PromoCell®

Differentiation of M1- or M2-Macrophages from PBMC/Monocytes

Application Note

Background

Macrophages are tissue-resident professional phagocytes and antigen-presenting cells (APC), which differentiate from circulating peripheral blood monocytes. They perform important active and regulatory functions in innate as well as adaptive immunity [1].

Activated macrophages of different phenotypes are routinely classified into M1-macrophages (CAM) and M2-macrophages (AAM). The classically activated M1-macrophages comprise immune effector cells with an acute inflammatory phenotype. These are highly aggressive against bacteria and produce large amounts of lymphokines [2]. The alternatively activated, anti-inflammatory M2-macrophages can be separated into at least three subgroups. These subtypes have various different functions, including regulation of immunity, maintenance of tolerance and tissue repair/wound healing [1,2]. Indeed, cells of the monocyte/macrophage lineage exhibit extraordinary plasticity in response to endogenous as well as exogenous stimuli, which can allow overriding of the initial M1/M2-polarization processes [2], for example M2-polarized macrophages can convert to the M1-activated status under certain conditions.

Primary human macrophages are difficult to isolate in sufficient amounts from tissue and do not proliferate in culture. In addition, it is commonly accepted that the obtained cells often exhibit significant phenotypical heterogeneity. Monocyte-derived Macrophages (MDM) provide an excellent alternative, since human blood monocytes are readily available in large numbers and can be differentiated into macrophages *in vitro*. Our Macrophage Generation Media were designed for the straight-forward and efficient differentiation of highly pure M1- or M2-macrophages directly from PBMC as a starting material (see figure 2). Prior monocyte purification is not necessary. As with all of our XF media series, the Macrophage Generation Media XF are serum- and xeno-free and thus provide a controlled culture environment – a significant benefit in terms of monocytes and macrophages standing for highly reactive immune cells. As a result, these media lack unwanted non-defined and deleterious effects attributable to FCS and therefore enable standardized and controlled macrophage differentiation.

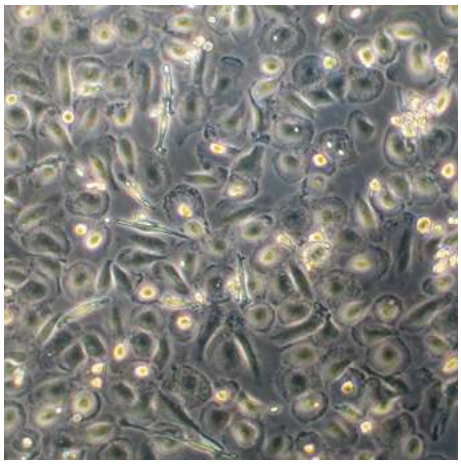


Fig. 1: Day 10 culture of activated M2-Macrophages differentiated in the our M2-Macrophage Generation Medium XF. M2a-activation was achieved by performing the optional activation step described in the protocol using 20 ng/ml IL-4. Note the typical "fried egg" morphology.

A commonly accepted marker profile for M1-macrophages is $CD68^+/CD80^+$, whereas M2-macrophages are characterized as $CD68^+/CD163^+$ [4].

In vitro differentiation of monocytes in the presence of our M1-Macrophage Generation Medium XF (C-28055, contains GM-CSF) leads to macrophages exhibiting a $CD68^+/CD80^+/CD163^-$/low M1-like polarized phenotype, whilst the M2-Macrophage Generation Medium XF (C-28056, contains M-CSF) promotes M2-like polarized $CD68^+/CD80^-$/low/$CD163^+$ macrophages (see figure 4 for exemplary flow cytometry plots). The Macrophage Base Medium XF (C-28057) represents the user-customizable version of this new product line. It comes without cytokines and therefore needs appropriate supplementation by the user.

If required, customized activation and subtype-specific polarization of the M1/M2-polarized macrophages may be performed by the user in an optional step of the protocol described below (also refer to figure 2/3 and step 8 of the protocol). Indeed, macrophage activation may elicit an altered expression pattern of certain markers as compared to non-activated cells [5].

