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Original Article

Recombinant Human IL-32θ Induces Polarization Into M1-like Macrophage in Human Monocytic Cells

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ABSTRACT

The tumor microenvironment (TME) is formed by several immune cells. Notably, tumor-associated macrophages (TAMs) are existed in the TME that induce angiogenesis, metastasis, and proliferation of cancer cells. Recently, a point-mutated variant of IL-32θ was discovered in breast cancer tissues, which suppressed migration and proliferation through intracellular pathways. Although the relationship between cancer and IL-32 has been previously studied, the effects of IL-32θ on TAMs remain elusive. Recombinant human IL-32θ (rhIL-32θ) was generated using an *Escherichia coli* expression system. To induce M0 macrophage polarization, THP-1 cells were stimulated with PMA. After PMA treatment, the cells were cultured with IL-4 and IL-13, or rhIL-32θ. The mRNA level of M1 macrophage markers (IL-1β, TNFα, inducible nitric oxide synthase) were increased by rhIL-32θ in M0 macrophages. On the other hand, the M2 macrophage markers (CCL17, CCL22, TGFβ, CD206) were decreased by rhIL-32θ in M2 macrophages. rhIL-32θ induced nuclear translocation of the NF-κB via regulation of the MAPK (p38) pathway. In conclusion, point-mutated rhIL-32θ induced the polarization to M1-like macrophages through the MAPK (p38) and NF-κB (p65/p50) pathways.

Keywords: IL-32θ; Inflammation; Tumor microenvironment; Tumor-associated macrophages; Immunotherapy

INTRODUCTION

Despite the extensive amount of studies conducted, cancer remains a serious disease and the leading cause of death in every country worldwide (1). Furthermore, during the coronavirus disease 2019 pandemic, cancer continued the second cause of death in the United States in 2020 (2). Therefore, continuing to develop innovative cancer treatments is essential. Recently, many researchers have focused on targeting the tumor microenvironment (TME) as a potential approach for cancer immunotherapy. The TME consists of several cell types, including endothelial cells, fibroblasts, immune cells, and soluble components, such as cytokines, chemokines, and growth factors (3). Tumor-associated macrophages (TAMs), the most numerous cells in the TME, have been shown to a significant role in inducing angiogenesis, metastasis, proliferation, and drug resistance in cancer (4). Additionally, TAMs have been found to inhibit cancer immunotherapy by directly or indirectly inactivating $CD8^+$

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
AKT, protein kinase B; iNOS, inducible nitric oxide synthase; rhIL-32θ, recombinant human IL-32θ; RT-qPCR, reverse transcriptase quantitative PCR; TAM, tumor-associated macrophage; TME, tumor microenvironment.

Author Contributions
Conceptualization: Park HM, Park JY, Yoon DY; Data curation: Park HM, Kim NY, Kim H; Formal analysis: Park HM, Park JY, Kim HG; Funding acquisition: Son DJ, Hong JT, Yoon DY; Investigation: Park HM, Park JY, Kim NY; Methodology: Park HM, Park JY, Kim H; Visualization: Park HM, Park JY, Kim HG; Writing - original draft: Park HM, Park JY; Writing - review & editing: Park HM, Park JY, Hong JT, Yoon DY.

T cells (5-7). Given the impact of TAMs on cancer progression, many studies have focused on developing therapies targeting these cells for cancer treatment.

IL-32 was first identified as a cytokine in 2005, since then 9 isoforms have been detected (8-10). Numerous studies have investigated its molecular structure and roles in the immune system and cancer biology (11-13). IL-32θ, one of the isoforms of IL-32, has been the focus of research due to its effects on human monocyte cell lines, as well as its potential anti-cancer effects in breast cancer and colon cancer cell lines (14-16). IL-32θ inhibits migration and invasion in breast cancer via binding to PKCδ (14). Additionally, in colon cancer, IL-32θ represses cancer stemness and epithelial-mesenchymal transition through inhibiting STAT3 pathway (17). Recently, a point mutation (A94V) was identified in IL-32θ in both cancerous and noncancerous human breast tissues (18). This point mutation inhibited the expression of inflammatory molecules by suppressing the nuclear translocation of NF-κB into the nucleus (18). Moreover, it inhibited tumor migration and proliferation in breast cancer cell lines and exerted anti-atherosclerotic effects on human endothelial cells (18,19). However, the previous studies investigating the effects of IL-32θ on human monocytes and cancer cell lines have only considered the intracellular signaling pathways using overexpressed cell lines. In this study, we investigated the regulatory effects of recombinant human IL-32θ (rhIL-32θ) on macrophage polarization in a human monocytic THP-1 cell line.

