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Generation of M2c Macrophages Using IL-10 Exposure In Vitro

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ABSTRACT

Background: Macrophages are essential cells of the innate immune system that highly adaptable and play vital roles in tissue homeostasis, immune regulation, and development. Among their various phenotypes, the M2c subtype induced by IL-10 and TGF-β is known for its regulatory functions, high phagocytic capacity, and pro-angiogenic potential. This study aimed to investigate whether M2c polarization via IL-10 stimulation occurs in a dose-dependent manner by differentiating human monocyte-derived macrophages using IL-10. **Methods:** M2c were activated with 5 ng/mL, 10 ng/mL, and 20 ng/mL hraIL-10 (5, 10, and 20 ng/mL) for 48 hours. IL-10 mRNA expression and protein concentration was analyzed using quantitative PCR and ELISA, respectively. **Results:** The results demonstrated a clear dose-dependent increase in IL-10 expression across both methods. ELISA measurements showed IL-10 levels increasing from a mean of 3.2±0.7 pg/mL in untreated controls to 19.34±2.3 pg/mL (5 ng/mL), 35.5±7 pg/mL (10 ng/mL), and 67.2±5.8 pg/mL (20 ng/mL). Similarly, qPCR analysis revealed a corresponding increase in IL-10 gene expression, with relative fold changes from 1±0 (untreated control) to 3.1±0.4, 6±1.3, and 10.1±1.6, respectively. **Conclusion:** These findings indicate that IL-10 induces M2c macrophage polarization in a dose-dependent manner, providing insight into optimized conditions for generating regulatory macrophage populations for potential therapeutic applications.

Keywords : Dose-dependent response, IL-10, M2c, Macrophage, Monocyte.

INTRODUCTION

Macrophages are key components of the innate immune system, forming dynamic three-dimensional networks across all tissues.[1] They engage in phagocytosis of apoptotic cells, cellular debris, immune complexes, pathogens, and metabolic waste.[2] Beyond their immunological roles, macrophages secrete growth factors and signalling molecules that are crucial for maintaining tissue homeostasis and supporting organ development.[3] Extensive research has demonstrated their involvement in fundamental physiological processes, including angiogenesis, metabolic regulation, adipogenesis, and the modulation of both central and peripheral nervous system functions.[4–7]

Once differentiated from monocytes, macrophages undergo significant metabolic reprogramming that directs their polarization toward either the pro-inflammatory (M1) or anti-inflammatory/regulatory (M2) phenotypes, depending on the cytokine milieu.[5] Stimulation with tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and lipopolysaccharide (LPS) promotes M1 polarization, which is associated with heightened microbicidal activity and the production of pro-inflammatory cytokines.[6,8] In contrast, M2 polarization is typically induced by cytokines such as interleukin-4 (IL-4), IL-13, IL-25, IL-10, and transforming growth factor-beta (TGF-β), leading to functions associated with tissue repair, immune regulation, and resolution of inflammation.[3,9] A more nuanced subclassification of M2 macrophages has been proposed, categorizing them into M2a and introduced additional subtypes, including M2b, characterized by activation through immune complexes and Toll-like receptor (TLR) signalling; M2c, identified as a deactivated macrophage subtype that downregulates pro-inflammatory cytokine production; and M2d, a regulatory macrophage variant commonly associated with tumour-associated macrophages (TAMs) and linked to immunosuppressive functions in the tumour microenvironment.[4,10]

One of the M2 macrophage subtypes is M2c, which is stimulated by IL-10 and TGF-β. Previous studies have demonstrated that M2c macrophages induced by IL-10 promote greater angiogenesis both in vitro and in vivo compared to M1 and M2a phenotypes. They also exhibit a higher phagocytic capacity for apoptotic cells in vitro.[4] Additionally, M2c macrophages have been shown to recruit stem cells associated with blood vessel formation under in vitro conditions. Furthermore, M2c macrophages express high levels of specific surface markers, including CD163, matrix metalloproteinases MMP7 and MMP8, and tissue inhibitor of metalloproteinases 1 (TIMP1).[11–14] Therefore, M2c macrophages play a crucial role in wound healing processes due to their regenerative and immunoregulatory properties.

