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# Establishment of macrophage polarization models adapted to TPOL

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## Abstract

**Currently, camphorquinone (CQ) is the preferred photoinitiator in dental restorative materials used in clinical settings. Nevertheless, its yellow hue may affect the appearance, therefore restricting its usage. Ethyl (2,4,6-trimethylbenzoyl) phenylphosphinate (TPOL) exhibits favorable photocuring and photobleaching properties, partially compensating for CQ's drawback. There is, to date, no reports involved in impacts of TPOL on macrophages and there lacks of a macrophage polarization model suitable for its research. In this study, MTT was utilized to investigate the cytotoxicity of TPOL, CQ and Phenylbis(acyl) phosphine oxides (BAPO) to two different types of macrophages. Flow cytometry was employed to identify phenotypes of macrophage M1 and M2. The findings indicate that BAPO exhibits the highest toxicity towards mouse macrophages, but the survival rates are above 80% in the cells treated by TPOL or CQ at concentrations of 1 μM, 5 μM, and 10 μM. The combination of 20 ng/mL LPS with 20 ng/mL IFN-γ is identified as the suitable induction factors for the M1 polarization model, while 50 ng/mL IL-4 is deemed to be appropriate for inducing the M2 polarization one. Therefore, the concentration of 10 μM TPOL is adapted to explore its influence and mechanistic process on macrophage polarization.**

## Keywords

**TPOL, CQ, BAPO , Cytotoxicity, M1 macrophage, M2 macrophage.**

## 1. Introduction

Photopolymerization, a rapidly advancing technique in polymer production, shows significant potential for various uses, especially in the biomedical field[1]. Due to its capacity to function at ambient temperature and swiftly trigger reactions, it is highly suitable for biomedical purposes[2]. In the field of biomedicine, photopolymerization is categorized into various subfields such as dentistry[3], tissue engineering[4], bioimaging[5], drug delivery system, and medical device[6]. For instance, in dentistry, light-cured polymer composites are commonly employed to fill cavities in hard dental tissues. The process of free radical photopolymerization involves initiation, propagation, and termination stages, ultimately resulting in the formation of oligomers or polymers[7]. The selection of appropriate photoinitiators, such as CQ, TPOL, and BAPO, is crucial for achieving the desired reaction rate and polymer properties. These photoinitiators must possess specific characteristics, including compatibility with light sources, high quantum efficiency, solubility in polymeric compositions, biocompatibility, non-cytotoxicity, resistance to yellowing, and thermal and temporal stability, especially for biomedical applications.

Materials implanted in the body, as foreign objects, will undoubtedly trigger an immune reaction[8]. Macrophages, the key cells in the body's defense system, have a vital regulatory function. These cells demonstrate notable flexibility, enabling them to take on various functional characteristics depending on the nearby environment and quickly shift between

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polarized conditions[9][10]. The specific phenotype of macrophages influences the healing outcomes of implants. Initially, following biomaterial implantation, the site is predominantly populated by pro-inflammatory classically activated M1 macrophages[11]. These cells release pro-inflammatory factors, reactive nitrogen, reactive oxygen species, and phagocytize pathogens, while also recruiting additional immune cells. The secretion of pro-inflammatory factors often coincides with the activation of numerous immune-related cells. Subsequently, as the inflammation resolves, macrophages are influenced by other immune cells and the microenvironment, transitioning into the anti-inflammatory M2 phenotype. In this state, they suppress inflammation, attract fibroblasts and stem cells, and release growth factors to facilitate tissue healing and repair[12].

## 2. Experimental procedures

### 2.1. Cell lines and culture conditions

In our laboratory, the RAW264.7 and J774A.1 murine macrophage cell lines underwent regular passages and were maintained. These cells were kept in DMEM medium containing 10% FBS and were grown in a 5% CO2 atmosphere at 37°C in an incubator.

