Immunobiology 220 (2015) 10–19

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

The activation status of human macrophages presenting antigen determines the efficiency of Th17 responses

Christina E. Arnold, Peter Gordon, Robert N. Barker [1], Heather M. Wilson [*,1]

Division of Applied Medicine, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen AB25 2ZD, UK

ARTICLE INFO

Article history:
Received 22 September 2014
Accepted 29 September 2014
Available online 13 October 2014

Keywords:
Antigen presentation
Human
Macrophage activation
T cell polarisation
Th17

ABSTRACT

Macrophages are antigen presenting cells that can adopt different activation states as directed by microenvironmental stimuli. It is well-recognised how CD4$^+$ T helper (Th) signals drive macrophage activation, but the ability of differentially activated human macrophages to stimulate the major types of CD4$^+$ T helper (Th) response by presenting antigen have not been well defined. Previous studies have focussed on murine cells, undifferentiated human monocytes, or macrophage products, and have been limited to non-physiological mitogenic Th responses. The aim was therefore to compare the Th cell polarising abilities of different human macrophage subsets when presenting specific antigen. We demonstrate for the first time that the way macrophages are activated, while naturally presenting antigen, has profound effects on downstream adaptive immune responses. In autologous co-cultures, LPS-activation was the most potent stimulus for antigen-loaded macrophages to drive Th17 polarisation from both unfractionated CD4$^+$ T-cells and the CD45RO$^+$ memory population, while IFNγ/LPS activated macrophages preferentially induced a Th1 phenotype. By contrast, IL-4-activated macrophages were ineffective in inducing responses by either Th subset. Although antigen-loaded dendritic cells were superior to macrophages in driving Th1 responses, the Th17 polarising capacity of the two antigen-presenting cell types was equivalent, and was strongly dependent on IL-1β secretion. Taken together, these results clearly demonstrate for the first time how differentially activated human macrophages present antigen to bias specific, rather than mitogen-driven, Th responses and lead us to propose that they impact adaptive immunity *in vivo*, particularly in determining Th17 polarisation within inflamed tissues.

Introduction

Macrophages are key features of inflammation, where their roles include elimination of infection, clearance of debris and restoration of tissue homeostasis (Murray and Wynn, 2011; Wilson, 2010). To fulfil these tasks, they can adopt different functional phenotypes dependent on the activating stimuli in their microenvironment. The extent of macrophage heterogeneity, and the polarising stimuli to which they respond, is subject to ongoing investigation, but distinct macrophage subsets have been described *in vitro* (Gordon and Taylor 2005; Martinez et al., 2008; Mosser and Edwards, 2008; Murray and Wynn, 2011). For example, classical or M1-activated macrophages, induced by microbial products in the presence or absence of IFN-γ, are microbicidal, tumoricidal and pro-inflammatory. Their dysregulation plays a central role in chronic inflammation and autoimmunity. In contrast, alternative or M2 activation by IL-4 and/or IL-13 gives rise to anti-inflammatory, tissue-remodelling cells. These subtypes are thought, however, to represent extremes of a continuum of activation states found *in vivo*. Although macrophages can act as professional antigen presenting cells (APC), and their heterogeneity impacts this role, it remains to be determined how efficiently each macrophage subset supports the responses of particular CD4$^+$ helper T (Th) subpopulations, notably the inflammatory Th17 type.

T cell differentiation is a central feature of adaptive immune responses, with polarisation into different subsets highly dependent on the APC and the cytokines they produce (Gutcher and Becher, 2007). CD4$^+$ helper T (Th) cells adopt different functional phenotypes characterised by cytokine secretion profiles, with, for example, Th1, Th2 and Th17 populations producing the respective signature cytokines interferon-γ (IFN-γ), interleukin-4 (IL-4) and IL-17A. Dendritic cells (DCs) are commonly viewed as the most

Abbreviations: APC, antigen presenting cell; DC, dendritic cell; KLH, keyhole limpet haemocyanin; PPD, purified protein derivative; SOCS, suppressor of cytokine signalling; TGM2, transglutaminase 2.

* Corresponding author. Tel.: +44 1224 437350; fax: +44 1224 437348.
E-mail address: h.m.wilson@abdn.ac.uk (H.M. Wilson).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.imbio.2014.09.022

potent APC, notably for naïve T cells (Iwasaki and Medzhitov, 2010), and are particularly effective at inducing Th1 responses. However, macrophages are typically the more abundant APC type within inflamed sites, where their numbers, but not those of DC, correlate with Th17 activity (Allam et al., 2011). One interpretation of these observations is that macrophages are at least as capable as DC in driving human Th17 responses, but this has not been tested directly, nor has the effectiveness of different macrophage subtypes been compared.

To date, the two-way relationships between different respective macrophage and Th subsets have been characterised largely from the perspective of the effects of Th cells on macrophages. Thus, it is well-reported that Th cell cytokines activate macrophages in inflamed sites, with Th1 and Th2 or T regulatory cells driving M1 and M2 macrophages, respectively (Gordon and Martinez, 2010; Murray and Wynn, 2011), but much less is known as to which of the macrophage subsets can drive different Th subpopulations, especially Th17 cells. In particular, key questions remain about the effects on the human Th response when each macrophage subtype presents antigen. Previous studies have either focussed on mice, or on LPS-activated human monocytes, macrophage culture supernatants or exogenously added examples of macrophage-derived cytokines when T cells respond to mitogenic stimuli such as anti-CD3/CD28 and/or PMA and ionomycin (Krausgruber et al., 2011; Mondal et al., 2010; Qin et al., 2012; Savage et al., 2008; Walter et al., 2013). Such approaches, although informative, do not fully recapitulate the natural interactions between the human cell types that stimulate Th cells specific for presented antigen. Moreover a direct comparison of differentially activated macrophages on autologous T cell responses has not been reported.

To address this, the aim was to determine how the activation state of human macrophages presenting antigen influences their ability to drive responses by different Th subsets. Our results identify the roles of differentially activated macrophages in shaping Th differentiation *in vitro*, and define the macrophage subsets most effective in supporting Th17 responses. These relationships have important implications *in vivo*, particularly for therapeutic manipulation of inflammation where macrophages are prominent.

Materials and methods

Cell isolation

Ethical approval for blood sampling from consenting healthy volunteers was granted by the Ethics Review Board of the College of Life Science & Medicine, University of Aberdeen in accordance with the declaration of Helsinki. Blood samples were taken by venepuncture and collected into EDTA vacutainers (Greiner Bio-one Ltd, Stonehouse, UK). Peripheral Blood Mononuclear Cells (PBMCs) were isolated by density gradient centrifugation using Lymphoprep (Axis-Shield, Dundee, UK) according to manufacturers' recommendation. Monocytes and T-cells were positively selected from PBMCs by magnetic-activated cell sorting (MACS), using anti-$CD14^+$ and anti-$CD2^+$ MicroBeads (Miltenyi Biotec, Surrey, UK). Memory T-cells were isolated by negative cell depletion using $CD45RO^+$ isolation kits (Miltenyi Biotec) and characterised by expression of CD45RO (Plebanski et al., 1992). Cell purity was confirmed by flow cytometry and was routinely ≥98% for positive cell selection and ≥95% for negative cell depletion.

Culture and differentiation of monocytes

Isolated monocytes were cultured in 24 well plates (4×10^5 cells/well) in DMEM (Lonza, Basel, Switzerland) supplemented with 2% penicillin/streptomycin (Life Technologies, Paisley, UK) and 10% heat-inactivated sterile filtered human AB^+ serum for 7 days to differentiate into macrophages (Arnold et al., 2014). Monocyte-derived dendritic cells (mo-DC) were generated by additional stimulation with 100 ng/mL human recombinant granulocyte macrophage colony-stimulating factor (GM-CSF) and 20 ng/mL human recombinant IL-4 (PeproTech, London, UK).

