Cell therapy

Differentiation and characterization of monocyte-derived dendritic cells and macrophages

Optimized dendritic cell and macrophage differentiation with CTS AIM-V Medium and CTS Immune Cell Serum Replacement

Introduction

Myeloid cells are gaining interest as promising candidates for immunotherapy due to their relative abundance and importance in inflammatory and cancerous tissues. Dendritic cells (DCs) and macrophages (Mϕ) are active players in modulating tissue immunity, and their inherent properties of phagocytosis, cytokine production, antigen presentation, and immune cell activation are being increasingly utilized for adoptive cell therapies.

DCs are unique antigen-presenting cells that act as a bridge between the innate and adaptive immune systems. Upon sensing foreign pathogens or endogenous danger signals, DCs upregulate co-stimulatory molecules, produce cytokines, take up and process antigens, migrate to lymph nodes, and present antigens to T cells to mount an immune response [1]. In the clinical setting, DCs are commonly being used as autologous (self-derived) cancer vaccines and immunotherapies [2-4]. Although there are many subsets of DCs that circulate in the blood, their small numbers have made them challenging candidates for cell therapies. Clinically relevant numbers of DCs are generally obtained by isolating monocytes from blood and differentiating them *ex vivo* in culture to generate mature DCs capable of inducing T cell activation and driving the anti-tumor response [5].

Macrophages are tissue-resident innate immune cells that perform a vital homeostatic role as phagocytic cells that clear pathogens and endogenous harmful materials, such as dead and apoptotic cells, amyloid beta, and surfactants, to maintain normal organ function. Macrophages exist in a dynamic state *in vivo* and can change their phenotype depending on environmental cues. *Ex vivo* polarized naïve or genetically modified macrophages are obtained by culture and differentiation of primary monocytes and are being investigated as autologous adoptive cell therapies for solid tumor immunotherapy and regenerative medicine [6-7].

Historically, DC and macrophage culture and differentiation protocols have used media with the addition of fetal bovine or human serum. Serum-free media are preferred for cell therapy applications because they help in achieving greater consistency and process control, and address performance, regulatory, and supply chain concerns. Gibco™ CTS™ AIM-V™ Medium is a serum-free medium, available in several formats for both research and manufacturing applications, and has been widely cited in peer-reviewed publications for multiple immune cell clinical applications [8-9]. Gibco™ CTS™ Immune Cell Serum Replacement (CTS ICSR) is a defined xeno-free supplement that has been widely used in serum-free T cell cultures and is available in both bottle and bioprocess container (BPC) formats. In this application note, we demonstrate the use of CTS AIM-V Medium and CTS ICSR in culturing functional monocyte-derived DCs and macrophages.

gibco

Materials and methods

Experimental overview

Monocytes were isolated on day –1 using the isolation workflow option for the Invitrogen™ Dynabeads™ Untouched™ Human Monocytes Kit and cultured in CTS AIM-V Medium and CTS ICSR with various growth factors and cytokines to create immature DCs or M0 Mϕ. From days 5–7, cells were cultured with or without various maturation (DC) or polarization (Mϕ) cocktails, and different endpoint functional assays were performed on days 7–12 (Figure 1).

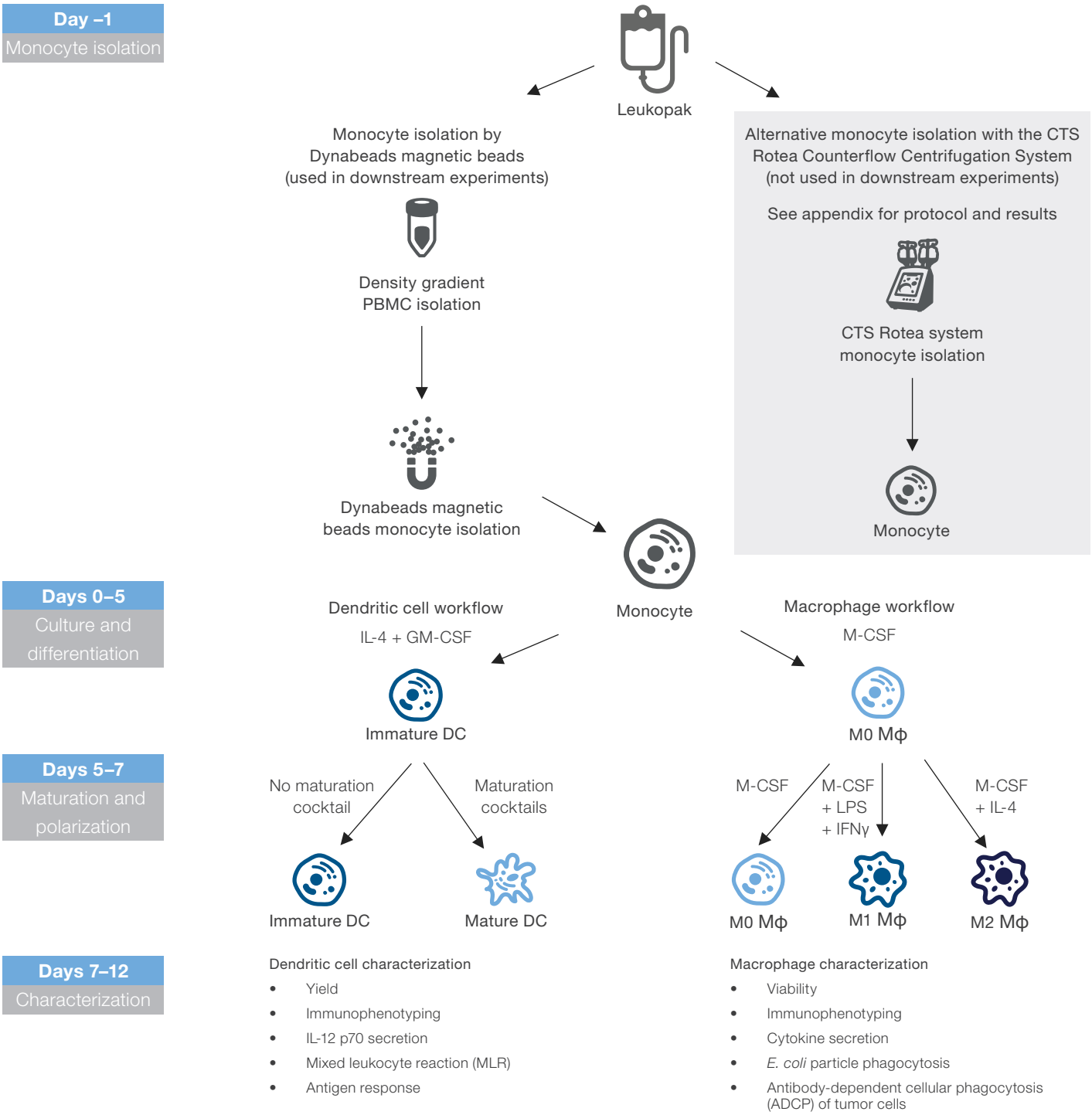


Figure 1. Experimental workflow. Monocytes were isolated on day –1 using the isolation workflow for the Dynabeads magnetic beads kit. Isolated monocytes were cultured in CTS AIM-V Medium and CTS ICSR with IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) for immature DCs and macrophage colony-stimulating factor (M-CSF) for M0 macrophages. On days 5–7, cells were cultured with or without various maturation (DC) and polarization (Mϕ) cocktails, with endpoint functional assays performed on days 7–12.

Monocyte isolation

Monocytes were enriched from the previously cryopreserved peripheral blood mononuclear cells (PBMCs) from healthy donors using the Invitrogen™ Dynabeads™ Untouched™ Human Monocytes Kit following the guidelines in the user guide. Purity and viability of isolated monocytes were determined by labeling with human anti-CD14 antibody and the Invitrogen™ LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, followed by flow cytometry analysis on an Invitrogen™ Attune™ CytPix™ Flow Cytometer. Viability and cell numbers of isolated cells were also independently measured using an automated cell counter.

Monocyte differentiation to DC and macrophage

Dentritic cell culture

Monocytes were seeded at 5 x 10^5 cells/well in 12-well Thermo Scientific™ Nunc™ Cell-Culture Treated Multidishes and cultured in CTS AIM-V Medium + 5% CTS ICSR or in another supplier's serum-free medium with 500 IU/mL interleukin-4 (IL-4) and 1,000 IU/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) for 7 days with fresh medium supplementation on days 3 and 5. On day 5, i) a traditional maturation cocktail first described by Jonuleit et al. [10], consisting of 10 ng/mL tumor necrosis factor alpha (TNFα), 10 ng/mL IL-1 beta, 15 ng/mL IL-6, and 1 µg/mL prostaglandin E2 (PGE2); or ii) an alternative maturation cocktail consisting of a toll-like receptor 4 (TLR4) agonist, monophosphoryl lipid A (MPLA) (5 µg/mL), and 2,000 IU interferon gamma (IFNγ) [11] was added in some samples to obtain mature DCs (mDCs). For $CD8^+$ T cell activation experiments, immature DCs (iDCs) were pulsed with 2 µg/mL viral antigen peptide mix from human cytomegalovirus, Epstein-Barr, and influenza virus (CEF) overnight before addition of maturation agents for 24 hours.

Dentritic cell characterization

mDCs and iDCs were harvested on day 7 and analyzed for DC yield and immunophenotyping characterization, and supernatant was harvested for IL-12 secretion. mDCs and iDCs were co-cultured with allogeneic or autologous T cells in CTS AIM-V Medium + 5% human AB serum at a ratio of ~1:20 for proliferation assays, and ~1:10 for activation assays. T cell proliferation was recorded in cell proliferation dye–stained T cells on day 4 of co-culture. T cell activation was analyzed by intracellular labeling of T cells on day 5 of co-culture.

