Cancer Immunology, Immunotherapy (2019) 68:1455–1465
https://doi.org/10.1007/s00262-019-02381-1

ORIGINAL ARTICLE

Melanoma-conditioned medium promotes cytotoxic immune responses by murine bone marrow-derived monocytes despite their expression of 'M2' markers

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Received: 4 February 2019 / Accepted: 16 August 2019 / Published online: 23 August 2019

Abstract

Macrophages have been shown to infiltrate a wide range of malignancies and are often considered to promote tumour survival, growth and spread. However, the source and behaviour of discrete tumour-associated macrophage populations are still poorly understood. Here we show a novel method for the rational development of bone marrow-derived monocytes appropriate for the study of processes which involve the contribution of circulating inflammatory monocytes. We have shown that in response to tumour-conditioned medium, these cells upregulate CD206 and CD115, markers traditionally associated with M2-type macrophages. Treated cells show reduced capacity for cytokine secretion but significantly impact $CD4^+$ and $CD8^+$ T-cell proliferation and polarization. Coculture with conditioned bone marrow-derived monocytes significantly reduced $CD4^+$ T-cell proliferation but increased $CD8^+$ T-cell proliferation and granzyme B expression with significant induction of IFNγ secretion by both $CD4^+$ and $CD8^+$ T cells, indicating that these cells may have a role in promoting anti-cancer immunity.

Keywords Inflammatory monocytes · Melanoma · Tumour immunology · Cytotoxic responses · Bone marrow-derived monocytes · T cells

Abbreviations

B16CM	B16F10-conditioned medium
BMDM	Bone marrow-derived monocytes
iNOS	Inducible nitric oxide synthase
mRPMI	Macrophage RPMI-1640
NTCM	No treatment concentrated medium
RANTES	Regulated on activation, normal T cell expressed and secreted
RBC	Red blood cell
TAM	Tumour-associated macrophage

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00262-019-02381-1) contains supplementary material, which is available to authorized users.

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Introduction

The difficulties of in vitro macrophage research have been documented at length [1]. At its heart are the inadequacies of current models and uncertainty over whether they translate to in vivo physiological behaviour [2].

In vivo research of macrophages continues to question basic physiological questions such as the native activation state of macrophage populations, the source of distinct macrophage populations and the epigenetic or other differences between macrophage populations that may influence their behaviour in response to specific environmental stimuli, all of which may affect the relevancy of previously published literature.

The M1/M2 dichotomy of macrophage polarization has been widely used in cancer research, with M1 macrophages considered to promote anti-cancer immune responses and M2 macrophages supporting tumour survival, growth and spread. Tumour-associated macrophages (TAMs) are generally considered to be M2-like in nature and thus a negative presence in tumour biology. This has been supported by a wide range of studies evaluating the presence and phenotypes of macrophages in human cancers, whereby TAMs express M2 markers and have been

associated with poorer prognostic outcomes, with the exception of colorectal and gastric cancers [3].

The protocols used for the generation of bone marrow-derived monocytes and macrophages differ widely across the literature. Limited guidelines are available for the development of BMDMs, including the growth factors to be used and the source of cells [1]. It has been suggested these guidelines in combination with comprehensive reporting standards will aid the process of unifying experimental protocols and increase the comparability and reproducibility of published research. However, there are still a wide range of protocol aspects which vary and which have significant impacts on the resultant populations [4–6].

Monocytes develop into macrophages, however, the distinction between monocyte and macrophage populations is unclear. Current theories suggest that monocytes migrate from the bone marrow to the circulation and exist as two distinct populations. One population is primed for inflammatory responses, these cells have been referred to as inflammatory monocytes and are defined as $CD11b^+$ $Ly6G^-$ $Ly6C^{hi}$ and $CCR2^+$. A second population defined as $CD11b^+$ $Ly6C^+$ and $CCR2^-$ are commonly referred to as non-classical monocytes and are primed for M2 responses [7]. It has been suggested that inflammatory monocytes over time or in the absence of inflammatory stimuli evolve into non-classical monocytes [8].

In addition to functional differences, these cells also differ in migration potential. Inflammatory monocytes rely on CCR2 to home towards the sites of inflammation, whereas non-classical monocytes rely predominantly on CX3CR1 and CCR5 expression to direct their homing capacity [9]. Both populations are likely to accumulate at tumour sites due to leaky vasculature and tumour expression of CCL2, CX3CL1 and RANTES (also known as CCL5), the reciprocal ligands for CCR2, CX3CR1 and CCR5, respectively. However, it is intuitive that variations will exist between tumour types and tumour models, depending on ligand expression and tumour accessibility and penetrability. Studies examining the presence of monocytes and macrophages in tumour lesions have frequently reported the presence of TAMs which have an M2-like phenotype. This may in part be due to the shorter half-life of inflammatory monocytes, recruitment of TAMs from tissue resident subsets rather than circulating monocytes, selective proliferation of M2-like TAMs in the tumour microenvironment or conditioning by the tumour of monocytes to an M2-like phenotype.

From a tumour immunology point of view, the relative contribution of these factors is poorly defined and may vary significantly between tumour types, but circulating monocytes have been shown to infiltrate murine tumours [10].

The nature of monocytes and macrophages infiltrating a tumour is likely to impact their functional contribution to tumour biology, thus for each tumour type and tumour model this must be individually assessed.

Here we present a concept to bench process for the rational development of an in vitro model for the study of monocytes and macrophages in a murine model of melanoma. We show the effect of tumour conditioning on these cells and their role in anti-cancer immunity.

Materials and methods

Cell line maintenance

The B16F10 cell line was maintained in RPMI-1640 supplemented with 10% FCS and 50 units/ml penicillin and 50 µg/ml streptomycin. Cells were cultured at 37 °C in a humidified chamber with 5% CO_2.

In vivo B16F10 subcutaneous tumour model

4–6-week-old female C57BL6J mice had their left flank shaved so that skin was visible. For tumour induction, 1×10^6 cells in 200 µl serum-free and antibiotic-free medium were injected subcutaneously. Tumour sizes were monitored on a daily basis and measured using a Vernier calliper. Blood samples were taken at various points of tumour growth, using volume as a measure of tumour development. Tumour volume measurements were calculated using the equation: Volume $= ab^2\pi/6$, where a is the largest diameter of the tumour and b is the greatest diameter of the tumour perpendicular to a.