Macrophage and mo-DC activation and T-cell co-culture

Macrophages or mo-DC were stimulated with 1 μg/mL LPS (OIII:B4 *E.coli*, Sigma Aldrich, Poole, UK) alone or in combination with 20 ng/mL IFNγ (R&D Systems, Abingdon, UK), or 20 ng/mL IL-4 for 5 h. Additionally cells were loaded with either recall antigen 5 μg/mL mycobacterial purified protein derivative (PPD) (Statens Serum Institute, Copenhagen, Denmark), or primary antigen 10 μg/mL keyhole limpet haemocyanin (KLH) (Calbiochem, Nottingham, UK). PPD readily provokes *in vitro* recall responses by T cells from donors who have been vaccinated with Bacillus Calmette-Guérin, whilst KLH elicits naïve responses unless subjects have been deliberately exposed to the antigen (Barker and Elson, 1994; Pickford et al., 2007). Control cells were left untreated or were loaded with antigen only in the absence of activating factors. Approximately 1×10^6 autologous T-cells were added per well. T-cell responses have been determined to peak at day 5 in response to PPD and at day 8 in response to KLH (Barker and Elson, 1994; Pickford et al., 2007).

T-cell proliferation assay

Cells from co-cultures were incubated with [^{3}H]-thymidine (Amersham, Bucks, UK) in 96-well plates in triplicates for 6 h. DNA of cells was harvested onto glassfibre-filtermats by vacuum-manifold, and proliferation measured with a Trilux-Beta-counter LKB-WALLAC, Turku, Finland) in corrected counts per minute (CCPM).

RNA isolation and qPCR

Total cellular RNA was isolated from untreated or cytokine-stimulated human monocyte derived macrophages using Trizol (Life Technologies) extraction and cleaned up using the RNeasy mini kit (Qiagen, Manchester, UK). The concentration of RNA was assessed by spectrophotometry using the NanoDrop-100. Five hundred nanograms of RNA was reverse transcribed into cDNA using Oligo dT 15 Primer (Promega, Southampton, UK) and Superscript II (Life Technologies). The following primers were used: *TNFα* forward (cgctccccaagaagacag); *TNFα* reverse (agaggctgaggaacaagcac); *SOCS3* forward (cttcgactgcgtgctcaa); *SOCS3* reverse (gtaggtggcgaggggaag); *IL-6* forward (gatgagtacaaaagtcctgatcca); *IL-6* reverse (ctgcagccactggttctgt); *HLA-DR* forward (acaactacggggttgtggag); *HLA-DR* reverse (gctgcctggatagaaaccac); *TGM2* forward (ggcaccaagtacctgctca); *TGM2* reverse (agaggatgcaaagaggaacg); *MRC1* mannose receptor forward (ttcagaaggttttacttggagtga); *MRC1* mannose receptor reverse (tctccataagcccagttttca); *CISH* forward (agccaagaccttctcctacctt); *CISH* reverse (tggcatcttctgcaggtgt). Quantitative real time PCR was carried out by Lightcycler 480 (Roche) with the Universal Probe Library system (Roche, West Sussex, UK). Gene expression was analysed using the comparative C_t method with target gene mRNA levels being normalised to GAPDH. All values represent the mean ± SD of differently activated macrophages compared to non-stimulated cells.

Cytokine analysis

ELISA: Supernatants were collected from macrophage/mo-DC-T-cell co-cultures after 5 or 8 days of culture. Concentrations of

IFNγ, IL-10 (matched Ab-pairs; BD Biosciences, Oxford UK) and IL-17A (matched Ab-pair eBioscience, Hatfield, UK) were determined by ELISA. 96-well/plates (NUNC) were coated with primary antibody at 2 μg/mL in 0.1 sodium carbonate buffer pH 9 for 1.5 h at 37 °C followed by blocking of non-specific binding sites with 2% bovine serum albumin (BSA) in PBS for 1 h at 37 °C. Plates were washed twice and 100 μL/well of culture supernatant was loaded onto ELISA plates in duplicates and incubated for 1.5 h at room temperature. Secondary antibody was applied at 1 μg/mL (IFNγ) and 0.5 μg/mL (IL-17A) in 0.2% BSA PBS for 1.5 h at room temperature. Plates were incubated with 100 μL/well ExtrAvidin (1:10,000) (Sigma Aldrich) for 1 h followed incubation with phosphatase substrate 1 mg/mL (Sigma Aldrich) until optimal development was achieved. Substrate development was measured with Labsystem Multiscan MS plate reader and cytokine levels were calculated from standard curves generated with Genesis software using a cubic spline smoothed function.

Cytometric bead array: Supernatants of macrophages and mo-DC was collected 24 h after activation and stored at −80 °C until assayed. Cytometric Bead Arrays were performed in parallel for IL-1β, IL-6 and IL-12p70 (all BD flex kits) according to manufacturer's instructions. The results were acquired on a BD FACSArray.

Phenotypic analysis of cells by flow cytometry

Macrophages and mo-DC were harvested with dissociation solution (HBSS 2 mM EDTA, 1 mM sodium citrate) on ice. Cells were stained with anti-CD14-APC (Miltenyi Biotec), anti-CD11b-PE, anti-CD11c-Pacific Blue, anti-CD1a-FITC and anti-HLA-DR-FITC (all BD Biosciences) in staining buffer (PBS 2%FCS 2 mM EDTA) for 30 min. Cytokines secretion from T-cells was blocked by addition of 2 mM monensin (eBiosciences) for 10 h prior to cell staining. T-cells were transferred to 5 mL flow cytometry tubes and were first stained for extracellular markers using anti-CD4-Pacific Blue, anti-CCR4-PE-Cy7, anti-CCR6-PerCP-Cy5.5, anti-CD45RA-AlexaFlour720, (eBiosciences) and anti-IL-23R-FITC (R&D Systems) on ice for 30 min. Cells were fixed and permeabilised for 20 min on ice. Cells were stained for intracellular markers with anti-IL-17A-APC (BD Biosciences) and anti-RORγt-PE (eBiosciences) antibodies for 30 min on ice. Fluorescence of cells was acquired on LSRII (BD Biosciences) and analysed with FCS Express (De Novo software, Los Angeles, CA).

Statistical analysis

Significant differences were calculated with PRISM 5.0 software (Graphpad, San Diego, CA, USA) using a non-parametric paired test (Wilcoxon-Signed-Rank-Test) or as indicated in Figure legends.

Results

Differentially activated human macrophages show distinct gene expression profiles

Few studies have compared directly the expression profiles of activation markers for different subsets of *in vitro* generated human monocyte derived macrophage mainly because these have not been scrutinised or cannot be agreed upon (Ambarus et al., 2012; Jaguin et al., 2013; Martinez et al., 2013; Murray and Wynn, 2011; Vogel et al., 2014). Therefore, we first confirmed the difference in phenotypes between LPS-, IFN-γ/LPS- and IL-4-activated human macrophages by analysing the gene expression levels of maturation markers that have been suggested to be upregulated (Ambarus et al., 2012; Arnold et al., 2014; Jaguin et al., 2013; Martinez et al., 2013; Murray and Wynn, 2011; Vogel et al., 2014). LPS induced the expression of TNF, SOCS3 and IL-6, as compared to non-stimulated cells (Fig. 1). Activation with IFN-γ/LPS showed a more potent effect, with greater mRNA expression of TNF, SOCS3, IL-6 and HLA DR than that of LPS stimulation alone. In contrast to LPS- and IFN-γ/LPS activated macrophages, IL-4 activation increased expression of the human M2 macrophage markers transglutaminase 2 (TGM2), CISH and macrophage mannose receptor (CD206) but not of the M1 markers SOCS3, TNF or IL-6. These results identify unique expression profiles that can classify differentially activated human macrophages, and point to phenotypic differences between our LPS, IFN-γ/LPS and IL-4 activated populations that could impact on their functions.