Schematic Overview

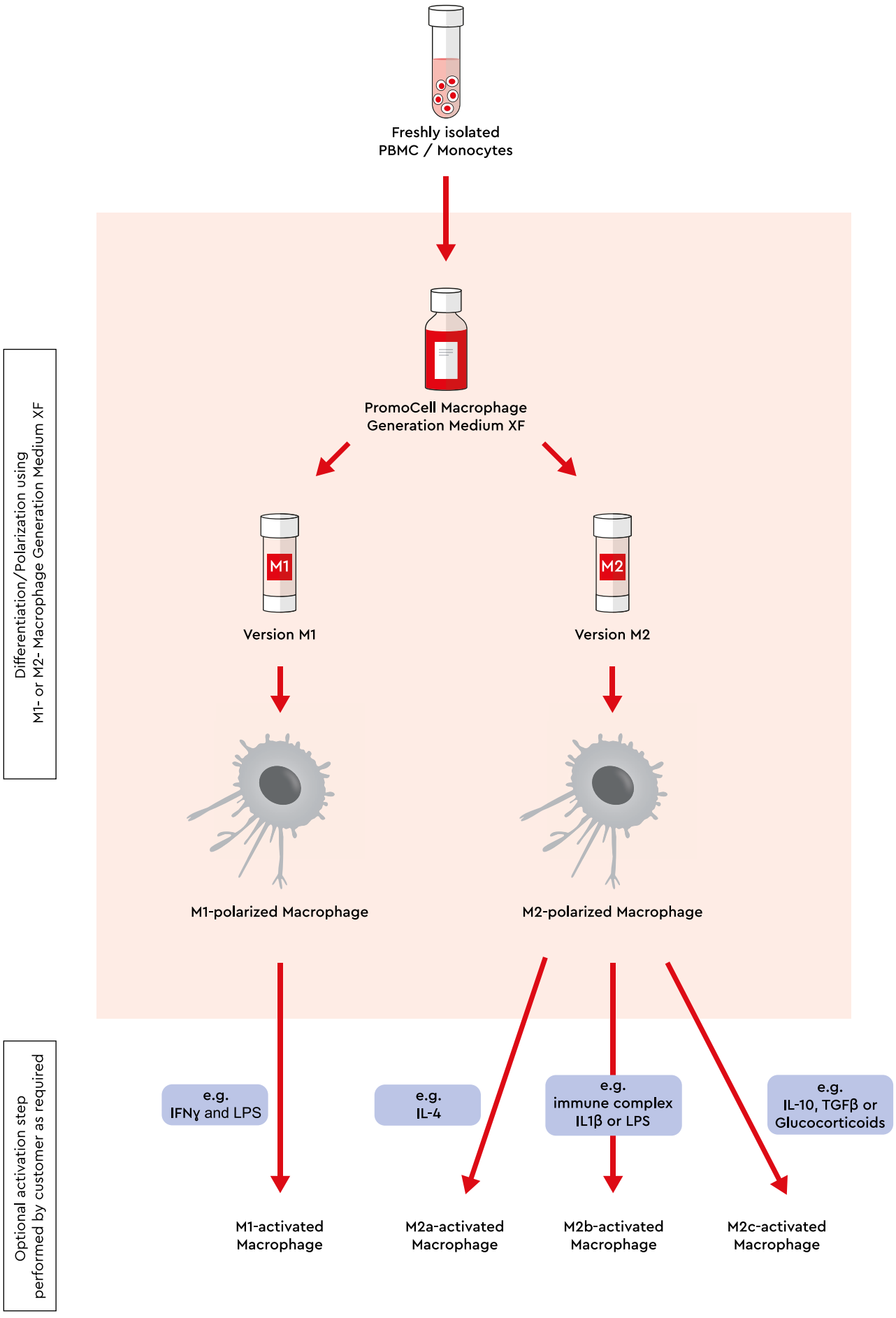


Fig. 2: Schematic overview on the user-customizable M1-/M2-Macrophage differentiation process using the PromoCell Macrophage Generation Media.

