Monocyte-derived macrophages: Customized in vitro large-scale differentiation from human PBMC

Application Note

Introduction

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 Base Medium XF in combination with the Monocyte Attachment Medium were designed as a complete system for easy and cost-efficient differentiation of pure monocyte-derived macrophages directly from fresh peripheral blood mononuclear cells (PBMC) as a starting material (see Figs. 1 and 3). Special equipment as well as prior monocyte purification, e.g. with magnetic beads, is not necessary saving time and costs.

The Macrophage Base Medium XF is the user-customizable version of our Macrophage Media. It comes without cytokines as a universally applicable MDM culture system featuring a fully user-customizable macrophage differentiation and activation process (see Tab. 1 for suggestions).

As with all our XF media series, the Macrophage Base Media XF exhibit a serum- and xeno-free formula. Thus, these media provide a controlled culture environment – a significant benefit in terms of monocytes and macrophages standing for highly reactive immune cells. Therefore, these media properties enable a standardized and customized macrophage differentiation and activation without the influence of undefined or animal-derived components.

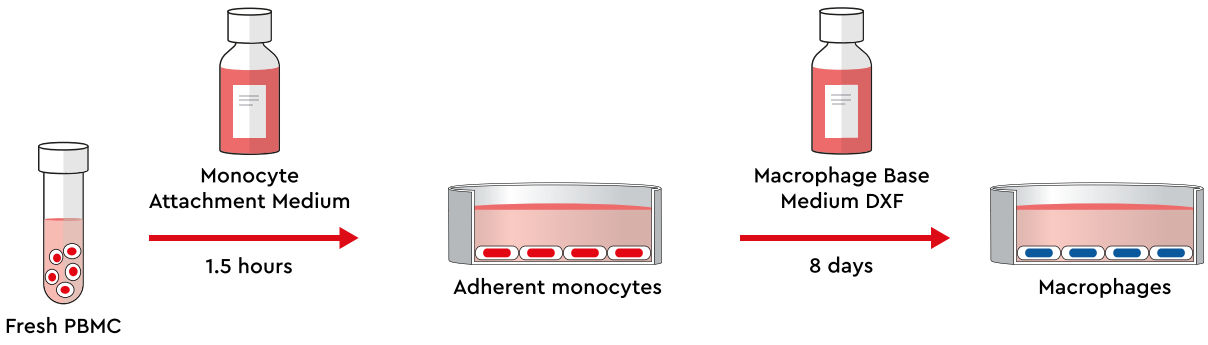

Fig. 1: Differentiation of monocyte-derived macrophages in 8 days directly from PBMC using the Monocyte Attachment Medium and the Macrophage Base Medium XF. *Activation of the culture can optionally be performed on day 7.*

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 [8]. Indeed, macrophages are involved in the outcome of many diseases, e.g., allergic and autoimmune disorders, cancer, diabetes, atherosclerosis, rheumatoid arthritis, and metabolic syndrome [9].

Traditionally, activated macrophages of different phenotypes have routinely been classified into M1- and M2-macrophages. 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 [10]. In contrast, the "alternatively activated" anti-inflammatory M2-macrophages comply with various regulatory functions of many kinds including regulation of immunity, maintenance of tolerance and tissue repair/wound healing [8, 10].
This functional heterogeneity of M2 macrophage functions lead to their allocation into three subgroups, i.e. M2a, M2b and M2c. Indeed, cells of the monocyte/macrophage lineage exhibit extraordinary plasticity in response to endogenous as well as exogenous stimuli potentially even leading to reversal of the initial M1/M2-polarization processes [2]. For example, M2 polarized macrophages can convert to the M1-activated status under certain conditions.

Therefore, it recently became common sense that the traditional M1/M2-model of macrophage polarization/activation is not satisfactory to reflect the whole complexity of activation states of this highly plastic cell lineage [11]. As a consequence, a group of international macrophage experts published a common framework proposal for macrophage activation nomenclature [7]. This new system stipulates the designation of *in vitro* macrophage activation states according to the stimulus used (e.g. 20 ng/ml recombinant human (rhu) IL-4) in combination with clear disclosure of differentiation factors employed for MDM generation (e.g. 100 ng/ml rhu M-CSF). See also Tab. 1.