Differential activation of macrophages presenting antigen shapes the type of Th response

A primary aim was to compare the efficiency with which antigen presentation by differentially activated human macrophages drives each of the major types of Th response *in vitro*. Our studies were deliberately designed to test the results of antigen-specific interactions between macrophages (as oppose to their monocyte precursors) and autologous Th cells, rather than relying on more contrived conditions such as unnatural mitogenic or anti-CD3 activation in the presence of macrophage-derived cytokines.

Macrophage presentation to stimulate primary responses

The first experiments determined the capacity of activated macrophages to drive naïve Th cell polarisation in response to a primary antigen, KLH (Barker and Elson, 1994; Pickford et al., 2007), since such naïve responses are most tractable in terms of their activation and differentiation. As shown in Fig. 2A, certain types of activated macrophage pulsed with KLH readily stimulated T cell proliferation by day 8 of co-culture, confirming the ability of our system to support responses to primary antigen presentation. Such relatively extended culture periods have previously been shown to allow naïve T cell responses to develop (Barker and Elson, 1994; Pickford et al., 2007). LPS or IFNγ/LPS-activated macrophages induced significantly higher T cell proliferation in response to KLH compared to those that had not been activated, or had been treated with IL-4. IL-10 activated macrophages supported only negligible responses (data not included). Thus, induction of primary T cell proliferation by macrophages is strongly dependent on its activation status.

Next, the ability of differentially activated, KLH antigen-loaded macrophages to polarise Th1, Th17 or anti-inflammatory cytokine responses was compared, by determining the concentrations of IFN-γ, IL-17A and IL-10, respectively, in culture supernatants. In all experiments, the levels of IL-4, indicative of Th2 polarisation, were low or undetectable (<3 pg/mL). Mirroring their ability to stimulate proliferation, LPS-, and more potently, IFNγ/LPS-activated macrophages induced significant IFNγ secretion in co-cultures (Fig. 2B), consistent with Th1 differentiation, whilst, in contrast, IL-4-activated macrophages supported only relatively weak IFNγ responses. However, a different profile of responsiveness to KLH presented by each activated macrophage subset was observed when IL-17A production was measured. Although again LPS- or IFNγ/LPS-, but not IL-4-, activated macrophages induced significant IL-17A secretion, LPS stimulation led to the most pronounced responses (Fig. 2C). IL-10 responses to KLH presentation by the differentially activated macrophages followed a different pattern, with LPS activation associated with the highest levels of the cytokine (Fig. 2D).

Overall, the results indicate that T cell responses to macrophages presenting primary antigen are strongly dependent on the activation state of macrophages, which dictates both the cytokine profile and amplitude of Th cell proliferation.

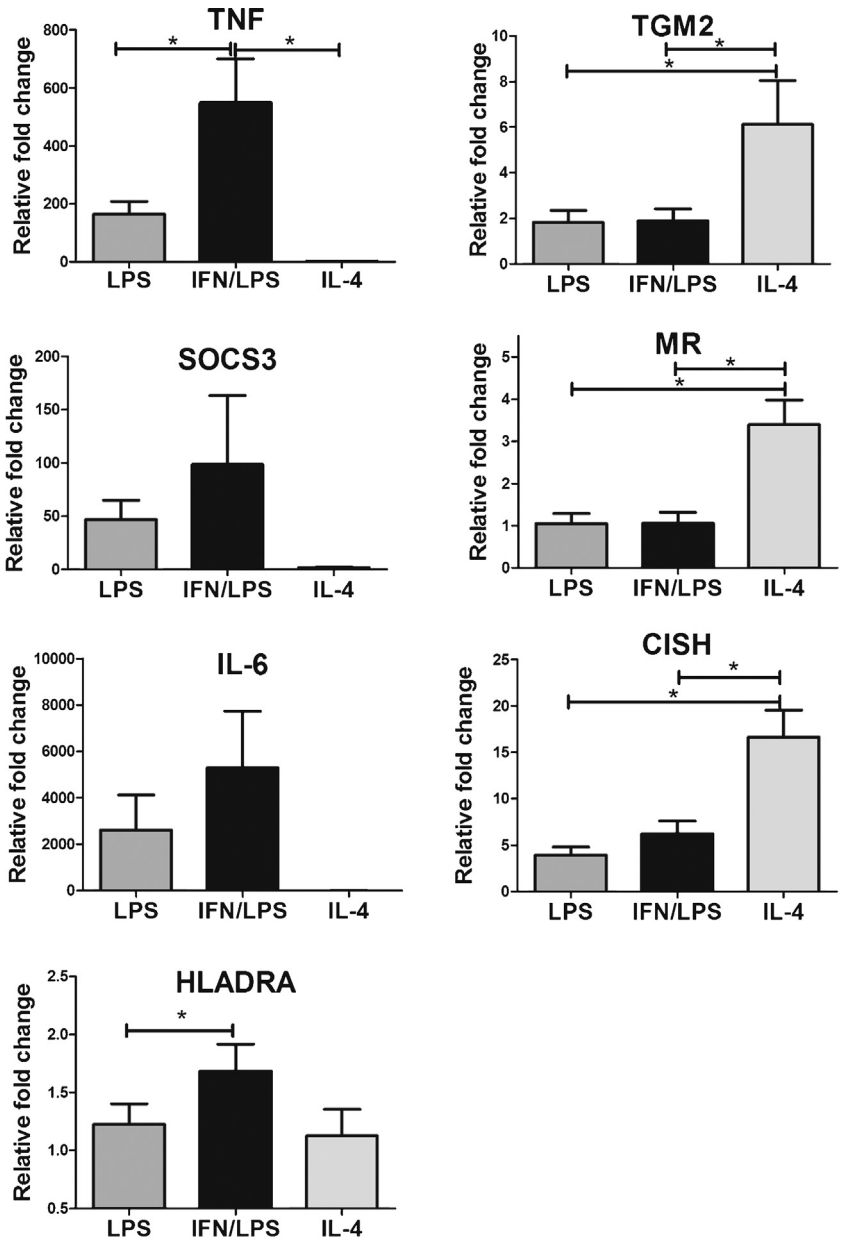

Fig. 1. Differentially activated human macrophages show distinct gene expression profiles. Human monocyte derived macrophages from the same donor preparation were cultured with LPS, IFN-γ/LPS or IL-4 for 5 h and mRNA expression was analysed by qPCR for TNF, SOCS3, IL-6, HLA DR, TGM2, mannose receptor (MR) or CISH. Values are normalised to a housekeeping gene (GAPDH) and shown as relative fold change in expression compared to non-stimulated cells. Values are mean ± SD; $n = 5$ different macrophage donor preparations; (*) <0.05.

Macrophage presentation to stimulate recall responses

Although we demonstrate above the differential ability of macrophages to drive polarisation of naïve T cells, *in vivo*, activated macrophages at sites of inflammation will predominantly contact memory T cells, the phenotypes of which are less easy to deviate. We therefore determined the capacity of differentially activated macrophages to drive T cell polarisation in response to a recall antigen. Macrophages generated from blood of Bacillus-Calmette-Guérin (BCG) vaccinated donors were activated with LPS, IFNγ/LPS or IL-4 to induce distinct phenotypes and loaded with mycobacterial Purified Protein Derivative (PPD), to elicit an antigen-specific recall T cell response.