Protocol Overview

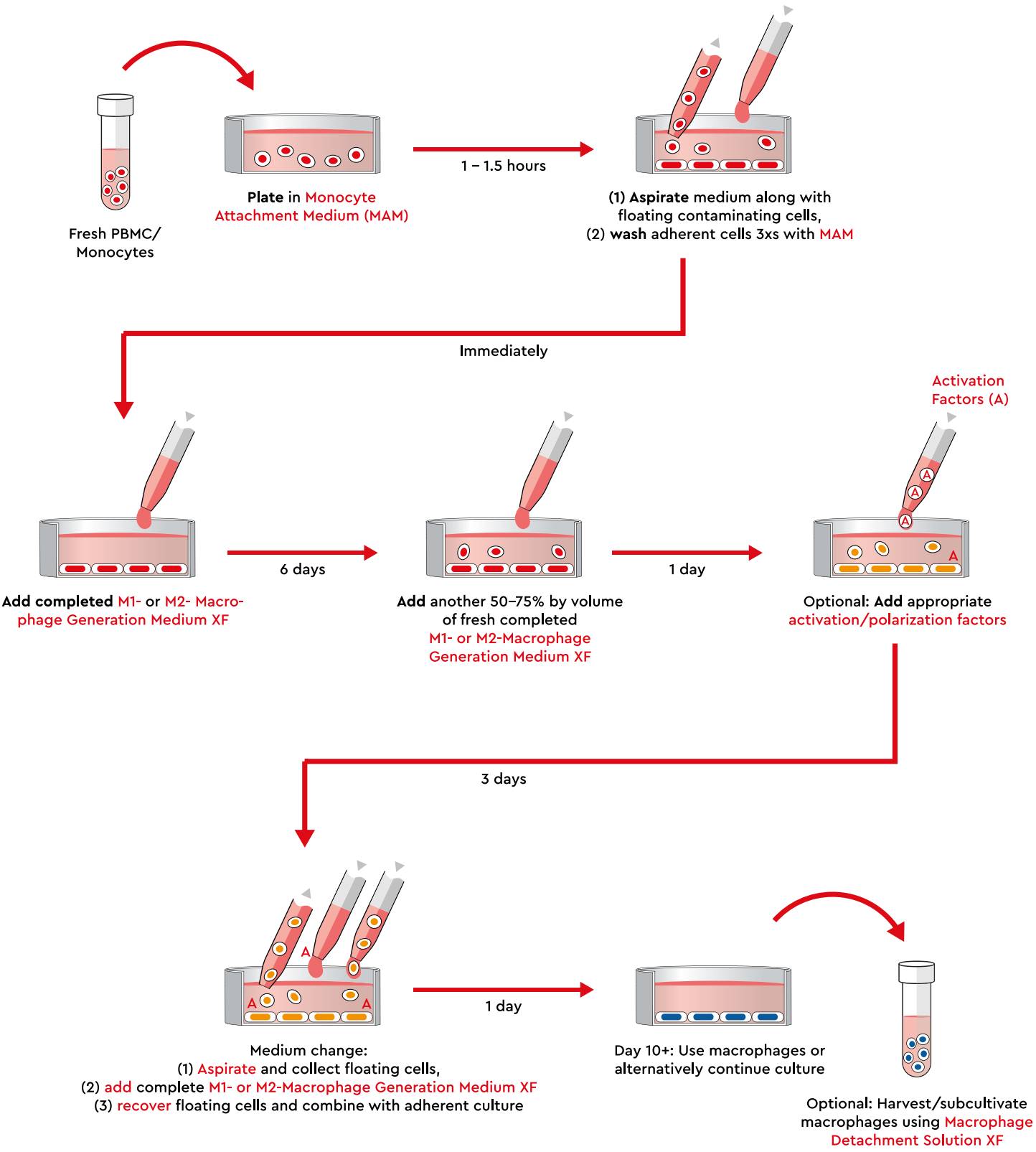


Fig. 3: Protocol overview using PromoCell M1-/M2-Macrophage Generation Medium XF (10 days).

