Remote control: the cancer cell-intrinsic mechanisms that dictate systemic inflammation and anti-tumor immunity

Wellenstein, M.D.

In vitro assessment of cancer cell-induced polarization of macrophages

Danique E.M. Duits[1], Max D. Wellenstein[1] & Karin E. de Visser[1,*]

Methods in Enzymology. 2020;632:133-154.
doi: 10.1016/bs.mie.2019.06.011

Affiliations:
[1] Division of Tumor Biology & Immunology, Oncode Institute, Netherlands Cancer Institute, 1066CX Amsterdam, The Netherlands

* Corresponding author

Abstract

The progression of cancer is strongly influenced by the crosstalk between cancer cells and immune cells. Immune cells can have both pro- and anti-tumor functions depending on the signals present in the environment. A significant proportion of the immune compartment of most solid tumors consists of tumor-associated macrophages. Although their abundance has been associated with poor prognosis in many solid tumor types, the molecular mechanisms by which cancer cells influence macrophage phenotype and function are largely unknown. In this chapter, we provide a detailed description of *in vitro* assays to study the impact of cancer cells on macrophages. We provide protocols to obtain macrophages from murine bone marrow and human peripheral blood, and to expose these macrophages to cancer cell-derived secreted molecules using conditioned medium from cancer cells. We describe several assays to assess cancer cell-induced polarization of macrophages. This experimental set-up can be utilized to gain molecular insights into how cancer cells influence macrophages.

1. Introduction

The development and progression of cancer strongly depends on interactions between cancer cells and their microenvironment[1]. Besides cancer cells, the tumor microenvironment (TME) consists of host stromal cells, extracellular matrix, vascular endothelial cells and immune cells. The immune landscape of tumors can differ tremendously between and within cancer types[2,3]. While intratumoral presence of some immune cell types can be favorable for disease prognosis, infiltration by others can promote tumor progression. For instance, increased influx of $CD8^+$ T cells is indicative of an anti-tumor immune response and as such is frequently associated with improved overall survival, whereas influx of regulatory T cells (Tregs) or myeloid cells provides an immunosuppressive environment which may promote tumorigenesis[2,3].

The cancer-immune cell crosstalk is a dynamic process that is influenced by multiple factors and determines whether a pro- or anti-inflammatory environment is established at the tumor site. The state of immune cells can dictate cancer cell behavior, but the phenotype of cancer cells also influences the composition and function of tumor-associated immune cells. A major part of the immune compartment of solid tumors comprises tumor-associated macrophages (TAMs), which play a key role in regulating inflammation, angiogenesis, remodeling of the extracellular matrix and cancer cell migration[4]. Due to their plasticity, TAMs can exert pro- or anti-tumorigenic functions depending on the cues they receive from their environment[4,5]. TAMs are able to dampen anti-tumor immune responses by inducing an immunosuppressive environment via multiple mechanisms. Direct interaction of TAMs with T cells via immune checkpoints, such as PD1-PDL1, results in immunosuppression by blocking anti-tumor immunity[6]. Indirectly, TAM-derived cytokines can induce Tregs or inhibit immune cell populations that are involved in anti-tumor immunity, such as dendritic cells (DCs) and $CD8^+$ T cells, thus leading to an immunosuppressive microenvironment[6]. On the other hand, phagocytosis and antigen presentation can be key anti-tumorigenic features of macrophages, although these functions are infrequently observed in TAMs[4,6]. Altogether, these findings indicate a crucial role for cancer-immune cell crosstalk in cancer progression. Better understanding of TAM regulation and biology could provide new targets to block pro-tumorigenic processes or to re-educate TAMs towards an anti-tumorigenic phenotype.

The clinical relevance of macrophages in cancer progression has been demonstrated in studies examining the correlation between macrophage abundance and disease outcome. In the majority of solid cancer types, such as breast, gastric, urogenital and head and neck cancer, as well as hematological cancers, high macrophage levels correlate with poor prognosis[7-11]. This suggests that in most cancer types, these cells play an adverse role in disease progression. Interestingly, in colorectal cancer patients, high macrophage infiltrate is associated with increased overall survival[9], underscoring the functional plasticity of these cells in different contexts and anatomical locations. In addition, recent evidence suggests that disease stage is another important factor in TAM phenotype[12]. Accumulation of macrophages has also been associated with poor response to certain anti-cancer therapeutics, such as chemotherapy[13], indicating that these cells can influence both cancer progression and therapy response. Therefore, macrophage-targeting agents may prove beneficial for the efficacy of conventional anti-cancer drugs[14]. These findings demonstrate that studying the crosstalk between macrophages and cancer cells will aid the development and improvement of agents targeting macrophages and their associated activating molecules, of which some are currently being tested in clinical trials[4,15].

Despite the apparent crucial role for macrophages in cancer progression, the molecular mechanisms of how cancer cells influence macrophage phenotype and function are largely unresolved. The *in vitro* generation of macrophages from human peripheral blood and mouse bone marrow to study cancer-immune cell interactions provides a relatively quick and easy approach to gain molecular insights into the crosstalk between cancer and immune cells. We here describe an *in vitro* assay focusing on how cancer cell-derived molecules can impact the phenotype and functional state of macrophages. By utilizing conditioned medium from cancer cell lines, we mimic paracrine signaling between cancer cells and TAMs occurring in the TME.