MATERIALS AND METHODS

Cell culture

The human monocytic cell line THP-1 (KCLB-40202; Korean Cell Line Bank, Seoul, Korea) was cultured in RPMI-1640 medium (HyClone, Logan, UT, USA). The medium was supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL Life Technologies, Rockville, MD, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C/5% CO_2.

Construction of rhIL-32θ expression vector

Construction of the rhIL-32θ expression vector has been previously described (19). Briefly, the rhIL-32θ sequence was synthesized by Bioneer (Daejeon, Korea) using HT-oligo™ synthesizer (Bioneer). The product was digested with NdeI and AgeI and ligated to a TEVSH vector (Addgene, Watertown, MA, USA) that had been digested with NdeI and AgeI. The mixtures were used to transform DH5α competent cells. Screening of transformant was completed with ampicillin (100 μg/ml). The endotoxin contamination level of rhIL-32θ was confirmed using endotoxin removal reagent polymyxin B (**Supplementary Fig. 1**).

Production and purification of His-tagged rhIL-32θ

Production and purification of the rhIL-32θ has been previously described (19). Briefly, *Escherichia coli Rosetta* transformed by the rhIL-32θ expressing vector was cultured in LB broth until an 0.5–0.6 value of OD_{600}. Following the addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.5 mM, the bacteria were incubated for 16 h at 16°C with rotation. The purification of rhIL-32θ was performed by Ni-NTA charged column (Thermo Fisher Scientific, Waltham, MA, USA), followed by CNBr Sepharose 4B coupled with KU32-52, an anti-IL-32 monoclonal antibody made as previously described (20). For in vitro experiments, purified rhIL-32θ was dialyzed by phosphate-buffered saline (pH 7.4) at 4°C and sterilized by filtration through 0.22 μm filters (Tisch Scientific, Miami, OH, USA).

Polarization of macrophage

For polarization of macrophage, THP-1 cells were seeded into 60 pi plates (3×10^5 cells/well) and stimulated with 100 nM PMA (Millipore Sigma, Burlington, MA, USA) for 72 h; after 24 h of the PMA treatment, the cells were treated with IL-4 (20 ng/ml) (PeproTech, Philadelphia, PA, USA) and IL-13 (20 ng/ml) (PeproTech) or rhIL-32θ (50 ng/ml) for another 48 h. The dose and time-dependent effects of rhIL-32θ on M1 macrophage related factors were represented in **Supplementary Fig. 2**.

Reverse transcription quantitative PCR (RT-qPCR)

The mRNA levels in the cells were measured using RT-qPCR. RT-qPCR was conducted with a relative quantification protocol using the BioFact™ 2X Real-time PCR Kit (BioFact, Daejeon, Korea) and Rotor-Gene 6000 series software 1.7 (Qiagen, Venlo, The Netherlands). The sequences of primer were listed in the **Supplementary Table 1**. The relative levels of mRNA were calculated using the $^{\Delta\Delta}$Ct method.