As expected for a recall response, proliferation of T cells peaked earlier than seen for macrophages presenting naïve antigen KLH, by 5 days of co-culture, and responses were substantially larger (Fig. 2E). LPS- or IFNγ/LPS-activated macrophages, loaded with PPD, clearly induced significant increases in proliferation, while IL-4-activated macrophages did not. Similarly, LPS- or IFNγ/LPS-, but not IL-4-, activated macrophages loaded with PPD stimulated significant increases in IFNγ secretion (Fig. 2F). The strong Th1 cytokine responses are a typical of mycobacterial challenge, but the PPD loaded macrophages were also capable of stimulating IL-17A production (Fig. 2G). As we saw for KLH stimulation, the ability to elicit IL-17A by presenting PPD depended on the activation state of the macrophage, and different macrophage subsets were most effective for IL-17A and IFNγ responses. Thus, similar to KLH loaded macrophages, LPS-activated macrophages presenting PPD induced the largest increase in IL-17A production as compared to the other macrophages types. IL-10 was also produced, and was up-regulated in T cell co-cultures with LPS-, IFNγ/LPS-, or IL-4-activated macrophages, with significant increases in the former two (Fig. 2H). The ability of LPS-activated macrophages that

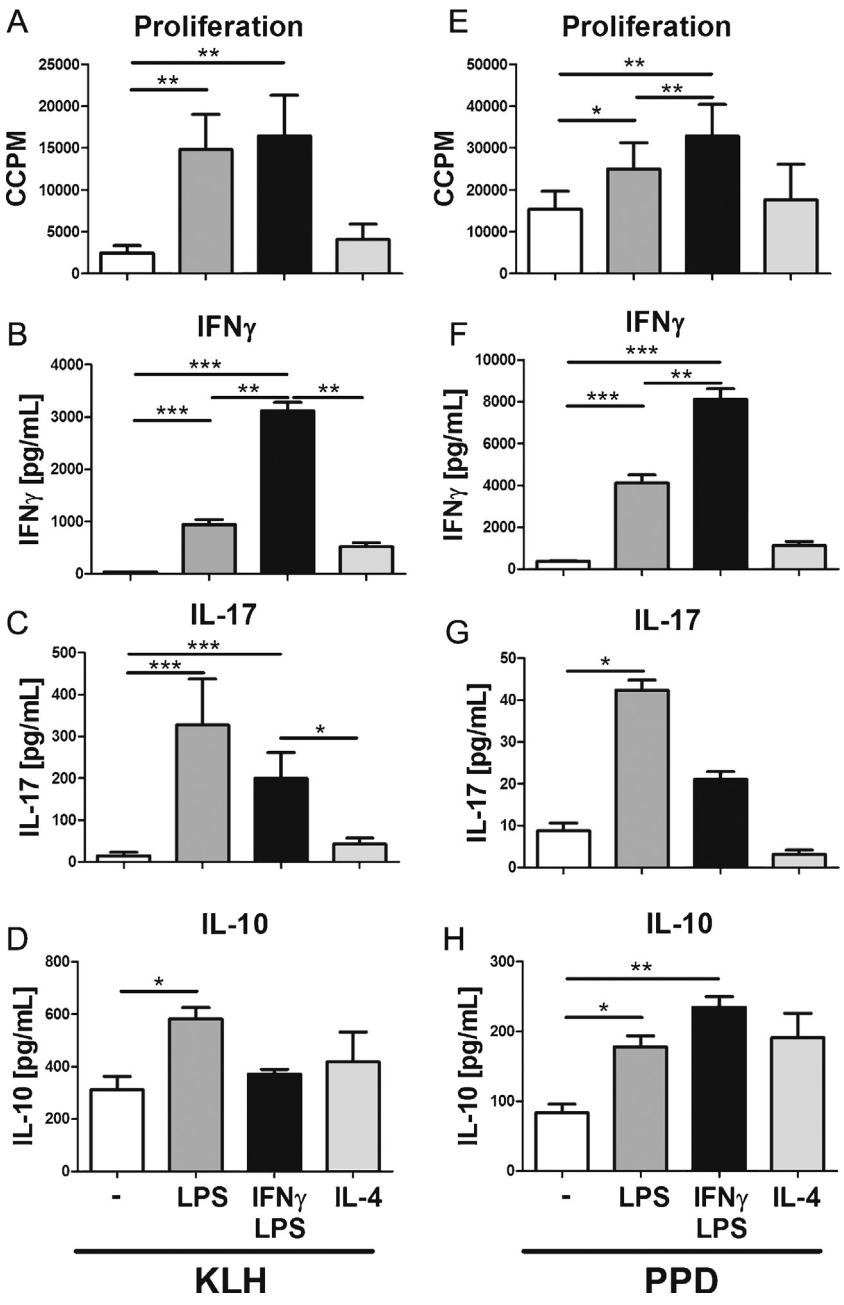

Fig. 2. Differential activation of macrophages presenting antigen shapes the proliferation and cytokine secretion of T cells after co-culture. Differences in proliferation and cytokine levels of individual donors were based on cultures of T cells and macrophages without antigen (control) (Δ = absolute value of sample with antigen minus absolute value of control without antigen). Differences where KLH was antigen (A–D); Differences where mycobacterial PPD was antigen (E–H). Mean values ± SEM, n = at least 7 different donor preparations. Significance was calculated by Wilcoxon-matched pairs test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

present KLH or PPD to drive IL-17A production is notable, and we confirmed that the cytokine was produced from a Th17 response, since none was detectable (<4 pg/mL) in cultures of LPS-activated macrophages alone, and flow cytometric staining of co-cultures identified increased proportions of $CD4^+$ cells with a CCR4, CCR6, IL-23R, RORγt, Th17 phenotype (*Supplementary Figure 1*).

In order to confirm that the responses to PPD were mediated by memory cells, we repeated the experiments with the $CD45RO^+$ T cell fraction, which represents the antigen-experienced compartment (Plebanski et al., 1992) (*Supplementary Figure 2*). Similar to the results obtained using unselected T cell populations, the $CD45RO^+$ fraction generated the highest IFNγ and IL-17A responses when presented with PPD by IFNγ/LPS- and LPS-activated macrophages, respectively.

Thus, in line with the experiments with KLH as antigen, we show that PPD loaded macrophages can induce T cell responses and their ability to do this is dependent on their activation status. For both antigens, IFNγ/LPS activation of macrophages most readily drives Th1 polarisation, while LPS activation most effectively induces Th17.

DCs and macrophages induce similar levels of Th17 polarisation.

The ready ability of appropriately activated macrophages to induce Th1, and particularly Th17 responses raised the question as to whether they are as effective as DCs, which are traditionally believed to be the major drivers of T cells due to their high expression of HLA-DR and antigen presenting ability (Steinman,