In this chapter, we provide a detailed overview of several *in vitro*-based approaches to study the impact of cancer cells on macrophages. Macrophages can be differentiated in a 7-day protocol from murine bone marrow or human peripheral blood mononuclear cells (PBMCs) to be used in a variety of experiments. In one to several days following differentiation, the impact of the cancer cell secretome on macrophage phenotype can be examined by exposing macrophages to the conditioned medium from cancer cells, followed by several assays to determine macrophage activation. By blocking or genetically disrupting the production and secretion of mediators of interest (e.g. cytokines or growth factors) by cancer cells, the impact of specific cancer cell-derived proteins on macrophage polarization can be studied. Although we describe a powerful method, it is limited to the assessment of paracrine signaling from cancer cells to macrophages. The complexity of an *in vivo* situation where cancer cell-macrophage interactions and paracrine signaling between both cell types impact cancer progression cannot be studied by solely using cancer cell conditioned medium. We will provide examples of experiments showing how cancer cells can influence macrophage phenotype in terms of activation state, intracellular pathway activity and expression of chemokines. In summary, our *in vitro* system can be utilized to gain molecular insights into cancer cell-induced polarization of macrophages.

2. Protocol

2.1. Isolation of murine bone marrow and differentiation into bone marrow-derived macrophages (BMDMs)

Researchers working with mouse models to study cancer can make use of the murine set-up of the culture method described in this chapter. In the murine setting, wild-type non-tumor-bearing mice are used to isolate the bone marrow to be differentiated into bone marrow-derived macrophages (BMDMs) by recombinant murine M-CSF stimulation for 7 days (**Fig. 5.1A, B**). We refer to section 2.2 for the isolation of macrophages from human peripheral blood. An overview of the average cell yield and the corresponding number of experiments that can be performed per mouse can be found in **Table 5.1**.

2.1.1. *Materials*

- Mouse bones (tibia and femora)
- Disposable scalpels (Swann-Morton)
- Clean paper towels
- 70% ethanol (diluted from 100% ethanol in H_2O)
- 10 mL syringe (BD Emerald)
- 27G x ½" 0.4 mm x 13 mm needles (BD Microlance 3)
- 70 µm cell strainer (Falcon)

- 50 mL tubes (Falcon)
- Full RPMI medium: RPMI medium 1640 (Gibco) supplemented with 10% Fetal Bovine Serum (Sigma), 100 IU mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Roche)
- Recombinant murine CSF-1 (Peprotech)
- MACS separation buffer, containing BSA (Miltenyi Biotec)
- PBS without Mg^{2+} and Ca^{2+} (Gibco)
- Non-tissue culture-treated 10 cm dishes (Corning, Sigma)
- Non-tissue culture-treated 24-well plates (Falcon)

Table 5.1. Indicative cell numbers upon differentiation

Average cell yield		Number of conditions	
		plated 300.000 cells per well in section 2.4.2	plated 500.000 cells per well in section 2.4.2
Murine bone marrow			
One leg	7-10 x 10^6 BMDMs	22-32 conditions	14-20 conditions
Two legs	15-20 x 10^6 BMDMs	50-65 conditions	30-40 conditions
Human blood			
50 mL of blood (buffy coat)	0.5-0.8x10^9 PBMCs = 50-80x10^6 $CD14^+$ cells = 25-40 x 10^6 MDMs	83-133 conditions	50-80 conditions
10 mL whole blood	1x10^7 PBMCs = 0.1 x10^7 $CD14^+$ cells = 5.000.000 MDMs	16 conditions	10 conditions

2.1.2. *Protocol for isolation of murine bone marrow and differentiation into macrophages*

All steps are performed at room temperature under sterile conditions.

Day 0

1. After euthanization of the wild-type mouse, collect tibia and femora by dissociating the femur head from the hip socket for both hind legs. Make sure the femur head stays attached to the femur to prevent loss of bone marrow. Remove the paw, skin and muscle tissue from the bones using a scalpel. Remove any residual tissue attached to the bones using clean paper towels soaked in ethanol (Note 1).
2. Detach the tibia from the femora and cut a small piece of both ends of the bones. Use a syringe with a 27G needle to flush the bones with full RPMI medium and strain the bone marrow through a 70 µm cell strainer (Note 2 and 3).
3. After collection of bone marrow, spin the cells down at 250 x g for 5 minutes and resuspend the cell pellet in 50 mL of full RPMI medium supplemented with 20 ng/mL murine M-CSF (Peprotech). Divide the bone marrow suspension over ten non-tissue culture-treated 10 cm dishes (5 mL per plate) and incubate at 37°C at 5% CO_2 (Note 4, 5, 6 and 7).

Day 3

4. Add 5 mL full RPMI medium supplemented with 20 ng/mL recombinant murine M-CSF to every 10 cm dish and incubate at 37°C until day 7 (**Fig. 5.1B**).

