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Serum Free Culture System for Myeloid Cell Therapies

Victor Chatterjee[1], Jeremy De Leon[1], Felizza Gunderson[2], Navjot Kaur[1].

Cell Biology, Life Sciences Solutions Group, Thermo Fisher Scientific, Frederick, MD[1], Carlsbad, CA[2], United States.

ABSTRACT

Myeloid cells have gained interest as promising candidates for cancer immunotherapy because of their relative abundance and importance in the tumor microenvironment. Macrophages and dendritic cells (DCs) are active players in modulating tissue immunity, and their inherent properties of antigen presentation, phagocytosis, cytokine production and immune cell activation are being increasingly utilized for oncologic immunotherapies. Macrophages and DCs used in clinical studies are commonly differentiated ex-vivo from monocytic precursors. In this study, we confirm the suitability of Cell Therapy Systems (CTS™) AIM-V medium supplemented with CTS Immune Cell Serum Replacement (ICSR) as a serum free culture system for monocyte derived macrophages and DCs.

Blood monocytes were differentiated either to unpolarized and polarized macrophages, or immature and mature DCs, by supplementing culture media with appropriate growth factors, cytokines, polarization, and maturation agents. Macrophages cultured in AIM-V medium supplemented with 5% ICSR demonstrated higher viability than other commercially available suppliers, expressed surface markers specific to polarization type, and exhibited robust functionality as evidenced by cytokine secretion, bacterial clearance, and antibody dependent cellular phagocytosis of HER-2 positive breast cancer cells. Similarly, DCs cultured in AIM-V medium supplemented with 5% ICSR showed comparable or improved yield than other suppliers, displayed strong expression of DC maturation markers, and induced allogeneic T-cell proliferation and activation in mixed leukocyte reaction assays. Additionally, mature DCs were functional in secretion of interleukin 12, and viral antigen pulsed mature DCs activated cytotoxic T-cells in autologous co-culture assays.

In conclusion, we demonstrate the use of CTS AIM-V and CTS ICSR as a reliable serum free media system for culture of functional monocyte- derived macrophages and DCs.

INTRODUCTION

Serum free media are preferred for cell therapy applications because they help in achieving greater consistency, process control, and address performance, regulatory and supply chain concerns. CTS AIM-V is a serum free medium widely used for multiple immune cell cultures and available in several formats for both research and manufacturing applications. CTS ICSR is a defined xeno-free supplement that has been widely used in serum free T-cell cultures. Recently, myeloid cells have gained interest as attractive candidates for therapies against solid tumors. Among many other myeloid cell types, monocyte derived macrophages and DCs and are widely used in immunotherapies and as cancer vaccines[1]. Here, we demonstrate that CTS AIM-V serum free medium with CTS ICSR supplementation is suitable for culture of functional macrophages and DCs.

METHODS

For macrophage culture: Untouched CD14⁺ monocytes (3 x 10^5 / well) were seeded in Nunclon™ Delta Surface 24 well plates and cultured in CTS AIM-V + 5% ICSR or other supplier's media with 50 ng/mL macrophage colony stimulating factor (M-CSF) and 50uM 2-mercaptoethanol (2-ME) for 7-8 days. Cells were fed with fresh media on day 3 followed by day 5 or 6 when 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 interferon gamma (IFNγ) - M1 polarization; and iii) 20 ng/mL interleukin-4 (IL-4) - M2 polarization. On Day 7/8, macrophages (Mφ) were harvested and analyzed for yield, phenotypic characterization and cytokine secretion. Bacterial phagocytosis was analyzed after co-incubating macrophages with 100ug/mL pH Rodo Deep Red E. coli particles in serum free IMDM media for 2 hours with or without pre-treatment by 10ug/mL cytochalasin-D, a phagocytosis inhibitor. Macrophages were also tested for phagocytosis of cancer cells. CFSE stained HER-2 expressing SKBR3 breast cancer cells were treated with 10ug/mL human IgG1 or trastuzumab (humanized HER-2 monoclonal antibody) for 1 hour, washed and then co-cultured with unpolarized macrophages in serum free IMDM medium for 2 hours. Antibody dependent cellular phagocytosis (ADCP) of cancer cells was analyzed by quantifying CFSE positive macrophages by flow cytometry and confirmed by immunofluorescent imaging of fixed cells after 2 hours of co-culture.