Macrophage Differentiation from freshly isolated PBMC

This protocol describes the *in vitro* differentiation of M1 or M2 macrophages directly from freshly isolated peripheral blood mononuclear cells (PBMC) using the Macrophage Generation Media

Differentiation protocol

Materials

- Monocyte Attachment Medium (C-28051)
- M1- or M2-Macrophage Generation Medium XF (C-28055 or C-28056)
- endotoxin-free PBS without Ca^{2+}/Mg^{2+}
- endotoxin-free PBS without Ca^{2+}/Mg^{2+}/2 mM EDTA/0.1% HSA
- optional: additional activation/polarization factors (refer to protocol step 8)
- optional: Macrophage Detachment Solution XF (C-41330, refer to protocol step 11)

Use aseptic techniques and a laminar flow bench.

1

Isolate Mononuclear Cells (Day 0)

Isolate fresh PBMC from buffy coats using your routine protocol or by using the Lymphocyte Separation Medium 1077. For the separation of vital mononuclear cells with the usage of the Lymphocyte Separation Medium 1077 follow the corresponding instruction manual which is available for download on our website www.promocell.com. Tuesday is a good day to start in order to avoid weekend work.

Note: Do not use buffy coats older than 20 hours, since this will significantly impair the experimental outcome. Buffy coats not older than 8 hours are optimalexperimental outcome.

2

Analyze Mononuclear Cells (Day 00)

Count and analyze the isolated PBMC for monocyte content, (e.g., using the FSC/SSC plot of a flow cytometer). Subsequently resuspend the cells at 100 million PBMC per ml in Monocyte Attachment Medium.

Note: The protocol may be performed without the determination of the monocyte content of the PBMC (see step 3). However, this might result in lower yield due to suboptimal initial plating density.

3

Let the monocytes attach (Day 0)

Plate freshly isolated PBMC in an appropriate amount of Monocyte Attachment Medium, e.g., 15 ml Medium per T-75 flask. Use a seeding density of 1 million/cm^2 for Mononuclear Cells with a monocyte content of ≥25% and 1.5 million/cm^2 for a monocyte content of <25%. Incubate for 1–1.5 hours at 5% CO_2 and 37°C in the incubator without any further manipulation.

Note: Use Nunc plasticware with NunclonTM surface for best results. Use a plating density of 1.5 million PBMC per cm^2 when step 2 was skipped.

4

Prepare the complete Macrophage Generation Medium XF (Day 0)

Prepare the Macrophage Generation Medium XF by adding the thawed SupplementMix aseptically to the Basal Medium. Swirl gently to obtain a homogeneous mixture. Then, add Cytokine Mix M1 or M2, respectively.

5

Wash the adherent cell fraction (Day 0)

By vigorously swirling the tissue culture vessel loosen non-adherent cells and aspirate them. Wash the adherent cells, i.e. monocytes, three times with warm Monocyte Attachment Medium by swirling the vessel and aspirating the supernatant.

Optional: Non-adherent cell fraction may be kept to isolate further blood cell types.

6

Start the macrophage differentiation (Day 0)

Add an appropriate amount of complete M1- or M2-Macrophage Generation Medium XF to the cells, e.g., 20 ml per T-75 flask and incubate for 6 days at 37°C and 5% CO_2 without medium change.

Note: The monocytes differentiate to M1-like or M2-like polarized macrophages under these conditions. If required, activation and subtype-specific polarization can be achieved by performing the optional activation step (refer to step 8).

7

Continue the differentiation process (Day 6)

Add another 50% to 75% by volume of fresh complete M1- or M2-Macrophage Generation Medium XF to the cells. Incubate the immature macrophages for another 3 days at 37°C and 5% CO_2.