Macrophage culture

Monocytes were seeded at 3 x 10^5 cells/well in 24-well Nunc Cell-Culture Treated Multidishes and cultured in CTS AIM-V Medium + 5% CTS ICSR or another supplier's medium with 50 ng/mL macrophage colony stimulating factor (M-CSF) and 50 µM 2-mercaptoethanol (2-ME) for 7–8 days. Cells were fed with fresh media on day 3. On day 5 or 6, cells were fed with fresh media supplemented as follows: i) without polarization agents—M0 polarization; ii) 10 ng/mL lipopolysaccharide (LPS) and 50 ng/mL IFNγ—M1 polarization; and iii) 20 ng/mL IL-4—M2 polarization.

Macrophage characterization

On days 7 and 8, macrophages were harvested and analyzed for viability, phenotypic characterization, and cytokine secretion. Bacterial particle phagocytosis was analyzed after co-incubating macrophages with 100 µg/mL Invitrogen™ pHrodo™ Deep Red *E. coli* BioParticles™ Conjugates for Phagocytosis in serum-free Gibco™ Iscove's Modified Dulbecco's Medium (IMDM) for 2 hours with or without pre-treatment for 30 minutes with 10 µg/mL Invitrogen™ cytochalasin D, a phagocytosis inhibitor. Macrophages were also evaluated for phagocytosis of cancer cells. Carboxyfluorescein succinimidyl ester (CFSE)-stained HER-2–expressing SKBR3 breast cancer cells were treated with 10 µg/mL human IgG1 or trastuzumab (humanized HER-2 monoclonal antibody) for 1 hour at 37°C, washed and then co-cultured with unpolarized macrophages in serum-free IMDM at a ratio of ~2:1 target vs. effector cell. Antibody-dependent cellular phagocytosis (ADCP) of cancer cells was analyzed by quantifying the percentage of CFSE-positive macrophages by flow cytometry and confirmed by immunofluorescence imaging of fixed cells after 2 hours of co-culture.

Characterization analysis instrumentation

Viable cell counts were analyzed with a trypan blue exclusion method on an automated cell counter. Brightfield and immunofluorescence imaging of cells were performed with live-cell imaging systems, including Invitrogen™ EVOS™ M7000 and M5000 imaging systems. Cytokine concentrations in cell culture supernatants were quantified using ELISA and measured with a microplate reader. Assessments of cellular surface markers, cell proliferation, intracellular cytokine production, and phagocytosis were performed on Invitrogen™ Attune™ CytPix™ and Attune™ NxT flow cytometers and analyzed by FlowJo™ software v10. Flow cytometry gating was performed using fluorescence minus one (FMO) controls.

Results

Monocyte enrichment

CD14+ monocytes enriched from PBMCs using the Dynabeads Untouched Human Monocytes Kit yielded 90% purity as analyzed by flow cytometric staining (Figure 2).

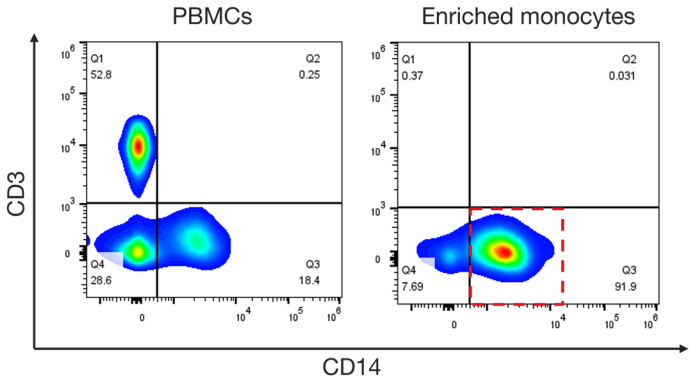


Figure 2. Monocyte enrichment from PBMCs. Representative flow cytometry dot plots showing monocyte enrichment from PBMCs with the Dynabeads Untouched Human Monocytes Kit.

DC yield

Across 6 donors, the yields of immature and mature DCs cultured in CTS AIM-V Medium + 5% CTS ICSR were comparable to or better yield than those of several products from other suppliers. Similar to cell morphology, yield was dependent on the agent used to drive maturation (Figure 4).

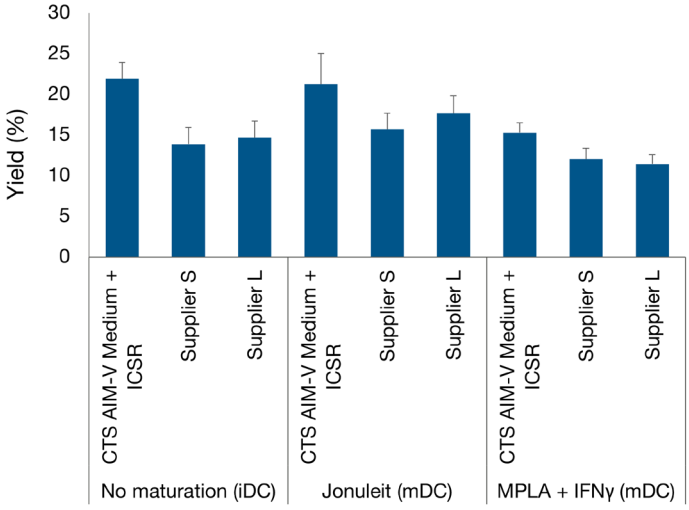


Figure 4. Yield of DCs cultured in various serum-free media and matured with different maturation agents. Percentage yield was calculated by dividing the number of viable DCs on day 7 by the number of viable monocytes seeded on day 0 (n = 6 donors, data expressed as mean ± standard error of mean).

DC morphology

iDCs differentiated in CTS AIM-V Medium + 5 % CTS ICSR showed flattened adherent morphology. On maturation with the Jonuleit's cocktail, mDCs were round, became loosely adherent, and showed dendritic processes characteristic of DCs. Conversely, alternative cocktails like MPLA + IFNγ generated mDCs that were elongated in shape and were more firmly adherent to the cell culture plate (Figure 3).

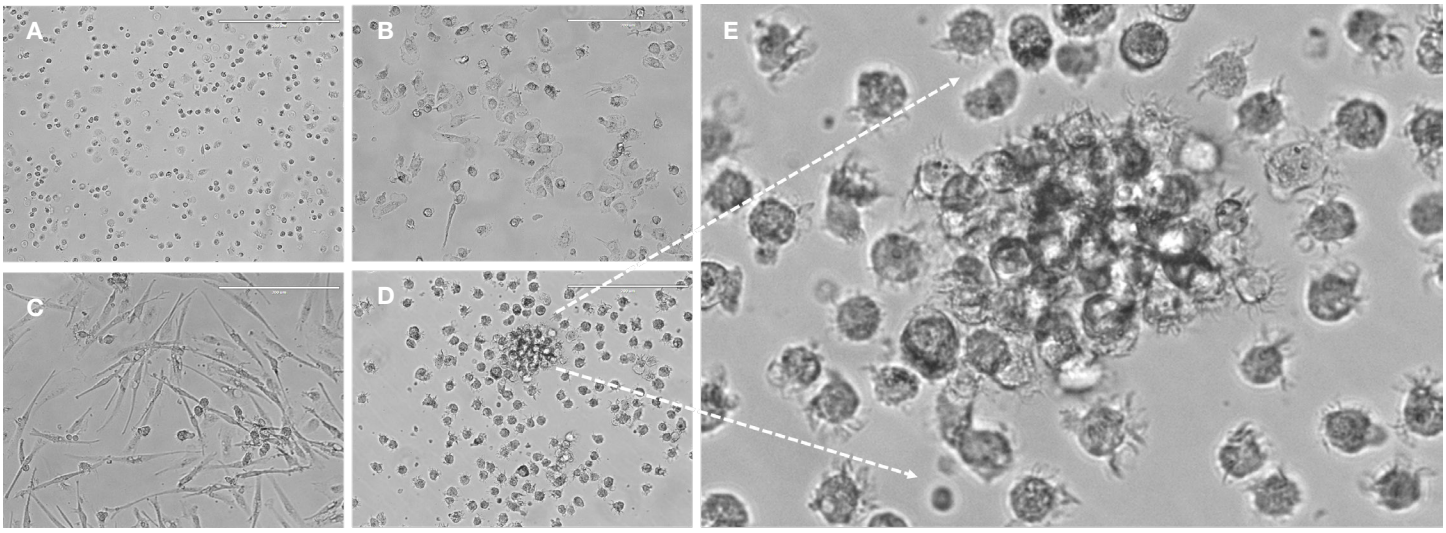


Figure 3. Morphology of cells cultured in CTS AIM-V Medium and CTS ICSR. (A) Monocytes on day 0, **(B)** immature DCs, **(C)** mature DCs from MPLA + IFNγ maturation, **(D)** mature DCs from Jonuleit's maturation, and **(E)** magnified image of Jonuleit's mature DCs.

DC phenotypic characterization

DCs traffic to the lymph nodes where cellular contact between T cells and DCs leads to antigen recognition through T cell receptor (TCR) interactions with peptide–MHC complexes on the DC surface. Additionally, T cell–DC contacts involve various receptor–ligand interactions, including those of co-stimulatory and adhesion molecules. Hence, mature functional DCs should not only express markers characteristic of activated antigen-presenting cells (APCs) like HLA-DR, CD83, and CD11c, but also show robust expression of co-stimulatory molecules like CD80/CD86 and CD40 that bind to CD28 and CD40L on T cells, respectively. Similarly, expression of the chemokine receptor CCR7 and CD209 (DC-SIGN) that binds to ICAM-3 on resting T cells is crucial for lymph node trafficking and T cell engagement, respectively. mDCs differentiated and matured in CTS AIM-V Medium + 5% CTS ICSR expressed elevated levels of DC maturation markers CD83, CD11c, and HLA-DR, and co-stimulatory molecules like CD80, CD86, and CD40 relative to molecules observed in monocytes and iDCs, accompanied with a decrease in CD14 expression. CD209 was increased in immature DCs, and CCR7 expression increased with Jonuleit's maturation agent only (Figure 5).