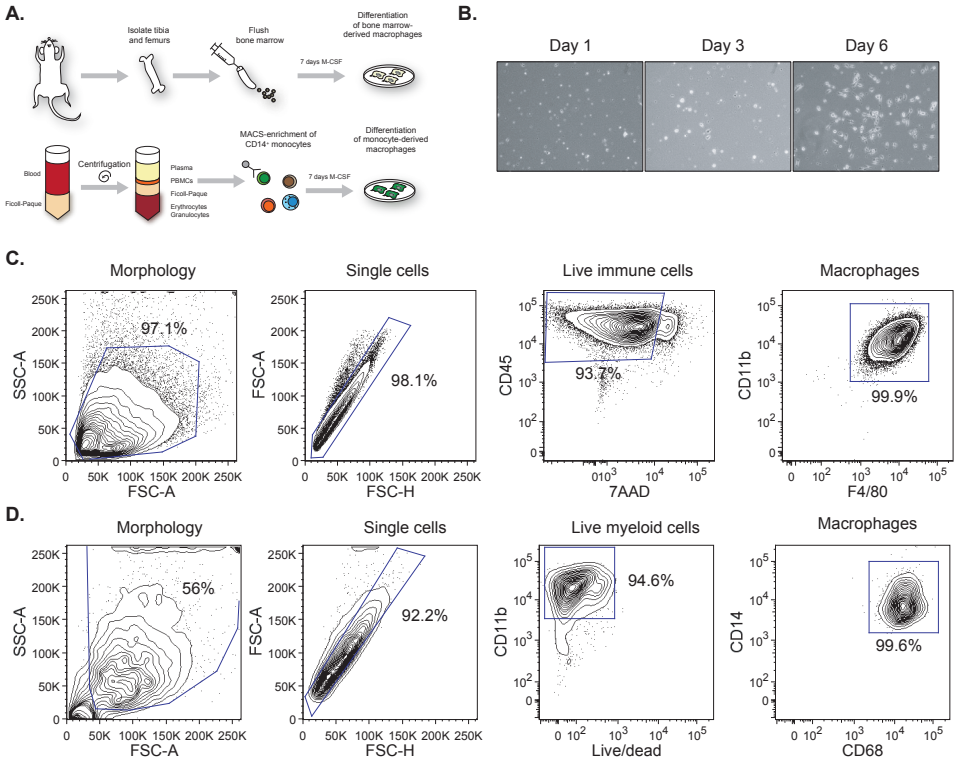

Figure 5.1. Isolation and purity of murine bone marrow-derived and human monocyte-derived macrophages. A. Schematic representation of isolation and differentiation protocol for murine bone marrow-derived macrophages (BMDMs) or human peripheral blood monocyte-derived macrophages (MDMs). For BMDMs, bone marrow is flushed from tibia and femora and cultured for 7 days in presence of recombinant murine M-CSF. For MDMs, peripheral blood mononuclear cells (PBMCs) are isolated using centrifugation over a Ficoll-Paque layer followed by enrichment of $CD14^+$ monocytes using magnetic beads, which are cultured for 7 days in recombinant human M-CSF. **B.** Microscopic images of differentiating BMDMs at day 1, day 3 and day 6. Morphological changes can be seen around day 6. **C.** Representative flow cytometry plots of differentiated BMDMs. After gating on single cells, live immune cells are identified as $7\text{-}AAD^-CD45^+$, and macrophages are identified as $CD11b^+F4/80^+$ cells. **D.** Representative flow cytometry plots of differentiated MDMs. After gating on single cells, live myeloid cells are identified as Live/dead-eFluor-780^-CD11b^+, and macrophages are identified as $CD14^+CD68^+$ cells.

Day 4 or 5

5. The preparation of conditioned medium from cancer cells starts on day 4 or 5 and is described in section 2.3.

Day 7

6. Discard the medium from all dishes and wash the cells with sterile PBS. Add 3 mL of MACS separation buffer (Miltenyi Biotec) to all dishes and incubate for at least 15 minutes at 37°C for cell detachment (Note 8 and 9).
7. Add 3 mL full RPMI medium to the plate with MACS separation buffer and mix to harvest the BMDMs. Collect the cells from all dishes in 50 mL tubes (Note 10). Spin

the cells down at 250 x g for 5 minutes and resuspend the cell pellet in 1 mL RPMI medium. Combine cell suspensions from one donor if more than one 50 mL tube was used to collect the cells from all dishes.

8. Count the cells using a manual counting chamber or an automated cell counter and proceed to section 2.4.2 (**Table 5.1**).

2.2. Isolation of human peripheral blood mononuclear cells and differentiation into monocyte-derived macrophages (MDMs)

To perform these experiments with human cells, peripheral blood from healthy donors can be used to isolate peripheral blood mononuclear cells (PBMCs) using density gradient centrifugation. By utilizing CD14 microbeads, CD14-expressing monocytes can be isolated from these PBMCs. These monocytes will be used to obtain monocyte-derived macrophages (MDMs) by stimulation of 7 days with recombinant human M-CSF (**Fig. 5.1A**). An overview of the average cell yield and the corresponding number of experiments that can be performed per buffy coat can be found in **Table 5.1**.

2.2.1. *Materials*

- Healthy donor blood (buffy coat or whole blood)
- PBS without Mg^{2+} and Ca^{2+} (Gibco)
- Ficoll-Paque Plus (GE Healthcare)
- 50 mL tubes (Falcon)
- NH_4Cl erythrocyte lysis buffer
- MACS separation buffer, containing BSA (Miltenyi Biotec)
- CD14 microbeads, human (Miltenyi Biotec)
- MACS Columns for magnetic cell isolation: columns for positive selection (usually MS or LS columns, see manufacturer's recommendations, Miltenyi Biotec)
- MACS magnetic separator (Miltenyi Biotec)
- 5 mL polystyrene round-bottom tube with cell strainer cap (Falcon)
- Recombinant human CSF1 (Peprotech)
- Full RPMI medium: RPMI medium 1640 (Gibco) supplemented with 10% Fetal Bovine Serum (Sigma), 100 IU mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Roche)

2.2.2. *Protocol for isolation of human monocytes and differentiation into macrophages*

All steps are performed at room temperature under sterile conditions, unless stated otherwise.

Day 0

1. Per 25 mL of a buffy coat, one 50 mL tube is needed. In a 50 mL tube, add 25 mL of a buffy coat to 25 mL PBS. Mix gently. Repeat this step until the entire buffy coat is diluted (Note 11).
2. Prepare 50 mL tubes with 25 mL Ficoll-Paque (GE Healthcare). For every 25 mL diluted blood, one tube with Ficoll is needed. Gently add 25 mL diluted blood to the tube containing the Ficoll: slowly pipet the blood via the side of the tube on top of the Ficoll without breaking the surface.
3. Centrifuge at room temperature at 350 x g for 20 minutes without brakes.