Note: Adherent as well as suspension cells may be present. Do not remove any of the used medium from the cells, just add the fresh medium.

8

Optional step: macrophage activation (Day 7)

For specific macrophage activation the whole volume of the culture is supplemented with adequate stimuli of the customers´ choice. Do not perform a medium change, just add the activation factors.
Examples of macrophage activation by defined stimuli (see also "Related products"):
Classically activated M1-macrophages can be generated by addition of IFN-γ (50 ng/ml) and LPS (10 ng/ml) to M1-macrophages. M2a-activation of M2-macrophages is achieved by 20 ng/ml IL-4. Supplementation with immune complexes and IL-1β or LPS will elicit M2b-activation, whilst IL-10, TGFβ or glucocorticoids lead to M2c-activation of M2-macrophages. An alternative type of M1-activated macrophage can be obtained by the activation of M2-macrophages with IFN-γ and LPS [2].

9

Optional activation step (Day 7)

Aspirate the medium including suspension cells and collect it in a centrifugation tube. Immediately, pipet fresh complete Macrophage Generation Medium XF supplemented with appropriate cytokines/activation factors to the cells. Centrifuge the cells in the tube for 15 minutes at 350 x g at room temperature. Discard the supernatant and carefully resuspend the cells in a small amount of fresh medium. Combine the resuspended cells in the tube with the adherent cells in the fresh medium contained in the tissue culture vessel. Incubate till the next day at 37°C and 5% CO_2.

Note: Adherent as well as non-adherent cells may be observed at this stage.

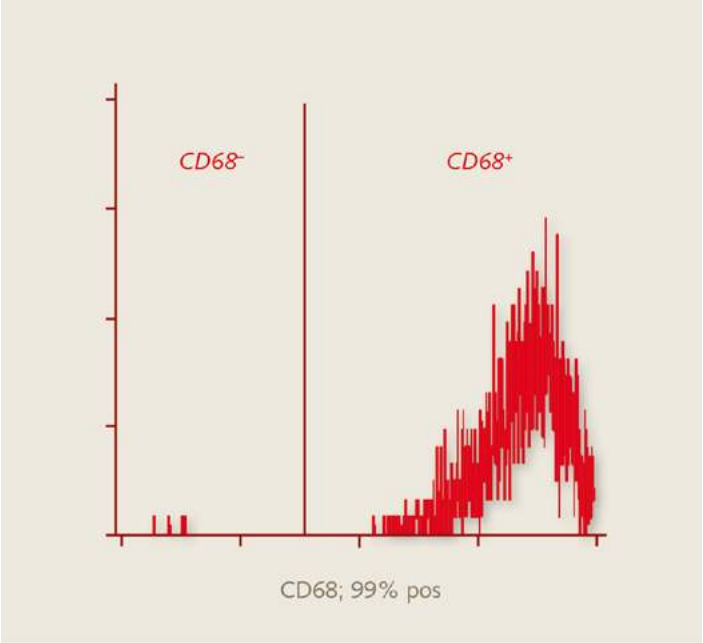
10

The macrophages are ready (Day 10)

The macrophages may now be used directly in the plates where they reside, e.g., when performing phagocytosis assays. Alternatively, they can be harvested (see instructions in optional step 11). Maintenance of the culture for up to three weeks by performing weekly medium changes with fresh complete Macrophage Generation Medium XF is possible.

Note: Macrophages appear as adherent cells with typical morphology: prominent nucleus with flatly outspread cytoplasm and multiple pseudopodia.