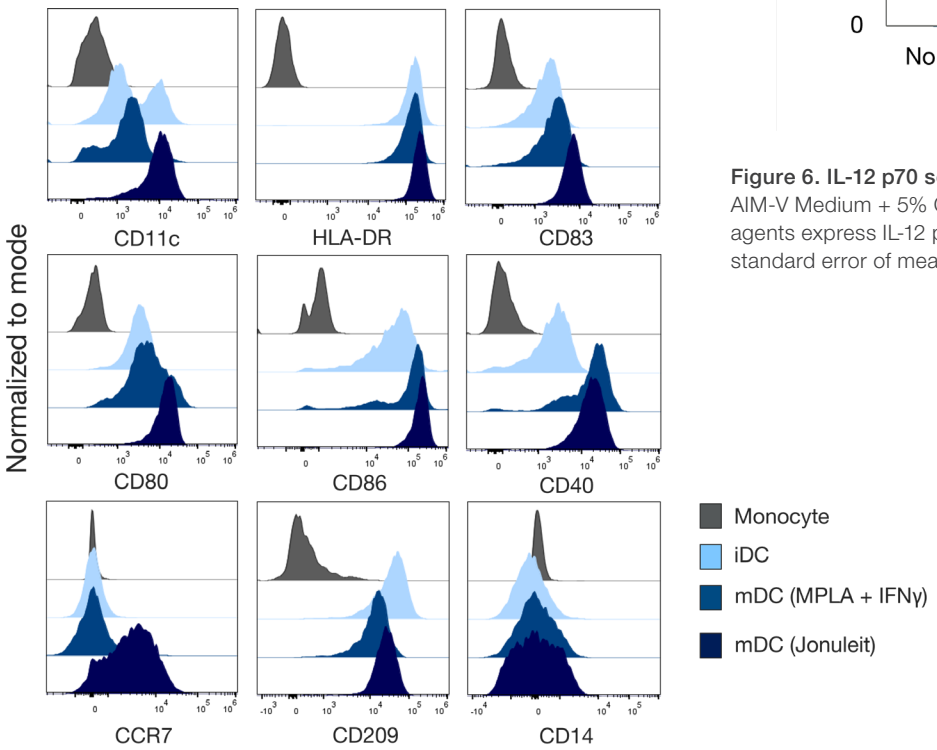


Figure 5. Immunophenotyping of monocyte-derived DCs. Representative flow cytometry histograms showing various maturation markers on monocytes, iDCs, and mDCs.

DC functionality: IL-12 p70 ELISA

Functional immunogenic DCs secrete IL-12 p70, which is responsible for promoting T helper 1 (Th1) cell polarization resulting in production of IFNγ from T cells. DCs cultured in CTS AIM-V Medium + 5% CTS ICSR and matured by MPLA + IFNγ increased IL-12 p70 secretion as compared to DCs matured by the traditional Jonuleit's maturation cocktail. Immature DCs showed negligible release of IL-12 p70 (Figure 6). This is consistent with findings in the literature showing inhibition of IL-12 production by PGE2 [12] (part of Jonuleit's maturation cocktail) and direct regulation of Th1 cytokines by MPLA through toll-like receptors [13].

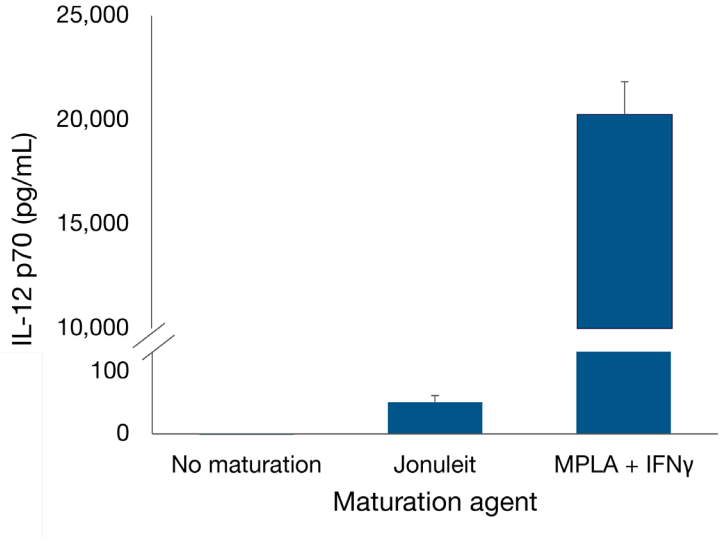


Figure 6. IL-12 p70 secretion. Monocyte-derived DCs cultured in CTS AIM-V Medium + 5% CTS ICSR and exposed to various maturation agents express IL-12 p70 (n = 3 donors, data expressed as mean ± standard error of mean).

DC functionality: T cell proliferation in MLR assay

The mixed lymphocyte reaction (MLR) assay is a commonly used technique to determine the immunogenicity of immune cells. We used a co-culture of purified allogeneic T cells with monocyte-derived DCs to study allogeneic T cell proliferation. DCs cultured in CTS AIM-V Medium + 5% CTS ICSR showed excellent functionality, as evidenced by increased T cell proliferation in allogeneic MLR co-culture assays compared to T cell–only negative controls. Both iDCs and mDCs were able to elicit T cell proliferation from HLA mismatched donors (Figure 7A, B).

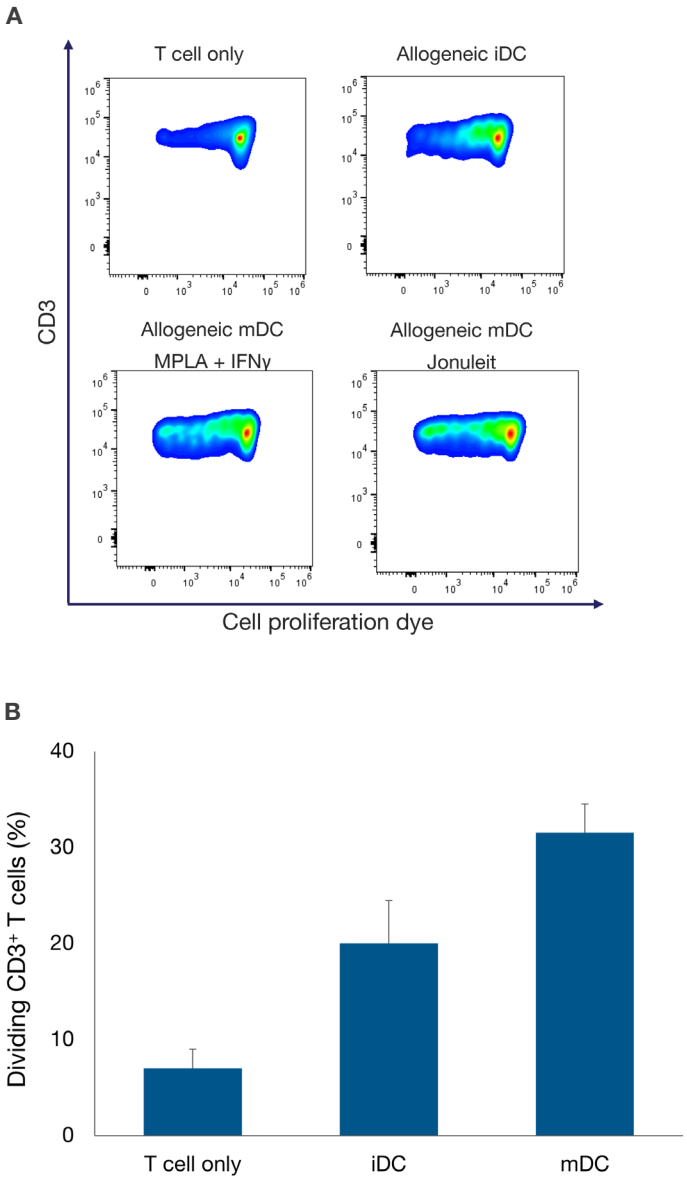


Figure 7. Allogeneic T cell proliferation. (A) Representative flow cytometry dot plots and **(B)** bar graphs showing increased T cell proliferation in DC–T cell co-cultures from different donors as compared to T cell only cultures. Data from DCs cultured in both MPLA + IFNγ and Jonuleit's maturation cocktail (n = 6 donors, data expressed as mean ± standard error of mean).

DC functionality: Th1 cell polarization in MLR assay

mDCs cultured in CTS AIM-V Medium + 5% CTS ICSR and matured in MPLA + IFNγ increased IFNγ production in helper T cells in MLR assays, indicative of Th1 cell polarization (Figure 8A, B). This effect is in line with increased IL-12 p70 production from mDCs cultured in CTS AIM-V Medium + 5% CTS ICSR. mDCs matured with the Jonuleit's cocktail did not show efficient Th1 cell polarization, probably because of lower IL-12 p70 production (data not shown).