### 2.2. Related reagents

The reagents CQ and BAPO were obtained from Aladdin; TPOL from Jiuri New Materials; FBS from Gibco; high-glucose DMEM medium and LPS from biosharp; CD80, CD86, CD11c, CD206, Fixed Membrane Rupture Kit from Thermo Fisher; IFN-γ from GenScript; IL-4 from Peprotech; MTT from Beyotime.

### 2.3. Preparation of photoinitiator

Photoinitiators were prepared by dissolving TPOL, CQ, and BAPO in DMSO to create a 50 mM mother solution. Fresh culture medium for dilution to working concentration was used during experiments, with the final concentration of DMSO not exceeding 1‰. It is important to minimize exposure to light during both preparation and use. TPOL has a molecular weight of 316.33. In each experiment, the photoinitiators were applied to the cells and allowed to incubate them for 30 minutes before being irradiated with light sources at wavelengths of 380 nm, 405 nm, and 455 nm to activate TPOL, CQ, and BAPO, respectively. The light source tip should be positioned 10 cm away from the sample.

### 2.4. Induction of M1 and M2 macrophages

During the logarithmic growth phase, cells were uniformly spread across a 6-well plate. After cells were adhered to the bottom of the well plated, LPS, IFN-γ, and IL-4 were respectively introduced at specific concentrations and incubated at 37°C for 24 hours to induce a response.

### 2.5. MTT assay

Application of MTT is effective in converting succinate dehydrogenase in living cell mitochondria into blue-purple crystals, allowing for the assessment of cell viability. After the drug was administered, it is recommended to rinse the cells thrice with PBS before exposing them to 5 mg/ml MTT for a duration of 4 hours, followed by dissolution in DMSO. Utilizing a microplate reader, the absorbance at 570 nm was determined. The percentage of cell survival was then computed as (sample well - blank well)/(control cell group - blank well)*100%.

### 2.6. Flow cytometry

After treatment with the drug, the cells were transformed into a single-cell state. Initially, they were blocked with 2% BSA for 30 minutes, followed by incubation with anti-CD80, anti-CD86, and anti-CD11c on ice for 40 minutes in an ice bath for surface staining. Subsequently, they were permeabilized using a fixed permeabilization kit, diluted with CD68 antibody in the

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permeabilization solution, and then incubated on ice for another hour. Finally, flow cytometry was performed.

After treatment with the drug, the cells were transformed into a single-cell state. Initially, they were blocked with 2% BSA for 30 minutes, followed by permeabilized using a fixed permeabilization kit, diluted with CD206 antibody in the permeabilization solution, and then incubated on ice for 1 hour. Finally, flow cytometry was performed.

### 2.7. Statistical analysis

The experiments were independently repeated three times, and the data are presented as mean ± standard deviation (x ± SD). Statistical analysis was conducted using GraphPad Prism 8.0 Software, with a one-way analysis of variance followed by a Tukey post-hoc test and t-test for comparing multiple groups. Statistical significance is defined as *p < 0.05 and **p < 0.01.

## 3. Results

### 3.1. TPOL has no obvious toxicity to J774A.1 cells

As a photoinitiator for clinical application, assessing lower cytotoxicity is crucial. The findings revealed that in J774A.1 cells (Fig.1), after 12 hours of exposure, TPOL at concentrations of 1 μM, 5 μM, and 10 μM exhibited significantly lower cytotoxicity compared to CQ ($p < 0.01$). After 24 hours, there was no notable difference between CQ and TPOL at all concentrations, except for BAPO, which showed significantly higher cytotoxicity than TPOL at 10 μM and 25 μM. By the 48-hour mark, the cytotoxicity of TPOL at 5 μM and 10 μM was significantly lower than that of CQ. These results demonstrate that BAPO has the highest toxicity in J774A.1 cells, while no significant difference was observed between CQ and TPOL, nor between TPOL and the control group. Notably, the cell survival rates exceeded 80% in the 12- and 24-hour low concentration groups (1 μM, 5 μM, 10 μM) and the control group, and there was no significant difference between TPOL and CQ. Therefore, three concentrations of 1 μM, 5 μM, and 10 μM TPOL can be selected to study its effect on J774A.1 cell polarization.

