophages could be measured, but were very low and near to the limit of detection of the assay. Nevertheless, ACh may reach sufficient concentrations to act locally and thus contribute to the inflammatory response. This is further substantiated by expression of muscarinic receptor mRNA in cells of the monocyte-macrophage lineage.

The present study demonstrated expression of mRNA for muscarinic M₁-M₅ receptors in human monocytes in contrast to a previous report [25]. This may reflect increased sensitivity of real-time PCR methodology. However, the present study showed increased expression of M₁, and M₃ mRNA in lung derived macrophages compared with monocytes. This may suggest that as monocytes differentiate towards a macrophage phenotype there is a concomitant change in expression of muscarinic receptors. However, lung tissue macrophages were obtained from the tissue of patients undergoing surgery for lung cancer. Although, the tissue was macroscopically normal, it is not known whether the tumour promoting environment may alter the expression of muscarinic receptors locally. Nevertheless, these data are consistent with reports of M₃ receptor expression in alveolar macrophages [12,13] and were further substantiated with immunocytochemistry. Using this technique, it was observed that despite little difference between MDM and lung

macrophages with respect to M₂ mRNA expression, there appeared to be increased protein expression in the MDM. Similarly, despite lung derived macrophages expressing significantly greater quantities of M₃ mRNA compared with MDM, protein expression by immunocytochemistry appeared reduced. This suggests that mRNA levels of muscarinic receptors may not reflect protein expression in these cell types. To address this further, we used FACS analysis of lung macrophage expression of M₂ and M₃ receptors and demonstrated expression of both receptors. Of note in induced sputum samples, muscarinic receptor M₂ and M₃ expression appeared to be restricted to the macrophage population. However, in contrast to a previous study [13] we did not see an increase in macrophage M₃ receptor expression in cells from COPD patients and this was corroborated in BAL macrophages.

Despite a lack of alteration of either M₂ or M₃ expression in macrophages from COPD patients, there is no doubt that these receptors are expressed by macrophages and MDM. These data led to a subsequent investigation into the role of these receptors on the macrophage surface. Stimulation of macrophages with carbachol did not stimulate the release of either IL-8 or IL-6 confirming a previous study whereby stimulation with ACh did not release IL-8 or monocyte chemotactic protein (MCP)-1 (CCL2) from alveolar macrophages [4]. Bovine alveolar macrophages produce LTB₄ following stimulation with ACh [12] and we demonstrated a similar effect of carbachol on lung derived macrophages. This appeared to be mediated via the M₃ receptor since this effect could be abrogated by 4-DAMP but not AF-DX116 or gallamine. It is possible that ACh may mediate inhibitory effects via nicotinic receptor activation, however Birrell *et al.*, 2008 [26] demonstrated that nicotine is not inhibitory in human lung macrophages. However, approximately 40% residual LTB₄ release was not affected by blockade of the M₃ receptor indicating that

LTB₄ release from lung macrophages stimulated with carbachol may invoke other pathways. For example, activation of the ERK pathway has been shown to be involved in LTB₄ release from ACh activated sputum cells from patients with COPD is isolated monocytes from healthy volunteers [13]. It is unlikely that this could be attributed to a feedback of LTB₄ stimulation on the macrophage as we have shown previously that this does not occur in lung macrophages [27]. The release of LTB₄ by carbachol stimulation was not observed in MDM despite expression of both M₂ and M₃ receptors on the surface of these cells. This may reflect uncoupling of these receptors from subsequent downstream signalling events. However, we have recently demonstrated that lung derived macrophages exhibit a greater capacity to synthesise and release LTB₄ when compared to MDM and therefore may not be the best cell type to use for study of these responses [27].