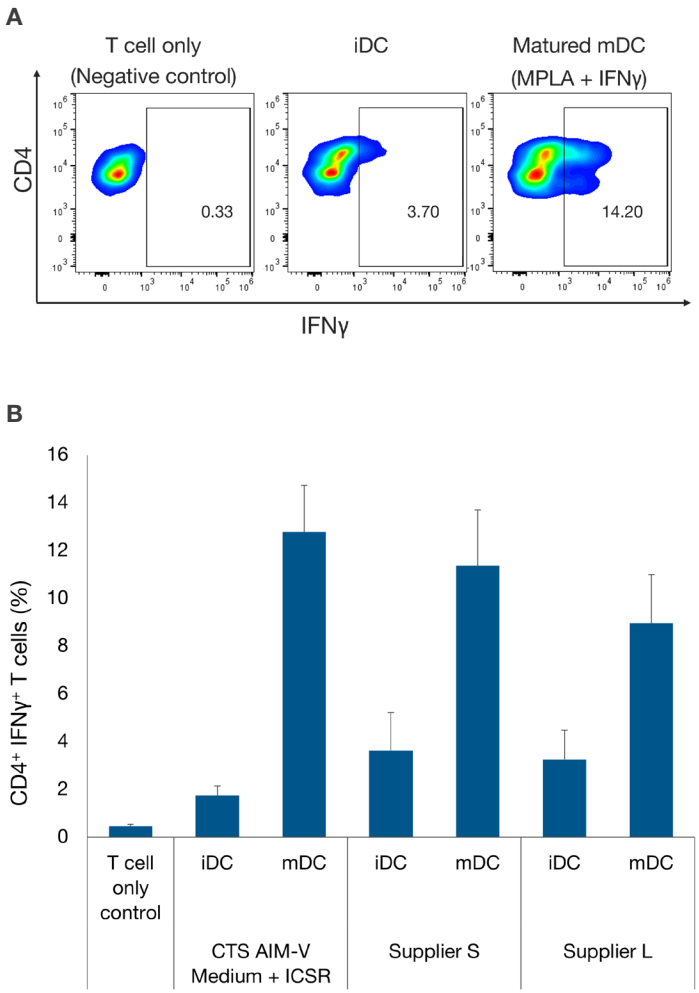


Figure 8. Allogeneic Th1 cell polarization. (A) Representative flow cytometry dot plots showing increased interferon gamma staining in T helper cells in allogeneic mDC–T cell co-cultures. **(B)** Bar graph showing percentage of IFNγ+ CD4+ T cells after co-culture with DCs differentiated in various serum-free media. Data from DCs cultured with MPLA + IFNγ maturation cocktail (n = 3 donors, data expressed as mean ± standard error of mean).

DC functionality: antigen response assay

DCs are classical antigen-presenting cells that can internalize, process, and present a foreign antigen to autologous T cells and activate them to mount an immune response. We tested this function by co-culturing autologous T cells with immature DCs and mature DCs (pulsed with the viral antigen peptide mix CEF, followed by maturation with MPLA + IFNγ). CEF is a pool of 32 well-defined HLA class I–restricted epitopes of cytomegalovirus (CMV), Epstein-Barr virus (EBV), and influenza virus that is commonly used to test activation of $CD8^+$ T cells. After 5 days of co-culture, we demonstrated that antigen-pulsed mature DCs showed significantly increased autologous $CD8^+$ T cell activation relative to T cell–only negative controls and co-cultures with immature DCs. Activation was evidenced by the increased percentage of cells that are double positive for granzyme B and IFNγ in $CD8^+$ T cells (Figure 9).

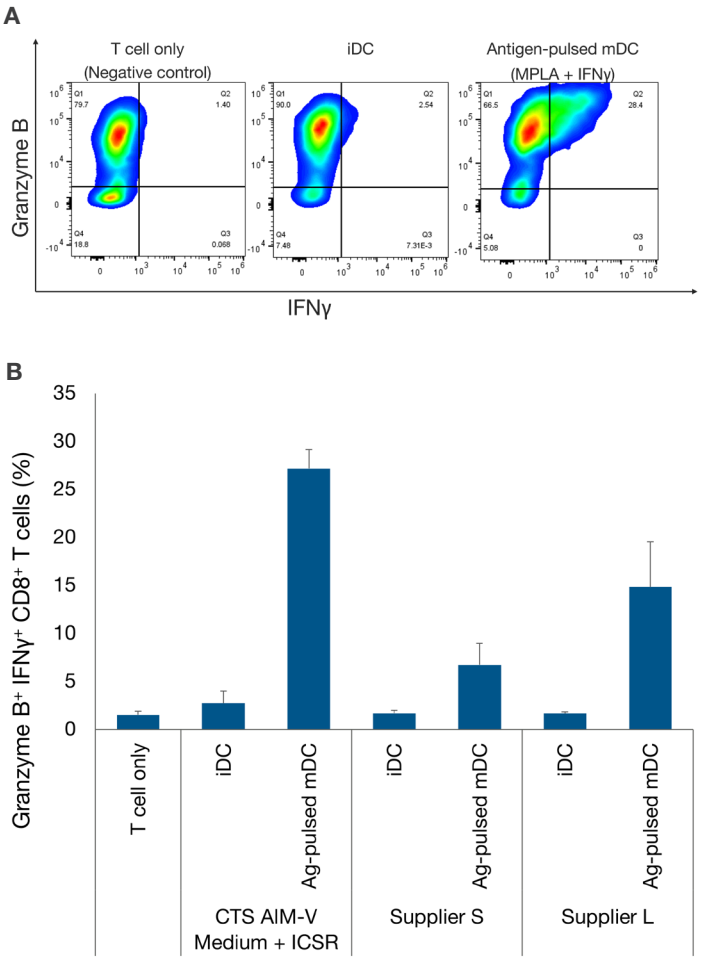


Figure 9. Antigen-pulsed mature DCs induce autologous $CD8^+$ T cell activation. (A) Representative flow cytometry dot plots and **(B)** bar graph showing increased granzyme B and IFNγ double positive autologous $CD8^+$ cytotoxic T cells by antigen-pulsed mDCs cultured in CTS AIM-V Medium + 5% CTS ICSR and matured with MPLA + IFNγ as compared to the T cell only negative control and immature DCs (n = 5 donors, data expressed as mean ± standard error of mean).

Macrophage morphology

Monocytes differentiated to macrophages in CTS AIM-V Medium supplemented with 5% CTS ICSR according to the experimental workflow (Figure 1) increase in size and show flattened and strongly adherent morphology (Figure 10).

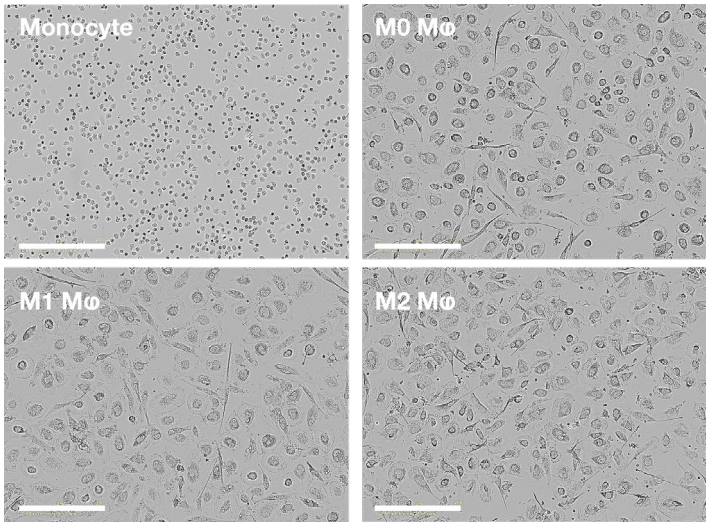


Figure 10. Monocyte and macrophage morphology. Monocytes, M0 macrophages (M0 Mϕ), M1 macrophages (M1 Mϕ), and M2 macrophages (M2 Mϕ) cultured in CTS AIM-V Medium + 5% CTS ICSR. Scale bar corresponds to 200 µm.

Macrophage viability

Across 7 donors, unpolarized or polarized macrophages cultured in CTS AIM-V Medium + 5% CTS ICSR showed improved viability compared to those cultured in another supplier's serum-free medium (Figure 11).

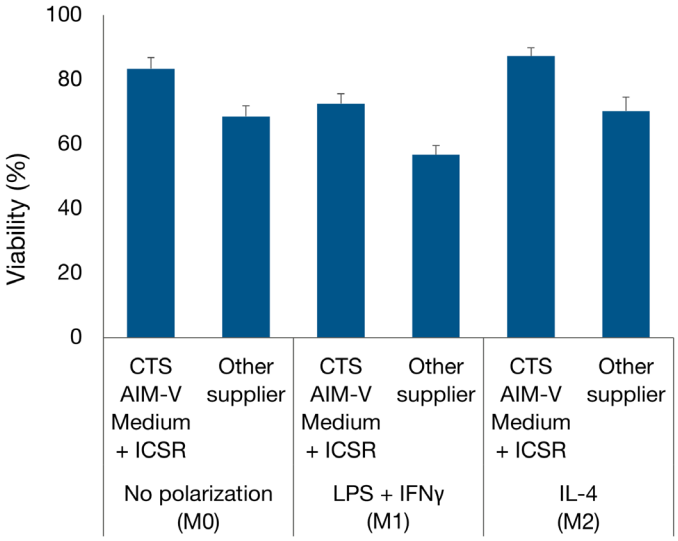


Figure 11. Macrophage viability. M0, M1, and M2 macrophages cultured in AIM-V Medium + 5% ICSR show improved viability relative to another supplier's serum-free medium (n = 7 donors, data expressed as mean ± standard error of mean).

Macrophage phenotype: cell surface markers

Unpolarized M0 macrophages differentiated in CTS AIM-V Medium + 5% CTS ICSR exhibited enhanced expression of macrophage surface markers as compared to monocytes, indicating adequate differentiation in our serum-free media system. Upon polarization, M1 macrophages expressed higher levels of M1 markers like CD80 and CCR7, and M2 macrophages exhibited higher levels of M2 markers like CD206 and CD209. M1 macrophages showed increased expression of CD64 and decreased expression of CD11b (Figure 12A).

Macrophage phenotype: cytokine secretion

Macrophages secrete appropriate cytokines according to specific triggers in the tissue environment. Pro-inflammatory or M1 macrophages secrete pro-inflammatory cytokines that attract and activate other immune cells, and pro-reparative macrophages release cytokines and mediators that help in tissue regeneration. These functions were confirmed by quantifying cytokine secretion by ELISA in polarized macrophages cultured in CTS AIM-V Medium + 5% CTS ICSR. M1 macrophages secreted M1 specific cytokines like IL-6 and TNFα selectively, while M2 polarized macrophages exhibited increased secretion of M2 specific cytokines and chemokines like CCL17 and IL-1ra (Figure 12B).