Exemplary flow cytometry analysis



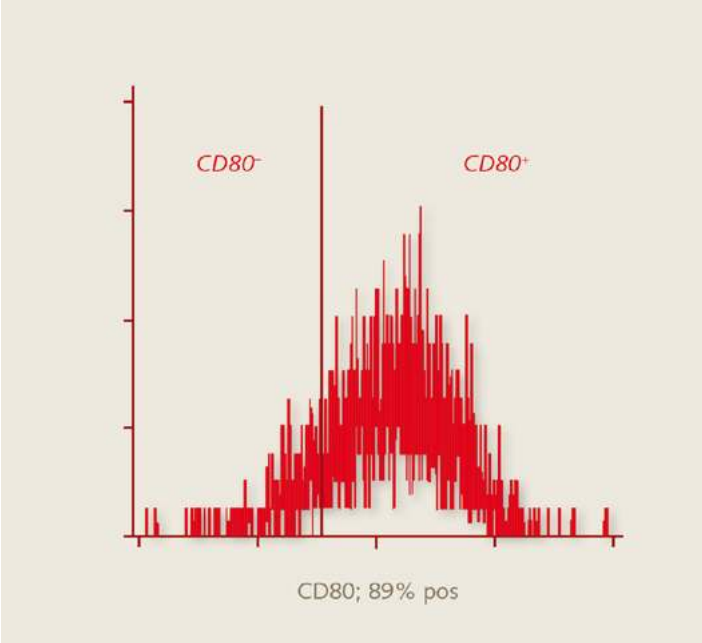
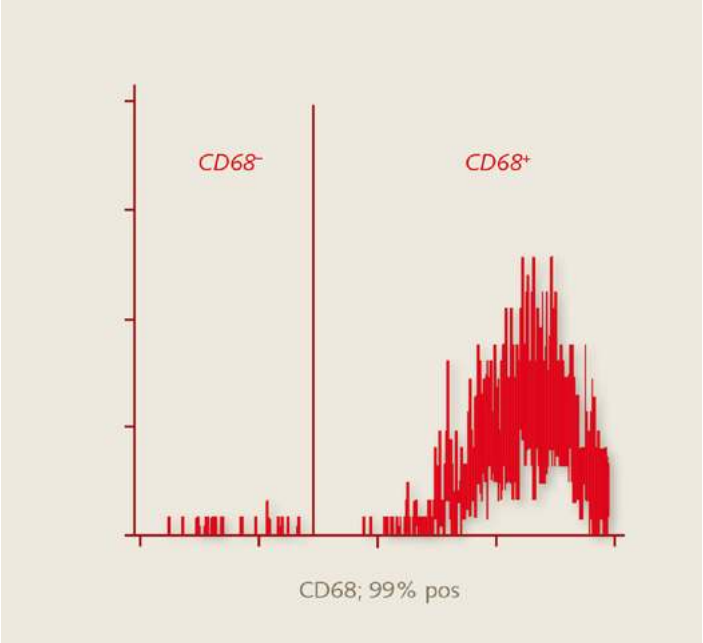


Fig. 4a: Exemplary flow cytometry analysis of day 10 M1-Macrophages generated in M1- Macrophage Generation Medium XF. Fresh peripheral blood mononuclear cells were plated in the Monocyte Attachment Medium. The purified monocytes were differentiated for 10 days without performing the optional activation step. Note that the cells exhibit the $CD68^+$ (99% positive) and $CD80^+$ (89% positive) marker profile, typical for M1-macrophages.