In summary, cells of the monocyte-macrophage lineage express components of the non-neuronal cholinergic system with the capacity to both synthesise and respond to ACh. The role of this system in regulating macrophage function is less clear but appears to regulate the release of LTB₄, in part, via the muscarinic M₃ receptor. Therefore, antagonists of the M₃ receptor might contribute to the control of inflammatory status such as the release of LTB₄ from macrophages in addition to the inhibitory effect of smooth airway contraction and suggest an additional role for these drugs in COPD and other inflammatory lung diseases [28].

Acknowledgements

This study was supported by Boehringer Ingelheim GmbH. It was supported by the NIHR Respiratory Disease Biomedical Research Unit at the Royal Brompton and

Harefield NHS Foundation Trust and Imperial College London. AK was supported by fellowships from Pfizer and Kanae Foundation for Life & Socio-medical Science, Japan.

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Figure legends

Figure 1. Expression of Muscarinic Receptor mRNA from Monocytes, MDM, Lung Derived Macrophages and Alveolar Macrophages

Expression of mRNA for muscarinic receptors M₁ (panel a), M₂ (panel b), M₃ (panel c), M₄ (panel d) and M₅ (panel e) from monocytes (mono) n=15-24, MDM n=33-54, lung macrophages (LM) n=22-28 or alveolar macrophages (AM) n=13-16 were analysed using real-time PCR and compared to the HPRT control gene. Data are presented as each individual data point and the horizontal line represents median values and * represents p<0.05 and *** p<0.001

Figure 2. Expression of M₂ and M₃ muscarinic receptors on MDM and Lung Macrophages

Panel a) MDM and lung macrophages were cultured on chamber slides and immunocytochemistry performed for either an isotype control (IgG) or M₂ and M₃ muscarinic receptor expression. The figure is representative of at least three independent samples. Panel b) A representative FACS histogram showing expression of M₂ and M₃ receptors on lung macrophages. Iso = isotype control.

Figure 3. Expression of M₂ and M₃ muscarinic receptors on cells from BAL.

BAL cells from non-smokers, smokers and patients with COPD were prepared as cytopins and immunocytochemistry performed for either an isotype control (IgG) or M₂ and M₃ muscarinic receptor expression. The figure is representative of at least three independent samples from each subject group.

Figure 4. Expression of M₂ and M₃ muscarinic receptors on cells from induced sputum.

Cells obtained from induced sputum from non-smokers, smokers and patients with COPD were prepared as cytopins and immunocytochemistry performed for either an isotype control (IgG) or M₂ and M₃ muscarinic receptor expression. The figure is representative of at least three independent samples from each subject group.

Figure 5. Release of LTB₄ from carbachol-stimulated MDM and lung macrophages.

MDM (panel a) or lung macrophages (panel b) were incubated in the absence (buffer) or presence of carbachol (100µM) for 30 min. Media was harvested and LTB₄ released into the cell media measured by EIA. Data are presented as each individual data point where * indicates p<0.05.

Figure 6. Effect of muscarinic receptor antagonists on the release of LTB₄ from carbachol-stimulated lung macrophages.

Lung macrophages were pre-treated for 30 min with either 4-DAMP (panel a) or AF-DX116 (panel b) or gallamine (panel c), prior to stimulation with carbachol (100µM) for 30 min. Media was harvested and LTB₄ measured by EIA. Data are normalised to carbachol stimulation (100%) and are presented as mean ± SEM, n=5-9.

Table 1. Expression of CHT1, ChAT and VACht mRNA in human cells of the monocyte-macrophage lineage

	CHT1	ChAT	VACht
Monocytes	1.6 ± 0.37 n=24	0.96 ± 0.25 n=25	22.9 ± 6.8 n=14
MDM	2.2 ± 1.1 n=53	0.78 ± 0.18 n=47	21 ± 5.7 n=21
Lung macrophages	6.8 ± 4.0 n=30	0.32 ± 0.09*, ++ n=27	35.1 ± 10.4 n=10
Alveolar macrophages	1.7 ± 0.6 n=15	0.47 ± 0.11 n=12	19.6 ± 13 n=7

Data are presented as mean ± SEM of the ratio of CHT1, ChAT and VACht gene expression compared with HPRT control gene, where * indicates p<0.05 for differences to monocytes and ++ p<0.01 for differences to MDM.