4. Using a pipette, carefully remove the plasma layer and collect the PBMCs from the interphase layer into a new 50 mL collection tube (**Fig. 5.1A**).
5. Fill tubes containing PBMCs with ice cold PBS, spin down 200 x g for 8 minutes (with brakes). Carefully pipet off the supernatant and resuspend the pellet in 1 mL PBS. Fill tubes with PBS, and repeat this wash step 2 times. After the wash steps, combine the separate tubes of one blood sample together, spin down 200 x g for 8 minutes and discard supernatant.
6. Add 5 mL of sterile NH_4Cl erythrocyte lysis buffer to the washed PBMCs, incubate for 5 minutes, and centrifuge at 200 x g for 8 minutes and discard supernatant.
7. Add 1 mL of sterile NH_4Cl erythrocyte lysis buffer to washed PBMCs and incubate for 5 minutes. Spin down 200 x g for 8 minutes and discard supernatant.
8. Resuspend pellet in 5 mL MACS buffer (Miltenyi Biotec) and count cells. Take a 30 µL sample from the cell suspension for later purity check by flow cytometry (step 19). Spin down 200 x g for 8 minutes and discard supernatant.
9. Resuspend pellet in 80 µL MACS buffer per 10^7 cells.
10. Add 20 µL MACS CD14 MicroBeads per 10^7 cells, mix and incubate on ice for 20 minutes.
11. Add 10 mL of MACS buffer to wash, spin down 250 x g for 8 minutes and discard supernatant. Resuspend cells in 500 µL of MACS buffer per 10^8 cells. To remove clumps, pass cell suspension through a 70 µM cell strainer. To ensure all cells came through, pass another 500 µL MACS buffer through the filter.
12. Depending on the number of cells, choose the appropriate Miltenyi magnetic column (see manufacturer's recommendations). Place the column in the magnetic separator above a 15 mL tube.
13. Wash column with 3 mL MACS buffer. Allow liquid to flow through.
14. When all liquid has passed the filter, add the cell suspension to the column.
15. Wash filter 3 times with 3 mL MACS buffer. The flow-through contains the CD14-negative fraction. Keep these cells for the purity check in step 19.
16. After washing, remove the column from the magnet and place it into a new 15 mL tube. Add 5 mL MACS buffer to the column and plunge cells through the column. This tube contains the CD14-positive fraction (Note 12). Count the cells.
17. Take 30 µl from the $CD14^-$ and $CD14^+$ fractions (from step 15 and 16, respectively) for later purity check (step 19).
18. To generate monocyte-derived macrophages, culture the $CD14^+$ fraction in 5 mL full RPMI medium supplemented with 20 ng/mL recombinant human M-CSF (Peprotech) in non-tissue culture-treated plates at a density of $5x10^6$ cells per 10 cm plate (Note 6 and 7).
19. Check the purity of your isolation by flow cytometry using the samples from the $CD14^-$ and $CD14^+$ fractions (from step 15 and 16, respectively) together with the total PBMC sample (from step 8) (see section 2.4.3.3).

Day 3

20. Supplement culture with 5 mL fresh full RPMI medium containing 20 ng/mL recombinant human M-CSF and incubate at 37°C until day 7.

Day 4 or 5

21. The preparation of conditioned medium of cancer cells starts on day 4 or 5 and is described in section 2.3.

Day 7

22. Discard the medium from all dishes and wash the cells with sterile PBS. Add 3 mL of MACS buffer and incubate at 37°C for 15 minutes (Note 9).
23. Collect the cells in a 50 mL tube by resuspending them in 3 mL of full RPMI (Note 10). Spin cells down at 250 x g for 5 minutes and resuspend the cell pellet in 1 mL RPMI medium. Combine cell suspensions if more than one 50 mL tube was used to collect the cells from the dishes.
24. Count the cells and proceed to section 2.4.2 (**Table 5.1**).

2.3. Preparation of conditioned medium from mouse/human cancer cell lines

2.3.1. *Materials*

- Cancer cell lines of interest
- Culture medium with FCS and additional supplements
- Culture medium without FCS (serum-free)
- PBS, without Mg^{2+} and Ca^{2+} (Gibco)
- 0.05% Trypsin-EDTA (Gibco)
- Tissue culture-treated 6-well plates (Greiner CELLSTAR)
- Inhibitory or activating agents of interest to target cancer cells or cancer cell-derived mediators

2.3.2. *Protocol for preparation of conditioned medium*

The conditioned medium is prepared on day 5-7 of the protocol that is described in sections 2.1 and 2.2.

Optional. To study the effect of cancer cell-derived proteins on macrophages, cancer cells can be treated with targeted therapies to block or activate these specific molecules prior to preparing conditioned medium from these cells. In case targeted therapies will be used in this assay, conditioned medium is prepared on day 4-7 of the protocol described in sections 2.1 and 2.2. In this setting, cancer cells are seeded on day 4 (see step 1 of this section) and the extra day (day 5) is used to perform the treatment of cancer cells for a pre-determined time (for example, 24 hours) to be followed by serum starvation at the end of day 6 (see step 2 of this section). Alternatively, cancer cells can be genetically manipulated, e.g. using CRISPR/Cas9 technology, to target specific genes of interest to assess how the proteins encoded by these genes are involved in paracrine activation of macrophages.

Day 5

1. Make sure the cancer cell lines are in good conditions for the experiment. Count the cancer cells and seed 200.000 cells per well in a 6-well plate (Note 13).

Day 6 (end of afternoon).

2. Discard the cell culture medium, add 1 mL serum-free medium to every well and incubate overnight for 16 hours (Note 14). Here, 1 mL of medium is used instead of the usual working volume of 2 mL for 6-well plates to concentrate the conditioned medium (Note 15).

Day 7 (morning).

3. Harvest the conditioned medium and spin down at 250 x g for 5 minutes to remove detached cancer cells. The supernatant is the conditioned medium to be used for macrophage activation in the subsequent experiment (see section 2.4, Note 16).