Serum- and xeno-free macrophage culture systems in combination with the published guidelines for unified experimental standards for *in vitro* macrophage activation constitute essential corner points for purposeful and effective progress in macrophage-related research.

	Activation state	Former designation	Differentiation factor (day 0+)	Activator (day 7+)	Activation process reference
M1	M(IFN-γ)	M1	GM-CSF (or M-CSF)	IFN-γ (50 ng/ml)	[1]
	M(LPS+IFN-γ)	M1	GM-CSF (or M-CSF)	IFN-γ (50 ng/ml) + LPS (10 ng/ml)	[1]
	M(LPS)	M1	GM-CSF (or M-CSF)	LPS (100 ng/ml)	[1]
	M(-)	M1, non-activated	GM-CSF	-	[2]
	M(-)	M0 / Mφ	2% human AB serum	-	[1, 3]
	M(-)	M2, non-activated	M-CSF	-	[2]
	M(GC)	M2c	M-CSF	DEX (100 nM)	[2]
	M(TGFb)	M2c	M-CSF	TGF-b1 (20 ng/ml)	[2]
	M(IL-10)	M2c	M-CSF	IL-10 (10 ng/ml)	[4]
	M(IC+LPS)	M2b	M-CSF	IgG (immobilized) + LPS (100 ng/ml)	[5]
M2	M(IL-4)	M2a	M-CSF	IL-4 (20 ng/ml)	[4, 5]
	TAM	M2-like	tumor microenvironment	tumor microenvironment	[6]

Table 1: Human macrophage activation reference table according to the common framework consensus nomenclature [7]. *The published differentiation factor/activator combinations are listed to serve as a basic guidance. Specific effects of activation on macrophages should be tested in comparison to the most appropriate non-activated M(-)-baseline variant as a control. DEX = dexamethasone, IC = immune complexes, IFN = interferon, IgG = immunoglobulin G, GC = glucocorticoids, (G)M-CSF = (granulocyte/) macrophage colony stimulating factor, IL = interleukin, LPS = lipopolysaccharide, TAM = tumor associated macrophages, TGF = transforming growth factor.*

*Use the differentiation factors M-CSF or GM-CSF at 50–100 ng/ml final concentration with the Macrophage Base Medium XF (= complete medium: see protocol step 4).