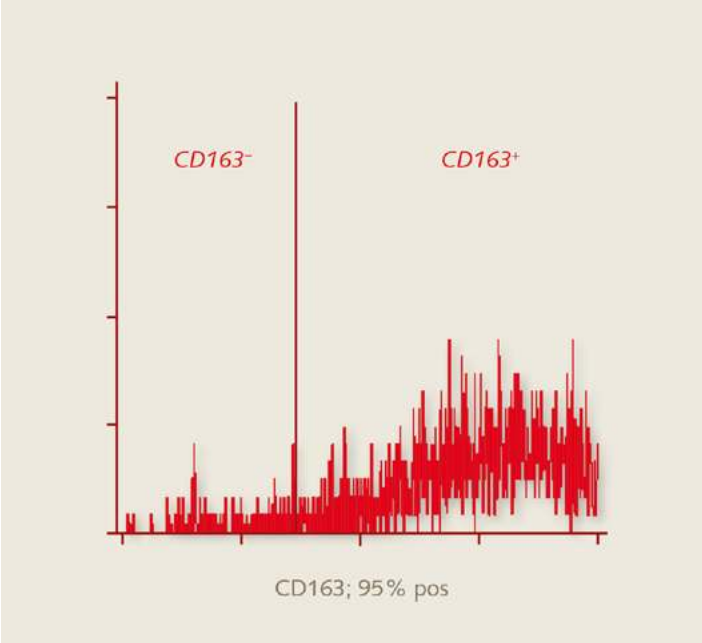


Fig. 4b: Exemplary flow cytometry analysis of day 10 M2-Macrophages generated in M2- Macrophage Generation Medium XF. Fresh peripheral blood mononuclear cells were plated in the Monocyte Attachment Medium. The purified monocytes were differentiated for 10 days without performing the optional activation step. Note that the cells exhibit the $CD68^+$ (99% positive) and $CD163^+$ (95% positive) marker profile, typical for M2-macrophages.

11

Optional step: Harvesting/subcultivation of macrophages (Day 10+)

Aspirate and discard the medium. Wash the adherent macrophages twice with endotoxin-free PBS without Ca^{2+}/Mg^{2+}. Immediately add an appropriate amount of cold (2–8°C) Macrophage Detachment Solution XF to the cells, e.g., 25 ml per T-75 flask. Seal the tissue culture vessel and incubate cells for 40 minutes at 2–8°C. If necessary, incubate another 20 minutes at room temperature to enforce cell release from the culture surface.
Firmly tap the tissue culture vessel to facilitate cell detachment. Make sure most of the cells have already detached or are only loosely adherent to the surface of the tissue culture vessel. Only then use a cell scraper to dislodge the remaining macrophages.
Collect the harvested macrophages in centrifugation tubes and dilute 1:1 with PBS/2 mM EDTA/0.1% HSA. Centrifuge cells for 15 minutes at 350 x g at room temperature. Apply two washes with PBS/2 mM EDTA/0.1% HSA to the cells and count them. The macrophages are now ready to be used for your experiments.

Note: The percentage of attaching cells after re-seeding depends on the overall health status of the macrophages before detachment and the successful performance of the detachment process itself. Thus, some degree of variation is unavoidable.