2.4. Readout assays to assess macrophage phenotype and activation status

A variety of methods can be used to assess macrophage phenotype and activation after exposure to cancer cell conditioned medium. Below, several assays are described to determine RNA or protein expression induced on *in vitro* generated macrophages.

2.4.1. *Materials*

Exposure of macrophages:

- Non-tissue culture-treated 24-well plates (Falcon)
- Full RPMI medium: RPMI medium 1640 (Gibco) supplemented with 10% Fetal Bovine Serum (Sigma), 100 IU mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Roche)
- Freshly prepared 2X concentrated conditioned medium (serum-free, see section 2.3.2)
- PBS without Mg^{2+} and Ca^{2+} (Gibco)
- Inhibitory or activating agents of interest targeting macrophages

RT-qPCR:

- 1,5 mL tubes (Eppendorf)
- RNA isolation kits, such as RNeasy Mini Kit (Qiagen) or ISOLATE II RNA Mini Kit (Bioline)
- Invitrogen AMV Reverse Transcriptase (Thermo Fischer) with Oligo(dT) primers
- SensiFAST™ SYBR® No-ROX Kit (Bioline)
- LightCycler 480 Thermocycler (Roche)
- Forward and reverse primers for genes of interest including reference gene (**Table 5.2**)

Table 5.2. Primers for RT-qPCR

Gene	Forward (5'–3')	Reverse (5'–3')
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Ccl2	GTTGGCTCAGCCAGATGCA	AGCCTACTCATTGGGATCATCTTG

Western blotting:

- RIPA buffer (20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl.)
- cOmplete™ Protease Inhibitor Cocktail (Sigma)
- Halt™ Phosphatase Inhibitor Cocktail (Thermo Fischer)

- Pierce BCA Protein Assay Kit (Thermo Fischer)
- LDS NuPAGE sample buffer (4X) (Thermo Fischer)
- 100 mM dithiothreitol (DTT)
- NuPAGE Bis-Tris gel (Thermo Fischer)
- Transblot Turbo nitrocellulose or PVDF blots (BioRad)
- Transblot Turbo Transfer system (BioRad)
- Ponceau S staining (Sigma)
- Western blot antibodies (**Table 5.3**)
- 1X TBS-Tween
- 10% and 5% Western blot blocking reagent (Roche) in TBS-T
- Optional: 3% Bovine Serum Albumin Fraction V (Roche) in TBS-T
- Optional: 5% non-fat milk in TBS-T

Table 5.3. Western blot antibodies

Antigen	Clone	Company	Dilution
β-actin	D6A8	Cell Signaling	1:5000
Total S6	5G10	Cell Signaling	1:1000
Phospho-S6$^{Ser235/236}$	D57.2.2E	Cell Signaling	1:1000
Total 4EBP1	53H11	Cell Signaling	1:1000
Phospho-4EBP1$^{Thr37/46}$	Thr37/46	Cell Signaling	1:1000

Flow cytometry:

- Clean paper towels
- 96-well cell culture plate, U bottom (Greiner CELLSTAR)
- FACS buffer: PBS supplemented with 0.5% BSA and 2 mM EDTA
- Fc Block (BD Biosciences)
- Fluorochrome-conjugated antibodies (**Table 5.4**)
- Cell viability staining (**Table 5.4**)
- Optional: Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences)
- Optional: Fluorochrome-conjugated antibodies for intracellular proteins

Table 5.4. Flow cytometry reagents

	Marker	Fluorochrome	Clone	Target species	Company	Dilution
Cell surface antigens	CD45	BUV395	30-F11	Mouse	BD Biosciences	1:400
	CD11b	BV650	M1/70	Mouse/human	eBioscience	1:400
	F4/80	FITC	BM8	Mouse	eBioscience	1:400
	MHC-II	APC-eFluor 780	M5/114.15.2	Mouse	eBioscience	1:200
	CD206	AF488	MR5D3	Human	AbD Serotec	1:200
	CD68	FITC	Y1/82A	Human	BD Biosciences	1:200
	CD14	BV421	M5E2	Human	BD Biosciences	1:400
Viability stains	7-AAD				eBioscience	1:20
	Fixable Viability Dye eFluor-780				eBioscience	1:1000

2.4.2. *Exposure of macrophages to cancer cell conditioned medium (Note 17)*

1. In a non-tissue culture-treated 24-wells plate, seed 300.000 differentiated BMDMs or MDMs per well in 250 µl full RPMI medium (Note 18). Keep 300.000 cells for purity check of the differentiated cells in section 2.4.3.3, step 2 (**Fig. 5.1C, D**).

2. Add 250 µl of freshly prepared conditioned medium to the macrophage culture. Add non-conditioned medium (i.e. medium that has not been cultured on cancer cells) to one well as a control. Exposure of BMDMs or MDMs to conditioned medium is performed for a pre-determined time. In our experience, an exposure time of 24 hours works well (Note 19 and 20).
3. **Optional**. Desired inhibitory or activating molecules targeting macrophages can be added to the culture at this point.
4. Proceed with section 2.4.3.1 for quantification of gene expression, with section 2.4.3.2 for western blotting of intracellular signaling pathways or with section 2.4.3.3, step 2 for assessment of macrophage phenotype using flow cytometry.

2.4.3. *Readout of macrophage polarization*

Upon exposure of macrophages with cancer cell conditioned medium, macrophage polarization can be analyzed using several techniques, examples of which are described below. These techniques include analysis of cancer cell-induced gene expression, intracellular protein signaling and regulation of cell surface markers.

