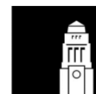


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# Standardized protocols for differentiation of THP-1 cells to macrophages with distinct M(IFNγ+LPS), M(IL-4) and M(IL-10) phenotypes

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## Abstract

*In vitro* models of differing macrophage functions are useful since human monocyte–derived macrophages are short–lived, finite and vary from donor to donor. Published protocols using the promonocytic cell line THP-1 have tended to result in cells that closely resemble classically-activated macrophages, differentiated in IFNγ and LPS. However, no protocol, to date, has fully recapitulated polarization of THP-1 to the M(IL-4) or M(IL-10) macrophage phenotypes seen when human monocyte-derived macrophages are exposed to each cytokine. Here we present protocols that can be used to prepare M(IL-4) polarized THP-1 that transcribe *CCL17*, *CCL26*, *CD200R* and *MRC1* and M(IL-10) cells which transcribe *CD163*, *C1QA* and *SEPP1*. We show that the inhibitory Fcγ Receptor IIb is preferentially expressed on cell surface of M(IL-4) cells, altering the balance of activating to inhibitory Fcγ Receptors.

Adoption of standardized experimental conditions for macrophage polarisation will make it easier to compare downstream effector functions of different macrophage polarisation states, where the impact of PMA exposure is minimized and rest periods and cytokine exposure have been optimized.


## Abbreviations

PMA: phorbol 12-myristate 13-acetate; LPS: lipopolysaccharide; DAG: diacyl glycerol; TNF: tumor necrosis factor; AML: acute monocytic leukaemia; TGM2: transglutaminase 2; M(IFNγ+LPS): macrophages differentiated in interferon gamma and lipopolysaccharide; M(IL-4): macrophages differentiated in interleukin 4; M(IL-10): macrophages differentiated in interleukin 10.


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## Highlights

THP-1 cells can be differentiated and polarised to produce both classically and alternatively–activated macrophages

THP-1 macrophages transcribe markers consistent with peripheral blood macrophages treated with IFNγ+LPS, IL-4 or IL-10

The activatory FcγRI is downregulated and inhibitory FcγRIIb is upregulated in M(IL-4) macrophages derived from THP-1 cells, altering the ratio of activatory to inhibitory FcγRs.

## 1 Introduction

Human circulating monocytes can be differentiated into macrophages and polarized into various functional subtypes. Macrophage polarization, formerly conceptualised as "classical" versus "alternative" activation, is now understood to comprise a wide spectrum of macrophage subtypes, including deactivation, depending on the combination of physiological or pharmacological stimuli [1]. Although many protocols exist for producing classically-activated or M(IFNγ+LPS) macrophages, to date attempts to recreate the full spectrum of macrophage polarization in human cell lines have met with limited success.

The THP-1 cell line is an immortalized, human AML–derived cell line [2]. THP-1 transcribe *MS4A3* that has been shown to be a marker for murine granulocyte monocyte progenitors (GMPs) and their progeny[3], suggesting that THP-1 cells are part of this lineage. Resting THP-1 cells have more phenotypic characteristics of monocytes than other myeloid cell lines, including the ability to differentiate to macrophages when treated with phorbol-12-myristate 13-acetate (PMA) [4 5]. Pertinently, THP-1 cells respond to lipopolysaccharide (LPS) by inducing TNF expression to high levels showing they have an intact Toll-like receptor 4/nuclear factor κB (TLR4 / NFκB) pathway (unlike the histiocytic cell line U937) [6]. It has also been shown that some of the MAP kinase pathways are intact in THP-1cells: anisomycin induces TNF expression that is sensitive to inhibitors of MEK1/2 and the human p38 analogue [7]. Because THP-1 cells retain most of the inflammatory monocyte signalling pathways, they were chosen as a suitable cell line model for hMDMs.

Treatment of THP-1 cells with PMA as a priming step, followed by rest and treatment with either proinflammatory or regulatory cytokines results in macrophage-like cells that express some of the marker genes typical of human monocyte-derived macrophages (hMDMs) [8-10]. The addition of PMA alone results in cells attaching to tissue culture plastic and adopting a stellate morphology. These cells express some macrophage differentiation markers (CD11β, CD14 and CD36) and can phagocytose and release TNF to a greater extent than untreated cells [11]. Resting the cells after PMA stimulation results in a phenotype more like M(IFNγ+LPS) hMDMs [9 12], with increased transcription of inflammatory cytokines, such as TNF and IL-1β [13].

Initial priming with a high concentration of PMA (100ng/ml) appears to compromise polarization to alternatively-activated macrophages (formerly known as M2a macrophages) when using IL-4, IL-13 or a combination of both these cytokines. These cells failed to upregulate M(IL-4)-specific chemokines and chemokine receptors (CCL1, CCL17, CCL22 and CCL24, CCR2, CXCR1 and 2) [14]. However, priming THP-1 cells with 10ng/ml PMA for 24 hours and then resting for 24 hours before exposing the primed cells to IL-4 for 48 hours resulted in cells which transcribed *MRC1* [15], which encodes the C-type lectin mannose receptor C 1 (CD206). This receptor has been consistently used as a marker of macrophages differentiated in both IL-4 and IL-10 [16].

The THP-1-derived M2 cells also upregulated CD163 that was previously widely used as a marker for anti-inflammatory (M2) macrophage subtypes [17]. However, it is now known that transcription of this haemoglobin scavenger receptor is increased in M(IL-10), but not M(IL-4) macrophages [18]. CD163 is also upregulated by glucocorticoids along with C1QA and IL-10 on macrophages of a "deactivated" phenotype [19], providing further support for the concept that it marks a population of cells distinct from the M(IL-4) population. CD163 is also upregulated on the surface of THP-1 cells primed with PMA and exposed to IL-10 [20]. We wished to develop protocols for differentiating THP-1 cells to distinct M(IL-4) and M(IL-10) phenotypes with the appropriate markers [21]. Although alternatively-activated macrophages (formerly known as M2b) can be differentiated in the presence of immune complexes (ICs), the difficulties of standardizing this reagent precluded development of this macrophage subset in this study.