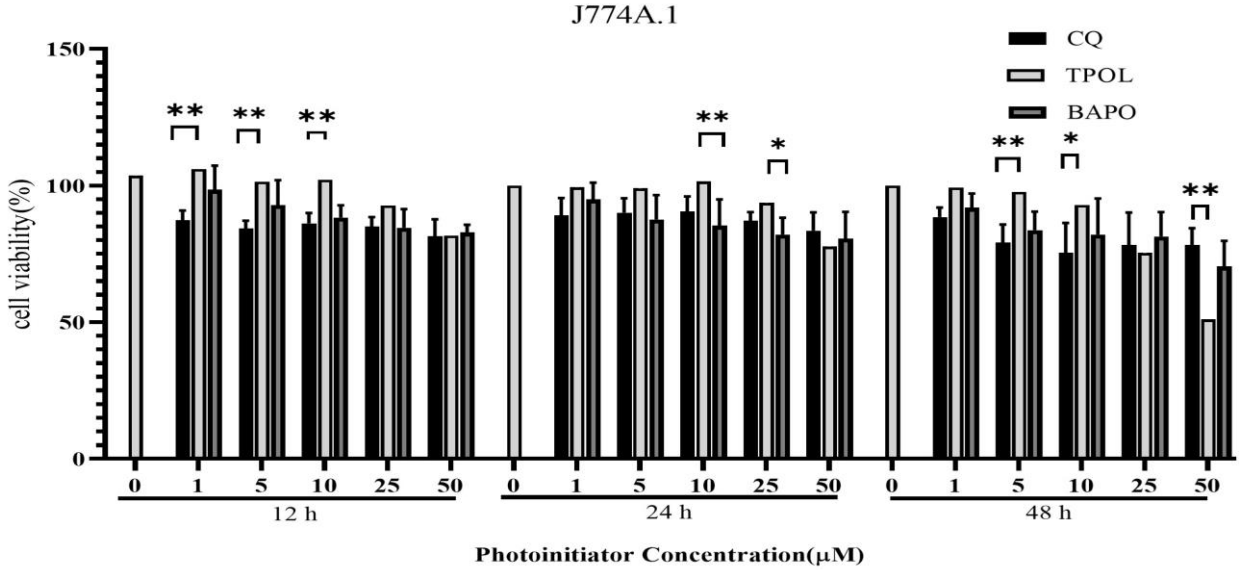


Fig.1 CQ and TPOL did not exhibit apparent toxicity towards J774A.1 cell at the 1 μM, 5 μM, and 10 μM concentrations. Cell viability percentages were evaluated through the MTT assay. *p < 0.05, **p < 0.01.

### 3.2. TPOL has no obvious toxicity to RAW264.7 cells

In Fig.2, the cytotoxicity of BAPO to RAW264.7 cells was notably higher than that of CQ and TPOL at the high concentrations of 25 μM or 50 μM, following a 12-hour incubation period.

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Subsequently, after incubated for 24 hours, BAPO displayed significantly greater cytotoxicity at the concentrations of 10 μM, 25 μM, and 50 μM compared to CQ and TPOL. These results suggest that BAPO exhibited the highest level of toxicity towards RAW264.7 cells, with no significant difference observed among CQ, TPOL, and the control group. Interestingly, there was no substantial variance between TPOL and CQ in the lower concentration groups (1 μM, 5 μM, 10 μM) at both time points, with cell survival rates exceeding 80%. Therefore, the concentrations of 1 μM, 5 μM, and 10 μM TPOL could be considered to explore its effect on RAW264.7 cell polarization.