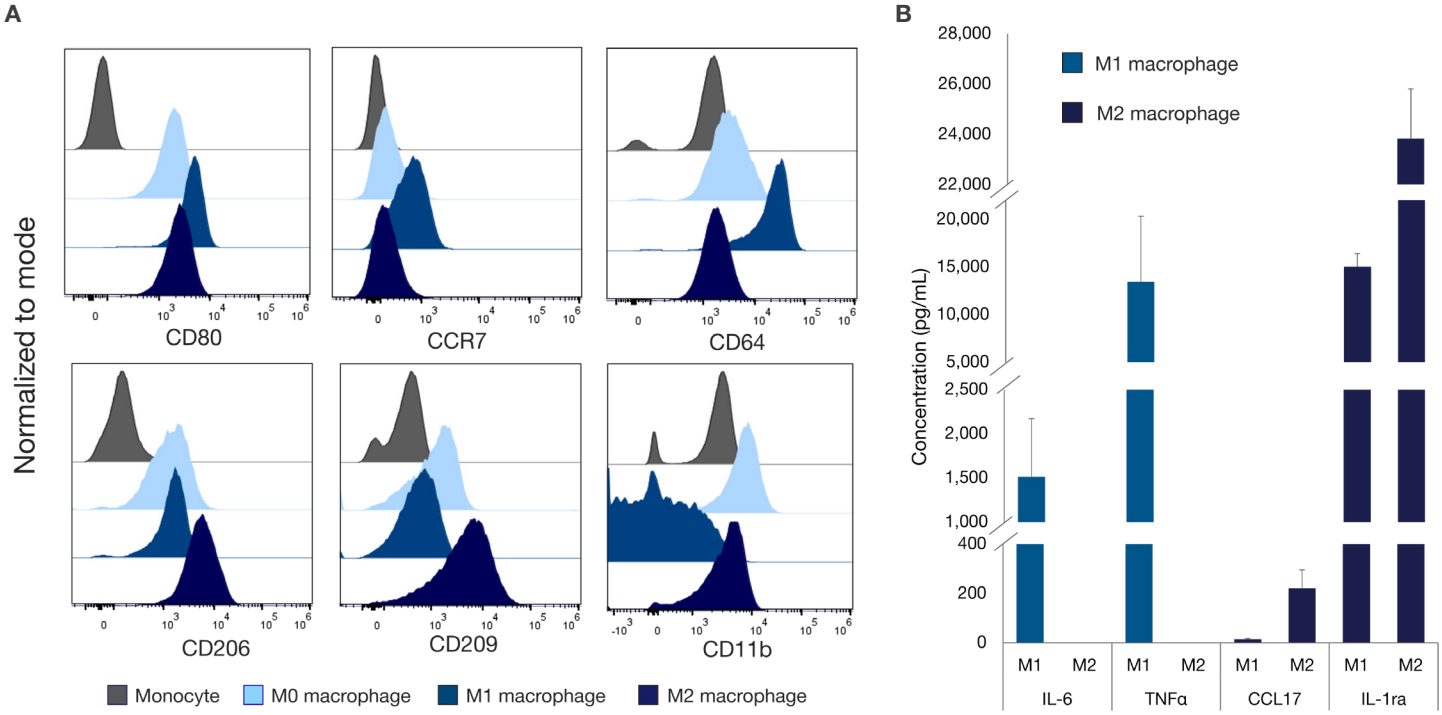


Figure 12. Macrophage immunophenotyping. (A) Representative flow cytometry histograms showing macrophages expressing markers specific to polarization [14] when cultured in CTS AIM-V Medium + 5% CTS ICSR. **(B)** Cytokine ELISA of macrophage supernatants showing cytokine secretion specific to polarization (n = 4–5 donors, data expressed as mean ± standard error of mean).

Macrophage functionality: *E. coli* phagocytosis

Macrophages are efficient phagocytes responsible for clearing tissues of foreign pathogens. This function was evaluated by quantifying *E. coli* particle uptake by polarized (M1 and M2) and unpolarized (M0) macrophages cultured in CTS AIM-V Medium + 5% CTS ICSR or in other suppliers' media, by flow cytometry. pHrodo dyes only emit fluorescence in an acidic environment and act as a surrogate marker for uptake in acidic endosomal and phago-lysosomal vesicles. Macrophages were incubated with pHrodo Deep Red *E. coli* BioParticles conjugates in the presence or absence of cytochalasin D, a phagocytosis inhibitor. As reported in the literature, M0 and M2 macrophages showed *E. coli* particle uptake that was inhibited in cytochalasin D–treated samples. Macrophages cultured in CTS AIM-V Medium + 5% CTS ICSR exhibited more robust uptake than those cultured in other supplier's serum-free media (Figure 13A, B). To confirm that *E. coli* particles have been truly phagocytosed by macrophages, immunostaining of *E. coli* particle–treated macrophages was performed. Macrophages that were pre-treated with cytochalasin D showed minimal uptake of *E. coli* particles as evidenced by a lack of red fluorescence. Macrophages that were not pre-treated with cytochalasin D showed robust uptake of pHrodo Deep Red *E. coli* BioParticles conjugates as evidenced by red fluorescence that co-localized with LAMP-1 staining, a phago-lysosomal marker, thus confirming phagocytosis (Figure 13C, D).

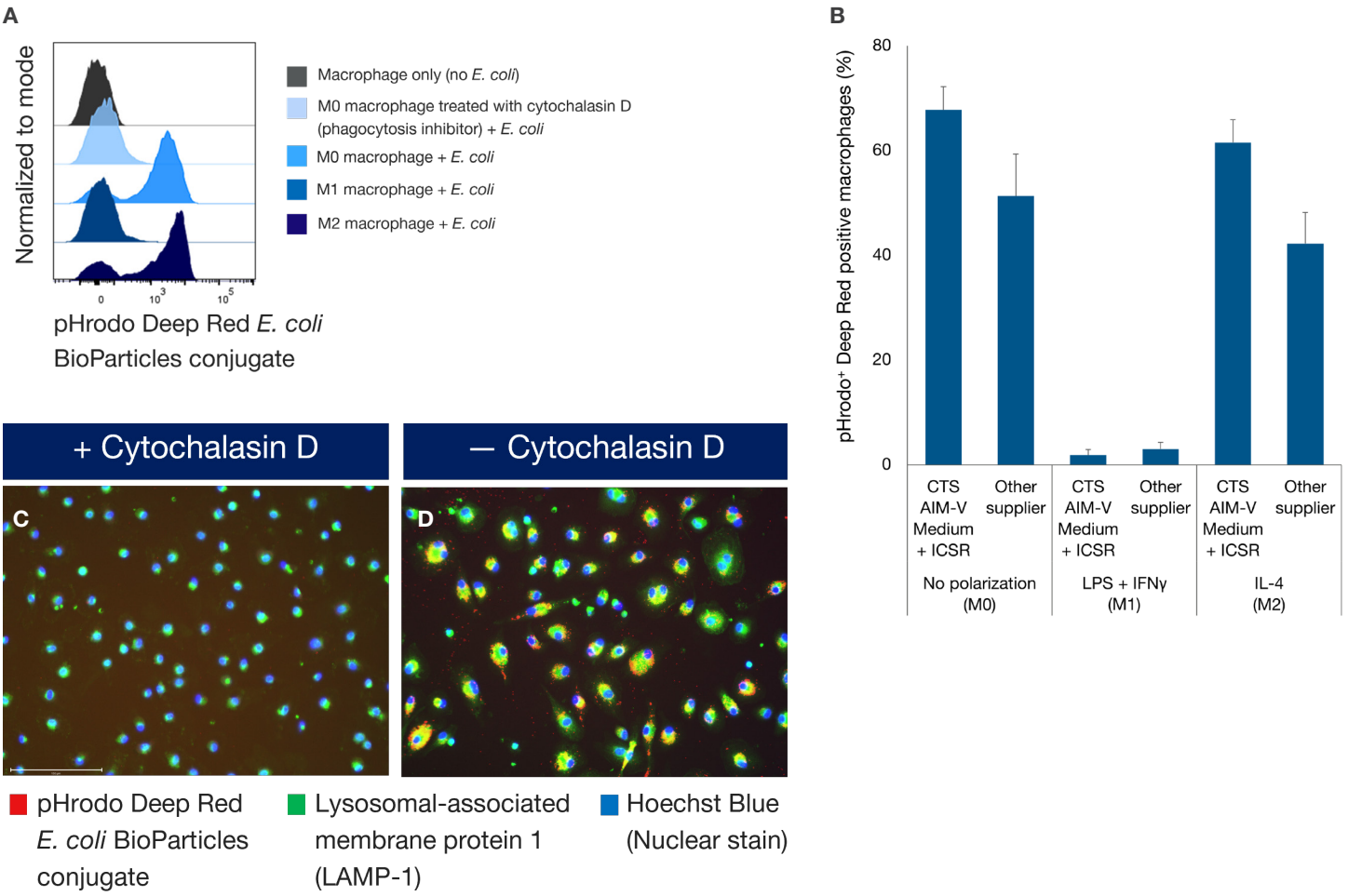


Figure 13. Phagocytosis of E. coli particles by macrophages. (A, B) Representative flow cytometry histograms and quantitative bar graphs showing increased *E. coli* particle clearance by M0 and M2 macrophages cultured in CTS AIM-V Medium + 5% CTS ICSR as compared to those cultured in another supplier's medium (n = 11 donors, data expressed as mean ± standard error of mean). **(C, D)** Phagocytosed *E. coli* particles (red) co-localized with intracellular LAMP-1 (green) indicating bacterial clearance by the phago-lysosomal pathway. There was minimal uptake of *E. coli* particles in cytochalasin D–treated macrophages. Scale bar = 150 µm.