We also sought to determine whether the THP-1 polarised cell lines could be used as models to study clinically-relevant macrophage functions mediated by Fcγ receptors (FcγRs). This receptor family has been postulated to be involved in many processes of health and disease [22] and is involved in the activation and regulation of macrophage functions following the binding of IgG and IgG-containing ICs. Activatory FcγRs are characterized by having an immunoglobulin receptor tyrosine activatory motif (ITAM) or interacting with an ITAM-bearing accessory protein when bound by ICs. Activating functions include phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), antigen presentation and release of pro-inflammatory mediators [23]. These functions are inhibited by ICs binding to FcγRIIb [24], which consists of an ectodomain highly homologous to FcγRIIa and an intracellular immunoglobulin receptor tyrosine inhibitory motif (ITIM)[25]. We were interested in whether the M(IL-4) or M(IL-10) macrophages expressed more of the inhibitory FcγRIIb and less of the activatory receptors than M(IFNγ+LPS) cells. Previous work showed that priming THP-1 cells with PMA decreased expression of the activatory FcγRIa and FcγRIIa receptors, but increased expression of both activatory FcγRIIIa and inhibitory FcγRIIb receptors [26]. Transcription of FcγRIIb was also upregulated in both M(IFNγ+LPS) and M(IL-4) cells derived from human blood monocytes [27]. The balance of activatory to inhibitory FcγRs, has been shown to be critically important in a variety of animal models of autoimmune diseases [28] and efficacy of therapeutic antibody therapies, confirmed in mice engrafted with human tumour cells [29].

Recent attempts to polarize the cell line THP-1 to both classically-activated M(IFNγ+LPS) and alternatively-activated M(IL-4) phenotypes similar to those of hMDMs have been broadly successful in producing cells with most of the appropriate cell surface proteins, when exposed to low doses of PMA [30]. However, the M(IL-4) cells did not upregulate CD206 on the surface and the THP-1 derived M(IL-10) cells did not show higher levels of CD163.

Here, we describe a set of protocols that can also be used with either IFNγ and LPS or IL-4 to produce macrophages transcribing appropriate classical activation markers (*CXCL9* and *10,*

*IRF1*) or alternative activation markers (*CCL17, ALOX15, CCL26, TGM2, CD200R* and *CD206*). Similar conditions can be used for polarizing PMA-primed THP-1 cells with IL-10 which result in cells that transcribe *CD163*, *C1QA* and the M(IL-10) marker gene *SEPP1* [31], but not *MRC1*. The M(IL-4) cells also express lower levels of FcγRIa and higher levels of the inhibitory FcγRIIb than other macrophage subtypes, supportive of inducing an immunomodulatory phenotype.

## 2 Materials and Methods

### 2.1 Macrophage Marker literature review

Transcriptional markers previously used to define different macrophage subsets were identified from the literature. Experimentally validated and frequently used transcripts were summarized into Table 1 (along with key references).

### 2.2 Cell culture

THP-1 cells (ECACC, Salisbury, UK) were counted and seeded at a concentration of 300,000 cells/ml in antibiotic-free RPMI media + 10% FCS (Sigma-Aldrich, St.Louis, MO) and maintained at 37ºC, 5% $CO_2$ in a humidified tissue culture incubator. THP-1 cells underwent mycoplasma testing as a quality control measure, and short tandem repeat (STR) profiling was performed to confirm cell type prior to experiments.

Human monocyte-derived macrophages from healthy controls, who had provided written consent, were used as positive controls for the polarization of THP-1 cells. Peripheral blood was collected from healthy volunteers by a trained phlebotomist and diluted 1:1 with PBS (Sigma-Aldrich, St.Louis, MO). The PBMCs were separated by spinning the diluted blood on top of a cushion of Lymphoprep density gradient medium according to Manufacturer's protocol (STEMCELL Technologies, Vancouver BC). The layer of mononuclear cells was taken and washed in 15ml PBS twice.

Macrophages were prepared from peripheral blood by plating out PBMCs in Corning 100mm tissue culture dishes. After two hours non-adherent cells were removed and the medium was replaced with fresh medium containing either IFNγ+LPS (20ng/ml and 250ng/ml respectively) or IL-4 (20ng/ml) or IL-10 (20ng/ml). The cells were maintained in medium with cytokines for seven days. RNA was extracted after the medium was removed by adding Qiagen RNeasy minikit lysis buffer (buffer RLT) directly to the adherent cells.

### 2.3 PMA exposure

All the differentiation conditions used PMA (Sigma-Aldrich, St. Louis, MO) added to RPMI media + 10% FCS for 24h to prime the THP-1 monocytes into macrophage-like cells followed by washing the PMA off and a 24h rest period in fresh media prior to exposure to polarizing cytokines. 100mm polystyrene dishes specially treated for tissue culture were used (Corning 430167 Sigma-Aldrich, St. Louis, MO). Initially, THP-1 cells were treated with 2.5, 5, 10, 20 and 50ng/ml PMA for the 24h priming period before being rested for 72h and exposed to polarizing cytokines. Micrographs were taken at 40x and 100x magnification.

The effective concentration for stimulation of Protein kinase C responsive genes was also investigated: THP-1 cells were treated with 0, 2.5, 5, 10, 20, 40, 80 and 160ng/ml PMA for 24h prior to 24h rest and subsequent RNA harvest.