For DC culture: Untouched CD14⁺ monocytes (5 x 10^5 / well) were seeded in Nunclon™ Delta Surface 12 well plates and cultured in CTS AIM-V + 5% ICSR or other supplier's media with 500 IU/mL IL-4 and 1000 IU/mL GM-CSF for 7 days with fresh media supplementation on days 3 and 5. On Day 5, i) a traditional maturation cocktail first described by Jonuleit *et al* [2], consisting of 10 ng/mL tumor necrosis factor alpha (TNFa), 10 ng/mL interleukin 1 beta, 15 ng/mL interleukin 6, and 1 µg/mL prostaglandin E2; or ii) an alternative maturation cocktail consisting of a toll like receptor 4 agonist, monophosphoryl lipid A (MPLA) (5 µg/mL), and 2000 IU interferon gamma (IFNγ)[3] was added in some samples to obtain mature DCs (mDCs). In some experiments, immature DCs (iDCs) were pulsed with 2 µg/mL viral antigen peptide mix (CEF) before addition of maturation agents. mDCs and iDCs were harvested on Day 7 and analyzed for DC yield, phenotypic characterization and interleukin 12 secretion. mDCs and iDCs were co-cultured with allogeneic or autologous T-cells 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.

Viable cell count was analyzed by a Vi-Cell Blu automated cell counter. Brightfield and immunofluorescent imaging of cells were performed with Incucyte® S3 and EVOS imaging systems. Cytokine ELISAs from cell culture supernatants were quantitated on a Perkin Elmer microplate reader. Assessment of cellular markers, cell proliferation and phagocytosis were performed on Invitrogen Attune CytPix and Attune NxT flow cytometers and analyzed by Flow Jo software v10. Data is represented as Mean ± Standard error of Mean.

RESULTS

1. Monocyte isolation from PBMCs

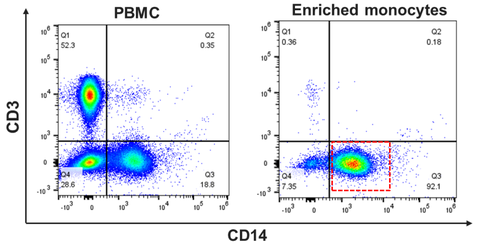

Fig. 1. Monocyte enrichment from PBMCs. Representative flow cytometry dot plots showing CD14⁺ monocyte enrichment (red box) from PBMCs by negative selection. >90% pure monocytes were isolated by using the Dynabeads™ Untouched™ Human Monocytes Kit.

MACROPHAGE CULTURE

2. Workflow for macrophage culture

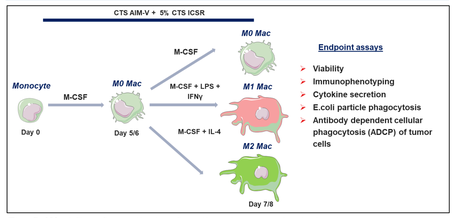

Fig. 2. Workflow for culturing functional monocyte-derived macrophages using AIM-V + 5% ICSR. Cell images were obtained from https://smart.servier.com/ and modified under Creative Commons Legal Code.

3. Macrophage morphology

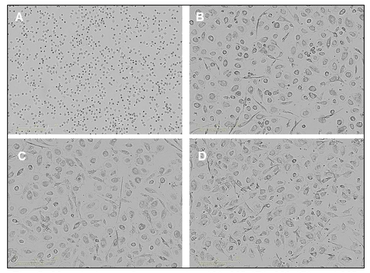

Fig. 3. Morphology of monocytes (A), M0 (B), M1 (C) and M2 (D) macrophages cultured in AIM-V + 5% ICSR. Scale bar corresponds to 200µm.

4. Macrophage viability

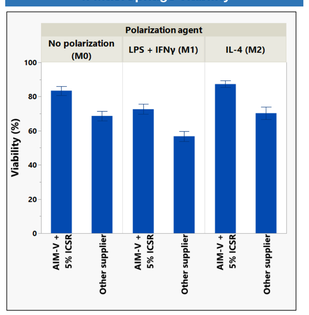

Fig. 4. Viability of macrophages. M0, M1 and M2 macrophages cultured in AIM-V + 5% ICSR show improved viability than other serum free supplier. N=7 donors.