2.4.3.1. Quantitative RT-PCR to detect gene expression in macrophages

This method is used to analyze cancer cell-induced gene expression in macrophages (**Fig. 5.2A**). Here, we provide one example of differential chemokine expression upon exposure to cancer cell conditioned medium (**Fig. 5.2A**). Other genes that can be of interest to be studied in this context are cytokines (genes encoding IL-1β, IL-6, IL-10, TGF-β, TNF-α), chemokines (genes encoding CCL2, CCL8, CCL17, CCL22), or enzymes (genes encoding Arginase-1, iNOS). In addition, expression of targets for checkpoint inhibition, such as PD-L1 or SIRP-α, can be determined.

1. After 24 hours of exposure to the conditioned medium, remove medium and wash cells once with PBS. Detach cells using 500 µL full MACS buffer per well and incubate at 37°C for 15 minutes (Note 9). Collect the cells in a 1,5 mL tube. Use a new tube for every well of plated cells. Spin down at 250 x g for 5 minutes and discard the supernatant.
2. RNA can subsequently be isolated from cell pellets either using Trizol-chloroform isolation, followed by DNase I (Invitrogen) treatment, or using RNA isolation kits (for example the Qiagen RNeasy kit, Note 21).
3. Determine RNA concentration and quality using NanoDrop 1000 UV-VIS Spectrophotometer (Thermo Fisher) or a similar device (Note 22). Quality is considered satisfactory when the A260/280 > 2.0.
4. Generate cDNA from a set input of RNA using AMV reverse transcriptase and Oligo(dT) primers (Invitrogen) or Maxima First Strand cDNA Synthesis Kit (Thermo Fisher). Usually, we use 20 ng of RNA input per gene that is tested. Analyze cDNA using SYBR-Green PCR with 500 nM primers for the gene of interest using a LightCycler 480 thermocycler (Roche). Include housekeeping gene for reference and perform cDNA analysis in technical duplicate (**Table 5.2**).

2.4.3.2. **Western blotting to determine intracellular protein levels in macrophages**

Western blotting is used to study intracellular signaling pathways in macrophages by antibody-based detection of phosphorylated proteins (**Fig. 5.2B**). A general overview of Western blotting is given, but similar protocols for this method can be used here.

1. After 24 hours of exposure to the conditioned medium, remove medium and wash macrophages once with PBS.
2. Detach cells from the plate using 500 µL full MACS buffer per well, incubate at 37°C for 15 minutes (Note 9), and collect the cells in a 1,5 mL tube. Spin down at 250 x g for 5 minutes and discard the supernatant.
3. Lyse cells by resuspending cell pellets in 50 µL RIPA buffer or equivalent containing protease and/or phosphatase inhibitors. Incubate for 45 minutes on ice.
4. Spin down 16.000 x g for 10 minutes at 4°C.
5. Harvest supernatant containing cell lysate and transfer to new tube. Measure protein concentration using the Pierce BCA Protein Assay Kit (Thermo Fisher).
6. Use equal amounts of protein per lysate (usually 20 – 40 µg) and add H_2O up to a total volume of 15 µL. Add 5 µL of 4X LDS NuPAGE sample buffer (Thermo Fisher) supplemented with 100 mM DTT to obtain a final volume of 20 µL per sample.
7. Denature the samples by boiling at 100°C for 5 minutes and spin down at 10.000 x g for 10 seconds.
8. Together with a molecular weight marker, load samples on a NuPAGE 4-12% Bis-Tris gel (Invitrogen) or any desired gel. Perform electrophoresis for approximately 45 minutes at 200 V. Depending on molecular weight of the protein to be analyzed, choose the appropriate gel to run your samples.
9. Transfer proteins from gel to membrane (nitrocellulose or PVDF). PVDF requires activation using 100% methanol. Transfer can be performed using the Trans-Blot Turbo system (Bio-Rad) according to the manufacturer's instructions.
10. **Optional**. After transfer of proteins, transfer efficiency can be checked using Ponceau S staining (Sigma).
11. Block membranes in 10% Western blot blocking reagent (Roche) in TBS-T for 1 hour at room temperature.
12. Incubate membranes overnight at 4°C with primary antibodies in 5% Western blot blocking reagent (Roche) in TBS-T (Note 23 and 24).
13. The next day, remove primary antibody mix and wash membranes three times for 10 minutes using TBS-T (**Table 5.3**).
14. Incubate membranes with the appropriate IRdye-conjugated secondary antibodies in 5% Western blot blocking reagent (Roche) in TBS-T for 1 hour at room temperature. Wash membranes three times for 10 minutes in TBS-T prior to visualization.
15. Infrared signals can be visualized using the Odyssey Imaging System (LI-COR, Note 25).

2.4.3.3. **Flow cytometry to assess macrophage purity and phenotype**

Flow cytometry is used to verify macrophage purity and to assess macrophage phenotype upon cancer cell-induced activation (**Fig. 5.2C**). For general macrophage identification, we use $CD11b^+F4/80^+$ for mouse and $CD11b^+CD14^+CD68^+$ for human. There are several markers used for looking at activation states, depending on the research questions. Often used markers include, but are not limited to, CD206, MHC-II/HLA-DR, PD-L1, Tie2, CD163 (human).

Verification of CD14 separation of human PBMCs

The three cell fractions from section 2.2.2 (step 19) are used to verify the purity of the isolated $CD14^+$ cells of human PBMCs. In this step, CD11b and CD14 are used to discriminate the different cell populations.

1. Plate the 30 µL samples of the PBMC, $CD14^-$ and $CD14^+$ fractions in a U-bottom 96-well cell culture plate. Proceed with step 5 of this section.

Verification of BMDM or MDM purity

After differentiation of murine bone marrow or human monocytes, macrophage purity is determined using flow cytometry. For BMDMs, the following markers are used: CD45, CD11b, F4/80 and the viability stain 7-AAD. For MDMs, antibodies against human CD11b, CD14, CD68 and a viability stain are used (**Fig. 5.1C, D**).