THP-1 cells were differentiated into M(IL-4) macrophages after priming with either 5 or 50ng/ml PMA for 24h before a 72h rest period and polarization with IL-4 (see below) before RNA harvest, cDNA synthesis and RT-PCR for M(IL-4) marker gene transcripts (see below).

### 2.4 Rest period

Post PMA priming rest time was increased incrementally to include 24h, 48h, 72h and 96h periods, prior to cytokine exposure for 48h. RT-PCR was performed to show transcripts from M(LPS+IFNγ) and M(IL-4) marker genes in the appropriate cell types.

### 2.5 Cytokine titration

Initial cytokine concentrations of 250ng/ml LPS (Peprotech, NJ, USA) and 20ng/ml IFNγ (Peprotech, NJ, USA) and 20ng/ml IL-4 (R&D Systems, MN, USA) were deduced from the literature.

For M(IFNγ+LPS) cells, IFNγ and LPS concentrations were kept consistent for the 48h cytokine exposure time. Potential M(IL-4) cells were treated with IL-4 concentrations of 20, 25 and 30ng/ml for the 48h polarization period. M(IL-10) macrophages were prepared by priming and resting as above, then treating the cells for 48h with IL-10 at 20ng/ml (R&D Systems, MN, USA).

### 2.6 Cytokine exposure time

THP-1 cells were initially incubated with IFNγ+LPS or IL-4 for different durations: 24h, 48h, 72h and 96h. We found 48h was optimal for these activators so IL-10 was subsequently applied for 48h to induce “deactivated” M(IL-10) cells in order to keep the exposure time for polarizing treatments consistent.

### 2.7 RNA extraction

Cells were washed in PBS and lysed using the Qiagen RLT plus buffer from the lysate according to the Qiagen RNeasy plus kit (Germantown, MD) protocol. Briefly, lysate was added to a genomic DNA exclusion column and centrifuged at 10,000xg to remove any genomic DNA. Lysate was then diluted at a 1:1 ratio with 70% ethanol and added to a mini-spin column before another 10,000xg centrifugation, adhering the RNA to the column membrane and removing the buffer. RNA was then washed once with RW1 buffer and twice in RPE buffer to remove impurities. RNA was then eluted using RNase-free deionized water,

RNA concentrations were measured and purity was checked using a Nanodrop-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples were diluted with UV treated $dH_20$ to 100ng/ml and then stored at -20°C for up to a week prior to cDNA synthesis.

### 2.8 cDNA synthesis

cDNA was produced according to the Invitrogen Superscript II protocol. Briefly 2.5µg RNA template was mixed with oligo dT (0.5µg) (Invitrogen, Carlsbad, (CA) and dNTPs (Bioline, London, UK) and heated to $65^0$C to remove secondary structures. Reactions were put on ice while First Strand buffer and DTT were added, and the mixture was heated to $42^0$C for 2 minutes so primers could anneal to RNA template. 1unit Superscript II enzyme was added and the reactions were heated to $42^0$C for 50 minutes. The reactions were stopped by heating to 65°C for 15 minutes prior to RT-PCR. Transcripts from a 60S subunit ribosomal protein gene (*RPL37A*) were generated as a control for the amount and quality of RNA in the cDNA synthesis.

### 2.9 Macrophage transcript analysis

Primers producing an amplicon of approximately 100bp were designed for genes identified as potential markers; the gene names and inducing agents are shown in Table 1. Primers were designed to span exon junctions so any genomic contamination produced a larger amplicon. Primers were designed to have a GC content of around 50%, Tm of approximately $60^0$C, and a two base GC clamp at the 3' end. Primers were checked for secondary structures using the basic Northwestern Oligocalc (http://www.basic.northwestern.edu/biotools/oligocalc.html) and specificity was verified using the UCSC *in silico* PCR tool (https://genome.ucsc.edu). Details of primers and amplicon lengths for M(IFNγ+LPS), M(IL-4) and M(IL-10) marker transcripts can be found in Table 2.

See Table 3 for details of reagents used. All PCRs were run using cycling conditions described in Table 4. Template and relevant primers were combined on ice with PCR buffer, magnesium chloride, dNTPs and *Taq* polymerase. Initial denaturation was performed at $95^0$C to remove secondary structures. A short denaturation was included at the start of each cycle, followed by 25 to 30 cycles including a 30" step at an empirically determined annealing temperature (see Table 4) and an elongation phase at $72^0$C.

For each transcript, cDNA was derived from human monocyte-derived macrophages polarized in either (IFNγ+LPS), IL-4 or IL-10

### 2.10 Agarose gel electrophoresis

All PCR products were run on a 3% agarose/tris acetate EDTA (TAE) gel for 75 minutes at 170V in TAE buffer, using a Bio-Rad Laboratories (CA, USA) electrophoresis system. Gels were imaged using a Bio-Rad "ChemiDoc MP" gel documentation system and ImageLab software.

### 2.11 Flow cytometry

Trypsin was added to flasks until cells became detached and RPMI media + 10% FCS was added to neutralize the effects of the enzyme. The cell suspension was centrifuged at 400xg for 5 minutes and re-suspended in a lower volume of media. Cell number was determined using the Countess automated cell counter (Invitrogen, Carlsbad, CA). 200,000 cells were added on to a 96 well plate for each sample, blocked in 2ng/ml IgG1 for 30 minutes on ice and stained with 100 times diluted FITC-conjugated anti-CD16 (DJ130c [FcγRIIIa], Merck Millipore, Massachusetts, USA) or FITC-conjugated anti-CD32 (IV.3 [FcγRIIa], StemCell Technologies, Vancouver, BC or 3D3 [FcγRIIb], BD Biosciences, CA) or PE-conjugated anti-CD64 [FcγRIa] (BioRad, CA USA) in the dark for 1h. Cells were subsequently washed in FACS buffer (PBS, 2% FCS, 2mM EDTA) and fixed in FACS fix (50% FACS buffer, 2% formaldehyde). Flow cytometry was performed using the plate reader arm of a Cytoflex cytometer (BD Biosciences, San Jose, CA) and data was recorded for the APC and FITC parameters. A total of four biological replicates were carried out as separate experiments.