Western blotting

The cell lysates were prepared in radioimmunoprecipitation assay buffer (iNtRON Biotechnology, Seongnam, Korea) containing 1× complete protease inhibitor cocktail and 1× PhosSTOP (Roche Diagnostics, Mannheim, Germany). To obtain nuclear or cytoplasmic proteins, the cells were fractionated using the NE-PER kit (Thermo Fisher Scientific), following the manufacturer's instructions. Samples were divided to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently transferred to 0.2 μm polyvinylidene difluoride membranes (Amersham Biosciences, Amersham, UK). Membranes were hybridized with the appropriate primary antibodies at 4°C overnight. The antibodies used in this study are listed in the **Supplementary Table 2**. Western blotting was performed using a chemiluminescence detection kit (Advanstar, Cleveland, OH, USA) and protein bands were captured using an EZ-capture MG protein imaging system (ATTO, Tokyo, Japan). The bands on the western blot were quantified using ImageJ open-source software (National Institutes of Health, Bethesda, MD, USA).

ELISA

For the measured of secreted protein, THP-1 (1×10^5 cells/well) cells were seeded into 24-well plates and treated under each condition. Cell culture supernatants were collected and analyzed using the ELISA kits for human IL-1β, and TNFα (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Re-polarization of macrophage

For macrophage re-polarization, THP-1 cells were seeded into 60 pi plates (3×10^5 cells/well) and stimulated with 100 nM PMA (Millipore Sigma) for 72 h; after 24 h of PMA treatment, the cells were treated with IL-4 (20 ng/ml) and IL-13 (20 ng/ml) for another 48 h. Subsequently, the culture medium was replenished with or without rhIL-32θ (100 ng/ml) for another 72 h.

Statistical analysis

One-way ANOVA with Tukey's honest test was used to compare groups in the *in vitro* experiments. Statistical analyses were performed using GraphPad Prism software version 9.0 (GraphPad Software, Boston, MA, USA). Results are presented as the mean ± SD of 3 experiments. All p-values were 2-sided, and $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$.

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RESULTS

rhIL-32θ induced M1 macrophage markers in M0 macrophage derived from THP-1 cells

To evaluate the effect of rhIL-32θ on M0 macrophages, we induced the polarization of M0 macrophages by treating THP-1 monocytes with PMA (100 nM) for 24 h. PMA-induced M0 macrophages were treated with IL-4/IL-13 (20 ng/ml) or rhIL-32θ (50 ng/ml) for 48 h (**Fig. 1A**). Macrophages treated with rhIL-32θ exhibited different morphological changes than M2 macrophages (**Fig. 1B**). Furthermore, the mRNA levels of M1 macrophage marker molecules including IL-1β, TNFα, and inducible nitric oxide synthase (iNOS), were predominantly expressed in rhIL-32θ-treated M0 macrophages. The mRNA levels of M2 macrophage-related molecules including CCL22, CCL17, TGFβ, and CD206 were increased in M2 macrophages. However, they were not altered by rhIL-32θ (**Fig. 1C**). Western blot and ELISA were performed to confirm the expression of IL-1β, TNFα, and iNOS (**Fig. 2**). These results revealed that treatment with rhIL-32θ significantly increased the expression of iNOS, IL-1β and TNFα.

rhIL-32θ promoted the phosphorylation of p38 MAPK and nuclear translocation of NF-κB

To investigate the effect of rhIL-32θ on MAPK phosphorylation in M0 macrophages, we examined MAPK phosphorylation levels in M0, M2, and rhIL-32θ-treated macrophages. By comparing phosphorylation levels under different conditions, we designed to determine the specific role of rhIL-32θ on MAPK signaling in M0 macrophages. In M2 macrophages, we observed a slight decrease in the phosphorylation of p38 MAPK, JNK, and ERK; conversely, protein kinase B (AKT) showed increased phosphorylation (**Fig. 3A**). In contrast, only the phosphorylation level of p38 MAPK was significantly enhanced in rhIL-32θ-treated macrophages (**Fig. 3A**). Furthermore, western blot analysis revealed that rhIL-32θ induced higher nuclear translocation of the NF-κB (p65/p50) than both M0 and M2 macrophages (**Fig. 3B**).