Development and culturing of bone marrow-derived monocytes (BMDM)

Healthy 4–6-week-old female C57BL6J were euthanized humanely by cervical dislocation. Tibia and femur bones were isolated. The bones were cleaned of as much muscles, cartilage and other material as possible using a standard aseptic dissection kit. Bones were submerged in ice-cold ethanol for 90 s to sterilize the surface of the bones and rinsed in PBS. Both ends of the bone were removed using sterile scissors. The bone marrow was then flushed from the bone with mRPMI (RPMI-1640 supplemented with 10% FCS, 1% P/S, 0.01 mM β-mercaptoethanol, 1 mM sodium pyruvate and 1 × Eagle's minimum essential medium non-essential amino acids) using a 26 gauge syringe and passed through a 70 µm filter to obtain a single cell suspension. The cells were washed in mRPMI. BMDMs were centrifuged at 270 g for 7 min for all washes. The cells were resuspended in red blood cell (RBC) lysis buffer at RT (room temperature) for 8 min with continuous agitation. Cells were washed and seeded in 10 cm bacteriological dishes, with 5×10^6 cells in

10 ml medium supplemented with 50 ng/ml M-CSF (Biolegend) added to each dish. Cells were left undisturbed at 37 °C in a humidified chamber with 5% CO_2 for 5 days unless otherwise specified. To harvest cells, the suspension cells were collected in the supernatant and adherent cells were isolated using gentle pipetting following 5–10 min incubation in ice-cold PBS with 5 mM EDTA.

Conditioned medium

2.5×10^6 B16F10 cells were seeded in a T175 flask in 20 ml RPMI-1640 supplemented with 2% FCS and 1% P/S. For no treatment controls, 20 ml of RPMI supplemented with 2% FCS and 1% P/S was added to a T175 and treated identically to the B16F10-conditioned medium in the absence of cells. The flasks were incubated for 48 h at 37 °C. Following incubation, the supernatant was isolated and centrifuged to remove any cells or cellular debris. Cell-free conditioned medium was stored at 4–8 °C for a maximum of 48 h. Conditioned or control medium was added to Vivaspin 20 ultracentrifugation tubes (GE Healthcare) with a molecular weight cutoff of 3 kDa and centrifuged at 8000 g for 1–3 h until medium was at least 20× concentrated. The volume of conditioned or control medium to be added was calculated as: (Volume obtained)/(Initial volume) × Final volume of treatment × Concentration required (1×).

Flow cytometry

For analysis of blood, blood was isolated by cardiac puncture and removed into heparinized tubes, while RBC lysis was performed prior to staining. Cells were washed in FACS buffer (PBS, 1 mM EDTA, 2% FCS, 0.1% sodium azide), resuspended in 500 µl blocking buffer (equal volumes of FACS buffer and FCS) and incubated for 10 min at RT. The cells were then washed twice. Fluorescently conjugated antibodies were then added to the cells and incubated for 20–30 min in the dark. The cells were washed twice, resuspended in FACS buffer and read on a custom BD flow cytometer. Where intracellular staining was required, following surface staining the cells were fixed in 2% paraformaldehyde for 20 min and washed in FACS buffer. The cells were then washed three times in intracellular staining permeabilization wash buffer (Biolegend), after which intracellular antibodies were then added and incubated in the dark for 20–30 min. The cells were washed twice in FACS buffer and then read by a flow cytometer. Antibodies used were CD11b-BV605 (M1/70), Ly6C-Pacific Blue (HK1.4), Ly6G-FITC (1A8), F4/80-PE (BM8), CD115-PE-Cy7 (AFS98), CD206-PerCP-Cy5.5 (C068C2), CCR2-APC (Clone #475301), CD4-PE (RM4-5), CD8-APC (53-6.7), CD25-APC PC61.5), CXCR3-PE/Cy7 (CXCR3-173) and granzyme B-PE/Cy7 (QA16A02). Compensation controls were performed using anti-rat anti-hamster Ig κ negative and positive CompBeads. Isotype controls and unstained controls were run for all experiments. Isotype controls and gating strategies for all flow cytometry graphs can be seen in supplementary figures 1–13. Results were analysed on FlowJo® software version 10.5.3.

Arginase assay

BMDMs were washed twice and lysed in 10 mM Tris–HCl pH 7.4 with protease inhibitors and 1% Triton X-100 for 30 min on ice with vigorous vortexing every 10 min. The samples were centrifuged at 13,000*g* for 20 min to remove debris. Protein content was normalized. Arginase activity was assayed using an Arginase Activity Assay Kit (Sigma). Briefly, 40 µL of cell lysate was added to each well in quadruplicate. 1 mM urea and ddH_2O were used as a standard and standard blank. 10 µl substrate buffer was added to each sample well and each sample blank well received no addition. Plates were sealed and incubated at 37 °C for 2 h. Urea reagent (200 µl) was added to all wells to stop the reaction and substrate buffer (10 µl) was added to the sample blank wells. Plates were incubated at RT for 60 min. If turbidity appeared in the wells, the plate was centrifuged and the supernatants were transferred to a new plate without agitation of the precipitate. The absorbance was read at 430 nm using a SpectraMax M2 (Molecular Devices).

Griess assay

Supernatants were isolated following treatment of BMDMs and centrifuged at 500 g for 5 min to remove debris. Nitrate standards were made (100–1.5 µM) using a 0.1 mM sodium nitrate solution. 50 µl of supernatant, standard and medium only blanks were added in triplicate to flat bottomed optically transparent 96-well plates. 50 µl of a 1% sulphanilamide solution was added to each well and stored in the dark at RT for 10 min. 50 µl 0.1% N-1-napthylethylenediamine dihydrochloride in ddH20 solution was added to each well and stored in the dark at RT for a further 10 min. The absorbance of the plate was read at 535 nm on a SpectraMax M2 (Molecular Devices) plate reader within 30 min.

qPCR

BMDMs were treated for 4 h or 24 h. RNeasy plus mini-kits (Qiagen) were used for RNA extraction. RNA was immediately normalized by nanodrop and reverse transcribed using Omniscript reverse transcriptase (Qiagen) with $Oligo(dT)_{15}$ primers (1 µM). cDNA was normalized to 60 ng/ml and qPCR was performed using Luna® Universal qPCR Master Mix. A full list of primers used is available in supplementary table 1.

$CD4^+$ and $CD8^+$ T-cell isolation, CFSE staining and coculture

BMDMs were treated for 24 h. Spleens were isolated from syngenic mice and passed through a 70 µM filter. RBCs were lysed in lysis buffer and $CD4^+$ or $CD8^+$ T cells were isolated using negative selection magnetic bead labelling kits (Miltenyi Biotec) according to the manufacturer's instructions. Separation was confirmed by purity analysis using flow cytometry. All cells were confirmed to be > 85% purity prior to use. Separated T cells were labelled in 5 µM CFSE in warm PBS for precisely 8 min. The reaction was stopped by adding ten volumes of complete medium. Cells were washed twice following stimulation with conditioned medium or cytokines and counted before being added to cocultures. 10^5 T cells and 10^5 macrophages were seeded in 96-well suspension plates in 200 µl and incubated for 5–7 days. Medium colour was observed daily to ensure no acidification of wells occurred.