Since the FcγRIIa expressed on THP-1 cells has histidine at position 131, the anti-CD32 monoclonal antibody 3D3 only recognizes FcγRIIb and FcγRIIc (which have arginine at the equivalent position [26]). THP-1 do not express FcγRIIc since both alleles contain stop codons, so any expression detected from 3D3 on THP-1 cells is FcγRIIb.

All analyses were performed using FlowJo software (BD Biosciences, San Jose, CA). Univariate distributions of flow cytometric data files from cells stained with the FcγRIIb–specific antibody (CD32-3D3) and the corresponding isotype control were compared by probability binning in 300 bins using FlowJo software, to obtain the T(x) metric [32].

### 2.12 Western Blotting

Cell extracts from each condition were made in RIPA buffer containing protease inhibitors (HALT protease inhibitors, Thermo Fisher Scientific Waltham, MA). Proteins were resolved by electrophoresis in precast gels (Bio-Rad, Hercules, CA) transferred to nitrocellulose membrane (GE Healthcare UK). After blocking in 5% non-fat milk powder the blots were probed with antibodies against TGM2 and β-actin as a loading control for the amount and quality of the extracted proteins. The antibodies used were as follows: TGM2 and β-actin Thermo Fisher Scientific (MA5-12739, MA5-15739). All primary antibodies were diluted 1:1000 before use and detected using HRP-conjugated secondary antibodies (Dako) according to Manufacturer's recommendations (Agilent, Santa Clara, CA).

## 3 Results

### 3.1 PMA concentration is critical for adherence, marker gene transcription and M(IL-4) polarization: higher PMA concentrations inhibit differentiation of THP-1 to cells transcribing M(IL-4) markers

Because previous attempts to polarize PMA-primed THP-1 cells to either a M(IFNγ+LPS) or a M(IL-4) phenotype were more successful using lower PMA concentrations [33], we used a range of PMA concentrations in order to establish the minimum concentration of PMA that would induce cell adhesion. Figure 1A shows micrographs of THP-1 cells primed with varying amounts of PMA, then differentiated with either IFNγ and LPS (top two rows) or IL-4 (bottom two rows). The magnification factors are shown for each row. Cells treated with 2.5ng/ml PMA adhered, but at concentrations less than 2.5ng/ml the cells did not stick to the plastic dishes used in this study. At higher concentrations cells with a spindle-like or stellate morphology were observed in both differentiation schemes, suggesting a more M(IFNγ+LPS)-like phenotype.

Because the initial priming signal induced transcripts known to be associated with M(IFNγ+LPS) cells (*IRF1* and *STAT1*), we tested for reduction of these transcripts in cells rested for 24h, 48h, 72h, 96h and 120h. *IRF1* and *STAT1* transcripts peaked at 48h and started to fall after 72h rest (see Figure 1B). We then tested for M(IL-4) transcripts in THP-1 rested for 72h after priming with 5ng/ml or 50ng/ml to confirm the inhibitory effect of higher concentrations of PMA on M(IL-4) polarization. Figure 1C shows RT-PCRs from THP-1 cells differentiated in IL-4 at 20ng/ml, 25ng/ml and 30ng/ml. Note that transcripts from M(IL-4) marker genes *ALOX15* and *CCL17* were greatly reduced in cells primed with the higher concentration of PMA. A concentration of 20ng/ml IL-4 was sufficient to induce transcription of the M(IL-4) marker genes following exposure to 5ng/ml PMA and a 72h rest period.

### 3.2 Rest period of 72 hours after PMA priming allows transcription of M(IL-4) marker genes

To further validate our optimisation protocol, we evaluated a wider range of M(IFNγ+LPS) and M(IL-4) transcripts. THP-1 were primed with PMA at a concentration of 5ng/ml and rested for 24h, 48h, 72h and 96h prior to 48h exposure to IFNγ and LPS (Figure 2A) or IL-4 at a concentration of 20ng/ml (Figure 2B). The M(IFNγ+LPS) markers *CXCL9* and *CXCL10* were transcribed in cells rested for 24h, but *IRF1* was more strongly transcribed in cells rested for 72h (Figure 2A). The presence of these transcripts suggests that priming with 5ng/ml PMA is sufficient for subsequent polarization to a M(IFNγ+LPS) phenotype. M(IL-4) transcripts were transcribed more strongly in cells rested for 48h or 72h than those rested for 24h. A rest period of 72h was chosen for use in subsequent experiments.

### 3.3 Cytokine exposure period following PMA priming and rest was critical for specific marker expression: marker genes were transcribed after 48 hours in macrophages differentiated in either LPS and IFNγ or IL-4.

Previous attempts to make polarized macrophages have used different cytokine exposure times. Shorter exposure times may have precluded identification of marker transcripts. The optimal exposure times for LPS+IFNγ and for IL-4 were investigated: THP-1 cells were primed with 5ng/ml PMA for 24h and rested for 72h before exposure to cytokines for 24h, 48h, 72h and 96h. Figure 3A shows M(IFNγ+LPS) markers *CXCL9* and *CXCL10* were transcribed after 24h, but *IRF1* showed the highest levels of transcription after 48h and 72h.