rhIL-32θ induced re-polarization of M2 macrophages into M1-like macrophages

In a previous experiment (**Fig. 1**), we found that rhIL-32θ induced the polarization of M0 into M1-like macrophages. Additionally, we confirmed whether rhIL-32θ could induce re-polarization into M1-like macrophages that were initially polarized into M2 macrophages by IL-4/IL-13. After inducing M2 macrophages using the previously described method, we treated them with rhIL-32θ (100 ng/ml) for 72 h (**Fig. 4A**). The effect of rhIL-32θ on M2 macrophages was investigated using RT-qPCR; this analysis revealed that rhIL-32θ treatment upregulated the expression of M1 macrophage markers, including IL-1β, TNFα, and iNOS (**Fig. 4B**). Conversely, M2 macrophage markers such as CD206, TGFβ, CCL17, and CCL22 were significantly downregulated (**Fig. 4B**). The protein expression levels of IL-1β, TNFα and iNOS were determined by western blotting or ELISA. The western blot confirmed that iNOS expression level increased after the rhIL-32θ treatment (**Fig. 5A**). Additionally, ELISA revealed that the levels of secreted IL-1β and TNFα were also increased by the rhIL-32θ treatment (**Fig. 5B**).

rhIL-32θ induced re-polarizing M2 into M1-like macrophages by increasing the phosphorylation level of p38 MAPK and nuclear translocation of NF-kB

We confirmed whether rhIL-32θ would induce the re-polarization of M2 to M1 macrophages by examining the phosphorylated MAPK level and nuclear translocation of NF-κB as shown in **Fig. 3**. The pAKT level in M2 macrophages was decreased, whereas p-p38 MAPK level was increased by the rhIL-32θ treatment (**Fig. 6A**). In addition, the nuclear translocation level of NF-κB (p65/p50) was significantly enhanced by the rhIL-32θ treatment in M2 macrophages (**Fig. 6B**).

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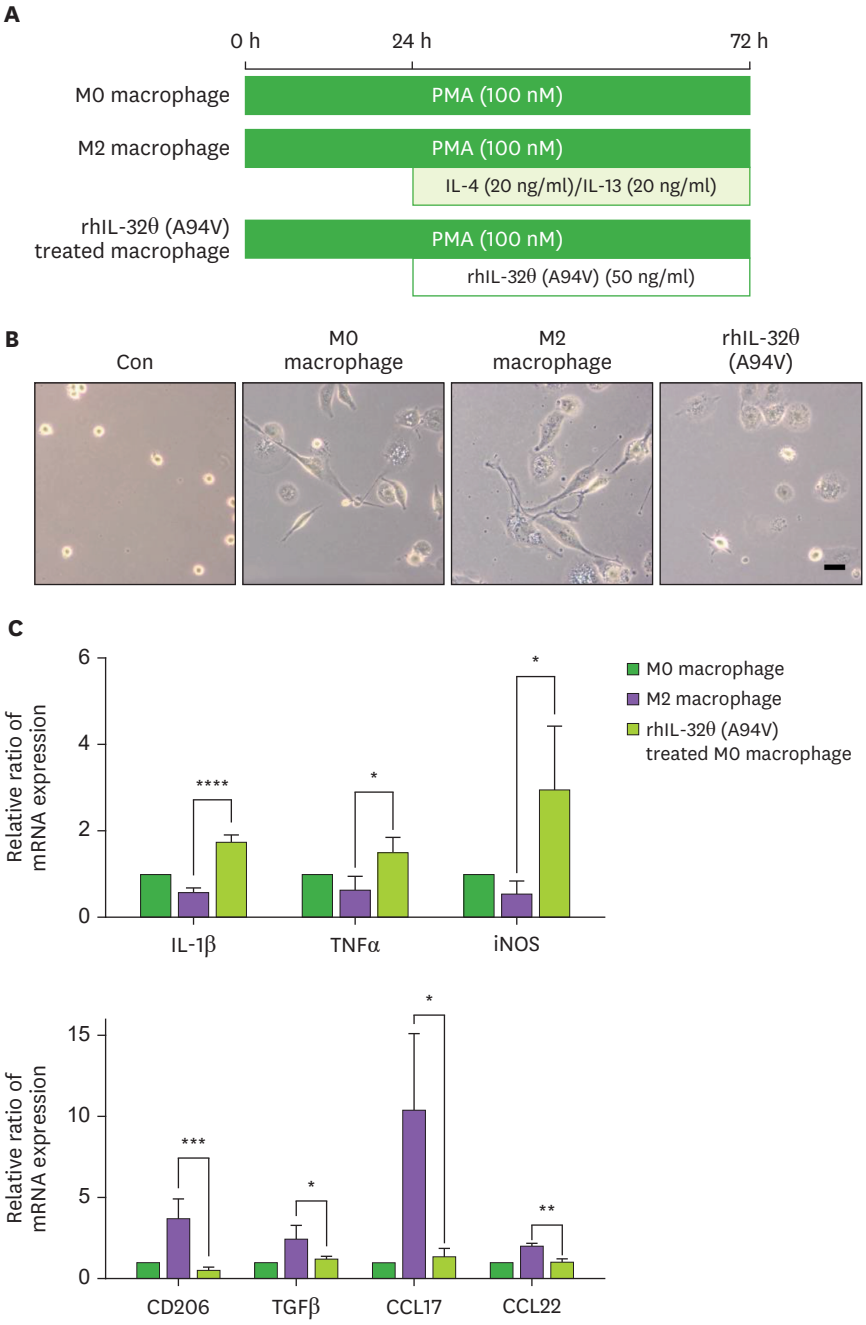