5. Macrophage phenotype

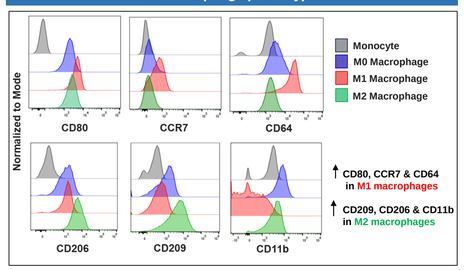

Fig. 5. Immunophenotyping of monocytes, unpolarized and polarized macrophages. Representative flow cytometry histograms showing macrophages expressing markers specific to polarization[4] when cultured in AIM-V + 5% ICSR.

6. Bacterial phagocytosis

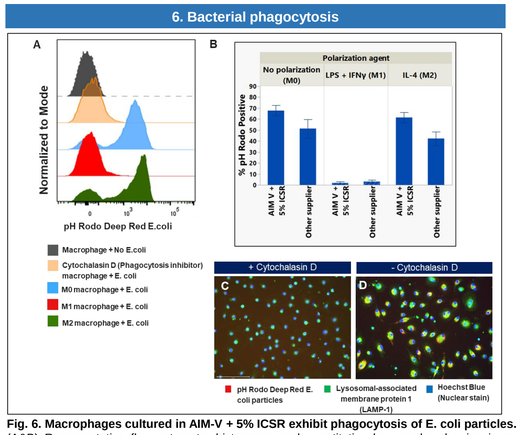

Fig. 6. Macrophages cultured in AIM-V + 5% ICSR exhibit phagocytosis of E. coli particles. (A&B) Representative flow cytometry histograms and quantitative bar graphs showing increased E.coli particle clearance by M0 and M2 macrophages cultured in AIM-V + 5% ICSR as compared to other supplier media. N=11 donors. (C&D) Phagocytosed E.coli particles (red) co-localized with intracellular LAMP-1 (green) indicating bacterial clearance by the phago-lysosomal pathway. There was no uptake of E.coli particles in cytochalasin-D treated macrophages. Scale bar = 150um.

7. Cytokine ELISA

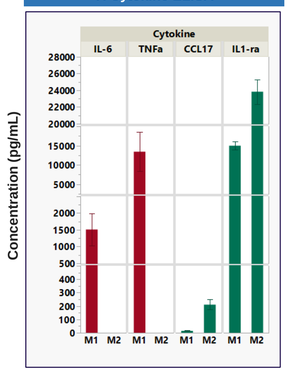

Fig. 7. Cytokine ELISA of macrophage supernatants showing selective secretion of cytokines specific to polarization. M1 macrophages secreted higher levels of IL-6 and TNFa, and M2 macrophages secreted increased levels of CCL17 and IL-1ra as compared to M1 macrophages. N = 4-5 donors.

8. Antibody dependent cellular phagocytosis (ADCP) of tumor cells

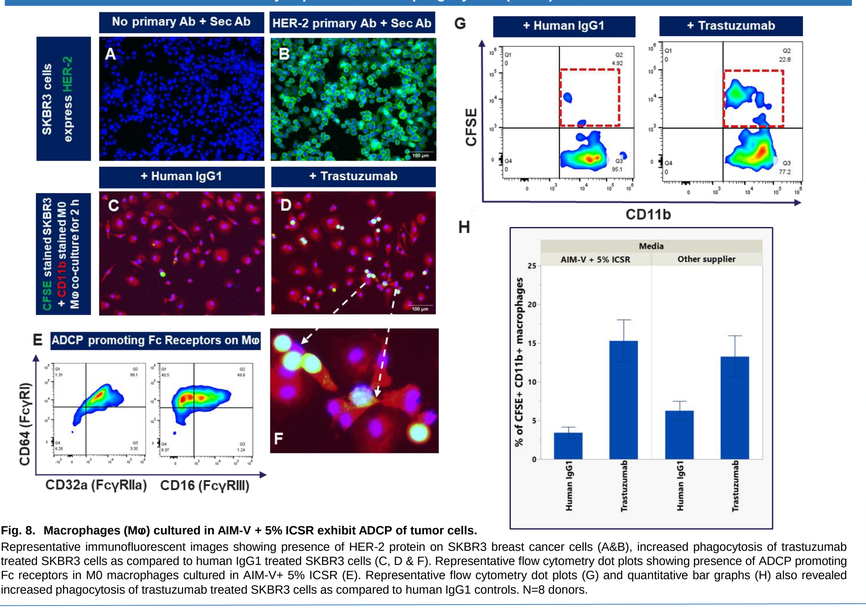

Fig. 8. Macrophages (Mφ) cultured in AIM-V + 5% ICSR exhibit ADCP of tumor cells.
Representative immunofluorescent images showing presence of HER-2 protein on SKBR3 breast cancer cells (A&B), increased phagocytosis of trastuzumab treated SKBR3 cells as compared to human IgG1 treated SKBR3 cells (C, D & F). Representative flow cytometry dot plots showing presence of ADCP promoting Fc receptors in M0 macrophages cultured in AIM-V+ 5% ICSR (E). Representative flow cytometry dot plots (G) and quantitative bar graphs (H) also revealed increased phagocytosis of trastuzumab treated SKBR3 cells as compared to human IgG1 controls. N=8 donors.