Figure 3B shows RT-PCR results for transcripts from M(IL-4) marker genes in cells differentiated in 20ng/ml IL-4 for 24h to 96h. Weak signals from *ALOX15* and *TGM2* transcripts were present in THP-1 differentiated in IL-4 for 24h, but *CCL17* transcripts were increased in cells after 48h of cytokine exposure following the rest period. 48h was chosen as the exposure time for both macrophage subsets so that THP-1 cells of both M(IFNγ+LPS) and M(IL-4) phenotypes could be obtained for further experiments on the same day.

### 3.4 Macrophages polarized under M(IFNγ + LPS), M(IL-4) and M(IL-10) conditions have unique transcript profiles

Having optimized each step of our THP-1 differentiation protocol we then sought to determine whether there was co-expression of markers between the different macrophage subsets. THP-1 cells were primed with 5ng/ml PMA and rested for 72h. The cells were then treated with IFNγ and LPS (20ng/ml and 250ng/ml) or IL-4 (20ng/ml) or IL-10 (20ng/ml) for 48h before harvesting, cDNA synthesis and RT-PCR.

M(IFNγ+LPS) transcripts (*CXCL9*, *CXCL10* and *IRF1*) were only observed in cells treated with LPS + IFNγ (Figure 4A). Having already shown our M(IL-4) protocol can be used to generate macrophages which transcribe the marker genes *ALOX15, CCL17* and *TGM2* (see Figures 2 and 3), we tested for the presence of transcripts from other well-known M(IL-4) marker genes. Transcripts from *MRC1* (encoding CD206) and *CD200R* were seen in M(IL-4), but not the other polarization conditions. *TGM2* transcripts were strongest in M(IL-4) cells, with lower levels of expression observed in other conditions (see Figure 4B). Western blotting demonstrated increased expression of TGM2 in THP-1 cells differentiated with IL-4 compared to THP-1 cells under other polarization conditions (see Supplementary Figure 1).

Our results suggest that it is possible to induce *CD163* transcription by exposing THP-1 cells to IL-10 for 48h after resting the cells for 72h. Transcripts from *CD163* were strongest in M(IL-10) cells, with only a trace in PMA-primed and M(IL-4) cells (see Figure 4C). Whilst *C1QA* was transcribed in THP-1 differentiated in IFNγ+LPS the transcription of this marker gene was higher in M(IL-10) cells. *SEPP1* was transcribed in PMA-primed cells as well as THP-1 treated with IL-10. Importantly, when the combination of the three M(IL-10) transcripts *CD163, C1QA* and *SEPP1* were considered, only M(IL-10) polarized THP-1 cells

expressed *CD163* in addition to *C1QA* or *SEPP1,* recapitulating the profile of markers observed in hMDM with an anti-inflammatory phenotype [34] .

To ensure reproducibility, mRNA was extracted from the macrophages from a replicate experiment and RT-PCR was carried out as previously, with similar results: the gels from this biological replicate are shown in Supplementary Figure 2.

### 3.5 Modulation of activatory and inhibitory FcγR expression: FcγRIa is downregulated in M(IL-4) and M(IL-10) cells but FcγRIIb is upregulated in M(IL-4) THP-1cells

The transcription of well-known marker genes led us to investigate expression of receptors relevant to key macrophage functions. Due to the high level of sequence homology, FcγR cell surface expression was evaluated by flow cytometry for each THP-1-derived macrophage population (Figure 5). FcγRIIa was expressed on all macrophage populations at a similar level to that seen in resting THP-1 cells, which have a monocytic phenotype. Downregulation of FcγRIa, the high affinity, activatory FcγR, was observed when THP-1 macrophages were polarised with either IL-4 or IL-10. The activatory receptor FcγRIIIa was not upregulated on any of the macrophage populations following priming with 5ng/ml PMA. The inhibitory FcγRIIb was expressed at higher levels on M(IL-4) cells than on other macrophage populations. This was seen in three other separate repeats of this experiment. Data from three biological replicates are shown in Supplementary Figure 3, which demonstrate that similar findings were observed.

In order to determine whether the apparent upregulation of FcγRIIb expression on M(IL-4) cells was significant and biologically meaningful, we compared distributions of the level of expression on each macrophage population with the matched isotype control using the Probability Binning Method [32]. For each experiment, we demonstrated upregulation of FcγRIIb on M(IL-4) when compared with paired isotype controls. The T(x) metric was at least two fold higher in M(IL-4) than any other macrophage population in each experiment (M(IL-4) T(x) 59-319, compared with M(IFNγ+LPS) T(x) 7.3-78).

## 4 Discussion

Our efforts to refine and standardize THP-1 differentiation and polarization protocols were based on sequential optimization of each stage in the protocol. Firstly, we applied reduced concentrations of PMA to prime the THP-1 cells for differentiation. We found that concentrations below 5ng/ml reduced the adherence of THP-1 cells to the tissue culture plastic and so 5ng/ml was chosen to be the minimum concentration. We showed PMA exposure in the absence of cytokines was sufficient to induce M(IFNγ+LPS) transcripts (*IRF1* and *STAT1*). These genes are also downstream of the IFNγ receptor[35] and so PMA would be expected to give a signal similar to IFNγ, skewing macrophage polarization towards a classical/M(IFNγ+LPS)-like phenotype. We showed washing the PMA off the cells after 24h exposure and resting them in fresh medium for 72h reduced transcription of *IRF1* and *STAT1*, which might explain why it was possible to induce M(IL-4) and M(IL-10) phenotypes following this rest period.