ELISA

IL-4, IL-10 and IFNγ Mouse ELISA Max™ Deluxe Sets (Biolegend) were used according to the manufacturer's instructions. Briefly, plates were blocked overnight at 4 °C in capture antibody diluted in coating buffer, all subsequent steps were performed at room temperature. Plates were emptied and washed. For each wash, plates were emptied, filled with PBS + 0.05% tween and allowed to soak for 30 s three times. The plates were then blocked for 1 h in assay diluent (from kit) with shaking. Plates were washed and samples, standards and controls were added to the plates for 2 h with shaking. Plates were washed and detection antibody was added for 60 min with shaking. The plates were washed and diluted avidin-HRP was added for 30 min with shaking. The plates were washed and freshly made substrate solution was added. The plates were incubated in the dark until a visible colour change had occurred. The reaction was stopped by adding an equal volume of 1 M HCl and the plates were promptly read at 450 and 570 nm. 570 nm readings were subtracted from 450 nm readings before analysis.

Data analysis

Statistical analysis was performed using paired and unpaired Student's *t*-tests for differences between groups. Flow cytometry data was analysed using FlowJo version 10.5.3. Statistical analysis was performed using Graphpad Prism version 5.03.

Results

Melanomas are one of the most immunogenic cancer types characterized by a high volume of tumour infiltrating leukocytes, and are consequently considered one of the tumours most likely to benefit from immunotherapy [11]. B16F10 cells are a highly aggressive cell line which can be used for the study of murine melanoma. Injected subcutaneously, they readily form primary tumours which metastasise. We used this model of tumour growth to examine the role of TAMs in murine melanoma. Circulating monocytes were analysed during tumour growth (Fig. 1). Monocytes were gated as $CD11b^+$ $F4/80^+$ $Ly6G^-$ and distinguished as $Ly6C^+$ $CCR2^-$ or $Ly6C^-$ $CCR2^-$ non-inflammatory monocytes or as $Ly6C^{hi}$ $CCR2^+$ inflammatory monocytes (gating shown in supplementary figure S1).

Inflammatory monocytes were found to be present in circulation and increased as a proportion of total $CD11b^+$ $F4/80^+$ $Ly6G^-$ monocytes during disease progression ($p < 0.01$) (Fig. 1). The proportion of other $CD11b^+$ $Ly6G^-$ monocytes decreased during tumour progression ($p < 0.01$). B16F10 tumours were excised, washed and plated for 24 h, tumours were confirmed to secrete CCL2 (> 1000 pg/ml/10^6 cells, data not shown), and the primary chemoattractant for CCR2.

In line with the three Rs of animal research, replacement, reduction and refinement, we next sought to develop monocytes in vitro from murine bone marrow which reflect the cells previously seen in vivo [12]. Bone marrow was cultured in the presence of M-CSF and analysed by flow cytometry daily to track their development (Fig. 2). Monocytic cells were defined as $CD11b^+$ $CD115^+$ $F4/80^+$ and with varying CCR2 and Ly6C expression [8]. Analysis of CD11b versus Ly6C allowed the identification of distinct monocyte subsets.

The proportion of monocyte subsets varied between suspension and adherent populations, however, once grouped on Ly6C and CD11b expression, other cell markers remained consistent indicating homogeneity of adherent and suspension populations, as such all cells were harvested for further use.

By day 5, non-monocytic $CD11b^-$ $Ly6C^-$ cells composed just 3 ± 0.89% of total cells. $CD11b^+$ $Ly6C^+$ cells were negative for CD115 and F4/80 (supplementary figure 2) and are likely to represent an immature population of monocytic cells at a midway point to development, by day 5, however, they represented a minimal proportion of the population at 10.47 ± 2.9%.

$CD11b^{hi}$ $Ly6C^-$, $CD11b^{hi}$ $Ly6C^+$ and $CD11b^+$ $Ly6C^{hi}$ populations were all positive for CD115 and F4/80 (supplementary figure 2). F4/80 is a murine marker of monocytes which is present on monocytes and highly expressed