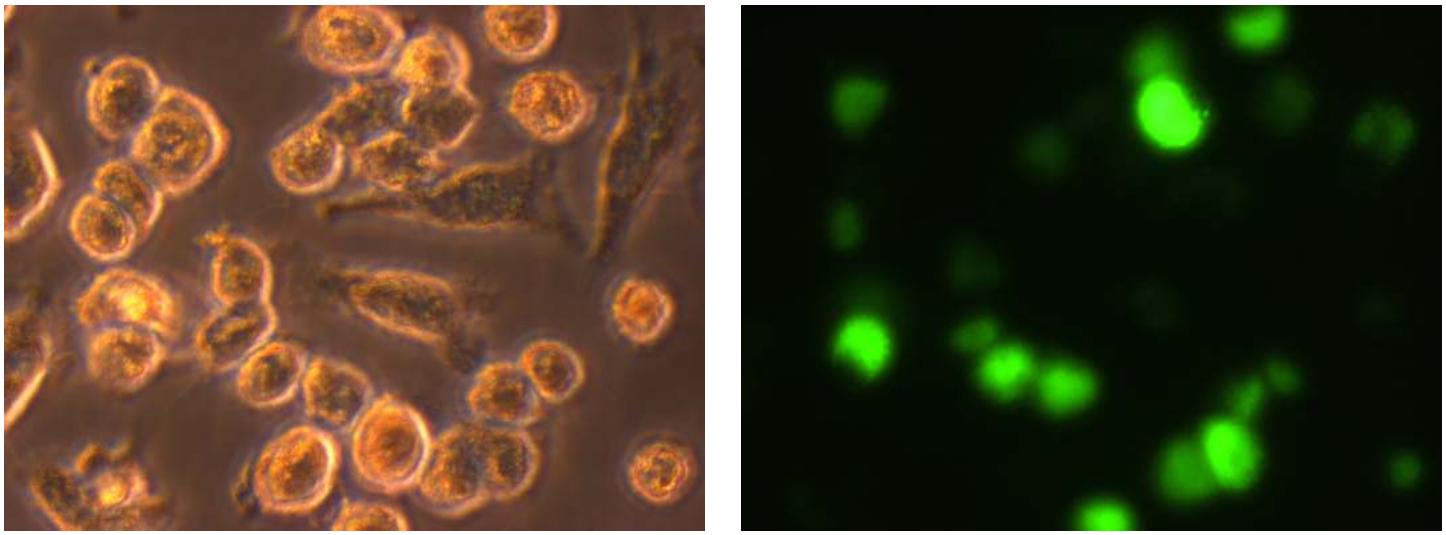


Fig. 5: Demonstration of the phagocytosis capabilities of Macrophages generated in the Macrophage Generation Media. Day 10 M2-macrophages were exposed to flourescently labelled E.coli for 24 hours before removing non-ingested bacteria. **Left:** Phase contrast image of M2-macrophages after phagocytosis of fluorescently labelled bacteria. Note that a portion of the cells already starts to detach from the culture surface, indicative for the approaching death of the cells. It is a commonly observed phenomenon, that a macrophage dies from its own toxic metabolic products, e.g., reactive oxygen species, after destroying approximately 50–100 bacteria. **Right:** Fluorescence image of the phagocytizing macrophages. Green fluorescence shows labelled bacteria ingested by the macrophages. Note that the roundish cells (macrophages starting to detach/dying) have phagocytized most bacteria, whereas the still attached macrophages exhibit reduced fluorescence because they have taken up less bacteria and are therefore still healthy.

Products

Media	Size	Catalog Number
Monocyte Attachment Medium (Ready-to-use)	250 ml	C-28051
M1-Macrophage Generation Medium XF	250 ml	C-28055
M2-Macrophage Generation Medium XF	250 ml	C-28056
Macrophage Base Medium XF	250 ml	C-28057
Macrophage Detachment Solution	250 ml	C-41330
Dulbecco's PBS, without Ca^{2+}/Mg^{2+}	500 ml	C-40232

Related Products

Media & Reagents	Size	Catalog Number
Human M1 Macrophages (GM-CSF), monocyte-derived, single donor (hMDM-GMCSF(-))	1.5 million cryopreserved cells	C-12914
Human M2 Macrophages (M-CSF), monocyte-derived, single donor (hMDM-MCSF(-))	1.5 million cryopreserved cells	C-12915
Human M1 Macrophages (GM-CSF), monocyte-derived, single donor (hMDM-GMCSF(-))	5 million cryopreserved cells	C-12916
Human M2 Macrophages (M-CSF), monocyte-derived, single donor (hMDM-MCSF(-))	5 million cryopreserved cells	C-12917
Cryo-SFM	125 ml	C-29912

References

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2. Murray PJ, Wynn TA (2011). Obstacles and opportunities for understanding macrophage polarization. J Leukoc Biol, 89(4):557–63.
3. Gordon S (2003). Alternative activation of macrophages. Nat Rev Immunol, 3(1):23–35.
4. Khramtsova G, Liao C, Khramtsov A, Li S, Gong C, Huo D, Nanda R (2009). The M2/Alternatively Activated Macrophage Phenotype Correlates with Aggressive Histopathologic Features and Poor Clinical Outcome in Early Stage Breast Cancer. Cancer Res, 69(24): Supplement 3, doi:10.1158/0008–5472.
5. Menzies FM, Henriquez FL, Alexander J, Roberts CW (2010). Sequential expression of macrophage anti-microbial/inflammatory and wound healing markers following innate, alternative and classical activation. Clin Exp Immunol, 160(3):369–79.

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