Our results suggest M(IL-4) polarization was inhibited when priming with higher concentrations of PMA, since *CCL17* and *ALOX15* transcripts were absent from cells differentiated in IL-4 exposed to 50ng/ml PMA. Our results also show an absence of M(IL-4) transcripts in cells rested for 24h rather than 72h. A review of previous protocols showed that short rest periods led to low M(IL-4) or M(IL-10) marker gene expression [36 37 30]. Published protocols used variable IL-4 concentrations and our results suggest a concentration of 20ng/ml is sufficient for expression of the well-known M(IL-4) transcripts *ALOX15*, *CCL17* and *TGM2*. We confirmed by immunoblotting that strong TGM2 protein expression was only observed in M(IL-4) cells.

After resting the PMA primed THP-1 cells for 72h, 48h of cytokine exposure was optimal for upregulation of both M(IFNγ+LPS) and M(IL-4) transcripts. Our final validation experiment also demonstrated that 48h of cytokine exposure resulted in cells expressing markers of a single macrophage polarization state. Of note, IL-4 exposure also induced *CD200R* and *MRC1* (CD206) transcripts, consistent with hMDMs exposed to IL-4. Previous studies had shown that increased time of exposure to IL-4 and IL-13 increased the transcription of *MRC1* in THP-1–derived macrophages, despite the high concentrations of PMA used to prime the THP-1 cells.[38]. However, these cells also transcribed slightly higher levels of *CD163*, a marker traditionally considered to be induced by IL-10 or by glucocorticoids [35]. The M(IL-4) cells should be *CD163* negative [21].

Our M(IL-4) protocol allowed generation of cells that transcribed *CD206,* but not *CD163.* By contrast we showed polarization of PMA-primed cells with IL-10 upregulated *CD163* far more than *CD206*. This is the first time that THP-1 macrophages have been polarized into both of these subtypes of alternatively–activated macrophages. Consequently, we suggest that transcription of *CD163* is a marker of M(IL-10) phenotype rather than for both alternatively-

activated and deactivated macrophages, (or M2a and M2c populations, using the previous macrophage nomenclature [37]). A recent report showed exposure of PMA-primed THP-1 cells to IL-10 upregulated mRNA and surface protein expression of CD163 in a similar manner to monocyte-derived macrophages [20].

Previous attempts using THP-1 cells primed with 1ng/ml PMA for several days and then exposed to IL-10 resulted in cells which did not express CD163 on the cell surface [30], but our macrophages transcribed the gene for this receptor. Our protocol included a shorter exposure to slightly higher PMA concentration (24h with 5ng/ml rather than 96h with 1ng/ml) extended rest period (72h vs 24h) and cytokine exposure time (48h vs 24h). We envisage that our protocol might be a starting point for studies of atheroprotective macrophages which express high levels of CD163 [39], or CD163 positive red pulp macrophages which are important in removing opsonized platelets via FcγRs in idiopathic thrombocytopenia purpura [40].

Correspondingly, the THP-1 cells differentiated in IL-4 may provide a good model for macrophage functions involved in the resolution of inflammation, including tissue remodeling and phagocytosis. Clearance of ICs and IC-opsonised cellular debris by phagocytosis is dependent on FcγRs [26,41]. Downstream signalling following IC stimulation is dependent on the balance of activatory to inhibitory FcγRs and we therefore investigated expression of these receptors on the surface of cells from different macrophage subtypes.

Expression of FcγRs is important for development of macrophage subtypes as shown previously using hMDMs [42]. We have previously shown that THP-1-derived macrophages exposed to higher levels of PMA (50ng/ml overnight followed by 24h of rest) expressed the activatory receptor FcγRIIIa along with FcγRIIa and FcγRIIb [26]. These cells secreted TNF on exposure to ICs and LPS; blocking each of the activatory receptors with specific $F(ab')_2$ fragments reduced the response [26]. The lower levels of PMA exposure and longer rest period of three days in our present study, however, resulted in cells that did not express substantial levels of FcγRIIIa so that FcγRIIa is the main low affinity activatory Fc receptor on M(LPS+IFNγ) cells. Using our optimized macrophage polarization protocols, we showed that FcγRIa was expressed on M(IFNγ+LPS) cells at similar levels to untreated THP-1 cells. However, M(IL-4) and M(IL-10) cells derived from THP-1 expressed FcγRIa at much lower levels. This is consistent with previous publications using hMDMs [27] and THP-1 [30].

We exploited the known THP-1 *FCGR2A*, *FCGR2B* and *FCGR2C* genotype to examine the expression of the class II FcγRs, using commercially available antibodies. FcγRIIa was expressed on all cell types in this study, in accord with previous work [27], whereas FcγRIIb was only expressed on M(IL-4) cells. Thus, the M(IL-4) cells have lower levels of activatory FcγRIa and increased levels of inhibitory FcγRIIb, consistent with a reduction of activatory signals through this pathway during the resolution phase of inflammation.

Previous studies have shown that M(IL-4) cells had the lowest levels of phagocytosis of opsonized cells[27]. Our new protocols will aid further investigation of the importance of FcγRIIb on macrophage populations, either when directly targeted by ICs or in conjunction with a variety of specific antibodies developed to complement therapeutic antibody strategies [29].

Having shown the development of protocols for differentiation of THP-1 cells using the standard stimuli (IFNγ+LPS and IL-4 or IL-10) resulted in the transcription of appropriate subset-specific transcripts, we propose that our THP-1 protocols might be considered for models of M(IL-4) and M(IL-10) subtypes. We show the THP-1 derived M(IL-4) cells are the only subtype that expressed TGM2 at high levels and that they also express the clinically important inhibitory receptor, FcγRIIb.

These new, optimised protocols will thus be useful for multiple downstream functional studies of M(IFNγ+LPS), M(IL-4) and M(IL-10) macrophages and more widespread adoption will allow greater harmonization between laboratories and an ability to directly compare different macrophage populations under differing experimental conditions. Furthermore, these protocols may be useful for further development of novel therapies that either harness or modulate the effect of FcγRIIb [29].