DENDRITIC CELL (DC) CULTURE

9. Workflow for DC culture

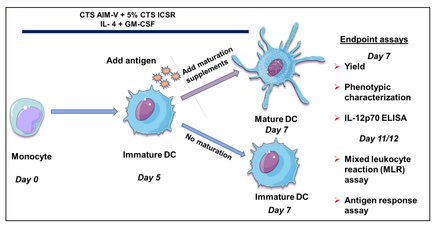

Fig. 9. Workflow for culturing functional monocyte derived DCs using AIM-V + 5% ICSR. Cell images were obtained from https://smart.servier.com/ and modified under Creative Commons Legal Code.

10. DC morphology

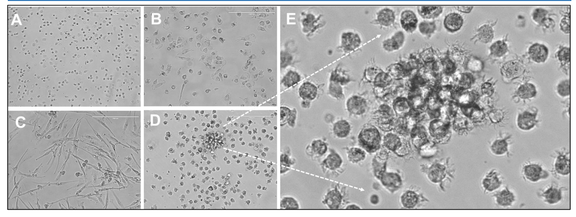

Fig. 10. Morphology of monocytes, immature and mature DCs cultured in AIM-V + 5% ICSR. A) Monocytes on Day 0, B) iDC, C) mDC (MPLA + IFNγ), D) mDC (Jonuleit's), E) enlarged image of mDC (Jonuleit's) on Day 7. Scale bar in A-D corresponds to 200 µm.

11. DC yield

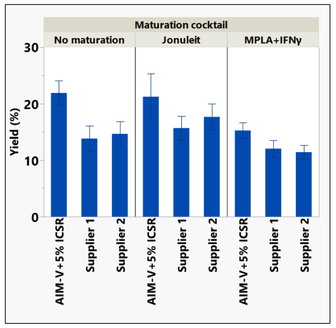

Fig. 11. Percentage yield of DCs cultured in various serum free media and matured with different maturation agents. Percentage yield was calculated by dividing number of viable DCs on day 7 by number of viable monocytes seeded on day 0, multiplied by 100. DCs cultured in AIM-V + 5% ICSR showed comparable or better yield than other supplier media. N=6 donors.

12. DC phenotype

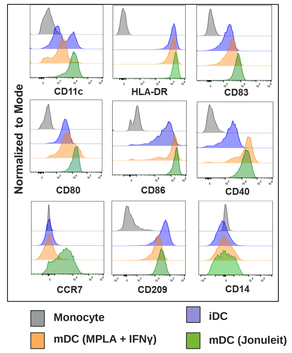

Fig. 12. Phenotypic characterization of DCs cultured in AIM-V + 5% ICSR. DC maturation markers (CD11c, HLA-DR, CD83); co-stimulatory molecules necessary for T-cell activation (CD80, CD86, CD40); C-C chemokine receptor 7 (CCR7) and adhesion molecules for T-cell engagement (CD209/DC-SIGN) are significantly elevated while CD14 (monocyte marker) is downregulated in DCs as compared to monocytes.

13. Cytokine ELISA

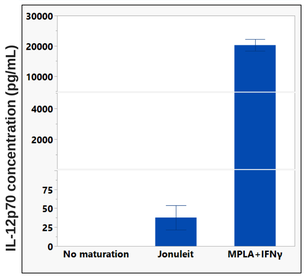

Fig. 13. Estimation of IL-12p70 levels in DC culture supernatants by ELISA. 5x10^5 monocytes were seeded in 1 mL of AIM-V + 5% ICSR per well on Day 0. IL-12p70 levels were obtained from iDC & mDC cell culture supernatants on Day 7. DCs matured by MPLA + IFNγ significantly increase IL-12 secretion compared to traditional Jonuleit's cocktail. iDCs express very low to undetectable levels of IL-12p70. N=4 donors.