Macrophage functionality: tumor cell antibody-dependent cellular phagocytosis (ADCP)

ADCP is the mechanism by which antibody-opsonized target cells activate the FcγRs on the surface of macrophages to induce phagocytosis, resulting in target cell death. This function of macrophages is being increasingly utilized in cancer immunotherapy. Many clinical trials for adoptive transfer of macrophages are investigating HER-2–expressing solid tumors (ClinicalTrials.gov ID NCT04660929 and NCT06224738). A clinically relevant assay was developed to test phagocytosis of HER-2–expressing SKBR3 breast cancer cells by macrophages cultured in CTS AIM-V Medium + 5% CTS ICSR. The presence of HER-2 protein (green) on the surface of SKBR3 cells was confirmed by immunofluorescence imaging (Figure 14A, B). Next, macrophages differentiated in CTS AIM-V Medium + 5% CTS ICSR were tested for their ability to perform ADCP of antibody-opsonized SKBR3 cells. After 2 hours of co-culture, macrophages showed increased ADCP of trastuzumab-opsonized (anti–HER-2 monoclonal antibody) SKBR3 cells as compared to hIgG1-opsonized controls, as evidenced by immunofluorescence imaging and flow cytometry analysis (Figure 14C, D, F, G). Next, the presence of the ADCP-promoting Fc receptors FcγRI (CD64), FcγRIIa (CD32a), and FcγRIII (CD16) on macrophages cultured in AIM-V Medium + 5% ICSR was confirmed (Figure 14F). ADCP efficiency was quantitatively estimated by calculating the percentage of CFSE⁺ (SKBR3) macrophages cultured in CTS AIM-V Medium + 5% CTS ICSR and another supplier's media. Macrophages cultured in AIM-V Medium + 5% ICSR showed ADCP of trastuzumab-opsonized SKBR3 cells comparable to that of macrophages cultured in serum-free medium from another supplier (Figure 14H).

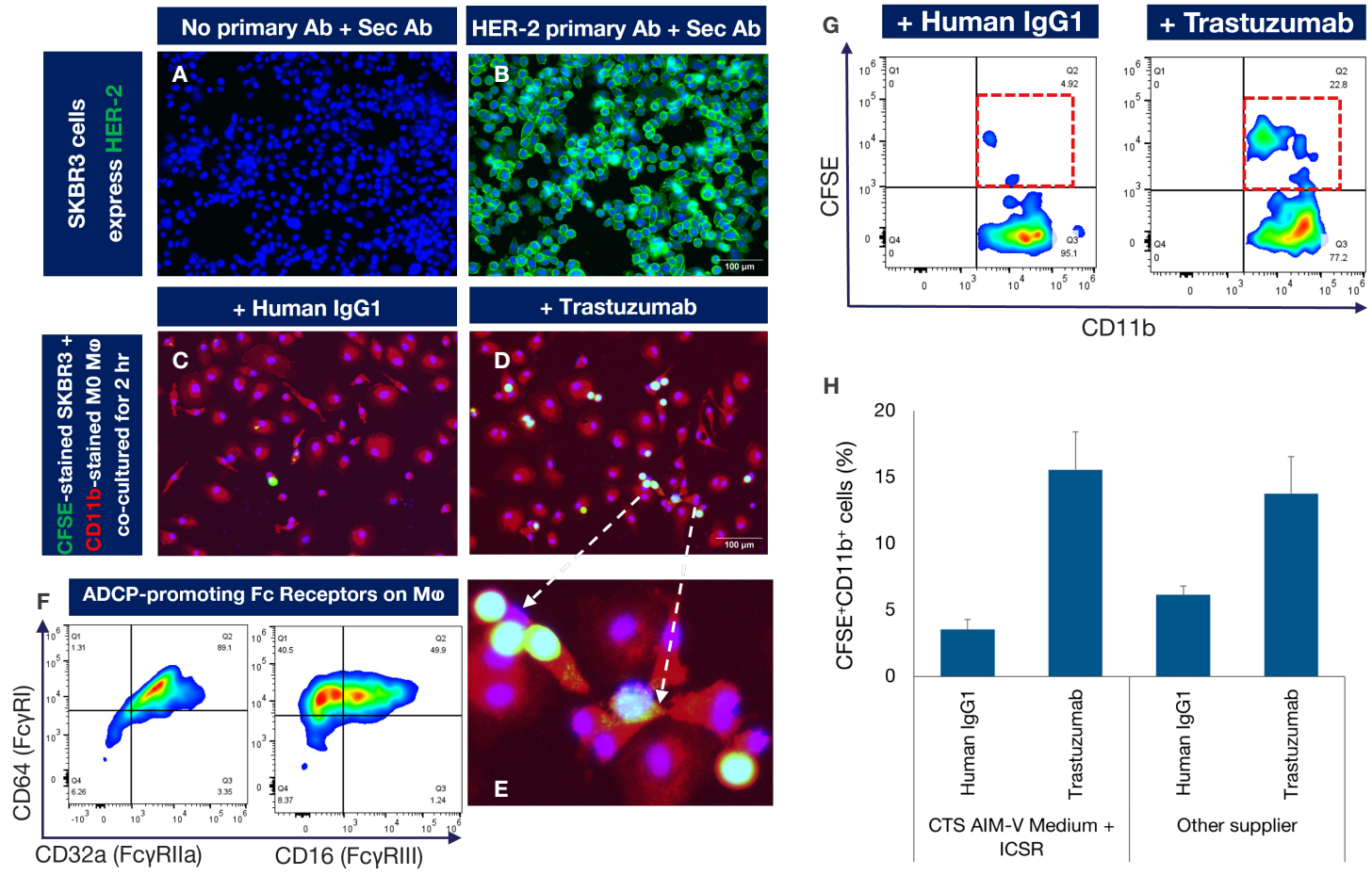


Figure 14. ADCP of tumor cells by macrophages. (A, B) Representative immunofluorescence images showing the presence of HER-2 protein on SKBR3 breast cancer cells and **(C, D, E)** increased phagocytosis of trastuzumab-treated SKBR3 cells as compared to human IgG1–treated SKBR3 cells. **(F)** Representative flow cytometry dot plots showing the presence of ADCP-promoting Fc receptors in M0 macrophages cultured in CTS AIM-V Medium + 5% CTS ICSR. **(G)** Representative flow cytometry dot plots and **(H)** quantitative bar graphs also revealed increased phagocytosis of trastuzumab-treated SKBR3 cells as compared to human IgG1 controls (n = 8 donors, data expressed as mean ± standard error of mean).

Discussion

The cell therapy industry has exploded over the past few years with many approved and numerous in-pipeline therapies, necessitating a significant need for scale-up, process validation, and critical raw material quality assurance as we move towards more Phase 3 clinical trials. To address this unmet need, innovation in cell culturing technologies, proper methods, and raw material sourcing and testing is desired. Currently, most clinical studies are still reliant on media supplemented with human or animal serum. This can introduce unwanted complexity during scale-up manufacturing and endangers reproducibility of clinical data mostly due to lot-to-lot variations. Serum supplementation also increases the danger of adventitious agents and enhances the possibility of supply chain risks as demand for serum increases. Modifications in cell culture conditions can also affect cellular physiology, thereby altering physical and performance characteristics and critical quality attributes. For the above reasons, serum-free media are preferred and are gradually becoming the gold standard for many cell therapy manufacturing workflows.

Dendritic cells and macrophages have become important candidates for cell therapy, vaccine development, drug delivery, and pharmaceutical testing. With the success of CAR-T cell therapies for hematological cancers, there is great interest in developing similar cell therapies against solid tumors. Approved personalized cell therapies against solid tumors like tumor infiltrating therapy (TIL) and T cell receptor (TCR-T) therapy rely on recognition and expansion of tumor antigen–specific T cells by professional antigen-presenting cells like DCs [15]. Additionally, DCs are extensively used as cancer vaccines [2-4] and for production of virus-specific T cells in the setting of hematopoietic stem cell transplantation (HSCT) for patients with malignancies [16] or patients with primary immunodeficiencies [17]. Similarly, *ex vivo* gene-engineered macrophages (CAR-Ms) are being investigated in solid tumors to utilize their inherent properties of tumor penetration, immune cell activation, and phagocytosis to kill tumor cells [7]. Adoptive transfer of naïve or gene-engineered macrophages is also being investigated for fibrotic diseases like cirrhosis and for regenerative medicine [6]. Since these newer cell types are becoming increasingly prevalent in upcoming cell therapy clinical pipelines, there is a need to validate serum-free media systems that can reliably culture these cell types.

CTS AIM-V Medium is a widely used serum-free medium that is closed system compatible, available in both bottle and BPC format, and has been extensively clinically proven by its use in multiple clinical trials worldwide. Similarly, CTS ICSR is closed system compatible, available in multiple formats, supports equivalent or improved results for multiple immune cell types when used at similar concentration as human serum, and comes with innovative packaging solutions for frozen supplements, including a high-density polyethylene (HDPE) secondary container designed to ensure integrity during freezing and thawing. Both CTS AIM-V Medium and CTS ISCR have been successfully used in commercially approved cell and gene therapies, which can reduce risk for investigational new drug (IND) and other regulatory applications.