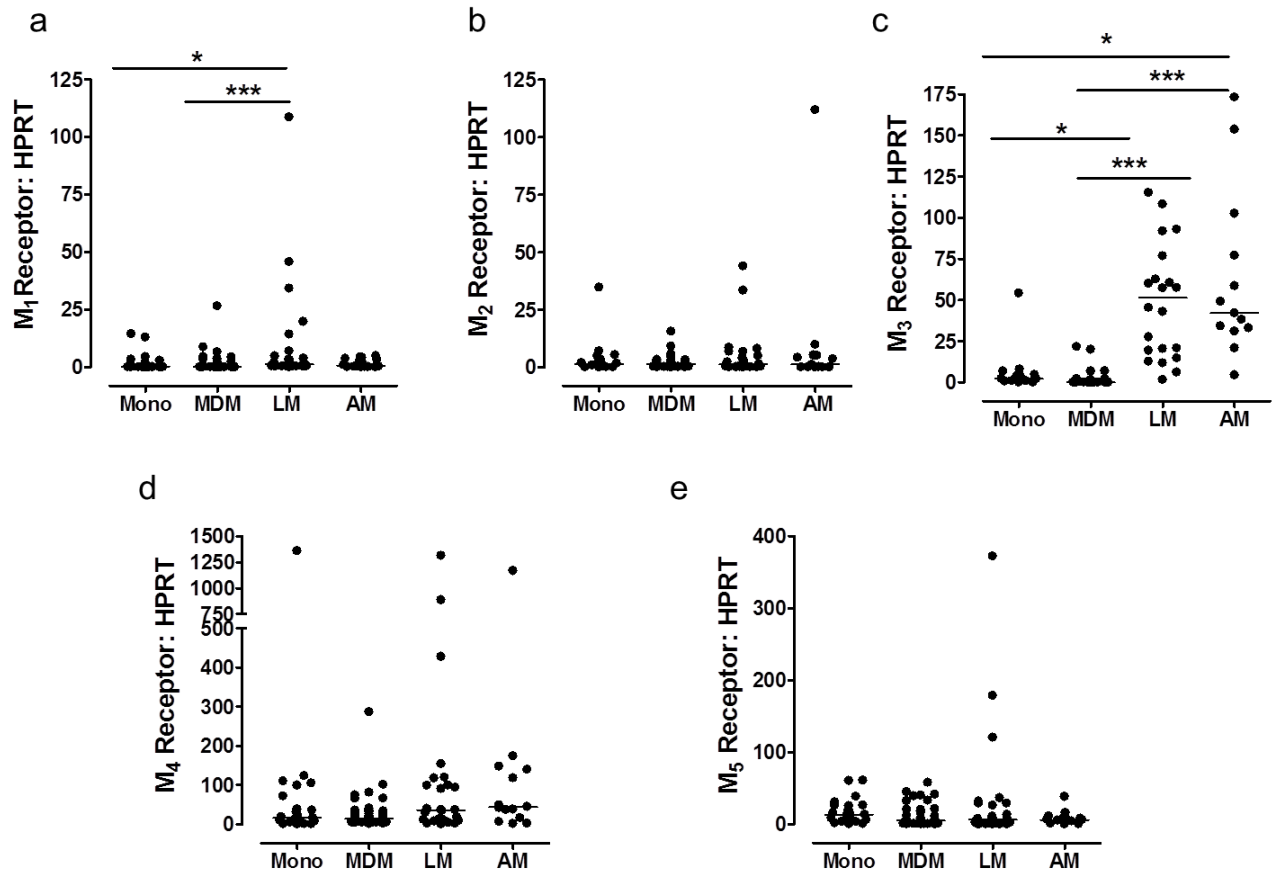


Figure 1