Protocol Overview

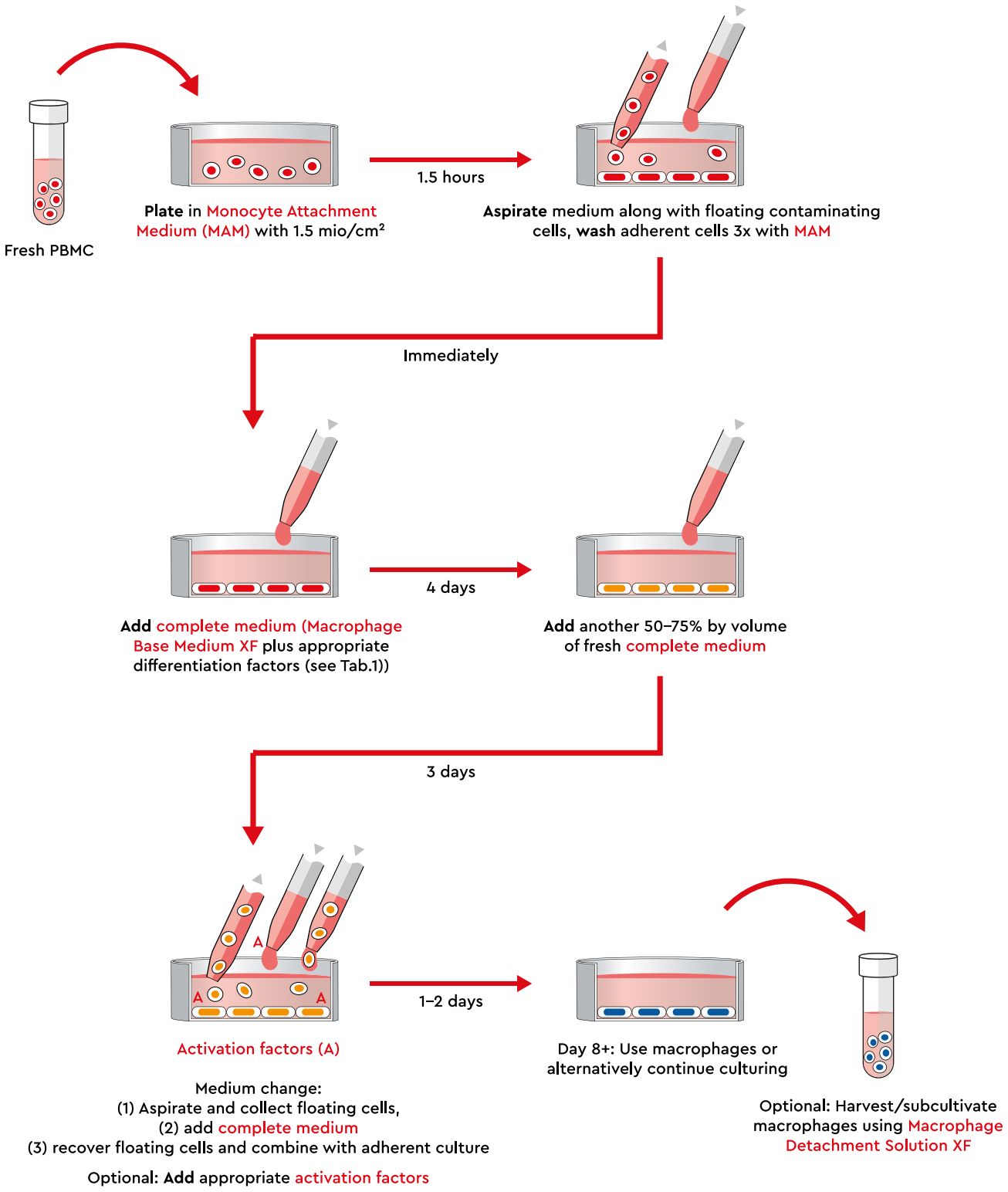

Fig. 2: Protocol overview using Macrophage Base Medium XF (8 days).

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 Base Medium XF.

Differentiation protocol

Materials

- Monocyte Attachment Medium (C-28051)
- Macrophage Base Medium XF (C-28057)
- Differentiation and activation factors (optional, refer to Tab. 1)
- PBS without Ca^{2+}/Mg^{2+} (C-40232)
- PBS without Ca^{2+}/Mg^{2+}/2 mM EDTA/0.1% HSA
- Optional: Macrophage Detachment Solution XF (C-41330, refer to protocol step 11)

Use aseptic techniques and a laminar flow bench.

1

PBMC isolation (day 0)

Isolate fresh PBMC from buffy coats using your routine protocol.

Note: Use buffy coats as fresh as possible. Do not use buffy coats older than 24 hours, since this will significantly impair the experimental outcome.

2

Count the PBMC (day 0)

Count the isolated PBMC and resuspend the cells at 100 million PBMC per ml in Monocyte Attachment Medium.

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 T75 flask. Use a seeding density of 1.5 million/cm^2. Incubate for 1.5 hours at 5% CO_2 and 37°C in the incubator without any further manipulation.

4

Prepare the complete Macrophage Base Medium XF (day 0)

Prepare the Macrophage Base Medium XF by adding the thawed SupplementMix aseptically to the Basal Medium. Swirl gently to obtain a homogeneous mixture.

Note: In order to promote survival and efficient differentiation of the monocytes in the given defined culture conditions, it is highly recommended to add a cytokine acting as a survival factor to the medium at this point, e.g. M-CSF or GM-CSF at 100 ng/ml. Alternatively, in order to generate non-activated/unpolarized M0 macrophages, add 2% human AB serum to the medium instead of M-CSF or GM-CSF. Macrophage Base Medium XF with added survival factors (M-CSF, GM-CSF or human AB serum) is referred to as "complete medium" in the following. Be aware that M-CSF produces a homogeneous population of differentiated MDM, while GM-CSF, although a stronger-acting survival factor, will yield a more heterogeneous population containing some moDC-like cells (myeloid dendritic cell-like cells) [7].