The generation of M2c macrophages through IL-10 stimulation has been studied.[15] However, whether this polarization occurs in a dose-dependent manner remains unclear. In this study, monocyte-derived macrophages will be differentiated into the M2c phenotype using only IL-10 at varying concentrations to investigate the dose-dependent effects of IL-10 exposure on M2c polarization. This approach aims to provide a better understanding of how different levels of IL-10 influence the efficiency of M2c macrophage generation, specifically by examining IL-10 expression-M2c specific marker.

MATERIAL AND METHODS

Study design

This post-test-only experimental study was carried out at the Stem Cell and Cancer Research (SCCR) Laboratory, located in Semarang, Central Java, Indonesia, between February and March 2025. The study used 3 mL human healthy blood, and the collection sample was adhered to the ethical standards and guidelines established by the Institutional Research Bioethics of Universitas Islam Sultan Agung.

Monocytes Preparation and Isolation

Monocytes isolated using human monocyte isolation kit by immunomagnetic cell sorting (Stem Cell Technologies, Canada). Unprocessed human whole blood was used. Peripheral blood collected using K2EDTA or K3EDTA as an anticoagulant. The sample was vortexed with RapidSpheres™ for 30 seconds to ensure even dispersion of particles. Next, 50 µL of Isolation Cocktail was added to the sample, and the sample was mixed gently by pipetting up and down 2–3 times. Afterward, 50 µL of

RapidSpheres™ was added, and the mixture was incubated at room temperature (RT) for 5 minutes. The recommended medium was added to the sample to top up to 4X the original sample volume, and the mixture was again gently pipetted. The tube was then placed in the magnet (without lid) and incubated at RT for 3 minutes. The magnet was picked up, and the tube was continuously inverted to pour the enriched cell suspension into a new tube. Another 50 µL of RapidSpheres™ was added to this new tube containing the enriched cells, and the sample was mixed and incubated at RT for 5 minutes. The tube was then removed from the magnet, and the new tube was placed back in the magnet (without lid) for a second separation, incubating at RT for 3 minutes. After this, the tube was removed, and the enriched cell suspension was transferred to a new tube. The magnet was again picked up, and the process of inversion and pouring the enriched cell suspension was repeated into a new tube. Finally, the isolated cells were obtained.

Macrophage Differentiation and IL-10 stimulation

Monocytes differentiated to macrophages using ImmunoCult™-SF Macrophage Medium (Stem Cell Technologies, Canada) with modification. ImmunoCult™-SF Macrophage Medium is thawed at room temperature (15–25°C) until just thawed and mixed thoroughly. Add 100 µL of 5 µg/mL Human Recombinant M-CSF to 10 mL of ImmunoCult™-SF Macrophage Medium, mixing thoroughly to achieve a final concentration of 50 ng/mL. Add purified human monocytes at 1 x 10^6 cells/mL to 5 mL of ImmunoCult™-SF Macrophage Differentiation Medium. After 4 days of incubation at 37°C, 2.5 mL of fresh medium is added to the flask, continue incubation at 37°C for an additional 2 days before activation. In day 8, M2c activated uses 5 ng/mL, 10 ng/mL, and 20 ng/mL hraIL-10. The cultures are then incubated at 37°C for 2 days. Macrophages are harvested by removing the supernatant, optionally centrifuging at 300 x g for 10 minutes for ELISA, and retaining the cell pellet. Macrophages are then resuspended in the appropriate medium or buffer for downstream applications.