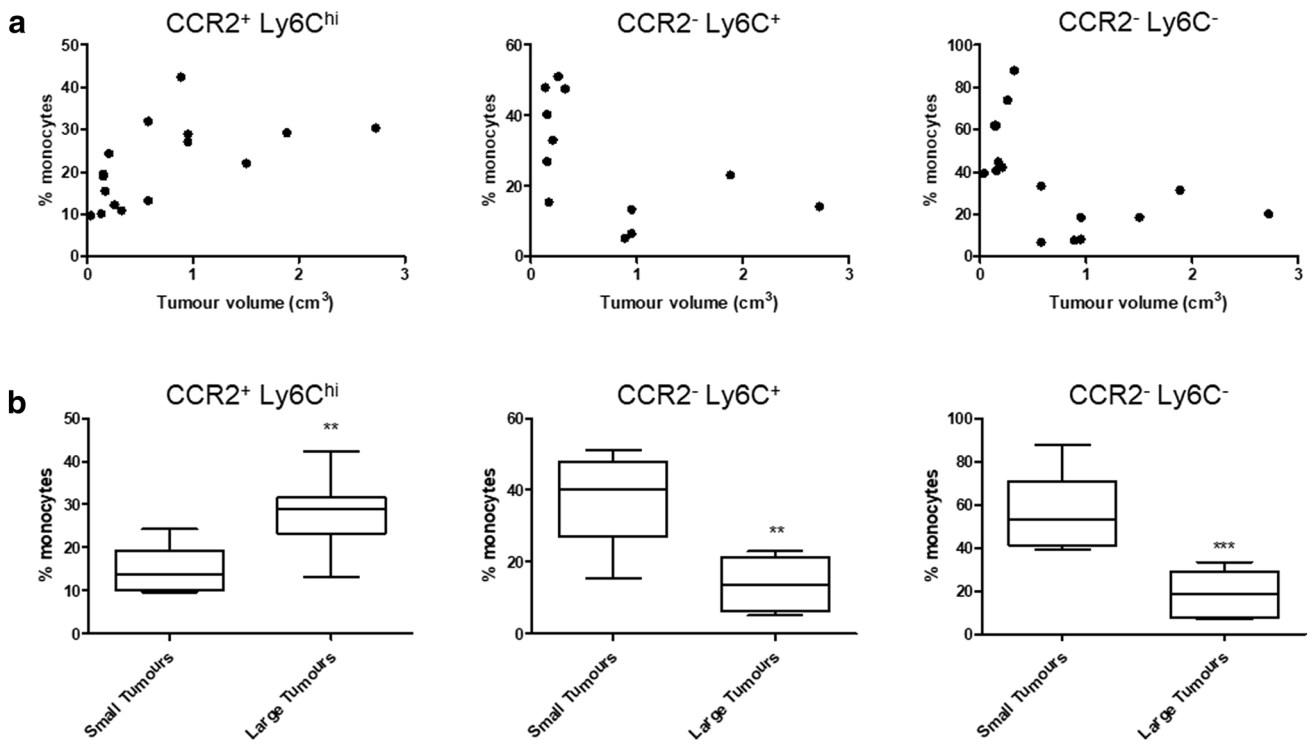

Fig. 1 Circulating monocyte populations in subcutaneous B16F10 tumour-bearing mice. Mice were subcutaneously inoculated with B16F10 cells. At various points of tumour development, blood was isolated and analysed by flow cytometry. Monocytes were gated according to forward scatter, side scatter and as $CD11b^+$, $F4/80^+$ and $Ly6G^-$. **a** Scatter plots of tumour size against the relative proportion of $CCR2^-$ $Ly6C^{hi}$ (left) $CCR2^-$ $Ly6C^+$ (middle), and $CCR2^-$ $Ly6C^-$ (right) in the blood. **b** Box plots of the data shown in (**a**) showing the proportion of cells when stratified between small (<0.5 cm^3) and large (>0.5 cm^3) tumours. Data shown are representative of $n=6$ per group. $^{**}p<0.01$, $^{***}p<0.001$ compared to small tumours

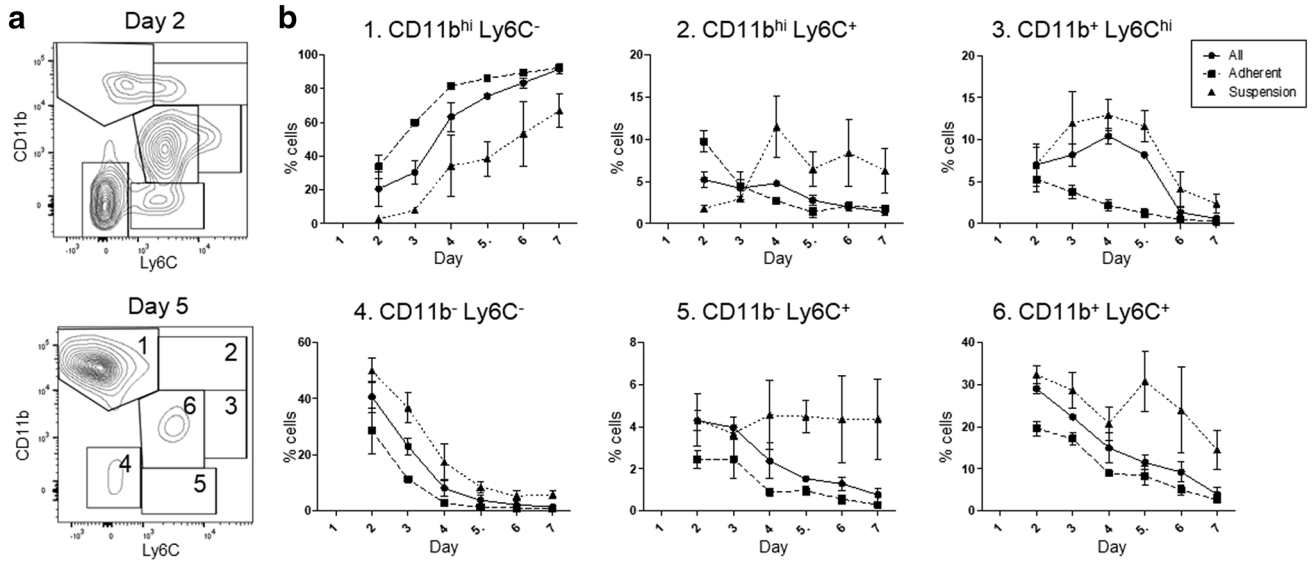

Fig. 2 The development of bone marrow-derived monocytes. Bone marrow was isolated and following red blood cell lysis was cultured in the presence of 50 ng/ml M-CSF between 2 and 7 days. **a** Representative graphs showing CD11b and Ly6C expression of whole BMDM populations on day 2 (top) and day 5 (bottom). **b** Adherent only, suspension only and mixed populations were each analysed by flow cytometry from day 2 to day 7 to track the development of BMDMS. Populations shown correspond to the gating shown in (**a**). Data shown are representative of $n=3$ per group

on macrophages. $CD11b^+$ $Ly6C^{hi}$ cells most accurately reflected inflammatory monocyte populations with CCR2 positivity and were $Ly6G^-$ (supplementary figures 3–5). These cells peaked in frequency at day 4, after which a gradual decline was seen from day 5 to day 7.

To study the effect of tumour conditioning on peripheral monocytes infiltrating into the tumour, day 5 BMDMs were used which were comprised of $77.6 \pm 3.38\%$ $CD11b^{hi}$ $Ly6C^-$cells, $2.76 \pm 0.97\%$ $CD11b^{hi}$ $Ly6C^-$cells and $8.17 \pm 0.07\%$ inflammatory monocyte-like $CD11b^+$ $Ly6C^{hi}$ cells. Thus, monocytic subsets represented $88.53 \pm 4.42\%$ of cell populations used for subsequent experiments.

To study the effect of tumour conditioning on monocytes, we treated BMDMs with concentrated melanoma-conditioned medium derived from B16F10 cells, termed B16-conditioned medium (B16CM). As this contained a low level of serum, which has been shown to influence macrophage polarization, our no treatment negative controls were treated with an equivalent amount of ultracentrifuged medium with serum which was not exposed to B16F10 cells. To clarify this distinction, we have labelled our no treatments as 'no treatment concentrated medium' (NTCM) [13]. Of note, FCS has been documented to contain varying cytokines, chemokines, growth factors and extracellular vesicles, all of which can influence macrophage polarization. To control for this, the FCS for each experiment was derived from the same batch and the same stock solution.

Treatments with either IFNγ and LPS or IL-4 and IL-13 were included as M1 and M2 controls, while their biological relevance is questionable, they have been included as assay positive controls and to give the results presented here context in comparison to other similar literature.

B16CM increased the expression of the 'M2' marker CD206 on $CD11b^+$ cells, although not to the extent of IL-4 and IL-13 conditioned BMDMs (Fig. 3) [14]. B16CM was also shown to increase CD115 expression on $CD11b^+$ cells to a similar extent as treatment with IL-4 and IL-13.