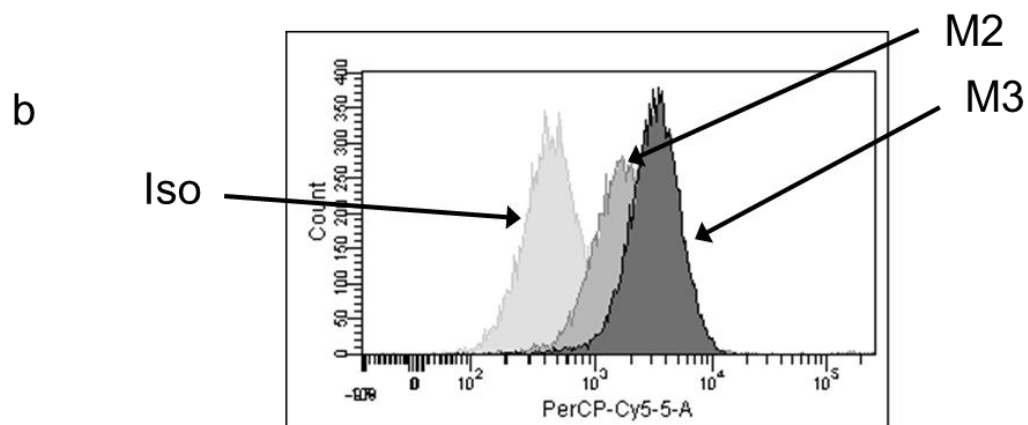
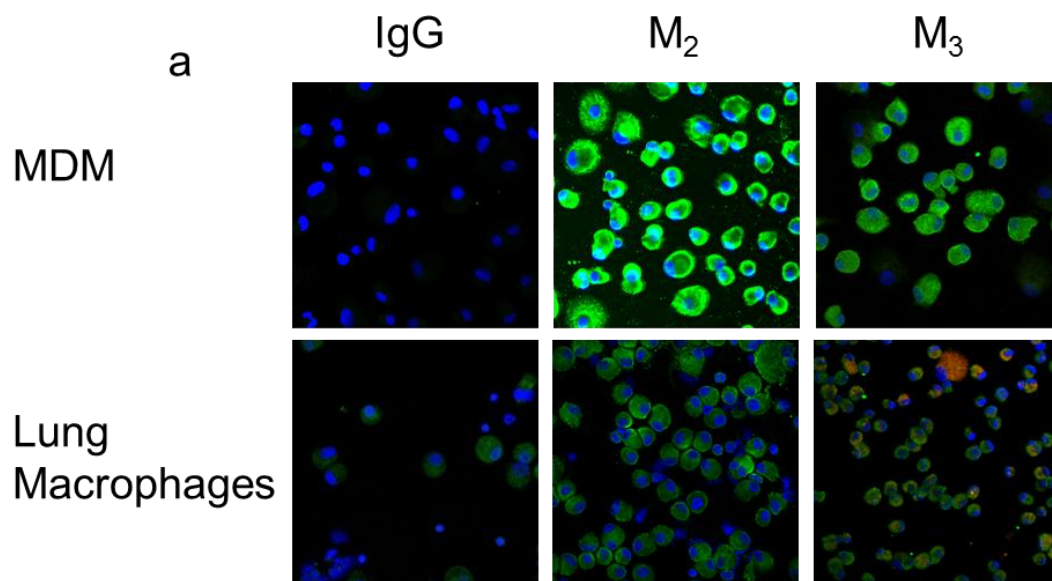