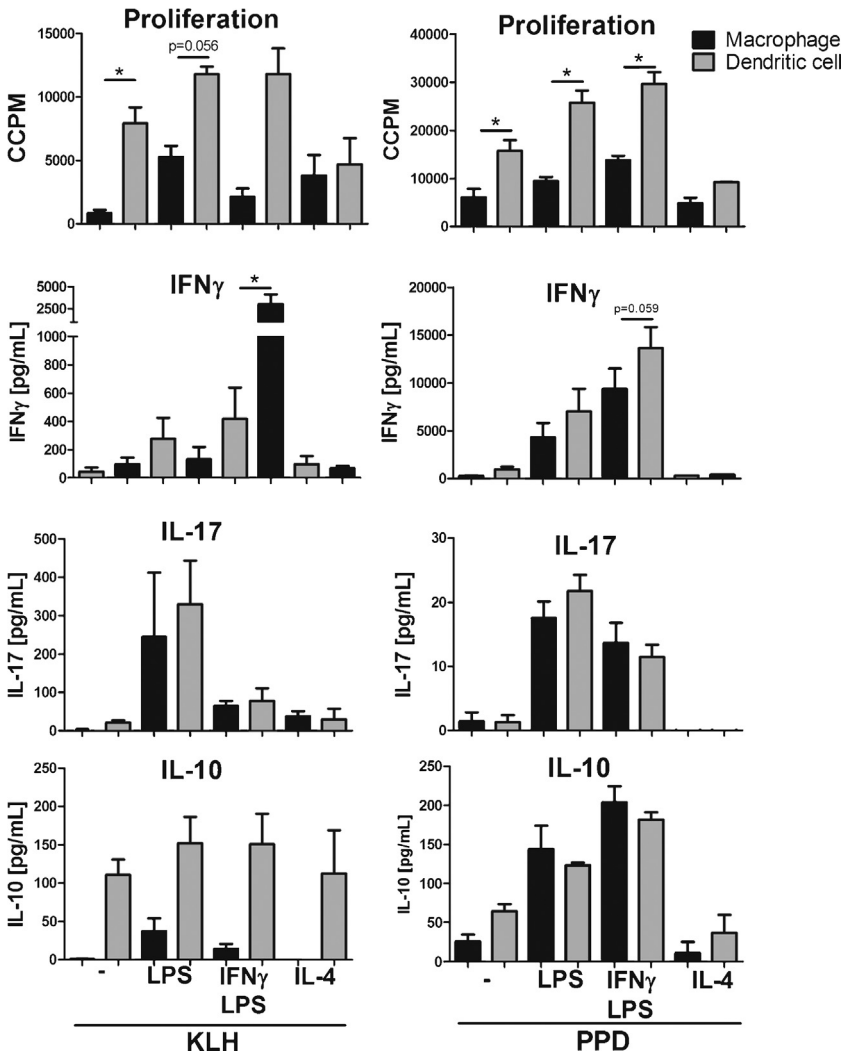

Fig. 3. Comparison of T cell responses from cultures with antigen-loaded macrophages or dendritic cells (DC). Differences in proliferation and cytokine levels (as described in materials and methods) of individual donors were based on cultures of T cells and macrophages or DCs without antigen (control). Results show absolute value of sample with antigen minus absolute value of control without antigen. Left panels: Differences where KLH was antigen; Right panels: Differences where mycobacterial PPD was antigen. Respective mean values ± SEM, $n = 5$ different donor preparations. Statistical significance was tested using paired t-tests ($^{*}p < 0.05$).

2012). Therefore, we compared the ability of monocyte derived DCs and differentially activated macrophages from the same donor to drive Th cell responses to presented antigens. Distinct differences in phenotypic (size, shape, adherence and CD14, HLA-DR, CD11b and CD11c expression) and functional characteristics (phagocytic activity) were confirmed for the macrophages and DCs we derived (*Supplementary Figure 3*). As expected, DCs activated with LPS or IFN-γ/LPS stimulated T cell proliferation in response to KLH or PPD, with IFNγ/LPS activation the most effective at inducing IFNγ (Fig. 3). Comparisons between respective cultures where the APC from the same donor preparation were either DC or macrophages and activated in the same way, supported the view that DCs are superior to macrophages in driving Th1 differentiation (Fig. 3). By contrast, however, despite much higher T cell proliferation induced by DCs, macrophages and DCs were equally efficient at driving Th17 differentiation based on secretion of IL-17A, which was highest when either cell type was activated with LPS. Even if the macrophage and DC phenotypes we have studied do not precisely recapitulate those *in vivo*, we have nevertheless established the principle that differential activation of the APC contributes to the type of T cell response. Thus, macrophages, which are more prominent at sites of inflammation, have the potential to be the most effective APC type in driving local Th17 responses.

Different activating stimuli trigger distinct levels of T cell polarising cytokines

Antigen-loaded human macrophages and DCs, activated by distinct stimuli, were shown to induce Th1 and Th17 cell responses with different efficiencies. To determine how this related to their secretion of polarising cytokines, the levels of IL-12 (associated with Th1) and IL-1β, IL-6, IL-23 (associated with Th17) were measured in supernatants of antigen-loaded, differentially activated macrophages or DCs 24 h post activation. IL-12 was upregulated only in the supernatants of either APC type after IFNγ/LPS-activation, consistent with the efficient polarisation to Th1 IFNγ responses seen under these conditions. Similarly, macrophages and DCs showed significant increases in IL-1β secretion following activation with LPS or IFNγ/LPS (Fig. 4), with LPS the more effective stimulus, mirroring the ability to induce Th17 responses. LPS or IFNγ/LPS also induced both APC types to secrete IL-6 and IL-23, with macrophages producing higher levels of IL-23 than DCs. By contrast, the activation of neither macrophages nor DCs with IL-4 upregulated secretion of any of the cytokines studied, as would be predicted from the poor ability of such cells to induce Th responses.

The role of IL-1β in driving human Th17 polarisation is supported by experiments based on anti-CD3/CD28 activation of T cells