2. Plate 300,000 cells in a U-bottom 96-well cell culture plate and proceed with step 5 of this section.

Assessment of macrophage phenotype upon conditioned medium exposure

For functional assessment of macrophages, cells are stained for general macrophage markers as described for verification of macrophage purity. In addition, expression of activation markers can be detected after gating on macrophage population to determine their phenotype. In **figure 5.2C**, we show an example of two activation markers, MHC-II and CD206, that can be assessed upon exposure of BMDMs and MDMs, respectively.

3. After exposure with cancer cell conditioned medium, remove medium and wash cells once with PBS. Detach cells from the plates using 500 µL MACS buffer by incubating at 37°C for 15 minutes followed by scraping off the cells (Note 9).
4. Plate the cells in a U-bottom 96-well cell culture plate and proceed with step 5 of this section.

Antibody staining

5. Incubate cells 15 minutes on ice with Fc Block (1:100 in FACS buffer, BD Biosciences, Note 26) followed by surface antibody staining in FACS buffer for 30 minutes on ice (see **Table 5.4** for a selection of commonly used markers to analyze macrophages).
6. Add 100 µl of FACS buffer to the samples and wash the cells by resuspending

three times. Spin down at 380 x g for 2 minutes and discard supernatant by inverting the plate and blotting the plate against clean paper towels.

7. Stain the cells with a viability dye to exclude dead cells. When only surface staining is applied to the cells, a non-fixable viability staining, such as 7-AAD (BD Biosciences) is used.
8. **Optional**. Intracellular staining of proteins of interest can be performed using an intracellular staining kit, such as the Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences, Note 27). In this case, a fixable viability staining has to be used followed by fixation of the cells according to manufacturer's instructions (**Table 5.4**). After fixation and permeabilization of the cells, intracellular staining of cytokines is performed.

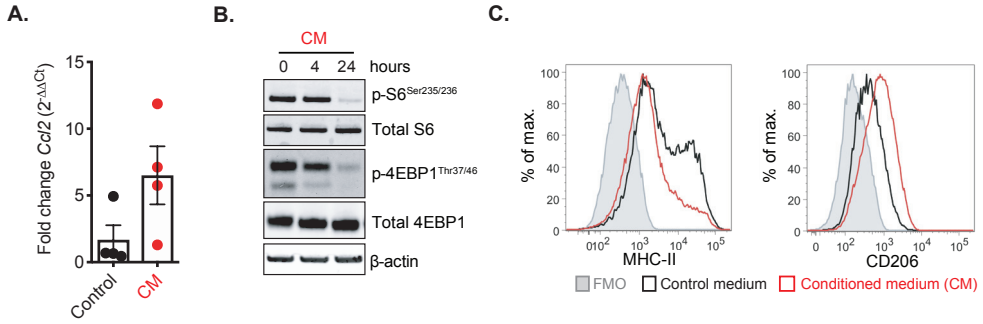

Figure 5.2. Examples of assays used to assess macrophage activation after exposure to cancer cell conditioned medium. A. Example of RT-qPCR analysis of BMDMs showing induction of *Ccl2* expression after exposure to cancer cell conditioned medium (CM), compared to control medium. **B.** Western blot showing down-regulation of mTOR signaling after 4- and 24-hour exposure of BMDMs to CM, as indicated by a decrease in phosphorylated S6 and phosphorylated 4EBP1. Beta-actin serves as a loading control. **C.** Flow cytometry histograms showing down-regulation of MHC-II and up-regulation of CD206 expression on bone marrow-derived macrophages (BMDMs) after exposure to CM (red), compared to control medium (black). An unstained sample is shown in grey. All data represent mean with S.E.M.; FMO = fluorescence minus one (unstained for the indicated protein).

3. Notes

1. Be careful with removing the tissue from the bones, as the bones could break when a high force is applied.
2. Flushing of the bones can also be performed using sterile PBS (without Mg^{2+} and Ca^{2+}). In that case, start using full RPMI medium when seeding the cells in culture plates.
3. At this point, bone marrow can be frozen down to be used for differentiation into BMDMs in future experiments. However, differentiation from fresh bone marrow and direct use of cells gives highest cell yield (**Table 5.1**). The use of frozen material usually gives only half of the amount of differentiated BMDMs.
4. As macrophages adhere very strongly to plastic wear, make sure to use non-tissue culture-treated plates in order to make the harvesting process less difficult.
5. When bone marrow is isolated from the two hind legs of the mouse, the average cell number that is reached is 50 million cells. We recommend to use ±5 million cells

per 10 cm plate, giving ten plates per mouse. When fewer cells are needed for the experiment (**Table 5.1**), freeze the remainder of the bone marrow for future use.