Conclusion

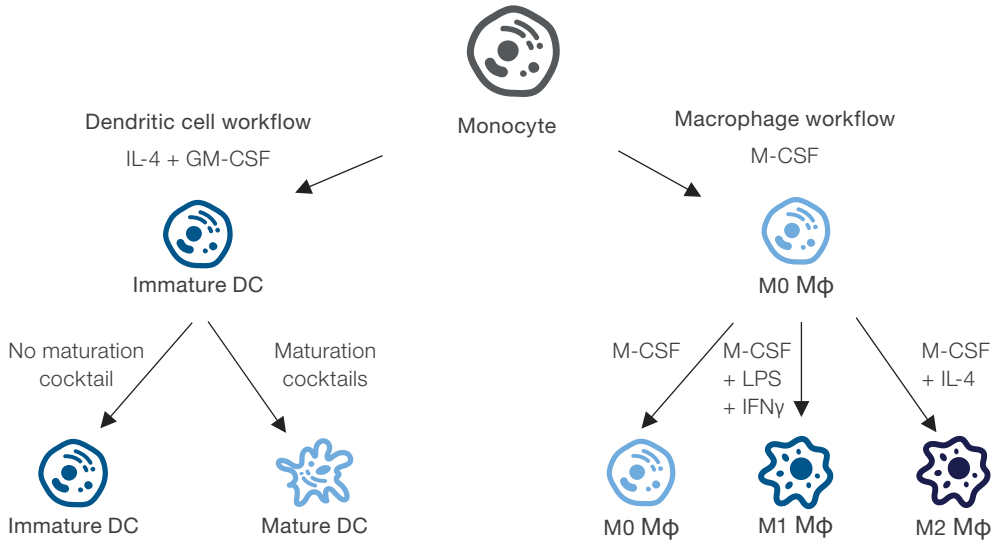
In this application note, we show the reliability of serum-free CTS AIM-V Medium supplemented with CTS ICSR for the culture of functional monocyte-derived DCs and macrophages. We demonstrate that monocyte-derived DCs cultured in CTS AIM-V Medium + 5% CTS ICSR show comparable or better yields of DCs than those cultured in other suppliers' media, express the desired surface markers of antigen-presenting cells, secrete IL-12, and induce robust T cell activation in allogeneic MLR and autologous antigen response assays.

The traditional maturation Jonuleit's cocktail induced elevated levels of DC maturation marker expression, characteristic morphology, and robust alloreactive T cell proliferation. However, this maturation protocol failed to produce sufficient levels of IL-12 p70 essential for inducing IFNγ production in helper and cytotoxic T cells. On the other hand, we confirmed that alternate maturation cocktails comprising TLR4 agonist MPLA and interferon gamma produce mature DCs that express co-stimulatory molecules and DC maturation markers, secrete higher levels of IL-12, and can induce T cell activation in allogeneic and autologous DC–T cell co-culture assays. Hence, choosing the optimal mix of maturation agents with the right media systems can yield functional DCs suitable for cell therapy applications.

We demonstrated that monocyte-derived macrophages cultured in CTS AIM-V Medium + 5% CTS ICSR show improved viability compared to those cultured in other suppliers' media. These macrophages express the desired surface markers of differentiated and polarized macrophages and secreted cytokines and chemokines specific to polarization. In addition, macrophages elicit robust functionality evidenced by increased bacterial particle clearance relative to those cultured in competitor media and significant increase in ADCP of trastuzumab-opsonized HER-2^{+} breast cancer cells as opposed to the human IgG1–opsonized control.

We conclude that CTS AIM-V Medium supplemented with 5% CTS ICSR is a reliable serum-free media system for culture of functional DCs and macrophages.

CTS AIM-V Medium and CTS ICSR support an optimized workflow for monocyte-derived DC and macrophage differentiation



CTS AIM-V Medium + CTS ICSR for monocyte-derived DCs

- Comparable or better yield of DCs than other commercially available media
- Differentiated DCs express surface markers present on antigen-presenting cells
- Secrete IL-12
- Induce robust T cell proliferation in allogeneic MLR assays
- Induce IFNγ production from T cells in both allogeneic MLR assays and autologous antigen response assays

CTS AIM-V Medium + CTS ICSR for monocyte-derived Mϕ

- Improved viability compared to other commercially available media
- Polarized macrophages express markers specific to M1 and M2 polarization
- Secrete cytokines and chemokines specific to M1 and M2 polarization
- Increased bacterial particle clearance
- Exhibit ADCP of trastuzumab-opsonized HER-2$^+$ breast cancer cells

References

1. Mellman. 2013. Dendritic cells: master regulators of the immune response. *Cancer Immunol Res.* 1(3):145-9. doi.org/10.1158/2326-6066.CIR-13-0102
2. Heras-Murillo et al. 2024. Dendritic cells as orchestrators of anticancer immunity and immunotherapy. *Nat Rev Clin Oncol.* 21:257-277. doi.org/10.1038/s41571-024-00859-1
3. Wculek et al. 2020. Dendritic cells in cancer immunology and immunotherapy. *Nat Rev Immunol.* 20:7-24. doi.org/10.1038/s41577-019-0210-z
4. Marciscano et al. 2021. The role of dendritic cells in cancer and anti-tumor immunity. *Semin Immunol.* 52:101481. doi.org/10.1016/j.smim.2021.101481
5. Liau et al. 2023. Association of autologous tumor lysate-loaded dendritic cell vaccination with extension of survival among patients with newly diagnosed and recurrent glioblastoma: A phase 3 prospective externally controlled cohort trial. *JAMA Oncol.* 9(1):112-121. doi.org/10.1001/jamaoncol.2022.5370
6. Na et al. 2023. A new era of macrophage-based cell therapy. *Exp Mol Med.* 55:1945-1954. doi.org/10.1038/s12276-023-01068-z
7. Li et al. 2024. A new era of cancer immunotherapy: Combining revolutionary technologies for enhanced CAR-M therapy. *Mol Cancer.* 23:117. doi.org/10.1186/s12943-024-02032-9
8. Trickett et al. 2002. Ex vivo expansion of functional T lymphocytes from HIV-infected individuals. *J Immunol Methods.* 262(1-2):71-83. doi.org/10.1016/s0022-1759(02)00018-2
9. Peng et al. 2005. Generation and maturation of dendritic cells for clinical application under serum-free conditions. *J Immunother.* 28(6):599-609. doi.org/10.1097/01.cji.0000175491.21099.04
10. Jonuleit et al. 1997. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur J Immunol.* 27:3135-3142. doi.org/10.1002/eji.1830271209
11. ten Brinke et al. 2010. Monophosphoryl lipid A plus IFNγ maturation of dendritic cells induces antigen specific CD8$^+$ cytotoxic T cells with high cytolytic potential. *Cancer Immunol Immunother.* 59:1185-1195. doi.org/10.1007/s00262-010-0843-z
12. Kalim et al. 2013. Prostaglandin E2 inhibits IL-23 and IL-12 production by human monocytes through down-regulation of their common p40 subunit. *Mol Immunol.* 53(3):274-82. doi.org/10.1016/j.molimm.2012.08.014
13. Komai-Koma et al. 2021. Monophosphoryl lipid A directly regulates Th1 cytokine production in human CD4$^+$ T-cells through Toll-like receptor 2 and 4. *Immunobiology.* 226(5):152132. doi.org/10.1016/j.imbio.2021.152132
14. Chen et al. 2023. Macrophages in immunoregulation and therapeutics. *Sig Transduct Target Ther.* 8:207. doi.org/10.1038/s41392-023-01452-1
15. Parkhurst et al. 2024. Adoptive transfer of personalized neoantigen-reactive TCR-transduced T cells in metastatic colorectal cancer: phase 2 trial interim results. *Nat Med.* 30:2586-2595. doi.org/10.1038/s41591-024-03109-0
16. O'Reilly et al. 2016. Virus-specific T-cell banks for 'off the shelf' adoptive therapy of refractory infections. *Bone Marrow Transplant.* 51:1163-1172. doi.org/10.1038/bmt.2016.17
17. Keller et al. 2020. Virus-specific T-cell therapies for patients with primary immune deficiency. *Blood.* 135(9):620-628. doi.org/10.1182/blood.2019000924

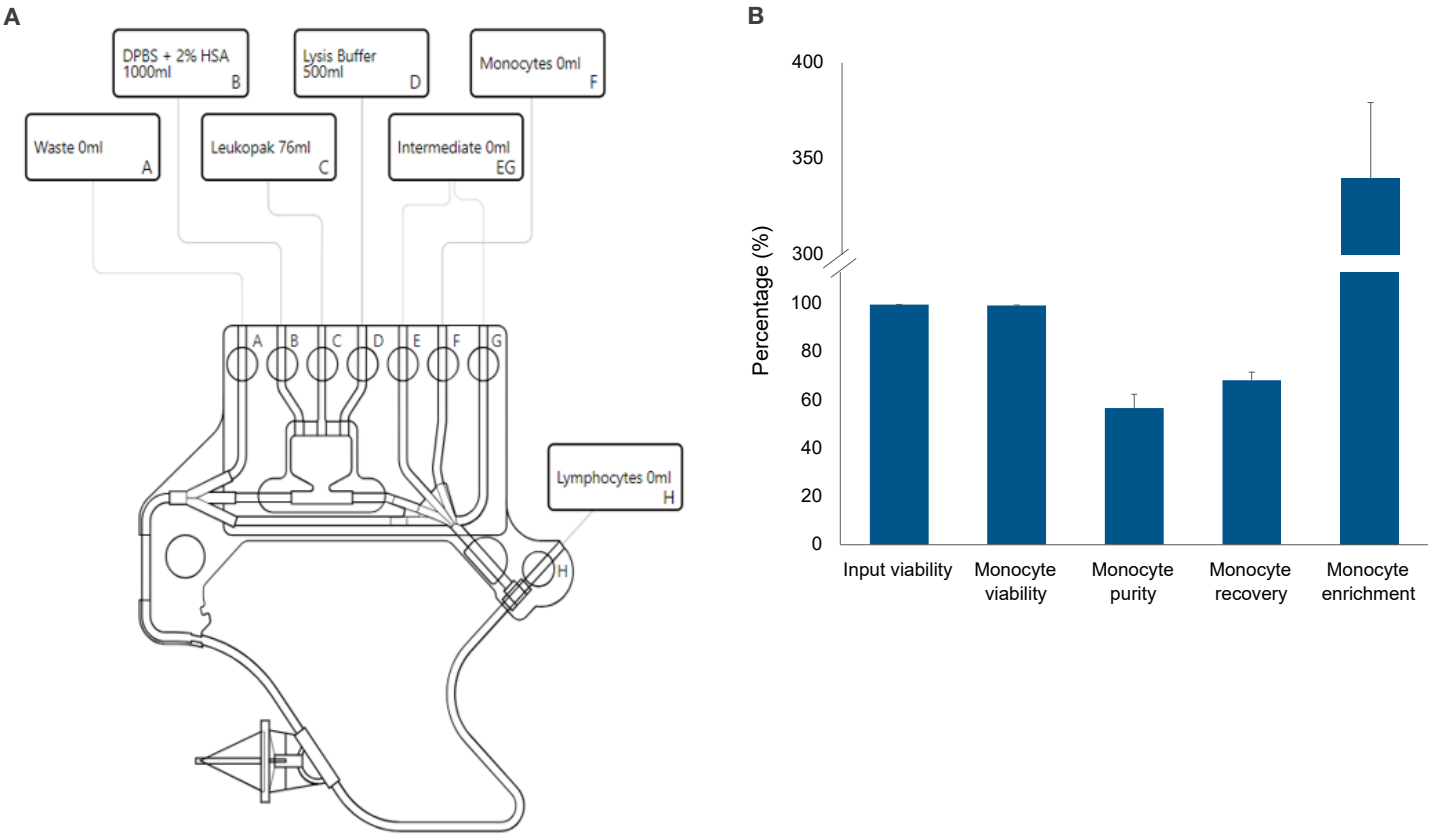
Appendix: CTS Rotea system monocyte isolation