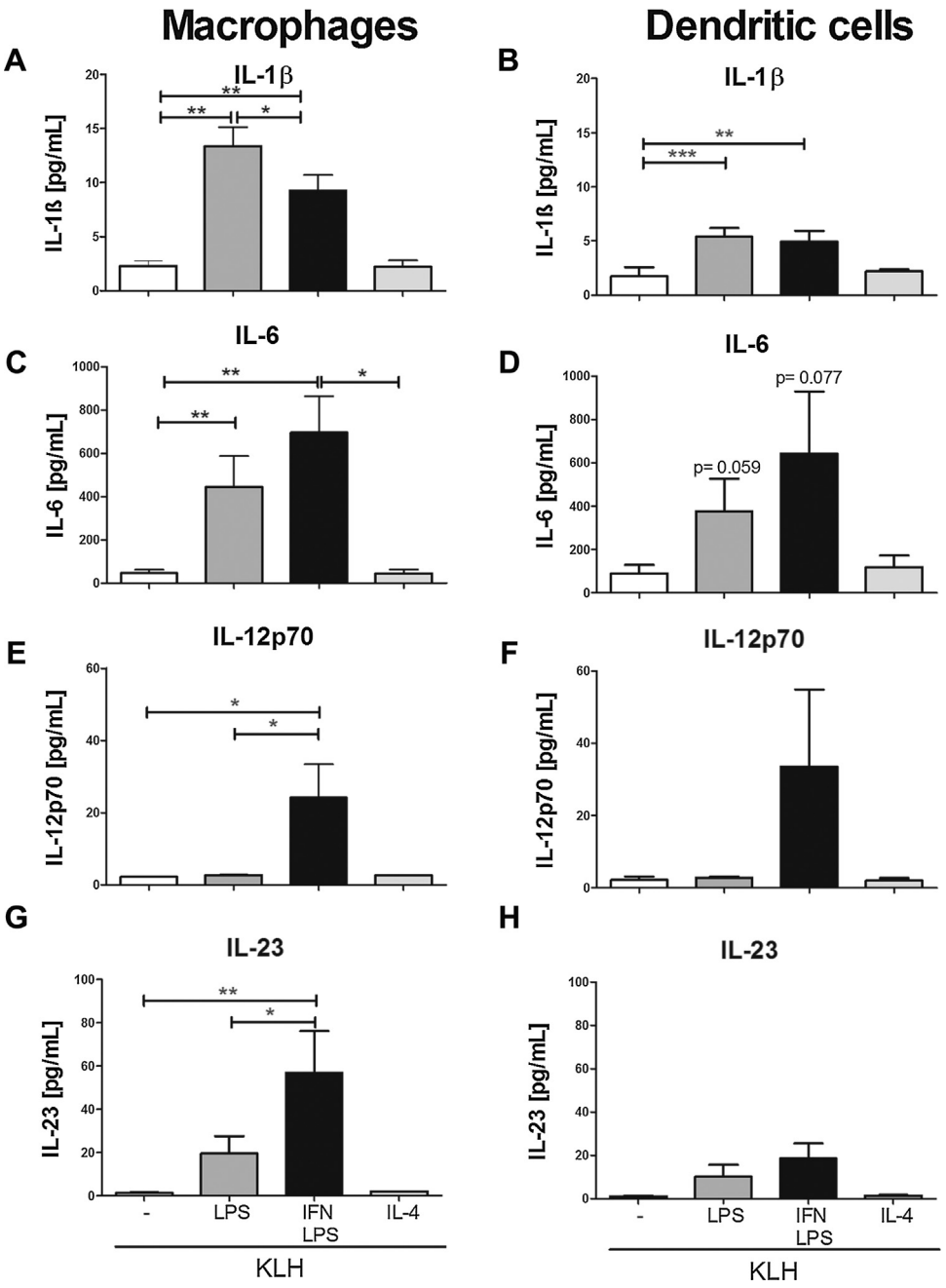

Fig. 4. Different activating stimuli trigger distinct levels of T cell polarising cytokines. T cell polarising cytokines in supernatants of activated macrophages (A, C, E & G) ($n = 8$) or DCs (B, D, F & H) ($n = 6$). Supernatants were harvested 24 h after stimulation and were analysed for cytokines by cytometric bead array (IL-1β, IL-6, IL-12p70) and ELISA (IL-23). Statistical significance was determined using a paired t-test ($^{*}p < 0.05$, $^{**}p < 0.01$) and differences represent levels of cytokine compared to antigen loaded, non-activated control macrophages or DCs.

with the addition of monocyte-derived supernatant or cytokines, in the presence or absence of IL-1β and/or IL-1β neutralising antibodies (Evans et al., 2009; Lee et al., 2010; Segura et al., 2013). To address whether the enhanced Th17 production with our LPS-activated macrophages was dependent on the high levels of IL-1β measured, the cytokine was neutralised with antibody. In LPS-activated macrophage co-cultures, which supported the highest Th17 responses, anti-IL-1β antibody significantly reduced mean IL-17A levels (Fig. 5). However, there was no such effect when macrophages were activated with IFNγ/LPS, where there is a more prominent Th1 response, IL-17a levels are lower, and IFNγ may suppress IL-1β production (Eigenbrod et al., 2013). The antibody alone did not activate macrophages to induce T cell responses (Fig. 5) and neutralising IL-1β in co-cultures had negligible effects on T cell proliferation, or the secretion of IFNγ or IL-10. Taken together, these results focus attention on the role IL-1β produced by LPS-activated human macrophages in preferentially polarising Th17 cells.

Discussion

This report addresses a gap in the understanding of the inter-relationships between macrophage and Th subsets, by directly comparing the efficiency with which antigen presentation by differentially activated human macrophages drives each of the major types of Th cell response. We demonstrated that the induction

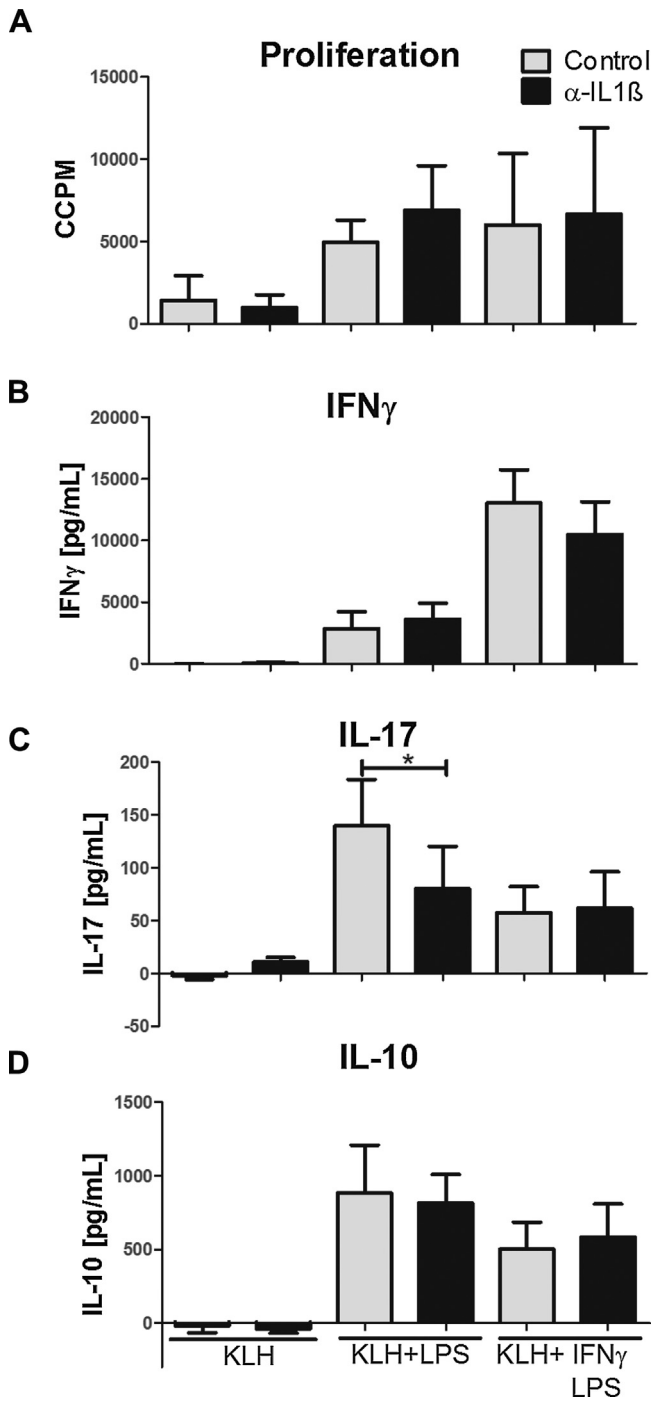

Fig. 5. Determination of the importance of IL-1β for human Th17 differentiation. Soluble IL-1β was inhibited by supplementation of anti-IL-1β antibody (10 μg/mL) in respective cultures at the beginning of co-culture. Results are mean ± SEM; $n = 7$. Statistical significance was determined using paired t-tests ($^{*}p < 0.05$).

of Th proliferation, and the bias towards particular Th subsets, is strongly dependent on the activation state of macrophages presenting antigen and the mediators they produce. In particular, LPS-activated macrophages were most efficient at stimulating IL-17A Th responses, associated with their production of IL-1β, while IFNγ/LPS activation induced the highest levels of IFNγ secretion by T cells. By contrast, IL-4 activated macrophages did not induce significant Th proliferation, or secretion of IFNγ or IL-17A, in line with their recognised anti-inflammatory and tissue reparative nature (Gordon and Taylor, 2005; Murray and Wynn, 2011). LPS-activated macrophages were at least as potent at inducing IL-17A as the corresponding DCs, raising the prospect that such macrophages make an important contribution to driving Th17 responses to antigen in inflamed sites *in vivo*. This is supported by the correlation reported between macrophage but not DC numbers and Th17 cells in inflammation (Allam et al., 2011).

The few studies to date on the role of activated human macrophages in driving Th cell responses have analysed mitogenic T cell activation/co-stimulation in the presence of polarising factors, rather than the specific responses to presented cognate antigen that are relevant *in vivo*. In one report, M2 macrophages suppressed $CD4^+$ T cell proliferation in mice by depleting arginine, which is required to induce such responses (Pesce et al., 2009), while another study demonstrated that human macrophages, in a mixed lymphocyte reaction, could induce both Th1 and Th17 subsets (Krausgruber et al., 2011). Other important studies showed that, monocytes activated with LPS or isolated from the inflamed synovium (synovial fluid) of arthritic patients supported efficient expansion of allogenic Th17, but not Th1 or Th2, responses to anti-CD3 antibody (Evans et al., 2007, 2009). In contrast, our study systematically compared the ability of antigen-loaded, differentially activated human macrophages to induce autologous specific Th cell responses. We showed that LPS- and IFN/LPS activated macrophages favoured Th1 and Th17 activation respectively against both primary and recall antigen, while IL-4 activated M2 type macrophages were relatively ineffective in eliciting these responses.