Figure 1. Effect of rhIL-32θ on the mRNA expression of macrophage polarization. (A) Schematic of the *in vitro* model using recombinant IL-4, IL-13, and IL-32θ in PMA-treated human monocytic THP-1 cell lines. (B) Cellular morphology of macrophage in polarized human monocytic THP-1 cells. Scale bar, 50 μm. (C) The effects of rhIL-32θ on M1 macrophage related genes (iNOS, IL-1β and TNFα) and M2 macrophage related genes (CD206, TGFβ, CCL17 and CCL22) expression in human monocytic THP-1 cells. The results are expressed as the mean ± SD of 3 independent experiments.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by one-way ANOVA with Tukey's honest test, n=3.

DISCUSSION

Macrophages eliminate neoplasms in the early stages of cancer. However, as cancer progresses, macrophages transform into TAMs owing to the formation of a TME (21).

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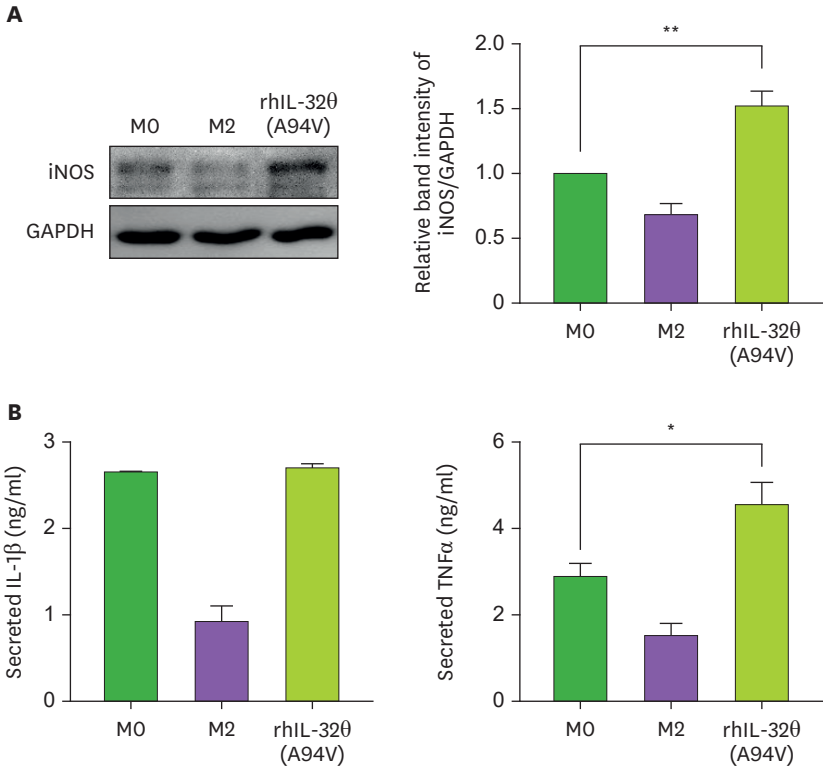