Figure 2

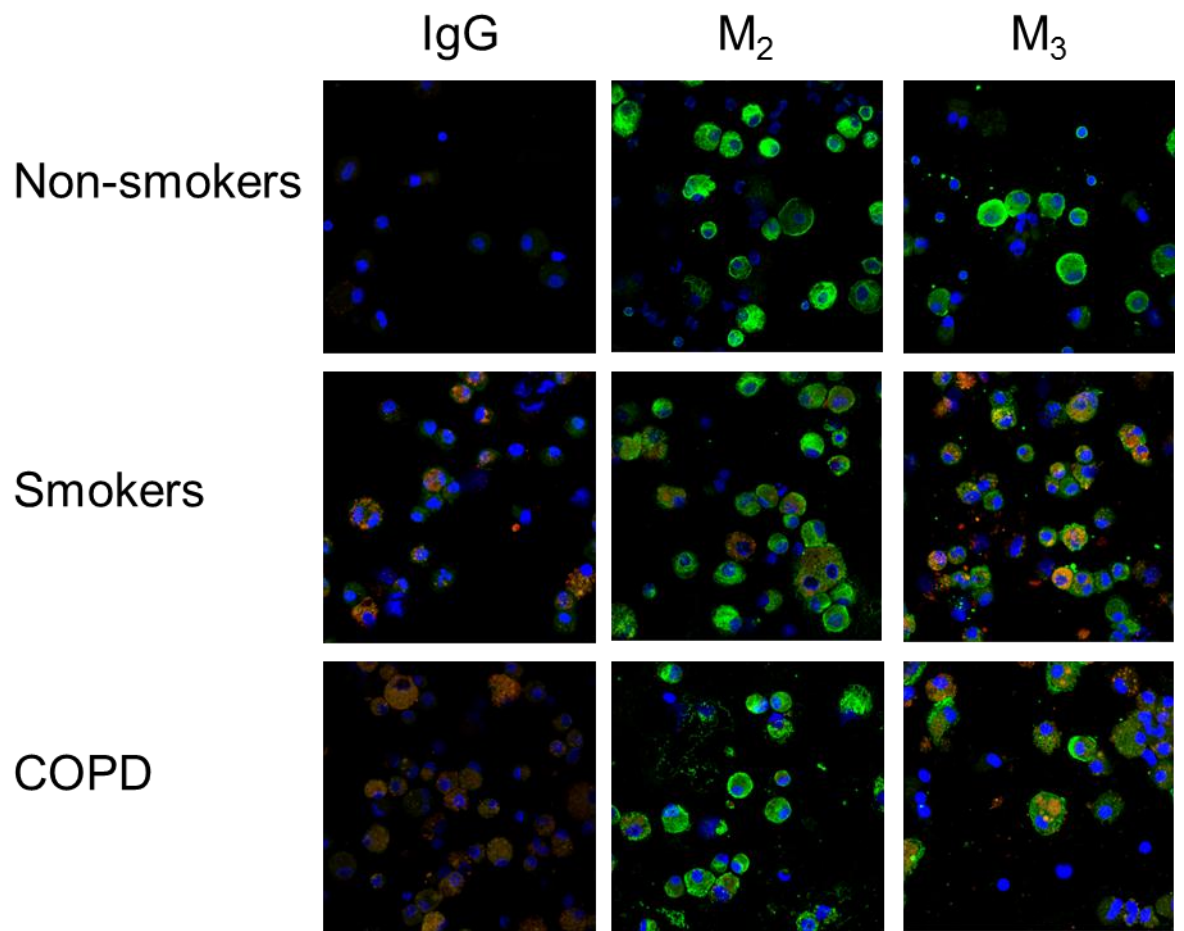


Figure 3