DCs are potent APCs and, as predicted, were more efficient than macrophages in inducing PPD- and KLH-specific T cell proliferation and IFNγ secretion, indicating their superiority in driving Th1 differentiation. Importantly, however, macrophages and DCs did not differ in their ability to drive antigen-dependent Th17 differentiation. This result, using specific antigen stimulation, is resonant of reports that human monocytes are more effective than DCs in supporting the expansion of allogenic lymphocytes stimulated with anti-CD3, and that monocyte-derived factors efficiently drive Th17 polarisation (Acosta-Rodriguez et al., 2007; Evans et al., 2007, 2009). However, monocytes and macrophages have different roles and locations *in vivo*, and our data show for the first time how differentially macrophages presenting antigen in, for example, inflamed sites, have the potential to bias the Th response.

One study that did directly investigate antigen-dependent Th17 polarisation by macrophages was performed on fungal-activated cells (Cheng et al., 2011). Fungal stimuli have a strong ability to bias antigen responses to Th17 (Vautier et al., 2010) through their ligation of innate pattern recognition receptors such as dectin-1, TLRs and the mannose receptor on APC. Similar to the LPS-activated macrophages we studied, fungally stimulated macrophages induced strong Th IL-17A production, but weaker IFNγ responses. Interactions between other TLR4 agonists such as saturated fatty acids and antigen loaded macrophages in peripheral tissues could therefore contribute locally to Th17 polarisation. Indeed, obesity is associated with increased adipose tissue macrophage infiltration, a switch to pro-inflammatory macrophages due, in part, to activation by excessive fatty acids, and greater numbers of IL-17A producing cells (Fabbrini et al., 2013; Winer et al., 2009).

During activation by presented antigen, the subset of human $CD4^+$ T lymphocytes is largely dictated by the cytokine micro-environment, with, for example, the ability of APCs to secrete IL-12p70, or a combination of IL-1β and IL-6, favouring Th1 and Th17 phenotypes, respectively. LPS stimulation induced a significant increase in IL-1β secretion from macrophages, which was higher than that from DCs, an observation which may help to explain the ability of these macrophages to support Th17 responses. The importance of IL-1β in driving Th17 differentiation in our

system is supported by other studies that highlight IL-1β as a key polarising factor for human Th17 in presence of mitogenic stimuli (Acosta-Rodriguez et al., 2007; Evans et al., 2009; Gerosa et al., 2008; Lasiglié et al., 2011), experiments based on *Mycobacterium tuberculosis*-dependent T cell polarisation (van de Veerdonk et al., 2010) and *C. albicans*-induced Th17 responses (Hise et al., 2009). IFNγ/LPS-activated macrophages secreted significantly lower levels of IL-1β, but higher levels of IL-12, than after LPS stimulation alone, again consistent with the shift from supporting Th17 to Th1 responses. One explanation for this fall in IL-1β production is provided by a recent report that IFN-γ suppresses the transcription of IL-1β, but not of TNF, IL-6, or IL-12p40 in LPS-activated macrophages, by selectively inhibiting binding of NF-κB p65 to the IL-1β promoter (Eigenbrod et al., 2013).

The identification of different levels of gene-specific mRNA expression by our LPS, IFNγ/LPS and IL-4 activated macrophages not only validates their distinct phenotypes, but also contributes to the sparse literature on polarisation markers for human macrophages (Ambarus et al., 2012; Jaguin et al., 2013; Vogel et al., 2014). We show strongly upregulated levels of TNF, IL-6, HLA DR and SOCS3 mRNA in LPS and IFNγ/LPS activated human macrophages, but not IL-4 activated cells, which are instead characterised by increased expression of TGM2, CISH and mannose receptor. These respective profiles are consistent with, but not identical to, murine M1 and M2 macrophage phenotypes. For example, although mannose receptor is a key murine M2 macrophage marker, we found its increased expression in IL-4 activated human macrophages was less extreme than in mouse cells, in line with previous reports that the precise profiles of activation markers differ between the two species (Ambarus et al., 2012; Jaguin et al., 2013; Murray and Wynn, 2011; Vogel et al., 2014).

In summary, the results presented here show key differences in the phenotype and function of activated macrophages that influence T cell responses. They are first to identify human macrophages as efficient APCs that can induce Th1 and Th17 polarisation from T-cells in the absence of mitogenic stimuli. This might explain the high predominance of Th17 at sites of inflammation where activated macrophages are present as has been suggested for mouse macrophages (Egan et al., 2008). The activation state of macrophages strongly influences their Th polarising capacity, raising the prospect that therapeutic interventions to skew macrophage phenotype could abrogate pathogenic Th1 or Th17 responses in autoimmune and inflammatory diseases, or conversely enhance their polarisation in fungal infection of cancer.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by Kidney Research UK (grant RP1/2012) and NHS Grampian Endowments Research Trust funding (grant 08/25).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imbio.2014.09.022.

References

Acosta-Rodriguez, E.V., Napolitani, G., Lanzavecchia, A., Sallusto, F., 2007. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. Nat. Immunol. 8, 942–949.

Allam, J.P., Duan, Y., Heinemann, F., Winter, J., Götz, W., Deschner, J., Wenghoefer, M., Bieber, T., Jepsen, S., Novak, N., 2011. IL-23-producing CD68(+) macrophage-like cells predominate within an IL-17-polarized infiltrate in chronic periodontitis lesions. J. Clin. Periodontol. 38, 879–886.

Ambarus, C.A., Krausz, S., van Eijk, M., Hamann, J., Radstake, T.R., Reedquist, K.A., Tak, P.P., Baeten, D.L., 2012. Systematic validation of specific phenotypic markers for in vitro polarized human macrophages. J. Immunol. Methods 375, 196–206.

Arnold, C.E., Whyte, C.S., Gordon, P., Barker, R.N., Rees, A.J., Wilson, H.M., 2014. A critical role for suppressor of cytokine signalling 3 in promoting M1 macrophage activation and function in vitro and in vivo. Immunology 141, 96–110.

Barker, R.N., Elson, C.J., 1994. Multiple self epitopes on the Rhesus polypeptides stimulate immunologically ignorant human T cells in vitro. Eur. J. Immunol. 24, 1578–1582.

Cheng, S.C., van de Veerdonk, F.L., Lenardon, M., Stoffels, M., Plantinga, T., Smeekens, S., Rizzetto, L., Mukaremera, L., Preechasuth, K., Cavalieri, D., Kanneganti, T.D., van der Meer, J.W., Kullberg, B.J., Joosten, L.A., Gow, N.A., Netea, M.G., 2011. The dectin-1/inflammasome pathway is responsible for the induction of protective T-helper 17 responses that discriminate between yeasts and hyphae of Candida albicans. J. Leukoc. Biol. 90, 357–366.

Egan, P.J., van Nieuwenhuijze, A., Campbell, I.K., Wicks, I.P., 2008. Promotion of the local differentiation of murine Th17 cells by synovial macrophages during acute inflammatory arthritis. Arthritis Rheum. 58, 3720–3729.

Eigenbrod, T., Bode, K.A., Dalpke, A.H., 2013. Early inhibition of IL-1β expression by IFN-γ is mediated by impaired binding of NF-κB to the IL-1β promoter but is independent of nitric oxide. J. Immunol. 190, 6533–6541.

Evans, H.G., Gullick, N.J., Kelly, S., Pitzalis, C., Lord, G.M., Kirkham, B.W., Taams, L.S., 2009. In vivo activated monocytes from the site of inflammation in humans specifically promote Th17 responses. Proc. Natl. Acad. Sci. U. S. A. 106, 6232–6237.