Figure 2. Effects of rhIL-32θ on the expression of M1 macrophage related proteins. (A) Protein levels of iNOS were analyzed by western blot. The graph shows the intensity of the iNOS bands normalized with the intensity of the GAPDH bands for each sample. Intensities were measured using the ImageJ software. (B) The secreted protein levels of IL-1β and TNFα were measured by ELISA. The results are expressed as the mean ± SD of 3 experiments. *p<0.05, **p<0.01 by one-way ANOVA with Tukey's honest test, n=3.

TAMs are primary targets of immunotherapy in cancer treatment (22). Unlike classical macrophages (M1 macrophages), TAMs play roles similar to M2 macrophages in reducing inflammation via the secretion of anti-inflammatory cytokines, chemokines, and growth factors (23). These secreted molecules inhibit immune cell reactions in the TME, including inflammation, antigen presentation, and phagocytosis (23); furthermore, they also play a crucial role in tumor migration, proliferation, and angiogenesis within the TME (4). Because of these factors, TAMs are a notable target for immunotherapy, and the re-polarization of macrophages may represent a fundamental treatment approach for several immunotherapies.

The macrophage is a crucial effect of the immune system, predominantly triggered to eliminate infections caused by bacteria, viruses, or neoplasms such as cancer cells (24). Previous research has revealed that differential factors between M1 macrophage and M2 macrophage. M1 macrophages express iNOS and secrete cytokines, including IL-1β and TNFα (25). These proteins are typically classified as pro-inflammatory proteins that stimulate an immune response to eliminate tumors in early-stage cancer (26,27). In contrast, M2 macrophages express CD206, which serves as their principal marker and secrete anti-inflammatory proteins and tumor-promoting factors such as TGFβ, CCL17, and CCL22 (23,28). Furthermore, M0 and M1 macrophages are morphologically characterized by their spheroid and radial shapes; in contrast, M2 macrophages are spindle-shaped (29). In our study, the macrophages treated with rhIL-32θ showed spheroid and radial shapes (**Fig. 1B**). RT-qPCR analysis revealed that the mRNA levels of genes associated with the inflammatory response (IL-1β, TNFα, and iNOS) were increased by rhIL-32θ in M0 macrophages (**Fig. 1C**).