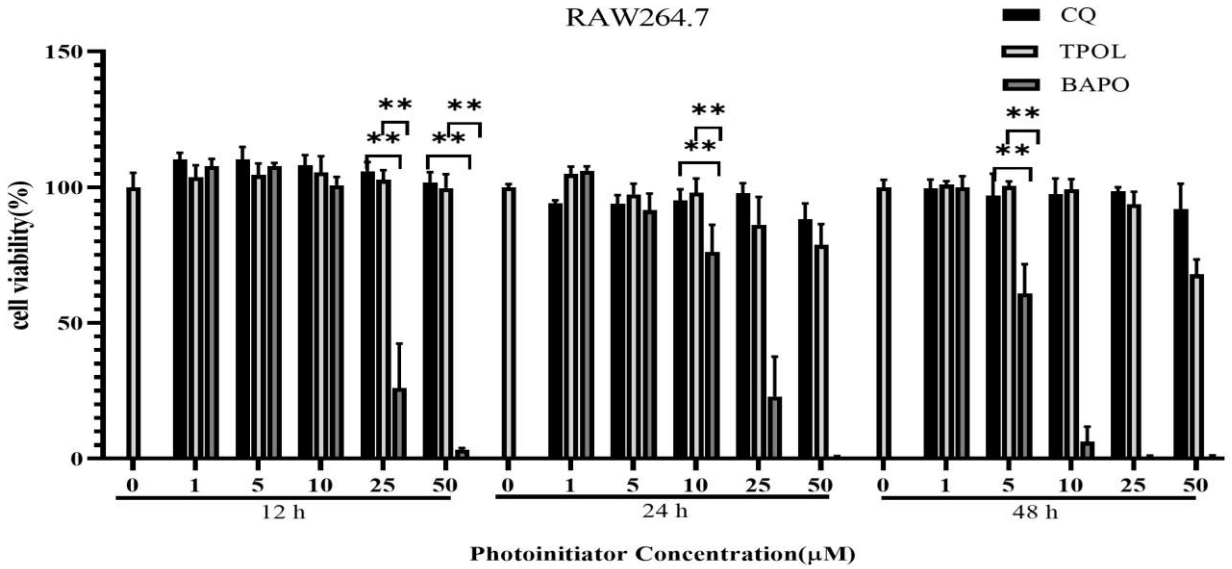


Fig.2 TPOL has no significant toxicity to RAW264.7 cells at the concentrations of 1 μM, 5 μM, and 10 μM. Cell viability percentages were evaluated through the MTT assay. **p < 0.01.

## 3.3. Induction of M1 and M2 macrophages in J774A.1 cells

Establishing M1 and M2 macrophage models in J774A.1 cell is essential for researching macrophage polarization (refer to Fig.3). The M1 phenotype is activated by LPS and IFN-γ, while the M2 phenotype is triggered by IL-4. CD68 serves as a crucial marker for macrophage, along with CD80, CD86, and CD11c for evaluating the M1 phenotype, and CD206 for the M2 phenotype. In J774A.1 cells, the expression levels of CD68CD80, CD68CD86, and CD68CD11c showed notable variances when exposed to a combination of 20 ng/mL LPS and 20 ng/mL IFN-γ compared to the M0 group. Similarly, the expression of CD206 was significantly altered when the cells were treated with 50 ng/mL IL-4. Consequently, a concentration of 20 ng/mL LPS and 20 ng/mL IFN-γ was selected for inducing the M1 phenotype, while 50 ng/mL IL-4 was chosen for inducing the M2 phenotype in J774A.1 macrophages.