To determine if this apparent 'M2'-like shift was reflected in arginine metabolism which has been proposed as a central feature of the M1 M2 dichotomy, we determined the arginase activity of cell lysates and the secretion of nitrate species. B16CM increased arginase activity but was significantly lower than IL-4/IL-13-treated BMDMs and comparable to IFNγ/LPS-treated BMDMs. All populations fell below the detectable limit of nitrate detection indicating little or no

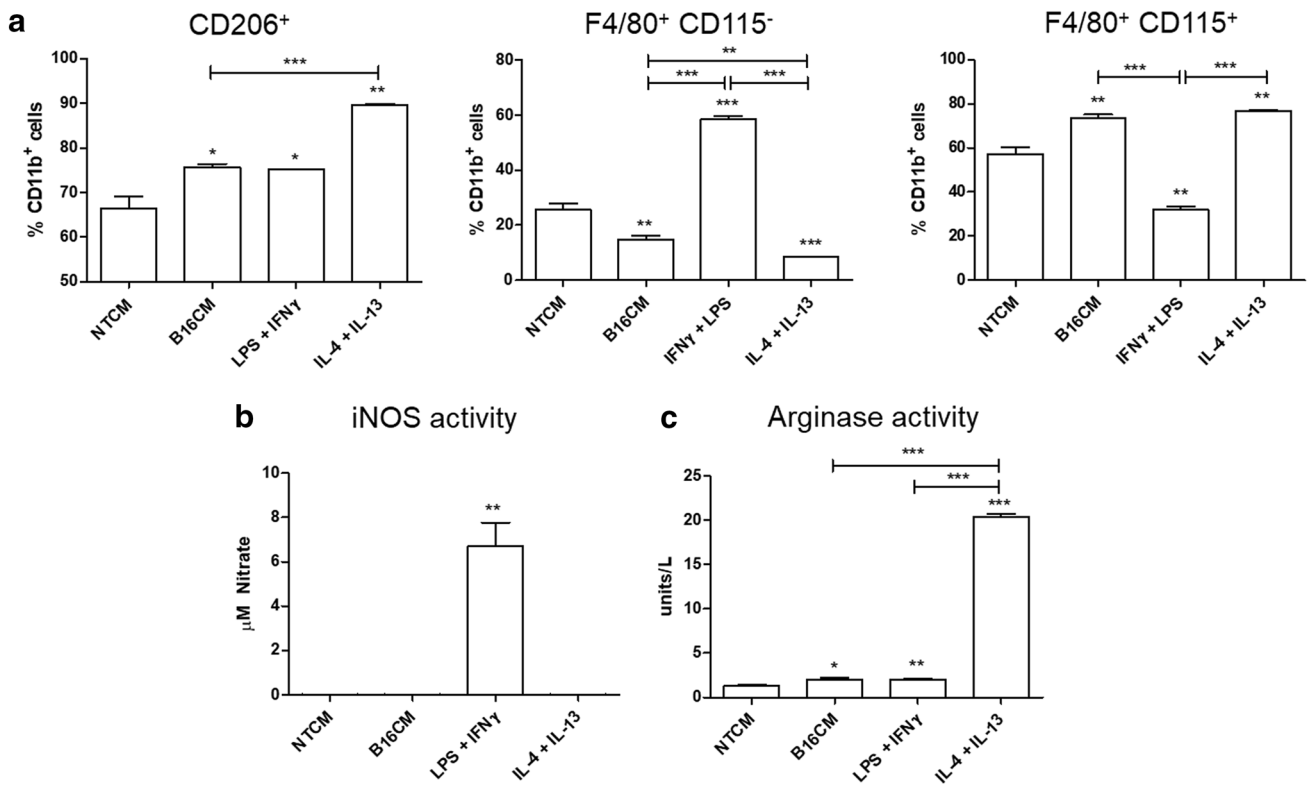

Fig. 3 The effect of B16CM on the expression of CD206 and CD115 and activity of iNOS and arginase by BMDMs. BMDMs were treated on day 5 with NTCM, B16CM, IFNγ/LPS or IL-4/13 for 24 h. **a** Cells were analysed by flow cytometry to determine the level of CD206 expression or CD115 expression on $CD11b^+$ BMDMs. **b** Culture supernatants were analysed by Griess assay to calculate the levels of iNOS activity. **c** Arginase activity was measured in cell lysates following treatment. Data shown are representative of $n = 3$ per group. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to NTCM unless specified

inducible nitric oxide synthase (iNOS) activity in these cells, except for IFNγ/LPS-treated BMDMs.

Thus while B16CM-treated BMDMs showed indication of an M2-like phenotype by CD206 and CD115 expression, supported by the absence of nitric oxide species secretion, the arginase activity was comparable to IFNγ/LPS-treated M1-like BMDMs and not IL-4/IL-13-treated M2-like BMDMs.

To determine the functional phenotype of these BMDMs, we next evaluated the gene expression of BMDMs 4 h and 24 h following treatment.

CD68 is a scavenger receptor expressed by monocytes and macrophages used to identify pan-macrophage populations, but is often upregulated on monocytes during activation [15]. In addition to the increase in surface markers CD206 and CD115 previously shown, gene expression of the surface markers, CD206 and CD68, was both shown to be increased by B16CM treatment after 4 h and an increase in CD68 expression was still visible at 24 h (Fig. 4).

Analysis of *ARG1* gene expression, the inducible form of arginase commonly seen in 'M2' macrophages revealed no observable increase in expression and at 24 h a significant decrease was observed.

No change in IL-10, IL-12 or IL-6 expression was found after B16CM treatment. IL-4/IL-13 treatment was shown to induce expression of TGFβ at 4 h after treatment and IL-6 24 h after treatment. IFNγ/LPS treatment showed no effect on TGFβ expression but increased IL-10, IL-12 and IL-6 expression 4 h after treatment. The inflammatory genes IL-6 and IL-12 still significantly increased 24 h following treatment.

Ym1 is a protein which has been linked to invasion. B16CM treatment was shown to reduce Ym1 expression after 24 h and was significantly lower than IL-4- and IL-13-treated BMDMs and IFNγ/LPS-treated BMDMs after 4 h.

To determine if the limited functional capacity seen in gene expression would translate to a reduced influence in determining tumour immunology, we next sought to identify the effect of B16CM-treated BMDMs on $CD4^+$ T-cell activity (Fig. 5).

NTCM-treated BMDMs were able to induce the proliferation of $CD4^+$ T cells, to a similar level as IL-4/IL-13-treated BMDMs, however, B16CM-treated BMDMs significantly decreased the proportion of activated T cells.