14. Mixed leukocyte reaction (MLR) assay

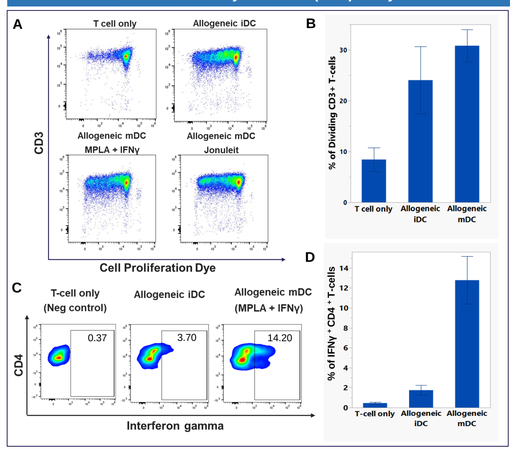

Fig. 14. DCs cultured in AIM-V + 5% ICSR increase allogeneic T-cell proliferation and Th1 cell polarization. (A) Representative flow cytometry dot plots showing increased T-cell proliferation after co-culture with DCs from HLA-mismatched donors. (B) Bar graph showing percentage of divided T–cells in allogeneic DC-T cell co-cultures. T-cells only with no DCs serve as negative controls. N=5 donors. (C) Representative flow cytometry dot plots showing increased interferon gamma staining in T helper cells in allogeneic mDC (MPLA + IFNγ) – T-cell co-cultures. (D) Bar graph showing percentage of interferon gamma positive T helper cells in allogeneic DC-T-cell co-cultures. N=3 donors.

15. Autologous DC-T cell antigen response assay

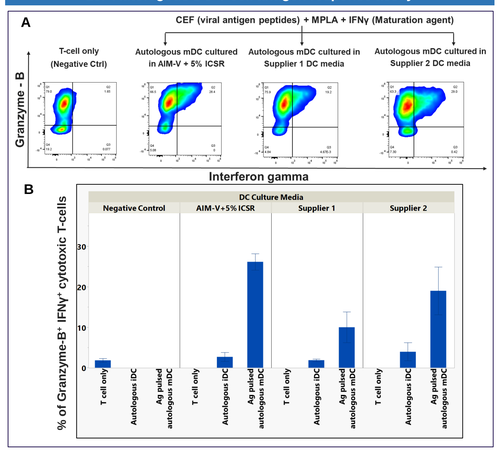

Fig. 15. Viral antigen pulsed mature DCs induce autologous CD8⁺ T-cell activation. A) Representative flow cytometry dot plots showing cytotoxic T-cell activation by viral antigen peptide mix (CEF) pulsed DCs matured with MPLA + IFNγ as compared to T-cell only negative control. Activation was evidenced by significantly increased Granzyme B and interferon gamma double positive CD8⁺ T-cells. mDCs cultured in CTS AIM-V + 5% ICSR show better, or comparable activation as compared to other supplier media. B) Bar graph showing data pooled from 5 donors.

CONCLUSIONS

AIM-V + 5% ICSR is a suitable serum free medium system for culture of macrophages and DCs

1. Cells cultured in AIM-V + 5% ICSR show better or comparable viability or yield than other serum free suppliers.
2. Cells cultured in AIM-V + 5% ICSR express appropriate markers of differentiation, polarization and maturation.
3. DCs are functional in IL-12 secretion, promote robust T-cell proliferation and Th1 polarization in MLR assays, and increased cytotoxic T-cell activation in antigen response assays.
4. Macrophages are functional in secreting cytokines specific to polarization, exhibit increased bacterial clearance by M0 & M2 macrophages, and promote ADCP of trastuzumab treated HER-2⁺ breast cancer cells.

REFERENCES

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2. Jonuleit, H., Kühn, U., Müller, G., Steinbrink, K., Paragnik, L., Schmitt, E., Knop, J. and Enk, A.H. (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.
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4. Chen, S., Saeed, A.F., Liu, Q. et al. Macrophages in immunoregulation and therapeutics. Sig Transduct Target Ther 8, 207 (2023).

PRODUCT INFORMATION

Product	Format	Catalog #
CTS™ AIM-V™ Medium	1 L bottle	A3830801
	2 L bag	A4672701
	10 L bag	A3830802
CTS™ Immune Cell Serum Replacement (ICSR)	50 mL bottle	A2596101
	250 mL bag	A4702901
	500 mL bottle	A2596102
	1 L bag	A4702902