J774A.1

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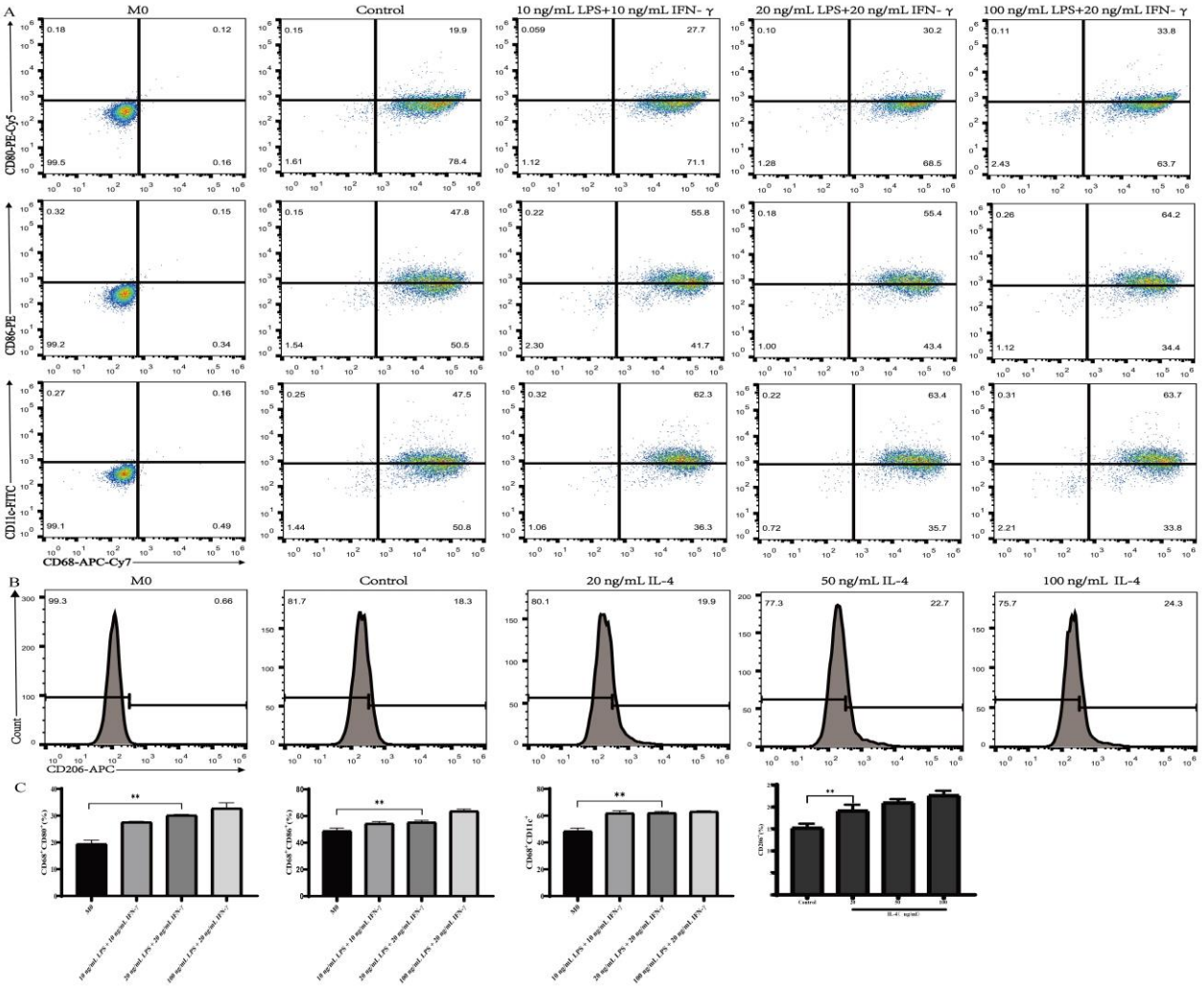


Fig.3 20 ng/mL LPS plus 20 ng/mL IFN-γ or 50 ng/mL IL-4 is the appropriate stimulation concentrations for induction of M1 and M2 polarization in J774A.1 cells, respectively. (A) The expression levels of $CD68^+CD80^+$, $CD68^+CD86^+$, $CD68^+CD11c^+$ were determined on the surface of the TPOL treated J774A.1 cells by flow cytometry. (B) The expressional level of $CD206^+$ on the surface of the TPOL treated J774A.1 cells was also measured by flow cytometry. (C) The percentages of $CD68^+CD80^+$, $CD68^+CD86^+$, $CD68^+CD11c^+$ and $CD206^+$ in J774A.1 cells were quantitatively analyzed. M0: growth medium. $^{**}p < 0.01$.