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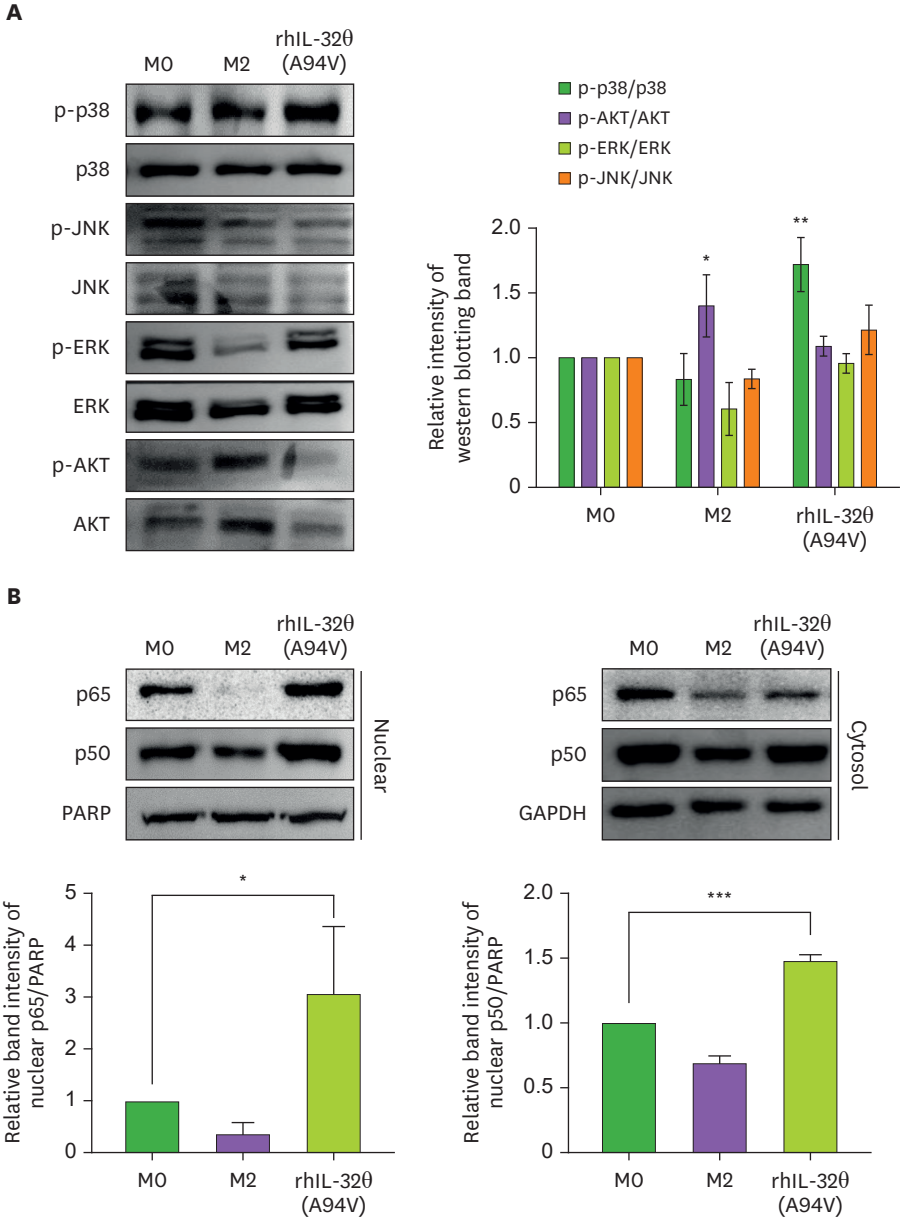

Figure 3. Modulatory effects of rhIL-32θ on the intracellular signaling pathways. (A) Western blot analysis of the AKT and MAPK (p38, ERK, and JNK) pathways in M0 polarized macrophages. The graph shows the intensity of the phosphorylated MAPK bands normalized with the intensity of the total MAPK for each sample. (B) Nuclear translocation of NF-κB was analyzed by western blot with nuclear-cytosol fractionation. The graph shows the intensity of the NF-κB bands normalized with the intensity of the internal control PARP for each sample. Intensities were measured using the ImageJ software. The results are expressed as the mean ± SD of 3 experiments.
PARP, poly (ADP-ribose) polymerase.
*p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with Tukey's honest test, n=3).

However, the expression of genes related to M2 macrophages (CD206, TGFβ, CCL17, and CCL22) was either unchanged or decreased upon treatment with rhIL-32θ (**Fig. 1C**). Moreover, the expression levels of the iNOS protein and the secretion levels of IL-1β and TNFα were increased by rhIL-32θ in M0 macrophages (**Fig. 2**).