**Tables**

**Table 1**. Transcript panel selected for optimization. Source refers to publications showing or recommending use of these genes for macrophage subtypes polarized by the inducing agents indicated

| **Gene** | **Inducing agents** | **Source** |
|---|---|---|
| *CXCL9* | M(IFNγ+LPS) | [21,43] |
| *CXCL10* | M(IFNγ+LPS) | [21,43] |
| *IRF1* | M(IFNγ+LPS) | [21,43] |
| *STAT1* | M(IFNγ+LPS) | [44] |
| *ALOX15* | M(IL-4) | [43] [21] |
| *CCL17* | M(IL-4) | [43] |
| *MRC1* | M(IL-4) | [21,43] |
| *CD200R* | M(IL-4) | [21,45] |
| *TGM2* | M(IL-4) | [21] |
| *CD163* | M(IL-10) | [46] |
| *C1QA* | M(IL-10) | [19] |
| *SEPP1* | M(IL-10) | [47] |

**Table 2** Sequences of transcript primers

| Gene | Forward primer sequence | Reverse primer sequence | length (bp) |
|---|---|---|---|
| ***CXCL9*** | dGCTGGTTCTGATTGGAGTGC | dGAAGGGCTTGGGGCAAATTG | 124 |
| ***CXCL10*** | dCCTTATCTTTCTGACTCTAAGTGG | dCTAAAGACCTTGGATTAACAGG | 107 |
| ***IRF1*** | dGGAAGGGAAATTACCTGAGG | dCTCCAGGTTCATTGAGTAGG | 101 |
| ***STAT1*** | dCAATGCTTGCTTGGATCAGC | dGTGATAGGGTCATGTTGCTAGG | 130 |
| ***ALOX15*** | dCAGATGTCCATCACTTGGCAG | dCTCCTCCCTGAACTTCTTCAG | 123 |
| ***CCL17*** | dCTTCTCTGCAGCACATCCAC | dCAGATGTCTGGTACCACGTC | 117 |
| ***MRC1*** | dCGAGGAAGAGGTTCGGTTCACC | dGCAATCCCGGTTCTCATGGC | 84 |
| ***CD200R*** | dCTGTACATAGAGCTACTTCCTGTTCC | dGCATTTCATCCTCCTCAACAACTGG | 187 |
| ***TGM2*** | dGCAGTGACTTTGACGTCTTTGCCC | dGTAGCTGTTGATAACTGGCTCCACG | 269 |
| ***CD163*** | dGAGGAGACCTGGATCACATGTGA | dGACCACAGCCAAGTTGTTGACACAC | 169 |
| ***C1QA*** | dGAGCATCCAGTTGGAGTTGAC | dCAAGTCCTCGGTCACCATAGA | 115 |
| ***SEPP1*** | dGTGGAGCTGCCAGAGTAAAAG | dCCAGGCTTCTCCACATTGCTG | 86 |
| ***RPL37*** **(control)** | dTTCCGCTCGTCCGCCTAATAC | dGGCCAGTGATGTCTCAAAGAG | 89 |

**Table 3** PCR reagents

Note that denaturation was at 95ºC for 2 minutes, annealing for 30 seconds and extension at 72ºC for 30 seconds.

| **Reagent** | **Concentration** | **Volume in 20µl reaction** |
|---|---|---|
| dH2O | N/A | 14.43 µl |
| Taq Buffer | 20x | 2 µl |
| Mg2+ (Applied Biosystems, CA, USA) | 50mM | 1.2 µl |
| dNTPs (Bioline, London, UK) | 0.4 µM | 0.4 µl |
| Forward primer (IDT, CA, USA) | 0.2µg/ml | 0.4 µl |
| Reverse primer (IDT, CA, USA) | 0.2µg/ml | 0.4 µl |
| Template | 2.5 µl | 1 µl |
| *Taq* polymerase | 1 unit | 0.17 µl |

**Table 4** Cycling conditions for marker PCRs

| **Gene** | **Annealing temperature (°C)** | **Number of cycles** |
|---|---|---|
| *CXCL9* | 63 | 25 |
| *CXCL10* | 55 | 25 |
| *IRF1* | 55 | 25 |
| *STAT1* | 60 | 30 |
| *ALOX15* | 60 | 30 |
| *CCL17* | 58 | 30 |
| *MRC1* | 60 | 30 |
| *CD200R1* | 60 | 30 |
| *TGM2* | 63 | 25 |
| *CD163* | 60 | 30 |
| *C1QA* | 60 | 30 |
| *SEPP1* | 60 | 30 |
| *RPL37A* | 63 | 25 |

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**Declarations of Interest:**

none

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**Figure Legends**

Figure 1: *Low concentrations of PMA should be used to prime THP-1 cells.*

(A) Photomicrographs taken after overnight exposure to varying concentrations of PMA, followed by a 72h rest and a 72h exposure to inducing agents. Higher PMA concentrations induce the stellate morphology typical of M(IFNγ + LPS) cells (top two rows), even in cells differentiated in IL-4 (bottom two rows). (B) shows RT-PCRs for transcripts from *IRF1* and *STAT1* from cells primed with varying concentrations of PMA; the bands seen in the positive control show transcripts from hMDMs differentiated in IFNγ and LPS. No reverse transcriptase (no RT) and no template negative controls are also included in the far right lanes. The housekeeping gene *RPL37A* acts as a loading control for cDNA synthesis. (C) shows transcripts for M(IL-4) macrophages, *ALOX15* and *CCL17,* when the cells are primed with 5ng/ml PMA (left panels). Fewer transcripts are seen when the cells are primed with 50ng/ml (right panels). Comparable levels of transcripts were seen at 20, 25 and 30ng/ml PMA.