Materials and methods

Monocytes can be enriched from a leukopak on the Gibco™ CTS™ Rotea™ Counterflow Centrifugation System (CTS Rotea system) using these protocol steps and a single-use kit setup (Appendix Figure 1A, C). Purity and viability of isolated monocytes were determined by labeling with human anti-CD14 antibody and the Invitrogen™ LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, followed by flow cytometry analysis on an Attune CytPix Flow Cytometer. Viability and cell numbers of isolated cells were also independently measured by an automated cell counter.

Results

For label-free enrichment of monocytes on the CTS Rotea system, leukopaks from 3 separate healthy donors were loaded into the Rotea chamber and monocytes were enriched by elutriating other cell types. Monocyte recovery was 68% with an enrichment of 340% from the input material, and monocyte viability was 99% post-elutriation with a purity of 58% as analyzed by CD14 positivity by flow cytometry (Appendix Figure 1B).



Appendix Figure 1. Rotea monocyte isolation protocol and results. Leukopaks from 3 separate donors were loaded into the Rotea chamber, and monocytes were enriched by elutriating other cell types using the protocol steps **(Appendix Table 1)** and **(A)** single-use kit setup with **(B)** results.

Table 1. CTS Rotea system monocyte isolation protocol.

Step	Description	Flow path	Centrifuge speed (x *g*)	Flow rate (mL/min)	Step type	Triggers
1	Pre-prime	B to A	0	100	Normal	Input bubble sensor, on
2	Lubricate rotary coupling	B to A	0	100	Normal	Volume: 15 mL
3	Prime chamber and line A	B to A	10	100	Normal	Volume: 40 mL
4	Add leukopak dilution volume	B to A	10	100	Normal	Volume: 1.5 x leukopak dilution mL
5	Prime bubble trap and line B	A to B	10	100	Normal	Volume: 15 mL
6	Prime line E	A to E	10	100	Normal	Volume: 5 mL
7	Prime line D	A to D	10	100	Normal	Volume: 5 mL
8	Dilute leukopak	A to C	10	100	Normal	Volume: 1 x leukopak dilution mL
9	Prime pause loop	J to K	10	25	Pause	Volume: 3 mL
10	Ramp G force	J to K	2,200	100	Pause	Time: 31 seconds
11	Ramp flow rate	J to K	2,200	32	Pause	Time: 31 seconds
12	Mix leukopak, “>>” to advance	J to K	2,200	32	Pause	------
13	Load leukopak and elutriate platelets	C to A	2,200	32	Normal	Input bubble sensor, on, pause Volume: 1 x leukopak aliquot mL
14	Pause	J to K	2,300	30	Pause	Time: 10 seconds
15	Wash cells to remove platelets	B to A	2,300	25	Nomal	Volume: 15 mL
16	Pause stable bed	J to K	2,300	25	Pause	Time: 20 seconds
17	Wash-2	B to A	2,300	25	Normal	Volume: 40 mL
18	Pause	J to K	2,300	25	Pause	Time: 10 seconds
19	Ramp speeds to elutriate lymphocytes and RBCs	J to K	500	15	Pause	Time: 30 seconds
20	Elutriate-1 CF 30	B to G	450	15	Normal	Volume: 70
21	Elutriate-2 CF 25	B to G	375	15	Normal	Volume: 70
22	Elutriate-3 CF 20	B to G	300	15	Normal	Volume: 70
23	Elutriate-4 CF 18	B to G	270	15	Normal	Volume: 70
24	Elutriate-5 CF 15	B to G	225	15	Normal	Volume: 70
25	Concentrate monocytes	J to K	2,600	20	Pause	Time: 15 seconds
26	Harvest monocytes	B to F	2,600	100	Harvest	Volume: 1 x harvest volume mL
27	Pause	J to K	2,600	40	Pause	Time: 10 seconds
28	Re-prime line E	B to E	2,600	40	Normal	Volume: 5 mL
29	Initiate lymphocytes and RBCs bed	E to G	2,600	25	Normal	Time: 2 mins
30	Load lymphocytes and RBCs	E to A	2,300	35	Normal	Input bubble sensor, on, pause Volume: 1 x intermediate draw volume mL
31	Pause before lysis step	J to K	2,300	20	Pause	Time: 10 seconds
32	Lysis step 1	D to A	2,300	20	Normal	Volume: 15 mL
33	Pause between lysis	J to K	2,300	20	Pause	Time: 20 seconds
34	Lysis 2	D to A	2,000	41	Normal	Time: 7 mins
35	Pause after lysis	J to K	2,500	22	Pause	Time: 10 seconds
36	Wash to remove RBCs and lysis buffer	B to A	2,000	41	Normal	Volume: 50 mL
37	Concentrate lymphocytes	J to K	2,600	20	Pause	Time: 15 seconds
38	Harvest lymphocytes	B to H	2,600	40	Harvest	Volume: 1 x harvest volume mL
39	Ramp down to remove aggregates	B to A	10	30	Pause	Time: 5 seconds
40	Ramp to stop	K to J	10	30	Pause	Time: 5 seconds

Ordering information

Description	Quantity	Cat. No.
Isolation		
Dynabeads Untouched Human Monocytes Kit	1 kit	11350D
Dynabeads Untouched Human T Cells Kit	1 kit	11344D
CTS Rotea Counterflow Centrifugation System	1 unit	A47679
CTS Rotea Single-Use Kit	5 kits	A49313
Culture, differentiation, and polarization		
CTS AIM-V Medium, without phenol red, without antibiotics	1 L	A3830801
CTS Immune Cell Serum Replacement (SR)	50 mL	A2596101
CTS DPBS without calcium chloride, without magnesium chloride	1 L	A1285601
CTS TrypLE Select Enzyme	100 mL	A1285901
Human GM-CSF, Animal-Free Recombinant Protein, PeproTech	100 µg	AF-300-03
Human M-CSF, Animal-Free Recombinant Protein, PeproTech	100 µg	AF-300-25
Human IL-4, Animal-Free Recombinant Protein, PeproTech	100 µg	AF-200-04
Human IL-2, Animal-Free Recombinant Protein, PeproTech	100 µg	AF-200-02
Human IFN-gamma, Animal-Free Recombinant Protein, PeproTech	100 µg	AF-300-02
Human IL-6, Animal-Free Recombinant Protein, PeproTech	50 µg	AF-200-06
Human TNF-alpha, Animal-Free Recombinant Protein, PeproTech	100 µg	AF-300-01A
Human IL-1 beta, Animal-Free Recombinant Protein, PeproTech	100 µg	AF-200-01B
Nunc Cell-Culture Treated Multidishes (12 well)	Case of 75	150628
Nunc Cell-Culture Treated Multidishes (24 well)	Case of 75	142475
Cell functionality and analysis		
Attune CytPix Flow Cytometer, blue/red/violet 6/yellow	1 each	A51849
Attune NxT Flow Cytometer, blue/red/violet/yellow	1 each	A24858
EVOS M7000 Imaging System	1 system	AMF7000
EVOS M5000 Imaging System	1 system	AMF5000
LIVE/DEAD Fixable Aqua Dead Cell Stain Kit	400 assays	L34966
LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit	400 assays	L34976
IL-6 Human ELISA Kit	96 tests	KHC0061
Human TNFα (Total) ELISA Kit	96 tests	BMS2034
Human TARC/CCL17 ELISA Kit	96 tests	EHCCL17
Human IL1RA ELISA Kit	96 tests	KAC1181
pHrodo BioParticles Conjugates for Phagocytosis and Phagocytosis Kit, for Flow Cytometry	5 x 2 mg	P35360
Cytochalasin D	1 mg	PHZ1063
eBioscience Cell Proliferation Dye eFluor 450	500 µg	65-0842-85
Trastuzumab Humanized Recombinant Human Monoclonal Antibody	100 µg	MA5-42305
eBioscience Lipopolysaccharide (LPS) Solution (500X)	4 x 100 µL	00-4976-03

Learn more at **thermofisher.com/ctstcell**