Analysis of culture supernatants showed NTCM-, B16CM- and IFNγ/LPS-treated BMDMs failed to generate IL-4 secretion by $CD4^+$ T cells, however, IL-4/IL-13-treated BMDMs resulted in a significant increase in IL-4 secretion.

While macrophages were able to directly secrete IFNγ (shown in grey, Fig. 5d, $p < 0.05$), coculture revealed significant additional IFNγ secretion from $CD4^+$ T cells.

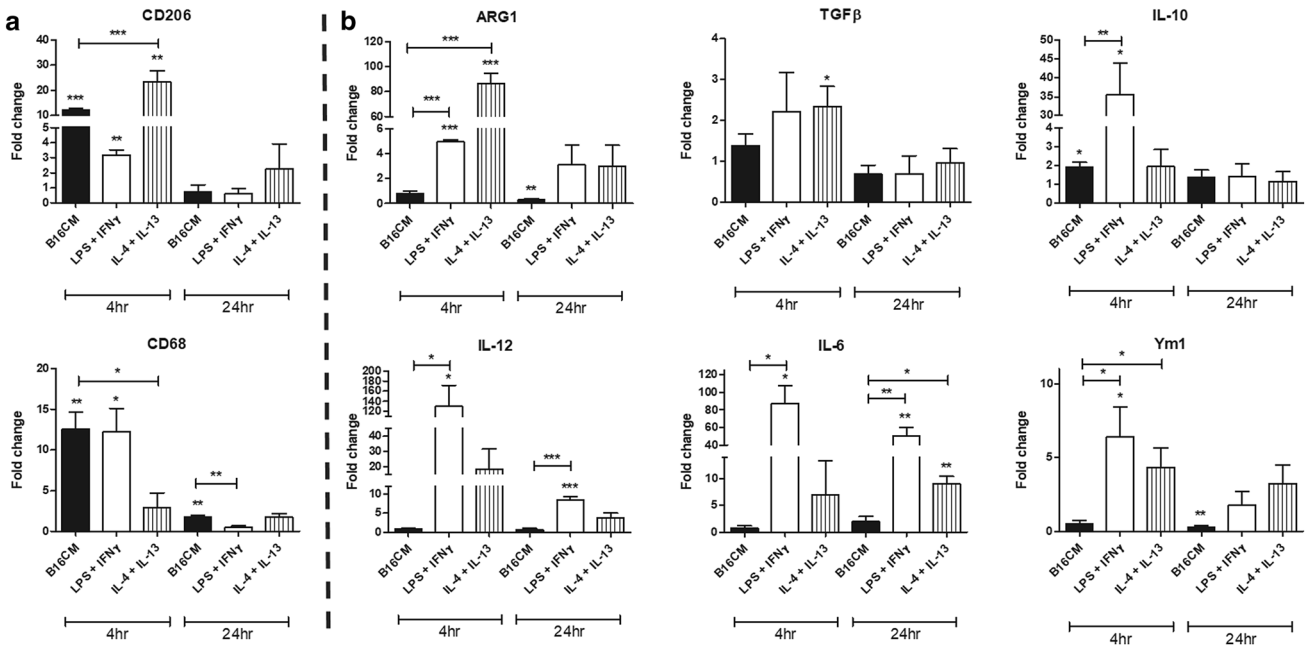

Fig. 4 The effect of B16CM on gene expression of BMDMs. BMDMs were treated on day 5 with NTCM, B16CM, IFNγ/LPS or IL-4/13 for 4 or 24 h. RNA was isolated and analysed for relative gene expression by qPCR. Results were normalized to NTCM only. **a** Gene expression for the surface receptors CD206 and CD68 was measured following treatment. **b** Gene expression of the functional proteins ARG1, TGFβ, IL-10, IL-12, IL-6 and Ym1 was measured following treatment. Data shown are representative of $n = 3$ per group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to NTCM (a mean value of one in all graphs due to normalization of data) unless specified

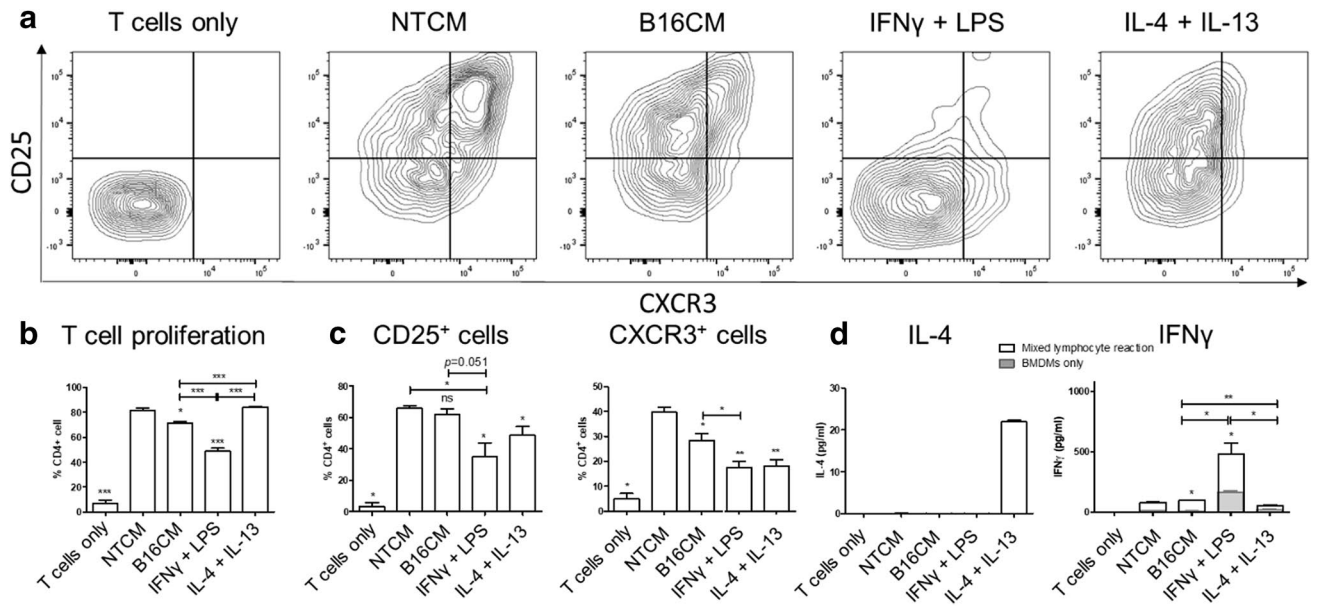

Fig. 5 The effect of treated BMDMs on $CD4^+$ T-cell expansion and polarization. Day 5 BMDMs were treated for 24 h. Following treatment, the cells were washed twice and cocultured with freshly isolated CFSE labelled splenic $CD4^+$ T cells from syngeneic mice for 7 days. Cells were analysed by flow cytometry and supernatants were analysed by ELISA. **a** T cells were gated for by CD4 expression. Representative graphs of CXCR3 against CD25 for each of the treatment groups are shown. **b** $CD4^+$ T-cell proliferation was determined by reduced CFSE staining. **c** Bar charts showing the number of $CD25^+$ and $CXCR3^+$ $CD4^+$ T cells using the gating shown in (**a**). **d** The IL-4 and IFNγ levels in supernatants following coculture are shown in white bar charts, while the level of the same cytokines following culture of treated macrophages alone is overlaid in grey. Data shown are representative of $n=3$ per group. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to NTCM unless specified