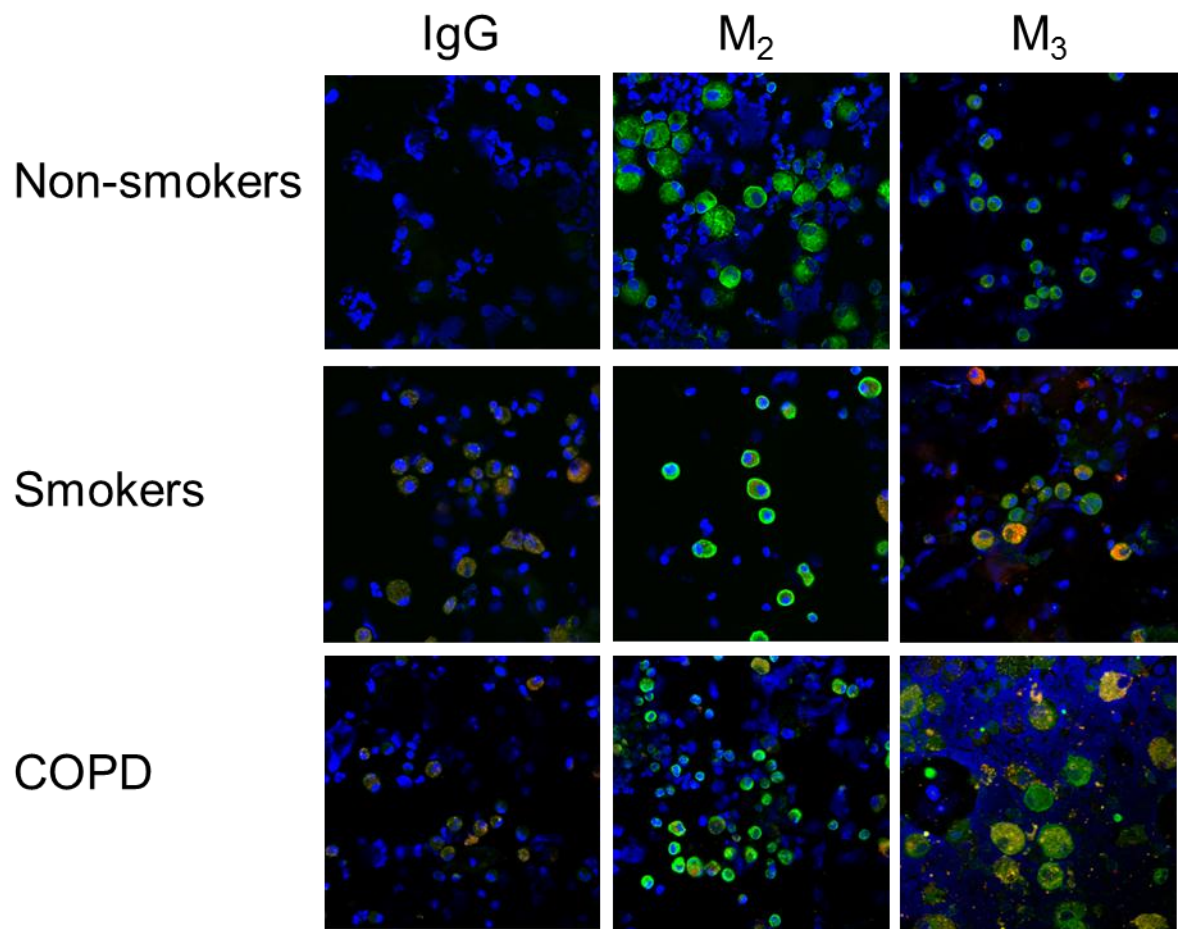


Figure 4

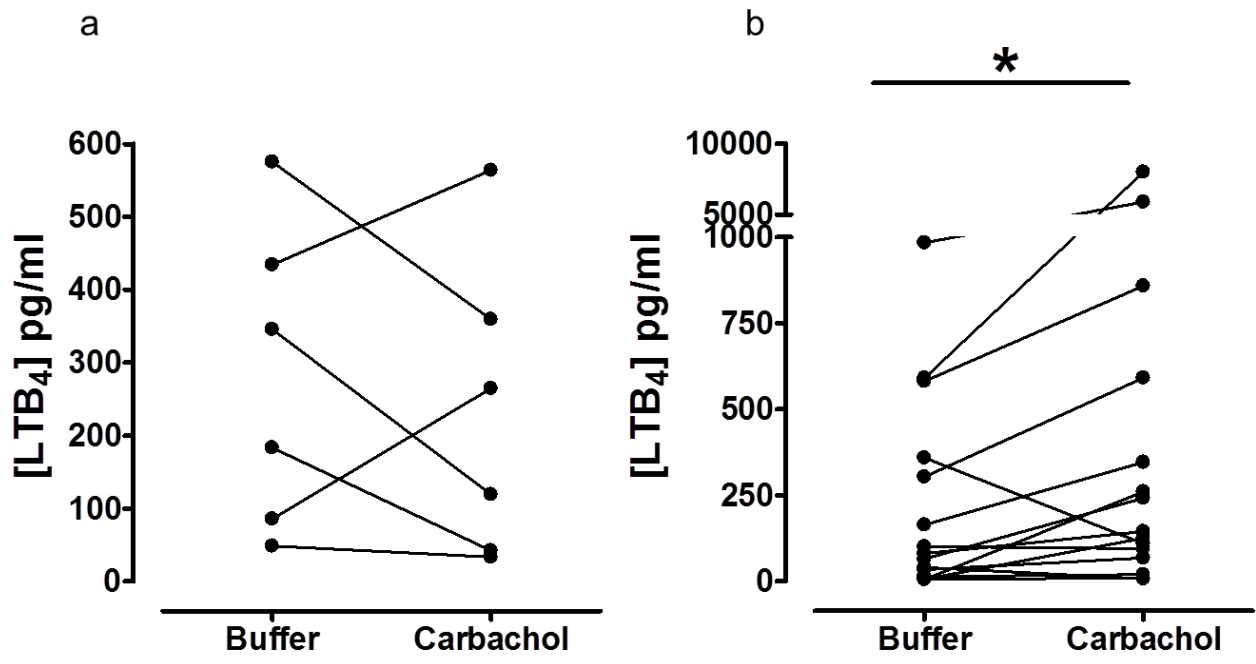