5

Wash the adherent cell fraction (day 0)

Vigorously swirl the tissue culture vessel and then aspirate the non-adherent cells. Wash the adherent cells, i.e., monocytes, three times with warm Monocyte Attachment Medium by thoroughly swirling the vessel and aspirating the supernatant.

6

Start the macrophage differentiation (day 0)

Add an appropriate amount of complete medium 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 into macrophages under these conditions. If required, activation can be achieved by performing the optional activation step (refer to step 9).

7

Add fresh complete medium (day 4)

Add another 50% to 75% by volume of fresh complete medium to the cells. Incubate the immature macrophages for another 3 days at 37°C and 5% CO_2.

Note: Do not remove any of the used medium from the cells, just add the fresh medium.

8

Medium change (day 7)

Aspirate the medium including suspension cells and collect it in a centrifugation tube. Immediately, pipet fresh complete medium to the adherent cells. Centrifuge the cells in the tube for 15 min at 350 x g at room temperature.
Discard the supernatant and carefully resuspend the cells in a small amount of fresh complete medium. Combine the resuspended cells in the tube with the adherent cells in the fresh complete medium contained in the tissue culture vessel. Incubate at least for another 24 hours at 37°C and 5% CO_2.

Note: Adherent as well as suspension cells may be present at this stage.

9

Optional activation step (day 7)

For specific macrophage activation, immediately after the prior media change, supplement the whole volume of the culture with adequate stimuli (refer to Tab. 1 for suggestions).

10

The macrophages are ready (day 8+)

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 several weeks is possible by performing weekly medium changes with fresh complete medium.

Note: Typically, macrophages appear as adherent cells with characteristic morphology: prominent nucleus with flatly outspread cytoplasm and multiple pseudopodia.

11

Optional step: Harvesting/subcultivation of macrophages (day 8+)

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 T75 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. Wash the cells twice with PBS / 2 mM EDTA / 0.1% HSA and count them subsequently. 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.

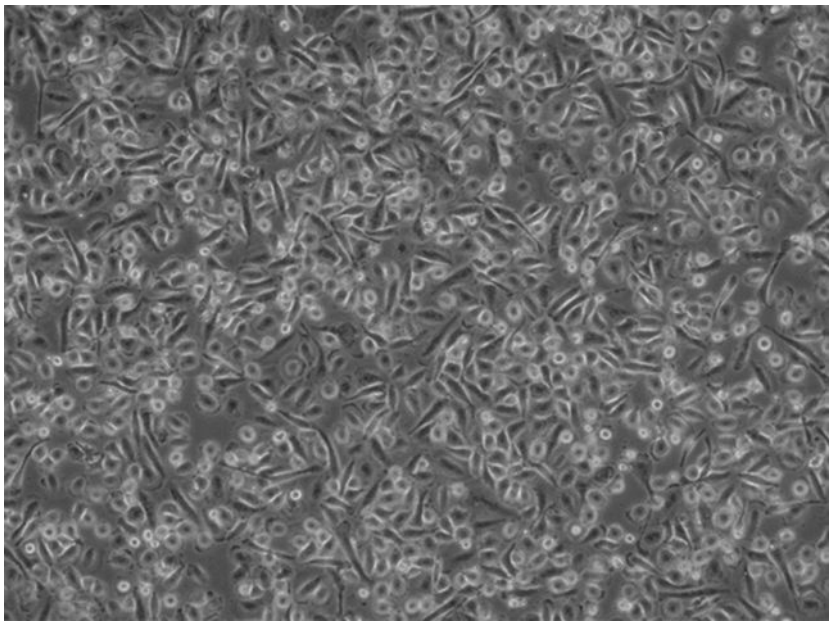

Fig. 3: Culture of non-activated human monocyte-derived macrophages (MDM) differentiated directly from PBMC as a starting material. *Monocytes were purified using the Monocyte Attachment Medium and differentiated in Macrophage Base Medium XF containing 100 ng/ml GM-CSF for 10 days.*

Products

Media	Size	Catalog Number
Monocyte Attachment Medium (Ready-to-use)	250 ml	C-28051
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
M1-Macrophage Generation Medium XF	250 ml	C-28055
M2-Macrophage Generation Medium XF	250 ml	C-28056
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

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07/2022