B16CM-treated BMDMs significantly increased secretion of IFNγ compared to NTCM- and IL-4/13-treated BMDMs but not to the extent of IFNγ/LPS-treated cells.

Flow cytometric analysis of activated $CD4^+$ T cells showed the induction of CXCR3 and CD25 on $CD4^+$ T cells by NTCM-treated BMDMs. B16CM-treated cells resulted in a decrease of $CXCR3^+$ T cells but had no effect on the number of $CD25^+$ T cells. Both IFNγ/LPS- and IL-4/IL-13-treated BMDM cocultures resulted in significantly lower numbers of $CD25^+$ and $CXCR3^+$ T cells compared to NTCM-treated cells. The trend indicated they were also lower than T cells cocultured with B16CM-treated cells, however, this trend was only significant for the expression of CXCR3 T cells cocultured with IFNγ/LPS-treated BMDMs.

While B16CM-treated BMDMs reduced $CD4^+$ T-cell proliferation and the level of activated T cells positive for the Th1 marker CXCR3, they also increased IFNγ secretion which is central to driving Th1 type anti-cancer immune responses. To further investigate the effect on the anti-tumour immune response we investigated the effect of treated BMDMs directly on $CD8^+$ T cells.

To determine if B16CM-treated BMDMs could have an effect on cytotoxic $CD8^+$ T-cell behaviour, we examined the proliferation of $CD8^+$ T cells cocultured with treated BMDMs.

B16CM-treated BMDMs were shown to significantly increase the proliferation of $CD8^+$ T cells in comparison to NTCM-, IFNγ/LPS- and IL-4/13-treated cells. IFNγ/LPS- and IL-4/13-treated cells had no significant impact on $CD8^+$ T-cell expansion (Fig. 6).

Along with increased $CD8^+$ T-cell proliferation, B16CM-treated BMDMs were found to increase the granzyme B levels of $CD8^+$ T cells. This increase was above the level seen following coculture with either IFNγ/LPS- or IL-4/13-treated BMDMs indicating that B16CM-treated BMDMs may prime $CD8^+$ T cells for cytotoxic responses.

All BMDMs were found to stimulate secretion of IFNγ by $CD8^+$ T cells. B16CM significantly increased IFNγ secretion, and this was also significantly higher than that induced by IL-4/IL-13-treated BMDMs, but was over ten-fold less than IFNγ secreted by cells cocultured with IFNγ/LPS-treated BMDMs.

Although B16CM-treated BMDMs significantly increased IL-10 levels compared to T cells alone, this was lower than the levels seen by B16CM-treated BMDMs alone in culture. This trend was seen in cocultures with all BMDM treatments except for cocultures with IFNγ/LPS-treated BMDMs which were significantly higher following inclusion of T cells ($p<0.01$) indicating $CD8^+$ T-cell

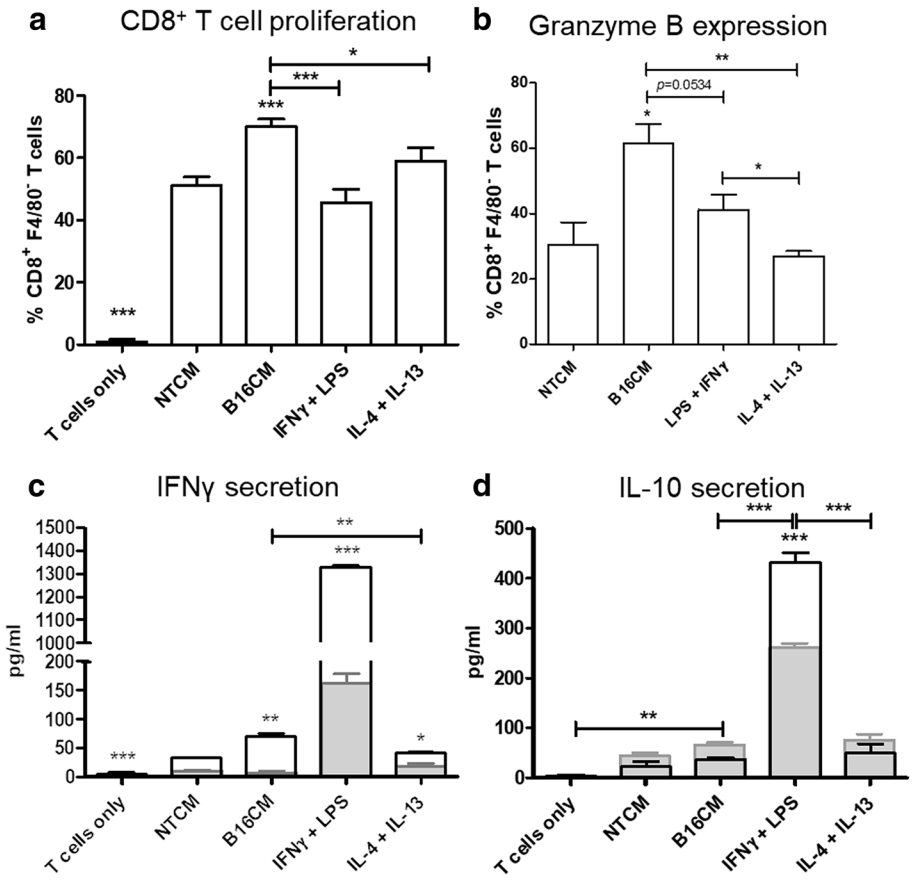

Fig. 6 The effect of treated BMDMs on $CD8^+$ T-cell expansion and polarization. Day 5 BMDMs were treated for 24 h. Following treatment, BMDMs were washed twice and cocultured with freshly isolated CFSE labelled splenic $CD8^+$ T cells from syngeneic mice. Cells were incubated for 5 days. **a** $CD8^+$ T-cell proliferation was determined by reduced CFSE staining. **b** Granzyme B levels were determined by flow cytometry. **c**, **d** The IFNγ and IL-10 levels in supernatants following coculture are shown in white bar charts, while the level of the same cytokines following culture of treated macrophages alone is overlaid in grey. Data shown are representative of $n=3$ per group. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to NTCM unless specified

secretion of IL-10 is absent except when cocultured with IFNγ/LPS-treated BMDMs.