6. In some studies, GM-CSF is used to induce macrophage differentiation. However, we recommend to only use M-CSF, because GM-CSF can induce different gene and protein expression profiles or even a mixed population of dendritic cells and macrophages, compared to M-CSF [16,17].
7. Culture in low volume could give issues such as liquid evaporation. In our protocol, low volume of medium (5 mL) is only used shortly (until day 3) and did not give any problems. Although, it is important to add 5 mL of supplemented medium to the culture at day 3 to prevent any issues during the second part of the culture.
8. Here, MACS separation buffer is used, but any other separation buffer containing EDTA should work as well.
9. Macrophages are hard to detach from the culture plates. In order to speed up the process, the dishes are incubated at 37°C for at least 15 minutes.
10. For the cells that are difficult to detach, it is possible to scrape them off the plate using the pipet or a cell scraper (optional). This process may increase cell yield, but also induces more cell death.
11. If whole blood is being used to isolate human monocytes, it needs to be diluted 1:3 or 1:4 in section 2.2.2, step 1.
12. At this stage, $CD14^+$ monocytes can be frozen down for future use. Note that freeze-thaw cycles can reduce total amount of cells.
13. Other culture plates can be used to prepare conditioned medium, depending on the amount of conditioned medium that is needed to expose the macrophages. For a 6-well plate, we seed 200.000 cells per well from which 1 mL of conditioned medium per well will be obtained. With 1 mL of conditioned medium, four wells with macrophages can be exposed (250 µl per well, see section 2.4.2).
14. Here, serum-free medium is used to specifically study the impact of cancer cell-derived molecules on macrophages. Researchers are free to use media containing serum, but keep in mind that the impact on macrophages can also be influenced by the factors present in serum (e.g. growth factors and cytokines).
15. We recommend to produce 2X concentrated conditioned medium. During the exposure step, conditioned medium will be added to macrophage culture in a 1:1 ratio (i.e. 250 µl conditioned medium to 250 µl RPMI medium). By utilizing 2X conditioned medium, the final concentration of the conditioned medium during the exposure of macrophages will be 1X.
16. At this point, conditioned medium can be frozen down for future use. From our experience, fresh preparation and direct use of conditioned medium gives the best results.
17. Biological replicates for these experiments are BMDMs or MDMs from additional donors.
18. If higher RNA or protein levels are desired for the readout assays in section 2.4.3, more macrophages can be seeded at this stage. **Table 5.1** shows the corresponding number of experiments that can be performed when 500.000 cells per condition are seeded.
19. We recommend to expose the macrophages up to a maximum of 48 hours starting at day 7, because viability of macrophages is limited. The optimal duration of the

exposure assay is 1-2 days.

20. To study whether the exposure of tumor-derived factors impacts differentiation into macrophages, cancer cell conditioned medium can be added at start of bone marrow- or monocyte differentiation. In this setting, we recommend to start with equal cell numbers per plate and to use 50% of conditioned medium diluted in full RPMI medium supplemented with M-CSF as described before. As a readout, the proportion of cells that have differentiated into macrophages can be assessed using both microscopy and flow cytometry by comparing cells that were exposed to cancer cell conditioned medium to cells cultured in control medium.
21. When using RNA isolation kits, the supplied lysis buffer can be added directly to the PBS-washed plate. This way, macrophages are lysed directly in the plate.
22. When seeding 300.000 macrophages, the total yield varies between 1600-3200 ng RNA.
23. For blocking of membranes and incubation of antibodies (next step), 3% BSA in TBS-T or 5% non-fat milk in TBS-T can also be used as reagent.
24. Phosphorylated proteins are known to be detected more easily when blocking buffers without phosphatases are used, such as BSA.
25. Other methods to visualize proteins of interest on the membrane, such as the chemiluminescent method, can also be applied here.
26. Macrophages can very potently aspecifically bind antibodies, so the use of Fc Block is essential here.
27. Depending on the research question, it can be relevant to perform intracellular staining of the cell, for instance to measure cytokines, but also other (intracellular) proteins can be visualized using flow cytometry.

References

1. Quail, D.F. & Joyce, J.A. Microenvironmental regulation of tumor progression and metastasis. *Nat. Med.* **19**, 1423-1437 (2013).
2. Gentles, A.J.*, et al.* The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat. Med.* **21**, 938-945 (2015).
3. Thorsson, V.*, et al.* The Immune Landscape of Cancer. *Immunity* **48**, 812-830.e814 (2018).
4. Mantovani, A., Marchesi, F., Malesci, A., Laghi, L. & Allavena, P. Tumour-associated macrophages as treatment targets in oncology. *Nat. Rev. Clin. Oncol.* **14**, 399-416 (2017).
5. Lewis, C.E. & Pollard, J.W. Distinct role of macrophages in different tumor microenvironments. *Cancer Res.* **66**, 605-612 (2006).
6. DeNardo, D.G. & Ruffell, B. Macrophages as regulators of tumour immunity and immunotherapy. *Nat. Rev. Immunol.* (2019).
7. Mahmoud, S.M.*, et al.* Tumour-infiltrating macrophages and clinical outcome in breast cancer. *J. Clin. Pathol.* **65**, 159-163 (2012).
8. Medrek, C., Ponten, F., Jirstrom, K. & Leandersson, K. The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. *BMC Cancer* **12**, 306 (2012).
9. Zhang, Q.W.*, et al.* Prognostic significance of tumor-associated macrophages in solid tumor: a meta-analysis of the literature. *PloS ONE* **7**, e50946 (2012).
10. Steidl, C.*, et al.* Tumor-associated macrophages and survival in classic Hodgkin's lymphoma. *N. Engl. J. Med.* **362**, 875-885 (2010).
11. Tan, K.L.*, et al.* Tumor-associated macrophages predict inferior outcomes in classic Hodgkin lymphoma: a correlative study from the E2496 Intergroup trial. *Blood* **120**, 3280-3287 (2012).
12. Singhal, S.*, et al.* Human tumor-associated monocytes/macrophages and their regulation of T cell responses in early-stage lung cancer. *Sci. Transl. Med.* **11**(2019).
13. DeNardo, D.G.*, et al.* Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov.* **1**, 54-67 (2011).
14. Salvagno, C.*, et al.* Therapeutic targeting of macrophages enhances chemotherapy efficacy by unleashing type I interferon response. *Nat. Cell Biol.* **21**, 511-521 (2019).
15. Brown, J.M., Recht, L. & Strober, S. The Promise of Targeting Macrophages in Cancer Therapy. *Clin. Cancer Res.* **23**, 3241-3250 (2017).
16. Lacey, D.C.*, et al.* Defining GM-CSF- and macrophage-CSF-dependent macrophage responses by in vitro models. *J. Immunol.* **188**, 5752-5765 (2012).
17. Helft, J.*, et al.* GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of CD11c(+)MHCII(+) Macrophages and Dendritic Cells. *Immunity* **42**, 1197-1211 (2015).