mRNA expression analysis

Total RNA from macrophage cells was extracted from medium using TRI Reagent (Sigma-Aldrich), following the manufacturer's protocol. Complementary DNA (cDNA) was synthesized using the Enhanced Avian First Strand cDNA Synthesis Kit (Sigma-Aldrich), adhering to the manufacturer's instructions. The reverse transcription reaction utilized oligo d(T) primers, with an incubation step at 70°C for 10 minutes, followed by 45°C for 15 minutes. Quantitative real-time PCR (qPCR) was performed in a two-step protocol using the Eco Real-Time PCR System (Illumina Inc., San Diego, CA, USA) and the KAPA SYBR® FAST Universal Kit (Sigma-Aldrich). A cDNA template of 3 ng was used for each reaction. The expression levels of IL-10 were assessed, with GAPDH serving as the reference gene. The primer sequences were as follows: IL10 F: 5'- TAA GCT CCA AGA CAA AGG GTG -3'; IL-10 R: 5'- GTC CTC CAG TCC AGT AGA TG -3'; GAPDH F: 5'- TCA AGG CTG AGA ACG GGA AG -3'; GAPDH R: 5'- CGC CCC ACT TGA TTT TGG AG -3'. The thermocycling conditions included an initial denaturation step at 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. Gene expression levels were quantified using the ΔΔCt method, analysed through the Eco Study Software (Illumina), and normalized to the β-actin housekeeping gene.

Intracellular ELISA

Protein concentrations were determined using UV-Vis spectrophotometry at 260 nm and 280 nm to ensure sample quality and consistency. Equal volumes of protein from each sample were loaded into

ELISA wells. The intracellular levels of IL-10 were measured using a specific ELISA kit (Elabscience, Texas, USA) according to the manufacturer's protocol. Standard curves were generated for each assay to calculate IL-10 concentrations. Colorimetric absorbance was recorded at a wavelength of 450 nm using a microplate reader. All measurements were conducted in triplicate to ensure the reliability and reproducibility of the results.

Data Analysis

All statistical analyses were conducted using SPSS version 26 (IBM, New York, USA). For data following a normal distribution, one-way analysis of variance (ANOVA) was performed, followed by Tukey's post-hoc test to assess pairwise group differences. Results are expressed as mean ± standard deviation (SD). A p-value of less than 0.05 was considered statistically significant.

RESULTS

Macrophage Differentiation

The macrophage differentiation process was carried out over eight days. On day one, successful monocyte incubation was observed (Fig. 1B), as indicated by an increased number of monocytes in the field of view compared to day zero (Fig. 1A). Cultured using ImmunoCult™-SF Macrophage Differentiation, monocyte differentiation into macrophages was confirmed on day 4 (Fig. 1C), and successful differentiation was evident from the amorphous-irregular morphology of macrophage cells.[16] Each IL-10 treatment dose given in day 6 to stimulate M2c differentiation, effectively stimulated M2c formation in day 7, as shown in the 5 ng/mL IL-10 (Fig. 1E), 10 ng/mL IL-10 (Fig. 1F), and 20 ng/mL IL-10 (Fig. 1G) groups compared to the untreated group (Fig.1D).