Figure 2: *A rest period of 72 hours after PMA exposure was optimal for M(IL-4) polarization*

The effect of a 72h rest period following PMA priming at 5ng/ml. The cells were washed and rested in fresh medium for the times indicated, prior to incubation in either IFNγ+LPS (20ng/ml and 250ng/ml) or IL-4 (20ng/ml). (A) shows RT-PCRs for the M(IFNγ+LPS) marker transcripts *CXCL9*, *CXCL10* and *IRF1* from (IFNγ+LPS)-treated THP-1 cells and hMDMs as the positive control. (B) shows RT-PCRs for the M(IL-4) marker transcripts *ALOX15*, *CCL17* and *TGM2* from IL-4 treated THP-1cells and hMDMs treated with IL-4 as a positive control. The highest level of transcription from *IRF1* can be seen in cells rested for 48 hours or more. We concluded that a 72 hour rest might be ideal for THP-1 differentiation into both M(IFNγ+LPS) and M(IL-4)-like macrophages, given the higher levels of transcription from *ALOX15* and *CCL17* in cells rested for 72h prior to treatment.

Figure 3: *Exposure to polarising agents was optimal at 48 hours*

cDNA was made from cells differentiated for 24h, 48h, 72h and 96h and RT-PCRs for – transcripts of interest were run out on agarose gels. Panel (A) shows *CXCL9* and *CXCL10* transcripts from M(IFNγ+LPS) are present after 24h exposure. Note the *IRF1* transcripts were strongest after 48h. Panel (B) shows that transcripts from M(IL-4) marker genes *ALOX15, CCL17* and *TGM2* are stronger after 48h of exposure.

Figure 4: *Transcripts from the M(IL-4) marker gene MRC1 are present only in IL-4 treated THP-1 cells and* CD163 *transcripts are preferentially upregulated in IL-10 treated macrophages*

(A) shows RT-PCRs M(LPS+IFNγ) transcripts *CXCL9*, *CXCL10* and *IRF1* from THP-1 cells differentiated using the conditions shown below each lane alongside hMDMs differentiated with LPS + IFNγ as a positive control. (B) shows M(IL-4) transcripts *MRC1*, *CD200R* and *TGM2* from THP-1 cells differentiated using the conditions shown alongside human MDMs differentiated using IL-4. (C) shows M(IL-10) transcripts *CD163*, *C1QA* and *SEPP1* shown alongside human MDMs differentiated using IL-10. *RPL37A* acts as a loading control for the amount and quality of the cDNA synthesis.

Figure 5: *FcγR expression on M(LPS + IFNγ), M(IL-4) and M(IL-10) polarised THP-1 macrophages.*

Flow cytometric analysis of FcγR expression in the different macrophage populations. This figure shows an example of staining seen in four separate experiments. Specific antibody binding is shown in blue whereas the binding to the isotype control is shown in pink for each antibody. Monocytes represent untreated THP-1 cells. Inhibitory receptor FcγRIIb is expressed at higher levels in M(IL-4) macrophages. This is shown as an increase in the T(x) metric shown in the top right corner of the overlays. The M(IL-4) and M(IL-10) cells express lower levels of the high affinity, activatory receptor FcγRIa.

Supplementary Figure 1: *Western blots show strong expression of TGM2 in PMA primed, 72 hour rested THP-1 treated with IL-4 for 48 hours.*

Western Bot for TGM2 shows low levels of expression in M(LPS =IFNγ), M(IL-4) and M(0) cells (primed with PMA, rested, but not treated with cytokines) compared with untreated THP-1 (monocytes). Expression of this marker is much higher in M(IL-4) cells than the other macrophage subtypes. β-actin is shown as a loading for the amount and quality of the extracted proteins added to each well.

Supplementary Figure 2: *Replicate transcript data demonstrating reproducibility of M(IFNγ+LPS), M(IL-4) and M(IL-10) polarisation*

Panel (A) shows RT-PCRs for M(IFNγ+LPS) transcripts *IRF1, CXCL10* and *CXCL9* from THP-1 cells differentiated using the conditions shown below each lane alongside human monocyte-derived macrophages (hMDMs) differentiated with IFNγ+LPS as a positive control. Panel (B) shows M(IL-4) transcripts *ALOX15*, *CCL17* and *TGM2* from THP-1 cells differentiated using the conditions shown alongside human MDMs differentiated using IL-4. Panel (C) shows M(IL-10) transcripts *SEPP1* and *CD163* shown alongside human MDMs differentiated using IL-10. *RPL37A* acts as a loading control for the amount and quality of the cDNA synthesis. Resting (THP-1 that have not been primed with PMA or polarised with cytokines), PMA-primed and cytokine polarised macrophages (M(IFNγ+LPS); M(IL-4); M(IL-10)), two negative controls are shown with no reverse transcriptase (RT) or no template, with MDM serving as positive controls, polarised as indicated above.

Supplementary Figure 3: *Replicate experiments demonstrating FcγR expression on M(IFNγ + LPS), M(IL-4) and M(IL-10) polarised THP-1 macrophages.*

Cells differentiated using the final macrophage differentiation and polarisation protocols were stained with antibodies specific for each FcγR, as indicated above each column. Flow cytometry histograms for specific antibodies were created after cell debris was gated out. Histograms for each specific antibody are shown in blue, overlying the isotype control in pink. The difference in the distribution between the antibody staining and respective negative control was quantitated using the Probability Binning method of Roederer *et. al.* to give the T(x) metric. This value is shown in the top right corner of each overlay for FcγRIIb.