## 3.4. Induction of M1 and M2 macrophages in RAW264.7 cells

In Fig.4, the rates of positive expression for CD68CD80, CD68CD86, and CD68CD11c in RAW264.7 cells showed significant differences when stimulated with 20 ng/mL LPS + 20 ng/mL IFN-γ compared to the M0 group. Moreover, the positive expression rate of CD206 also differed significantly from that of the M0 group when the cells were stimulated with 50 ng/mL IL-4. Therefore, 20 ng/mL LPS plus 20 ng/mL IFN-γ is adapted to induce M1-type RAW264.7 cells, while 50 ng/mL IL-4 4 to induce M2-type in RAW264.7 cells.

RAW264.7

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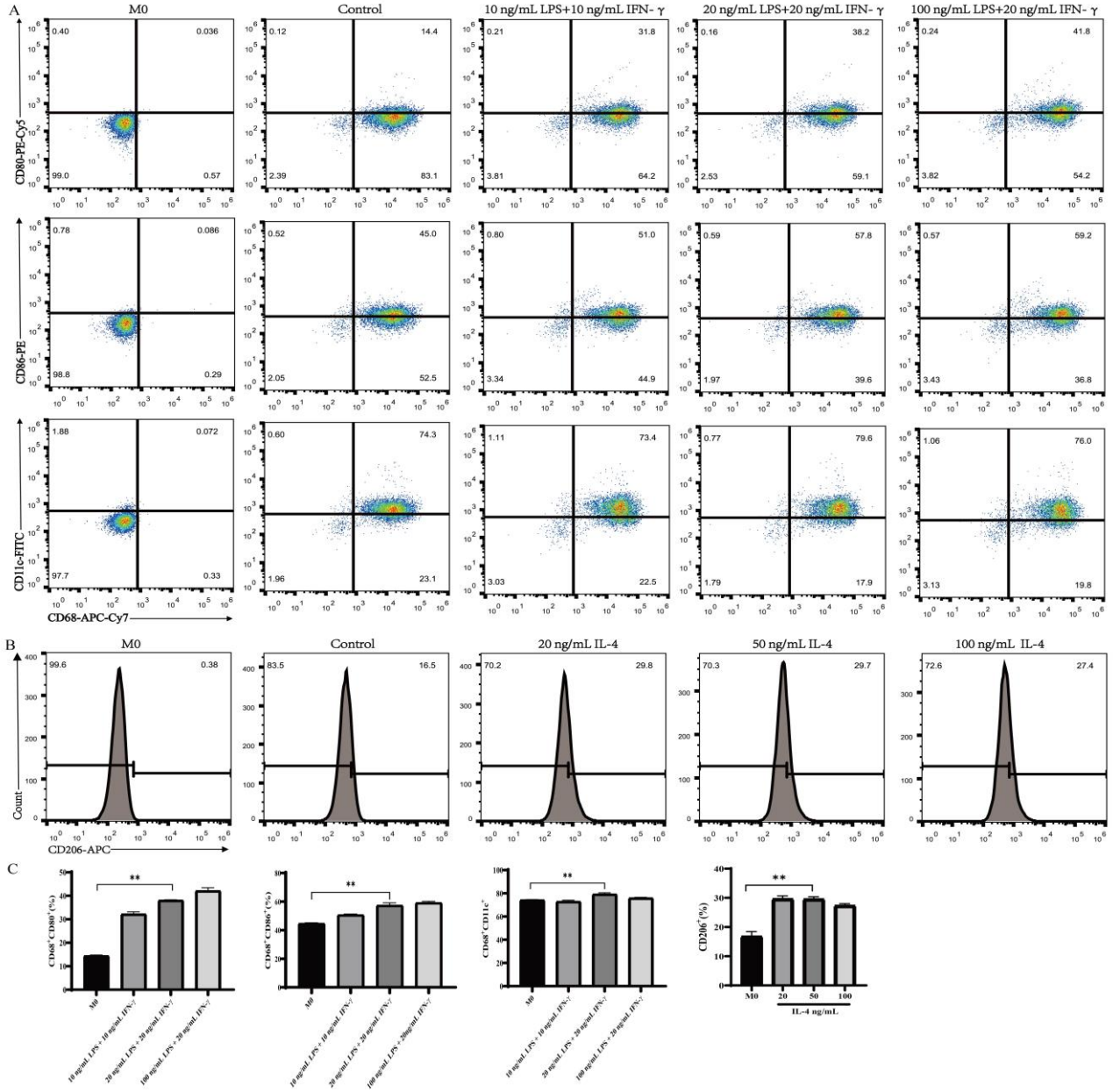