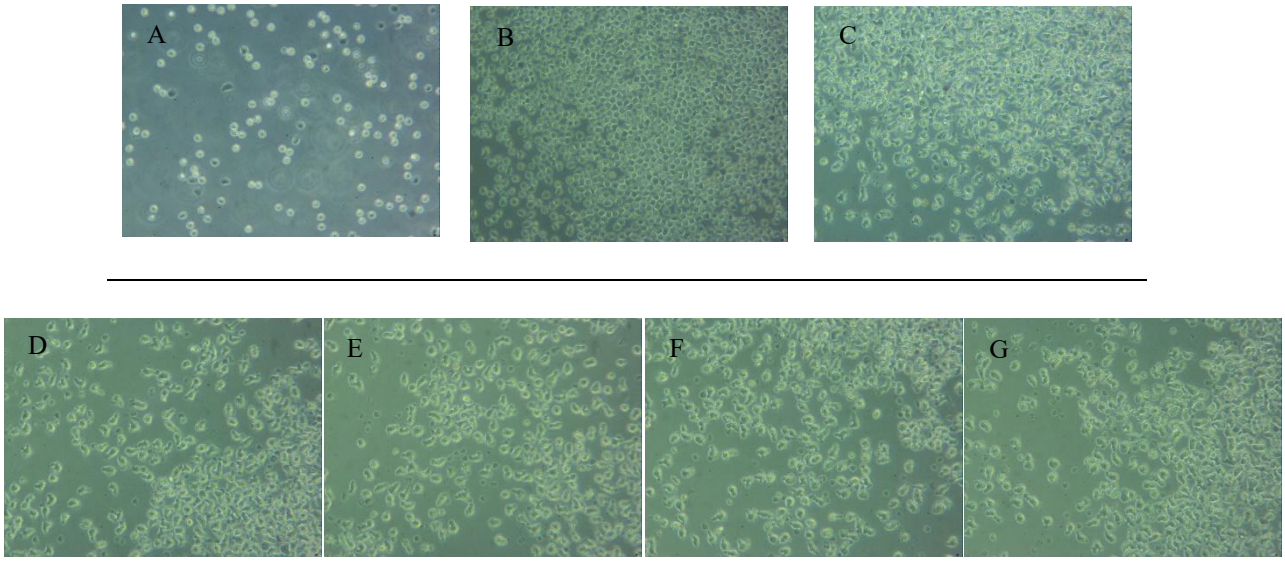

Figure 1. Macrophage Differentiation. (A) Day 0: Monocytes isolation was successfully conducted. **(B)** Day 1: Monocytes was successfully cultured. **(C)** Day 4: Monocyte was successfully differentiated to macrophages, shown by irregular-amorphs morphology of cells. **(D)** Macrophage without treatment IL-10 in day 7. **(E)** M2c stimulated-Macrophage with 5 ng/mL IL-10 treatment in day 7. **(F)** M2c stimulated-Macrophage with 10 ng/mL IL-10 treatment in day 7. **(G)** M2c stimulated-Macrophage with 20 ng/mL IL-10 treatment in day 7.

Effect of M2c induced IL-10 dose-dependent on IL-10 mRNA Relative Expression

IL-10 treatment macrophage showed a significant increase in IL-10 mRNA expression compared to untreated macrophage ($p \leq 0.01$), reflecting M2c differentiation. Figure 2 shows the treatment of higher dosage of IL-10 treatment resulted in significance increased IL-10 mRNA expression compared to lower dosage IL-10 treatment. Corresponding significant increase observed in IL-10 mRNA expression, with relative fold changes from 1±0 (control) to 3.1±0.4, 6±1.3, and 10.1±1.6, respectively. This finding highlights the effect of different IL-10 dosages on the expression of IL-10 mRNA in M2c.

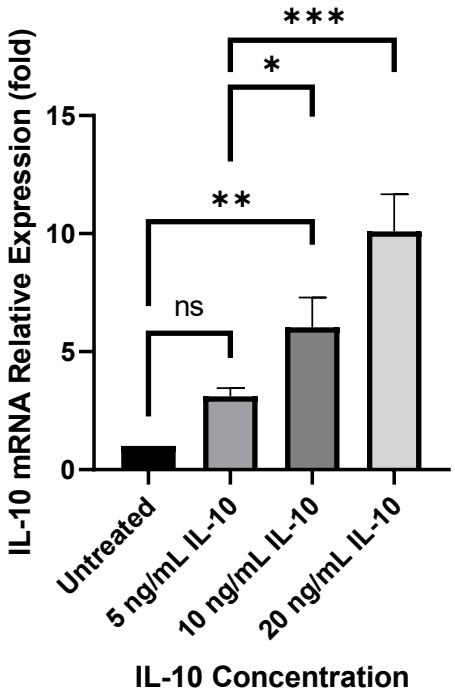

Figure 2. IL-10 mRNA Expression. Higher dosage of IL-10 treatment showed significant increase in IL-10 mRNA expression compared to lower dosage IL-10 treatment. Higher dose IL-10 treatment macrophages group showed a significant higher level in IL-10 mRNA expression compared to the untreated macrophage group. Ns: $p \geq 0.05$, *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.