Discussion

It has been noted that M-CSF-derived BMDMs adopt an M2-like phenotype. This polarization phenomenon cannot be avoided with the use of BMDMs derived from M-CSF of GM-CSF (GM-CSF-derived cells conversely adopt a relative M1-like phenotype and the inclusion of a dendritic cell population). Many protocols involve the culturing of cells for 7–14 days, however, it is likely that this may be subversive in dissecting the minutiae of monocyte/macrophage function, as it often requires powerful signalling to see any shift away from an M-CSF-induced M2-like phenotype. Other studies have used M-CSF withdrawal or GM-CSF pulsing prior to stimulation to induce a 'M0' phenotype prior to activation, however, all starting states are subjective to the protocol used and the results presented should be noted in that context. To aid reproducibility, we have retained a simplified protocol, but future work is required to determine if the results presented can be recapitulated under different BMDM culturing conditions [1].

Due to the leaky vasculature of tumour tissue, it was traditionally presumed that peripheral circulating monocytes were readily trafficked in the tumour microenvironment. However, it has been shown in a murine model of colon adenoma that the tumour may contain a dominant population of self-maintaining macrophages distinct from circulating populations [16]. However, using a subcutaneous B16F10 model of murine melanoma which secretes CCL2, we have shown that the levels of circulating $CCR2^+$ inflammatory monocytes increase during tumour progression. Using the B16 cell line variant B16BL6 tumour model, it has been shown that the level of monocytic proliferation in the tumour is low and that bone marrow is likely to provide a continuous flow of poorly proliferating monocytes to the tumour [17]. A recent paper has also shown that circulating inflammatory monocyte levels correlate to both intratumoural macrophage infiltration and patient survival in colorectal cancer [18].

To form an in vitro model to study the effect of B16F10 tumours on these cells, we opted to treat M-CSF-derived BMDMs on day 5 with conditioned medium. This population, which is $88.53 \pm 4.42\%$ mature monocytic cells, is enriched for inflammatory monocyte-like cells and limits the M2 polarizing effect of M-CSF itself.

The model contains inherent limitations including the purity of the cell population and also the effect of tumour

conditioning is affected by B16F10 secreted factors only and does not account for any cell–cell interactions or biochemical effects such as hypoxia or low pH commonly exerted in tumour microenvironments.

Due to limited availability of clinical tissue, the function of TAMs is often inferred from surface markers commonly expressed by M2-like cells seen during examination of formalin-fixed paraffin-embedded tissue [3].

Here we have shown that expression of cell surface markers traditionally associated with 'M2' macrophages may not reflect functional activity and these cells expressing 'M2' markers may instead promote anti-cancer immune responses via $CD8^+$ T-cell expansion, IFNγ expression and increased cytotoxicity. B16CM increased the expression of cell surface markers CD206, CD115 and CD68, but this did not translate into a change in IL-10 or IFNγ production as determined by ELISA or IL-6, IL-10, IL-12 or TGFβ expression as determined by qPCR. These results reflect previous studies showing that B16F10-conditioned medium can inhibit cytokine production by murine lymphocytic preparations and that melanoma-derived exosomes can promote mixed M1 M2 polarization in macrophages, although melanoma-derived exosomes were able to augment the production of cytokines such as TNFα, IL-1β and IL-10 [19, 20].

TGF-β can be secreted by M2-like macrophages and can promote tumour cell stemness and migration, while both TGF-β and IL-10 are potent inhibitors of effective T-cell responses [21, 22]. A reduction in these immunosuppressive mediators is positive in an anti-cancer context, but the inability of B16CM-treated BMDMs to produce IL-6 or IL-12 which are both potent inflammatory mediators required for the induction of IFNγ secreting T cells indicates a more ambiguous role in disease [23].

Coculture with T cells showed a marginal decrease in $CD4^+$ T-cell expansion by B16CM-treated BMDMs but an increase in $CD8^+$ T-cell expansion. Coculture of B16CM-treated BMDMs with either $CD4^+$ or $CD8^+$ coculture also increased IFNγ secretion by T cells, which has been identified as a central cytokine in driving anti-cancer Th1 immune responses.

These results are in contrast to other studies which have shown that direct treatment of T cells with cell line-conditioned medium or fractionated exosomes from conditioned medium reduces T-cell proliferation and induces a suppressive $CD8^+$ T-cell phenotype [24].

Intratumoural depletion of regulatory T cells has been shown to control the growth of murine models of melanoma, as these cells can inhibit the cytotoxic anti-tumour effect of $CD8^+$ T cells [25, 26]. Regulatory T cells have also been shown to correlate to prognosis in human melanoma indicating that they play an influential role in disease [27–29]. There is intimate cross-talk between melanoma-derived factors, including melanoma-derived exosomes and cytokines, and T cells that can affect their function [25, 30–33]. The ability of B16CM-treated BMDMs to induce $CD8^+$ T-cell proliferation and cytotoxicity without increasing the frequency of $CD25^+$ T cells may indicate that these cells could counteract the direct inhibitory effect of conditioned medium on T cells.

Similar factors such as cytokines and exosomes are present in FCS, and while controlled for in this study, serum-free growth media are becoming increasingly available and may permit more sensitive studies to be performed on the effect of cell line-conditioned media or their constituents.

Due to their role in a wide range of tumour promoting activities, macrophages have been viewed as actionable targets in the development of novel anti-cancer therapeutics, however, these efforts have been largely unsuccessful. Understanding the role of TAMs is essential for the development of effective therapeutics, these results suggest that the presence of M2 markers on macrophages, as are commonly reported on TAMs, does not prohibit them from promoting T-cell responses.

Author contributions LFT: study design, wet lab work and paper writing. ACM and PFF: study design, evaluation of results and paper writing.

Funding We would like to thank Breakthrough Cancer Research who funded this research.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval and ethical standards All animal husbandry and experimental procedures were approved and licensed by the Animal Experimentation Ethics Committee (AEEC) in University College Cork under licence 2012-047 and performed according to the Irish Cruelty to Animals Act, 1876.

Animal source All animals were purchased from Envigo in the UK.

Cell line authentication The B16F10 cell line was purchased from and authenticated by the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis Tumor Repository.

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