Figure 5

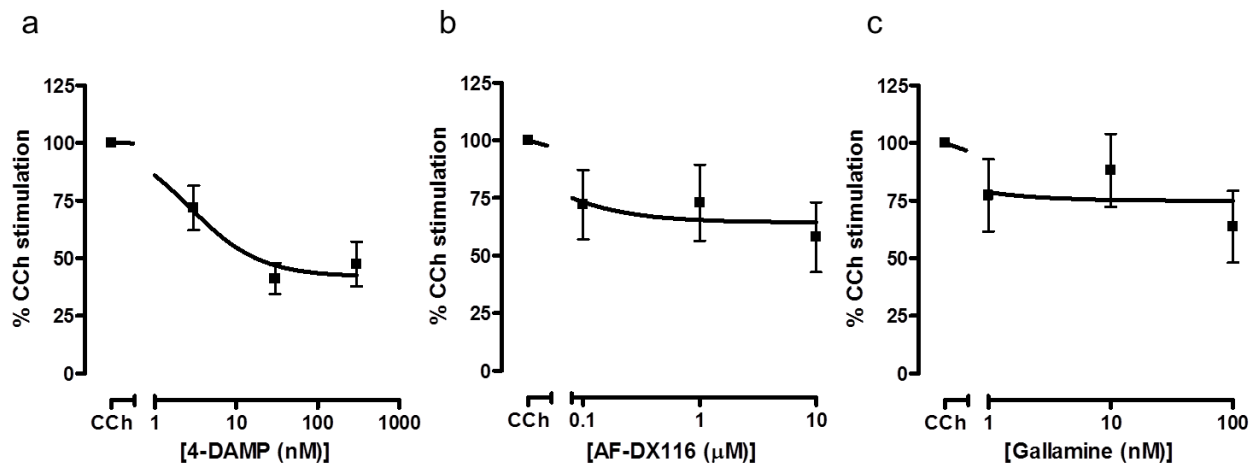


Figure 6