Effect of M2c induced IL-10 dose-dependent on IL-10 Protein Level

To ensure the successful induction of M2c macrophages and evaluate the effect of different IL-10 doses, IL-10 protein levels were measured using ELISA. This analysis aimed to assess whether increasing concentrations of IL-10 stimulation would result in enhanced expression of IL-10 protein, serving as an indicator of M2c polarization. The results (Fig. 3) demonstrated a clear dose-dependent trend in IL-10 protein production. In the untreated control group, baseline IL-10 levels remained low, with an average concentration of 3.2±0.7 pg/mL. Upon stimulation with 5 ng/mL IL-10, IL-10 protein levels increased significantly to a mean of 19.34±2,3 pg/mL, indicating an early induction response. Further increases in IL-10 dosage led to even higher protein expression: 10 ng/mL IL-10 resulted in an average of 35.5±7.0 pg/mL, while the highest dose of 20 ng/mL IL-10 yielded the greatest IL-10 expression, with an average of 67.2±5.8 pg/mL.

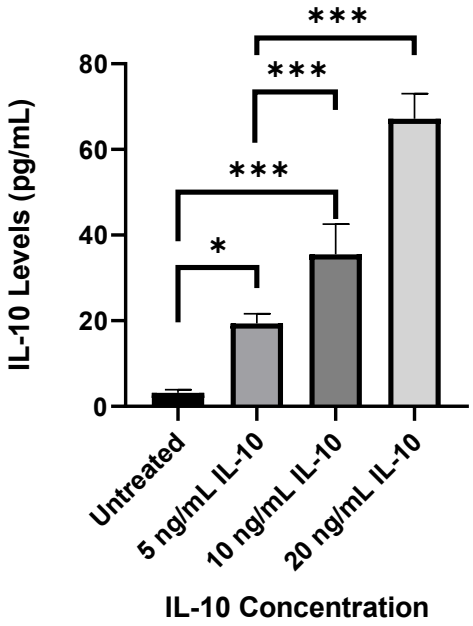

Figure 3. IL-10 Protein Level. Higher dosage of IL-10 treatment showed significant increase in IL-10 level compared to lower dosage IL-10 treatment. IL-10 treatment macrophages group showed a significant higher level in IL-10 level protein compared to the untreated macrophage group. *: $p \leq 0.05$; ***: $p \leq 0.001$.

DISCUSSION

Our study demonstrates that IL-10 administration to macrophages promotes their polarization towards the M2c phenotype. This finding is consistent with previous research showing that macrophages stimulated with IL-10 differentiate into M2c cells.[15] During M2c polarization, IL-10 binds to the IL-10 receptor complex, inducing the phosphorylation of the tyrosine kinases Jak1 and Tyk2.[17] These kinases subsequently phosphorylate the IL-10 receptor, leading to the activation of STAT3. STAT3 then forms a homodimer and translocate to the nucleus, where it binds to STAT-binding sites in the promoters of IL-10-responsive genes.[18] This process drives the expression of genes that suppress various inflammatory pathways, contributing to the anti-inflammatory effects of the M2c macrophage phenotype.