Fig.4 In RAW264.7 cell, 20 ng/mL LPS + 20 ng/mL IFN-γ and 50 ng/mL IL-4 were the appropriate stimulation concentrations for inducing M1 and M2 models, respectively. (A) Expression level of $CD68^+CD80^+$、$CD68^+CD86^+$ 、$CD68^+CD11c^+$ in RAW264.7 cell stimulated. (B) Expression level of $CD206^+$ in RAW264.7 cell stimulated. (C) The percentages of $CD68^+CD80^+$、 $CD68^+CD86^+$ 、 $CD68^+CD11c^+$ and $CD206^+$ in RAW264.7 cell were quantitatively analyzed. M0: growth medium. **p < 0.01.

## 4. Discussion

CQ is a biologically active yellow crystalline powder with a strong absorption peak at 360-510 nm[13]. It is sensitive to visible light, and the initiation of photopolymerization requires the co-initiator EDB. Studies have shown that elevating the level of CQ within a specific range can improve the rate of conversion and mechanical characteristics of the resulting polymer. However, limitations such as insufficient light penetration may hinder the reaction of the photoinitiator at the bottom. This can lead to unreacted CQ, which imparts a yellow color to the polymer, with higher concentrations intensifying the yellowing effect over time, posing challenges for color matching in dental applications[14]. Apart from color issues, unreact amines

in CQ and EDB may also present toxicity concerns. On the other hand, TPOL is a slightly yellow liquid with maximum absorption at 273 nm and 373 nm. It can independently initiate photopolymerization reactions without the need for an amine co-initiator, offering lower yellowing effects compared to CQ and resulting in a transparent polymer. Our study found that both CQ and TPOL exhibited similar toxicity in macrophages, with TPOL showing comparable mechanical properties to CQ. Therefore, TPOL emerges as a promising photoinitiator candidate for dental composites. While BAPO is often seen as a substitute for CQ[15], our research demonstrated that BAPO displayed higher cytotoxicity than TPOL and CQ in macrophages cells. Furthermore, studies have revealed that BAPO exhibits greater cytotoxicity and genotoxicity than CQ in human oral glial cells[16][17].

Inducing macrophage polarization in vitro using stimulants offers advantages such as a short time frame, simplicity, and cost-effectiveness. Typically, LPS and IFN-γ are utilized to trigger M1 type macrophage activation, whereas IL-4/IL-13 prompts M2 type macrophage development[18]. This approach replicates the interaction of macrophages with activated $CD4^+$ T cells, which release diverse cytokines such as IFN-γ from TH1 cells and IL-4 or IL-13 from TH2 cells. In contrast to stable cell phenotypes like Th1 and Th2, M1 and M2 macrophages display fluid characteristics. Our investigation demonstrated that three different concentrations of initiators (1 μM, 5 μM, 10 μM) did not exhibit notable cytotoxicity towards macrophages, and were thus chosen for studying their influence on macrophage polarization transition. The models representing M1 and M2 macrophages are successfully established using a combination of 20 ng/mL LPS with both 20 ng/mL IFN-γ and 50 or ng/mL IL-4, respectively, which serves undoubtedly as an important tool for future exploration into impacts of these photoinitiators on macrophage polarization.

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