Evans, H.G., Suddason, T., Jackson, I., Taams, L.S., Lord, G.M., 2007. Optimal induction of T helper 17 cells in humans requires T cell receptor ligation in the context of Toll-like receptor-activated monocytes. Proc. Natl. Acad. Sci. U. S. A. 104, 17034–17039.

Fabbrini, E., Cella, M., McCartney, S.A., Fuchs, A., Abumrad, N.A., Pietka, T.A., Chen, Z., Finck, B.N., Han, D.H., Magkos, F., Conte, C., Bradley, D., Fraterrigo, G., Eagon, J.C., Patterson, B.W., Colonna, M., Klein, S., 2013. Association between specific adipose tissue CD4+ T-cell populations and insulin resistance in obese individuals. Gastroenterology 145, 366–374.

Gerosa, F., Baldani-Guerra, B., Lyakh, L.A., Batoni, G., Esin, S., Winkler-Pickett, R.T., Consolaro, M.R., De Marchi, M., Giachino, D., Robbiano, A., Astegiano, M., Sambataro, A., Kastelein, R.A., Carra, G., Trinchieri, G., 2008. Differential regulation of interleukin 12 and interleukin 23 production in human dendritic cells. J. Exp. Med. 205, 1447–1461.

Gordon, S., Martinez, F.O., 2010. Alternative activation of macrophages: mechanism and functions. Immunity 32, 593–604.

Gordon, S., Taylor, P.R., 2005. Monocyte and macrophage heterogeneity. Nat. Rev. Immunol. 5, 953–964.

Gutcher, I., Becher, B., 2007. APC-derived cytokines and T cell polarization in autoimmune inflammation. J. Clin. Invest. 117, 1119–1127.

Hise, A.G., Tomalka, J., Ganesan, S., Patel, K., Hall, B.A., Brown, G.D., Fitzgerald, K.A., 2009. An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen Candida albicans. Cell Host Microbe. 5, 487–497.

Iwasaki, A., Medzhitov, R., 2010. Regulation of adaptive immunity by the innate immune system. Science 327, 291–295.

Jaguin, M., Houlbert, N., Fardel, O., Lecureur, V., 2013. Polarization profiles of human M-CSF-generated macrophages and comparison of M1-markers in classically activated macrophages from GM-CSF and M-CSF origin. Cell Immunol. 281, 51–61.

Krausgruber, T., Blazek, K., Smallie, T., Alzabin, S., Lockstone, H., Sahgal, N., Hussell, T., Feldmann, M., Udalova, I.A., 2011. IRF5 promotes inflammatory macrophage polarization and T(H)1-T(H)17 responses. Nat. Immunol. 12, 231–238.

Lasiglié, D., Traggiai, E., Federici, S., Alessio, M., Buoncompagni, A., Accogli, A., Chiesa, S., Penco, F., Martini, A., Gattorno, M., 2011. Role of IL-1 beta in the development of human T(H)17 cells: lesson from NLPR3 mutated patients. PLoS One 6, e20014.

Lee, W.W., Kang, S.W., Choi, J., Lee, S.H., Shah, K., Eynon, E.E., Flavell, R.A., Kang, I., 2010. Regulating human Th17 cells via differential expression of IL-1 receptor. Blood 115, 530–540.

Martinez, F.O., Helming, L., Milde, R., Varin, A., Melgert, B.N., Draijer, C., Thomas, B., Fabbri, M., Crawshaw, A., Ho, L.P., Ten Hacken, N.H., Cobos Jiménez, V., Kootstra, N.A., Hamann, J., Greaves, D.R., Locati, M., Mantovani, A., Gordon, S., 2013. Genetic programs expressed in resting and IL-4 alternatively activated mouse and human macrophages: similarities and differences. Blood 121, e57–e69.

Martinez, F.O., Sica, A., Mantovani, A., Locati, M., 2008. Macrophage activation and polarization. Front. Biosci. 13, 453–461.

Mondal, A., Sawant, D., Dent, A.L., 2010. Transcriptional repressor BCL6 controls Th17 Responses by controlling gene expression in both T cells and macrophages. J. Immunol. 184, 4123–4132.

Mosser, D.M., Edwards, J.P., 2008. Exploring the full spectrum of macrophage activation. Nat. Rev. Immunol. 8, 958–969.

Murray, P.J., Wynn, T.A., 2011. Protective and pathogenic functions of macrophage subsets. Nat. Rev. Immunol. 11, 723–737.
Pesce, J.T., Ramalingam, T.R., Mentink-Kane, M.M., Wilson, M.S., El Kasmi, K.C., Smith, A.M., Thompson, R.W., Cheever, A.W., Murray, P.J., Wynn, T.A., 2009. Arginase-1-expressing macrophages suppress Th2 cytokine-driven inflammation and fibrosis. PLoS Pathog. 5, e1000371.
Pickford, W.J., Watson, A.J., Barker, R.N., 2007. Different forms of helper tolerance to carcinoembryonic antigen: ignorance and regulation. Clin. Cancer Res. 13, 4528–4537.
Plebanski, M., Saunders, M., Burtles, S.S., Crowe, S., Hooper, D.C., 1992. Primary and secondary human in vitro T-cell responses to soluble antigens are mediated by subsets bearing different CD45 isoforms. Immunology 75, 86–91.
Qin, H., Holdbrooks, A.T., Liu, Y., Reynolds, S.L., Yanagisawa, L.L., Benveniste, E.N., 2012. SOCS3 deficiency promotes M1 macrophage polarization and inflammation. J. Immunol. 189, 3439–3448.
Savage, N.D., de Boer, T., Walburg, K.V., Joosten, S.A., van Meijgaarden, K., Geluk, A., Ottenhoff, T.H., 2008. Human anti-inflammatory macrophages induce Foxp3+ GITR+ CD25+ regulatory T cells, which suppress via membrane-bound TGFbeta-1. J. Immunol. 181, 2220–2226.
Segura, E., Touzot, M., Bohineust, A., Cappuccio, A., Chiocchia, G., Hosmalin, A., Dalod, M., Soumelis, V., Amigorena, S., 2013. Human inflammatory dendritic cells induce Th17 cell differentiation. Immunity 38, 336–348.
Steinman, R.M., 2012. Decisions about dendritic cells: past, present, and future. Annu. Rev. Immunol. 30, 1–22.
van de Veerdonk, F.L., Teirlinck, A.C., Kleinnijenhuis, J., Kullberg, B.J., van Crevel, R., van der Meer, J.W., Joosten, L.A., Netea, M.G., 2010. Mycobacterium tuberculosis induces IL-17A responses through TLR4 and dectin-1 and is critically dependent on endogenous IL-1. J. Leukoc. Biol. 88, 227–232.
Vautier, S., Sousa Mda, G., Brown, G.D., 2010. C-type lectins, fungi and Th17 responses. Cytokine Growth Factor Rev. 21, 405–412.
Vogel, D.Y., Glim, J.E., Stavenuiter, A.W., Breur, M., Heijnen, P., Amor, S., Dijkstra, C.D., Beelen, R.H., 2014. Human macrophage polarization in vitro: maturation and activation methods compared. Immunobiology 19, 695–703.
Walter, G.J., Evans, H.G., Menon, B., Gullick, N.J., Kirkham, B.W., Cope, A.P., Geissmann, F., Taams, L.S., 2013. Interaction with activated monocytes enhances cytokine expression and suppressive activity of human CD4+CD45ro+CD25+CD127(low) regulatory T cells. Arthritis Rheum. 65, 627–638.
Winer, S., Paltser, G., Chan, Y., Tsui, H., Engleman, E., Winer, D., Dosch, H.M., 2009. Obesity predisposes to Th17 bias. Eur. J. Immunol. 39, 2629–2635.
Wilson, H.M., 2010. Macrophages heterogeneity in atherosclerosis—implications for therapy. J. Cell Mol. Med. 14, 2055–2065.