The differentiation of macrophages into the M2c phenotype can be identified by the secretion of M2c polarization markers such as SOCS3, BCL3, and IL-10.[19] In this study, to assess M2c macrophage polarization, we measured the mRNA expression levels of IL-10 as well as the IL-10 protein levels. IL-10 stimulation was applied in varying doses, which has not been explored in previous studies, to evaluate its impact on IL-10 expression in M2c macrophages. The results indicated that IL-10-induced M2c macrophages were capable of secreting IL-10 protein and expressing IL-10 mRNA. This is in accordance with prior studies, which demonstrated that M2c-stimulated IL-10 exhibits higher IL-10 expression.[15]

What is particularly interesting in this study is that different IL-10 doses led to varying IL-10 expression levels. ELISA measurements showed a dose-dependent increase in IL-10 levels, with

values rising from a mean of 3.2±0.7 pg/mL in untreated controls to 19.34±2.3 pg/mL (5 ng/mL), 35.5±7 pg/mL (10 ng/mL), and 67.2±5.8 pg/mL (20 ng/mL). Similarly, qPCR analysis demonstrated a corresponding increase in IL-10 gene expression, with relative fold changes from 1±0 (untreated control) to 3.1±0.4, 6±1.3, and 10.1±1.6, respectively. The significant upregulation of IL-10 mRNA in response to treatment suggests the involvement of a positive feedback loop, wherein IL-10 promotes its own expression. These findings indicate that administering varying doses of IL-10 to induce M2c-polarized macrophages plays a critical role in modulating IL-10 gene expression and enhancing IL-10 protein production. This dose-dependent response underscores the reliance of M2c phenotype formation on the strength of IL-10 signalling, emphasizing the importance of optimizing IL-10 dosage for therapeutic applications.

Interleukin-10 (IL-10) is an anti-inflammatory cytokine that plays a crucial role in regulating the immune system balance by controlling inflammatory responses to prevent excessive reactions and maintain immune homeostasis.[4,20] Produced by regulatory T cells, B cells, and macrophages, IL-10 inhibits the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, while also reducing the expression of major histocompatibility complex (MHC) class II molecules on antigen-presenting cells (APCs), thereby limiting T cell activation.[20,21] IL-10 also supports the activity of regulatory T cells, which prevent autoimmune diseases by maintaining immune tolerance.[22] In the context of infections, IL-10 regulates inflammation to prevent damage to healthy tissues from excessive immune responses, and it modulates B cells and T cells to avoid unnecessary tissue damage.[23] Additionally, IL-10 plays a role in chronic inflammatory diseases such as Crohn's disease and rheumatoid arthritis by reducing inflammation, demonstrating therapeutic potential in treating autoimmune diseases, chronic inflammation, and immunopathological conditions through immune modulation.[20,24,25] Enhancing IL-10 through the creation of a positive loop via M2c induction, it is expected to have a positive impact on regulating the immune response more effectively.

This study has limitations, including the limited sample size, also the lack of long-term monitoring of the effects of IL-10 enhancement through M2c induction, which could provide a more comprehensive understanding of its impact over a longer period. The absence of in-depth analysis of the interactions between IL-10 and other factors in the immune response, which could influence a more complete understanding of the mechanisms. Lastly, the limitations in the variety of experimental models used, which may not fully reflect the complexity of the immune response in humans.

CONCLUSIONS

Our study demonstrates that IL-10 treatment effectively induces M2c polarization in macrophages in a dose-dependent manner. Through administering varying doses of IL-10, we observed significant increases in both IL-10 mRNA expression and protein production, highlighting the critical role of IL-10 in driving M2c macrophage differentiation. ELISA measurements showed a dose-dependent increase in IL-10 levels, with values rising from a mean of 3.2±0.7 pg/mL in untreated controls to 19.34±2.3 pg/mL (5 ng/mL), 35.5±7 pg/mL (10 ng/mL), and 67.2±5.8 pg/mL (20 ng/mL). qPCR analysis demonstrated a corresponding increase in IL-10 gene expression, with relative fold changes from 1±0 (untreated control) to 3.1±0.4, 6±1.3, and 10.1±1.6, respectively. This

dose-dependent response suggests the existence of a positive feedback loop that amplifies IL-10 expression, further enhancing the anti-inflammatory functions of M2c macrophages.

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Competing Interests

There is no conflict of interest.

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