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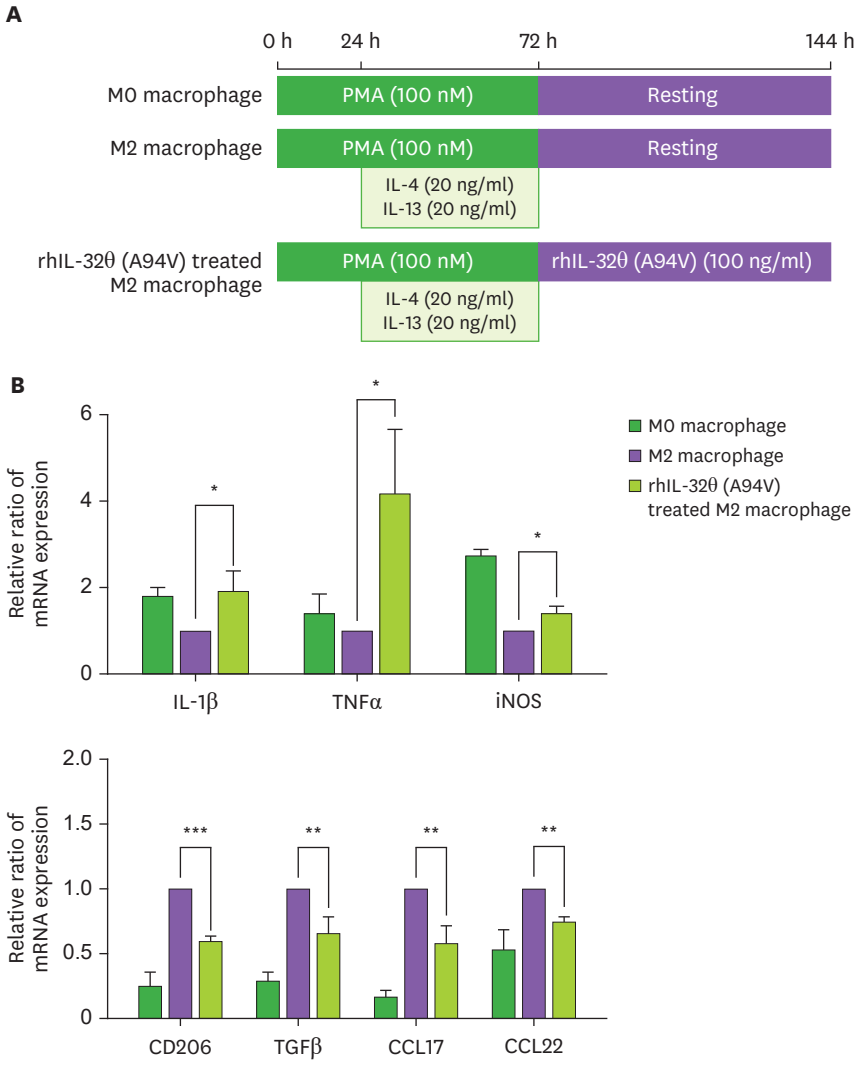

Figure 4. Effect of rhIL-32θ on the mRNA expression of polarization related gene in M2 macrophage cells. (A) Schematic of the *in vitro* model using rhIL-32θ in M2 macrophages derived from THP-1 cells. (B) The effects of rhIL-32θ on mRNA expression levels of M1 macrophage genes (iNOS, IL-1β and TNFα) and M2 macrophage related genes (CD206, TGFβ, CCL17 and CCL22) in M2 macrophages derived from THP-1 cells. The results are expressed as the mean ± SD of 3 independent experiments.
*p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with Tukey's honest test, n=3.

Inflammation is strongly associated with the activated MAPK and the nuclear translocation of NF-κB (30). Phosphorylated MAPK leads to the nuclear translocation of NF-κB that induces pro-inflammatory cytokines, chemokines, and several inflammatory molecules (30). Moreover, the MAPK pathway and NF-κB nuclear translocation have been implicated in the polarization of M0 macrophages to the M1 macrophage phenotype (31). In contrast, AKT phosphorylation is associated with the polarization of M2 macrophages (31). Western blot analysis indicated that the levels of phosphorylated p38 MAPK were increased by rhIL-32θ (**Fig. 3A**). However, AKT phosphorylation was not significantly altered (**Fig. 3A**). Additionally, the nuclear translocation of NF-κB (p65 and p50) into the nucleus was increased by rhIL-32θ (**Fig. 3B**). These results demonstrate that rhIL-32θ induced the polarization of M0 macrophage into M1-like phenotype, rather than M2 phenotype (**Figs. 1-3**).

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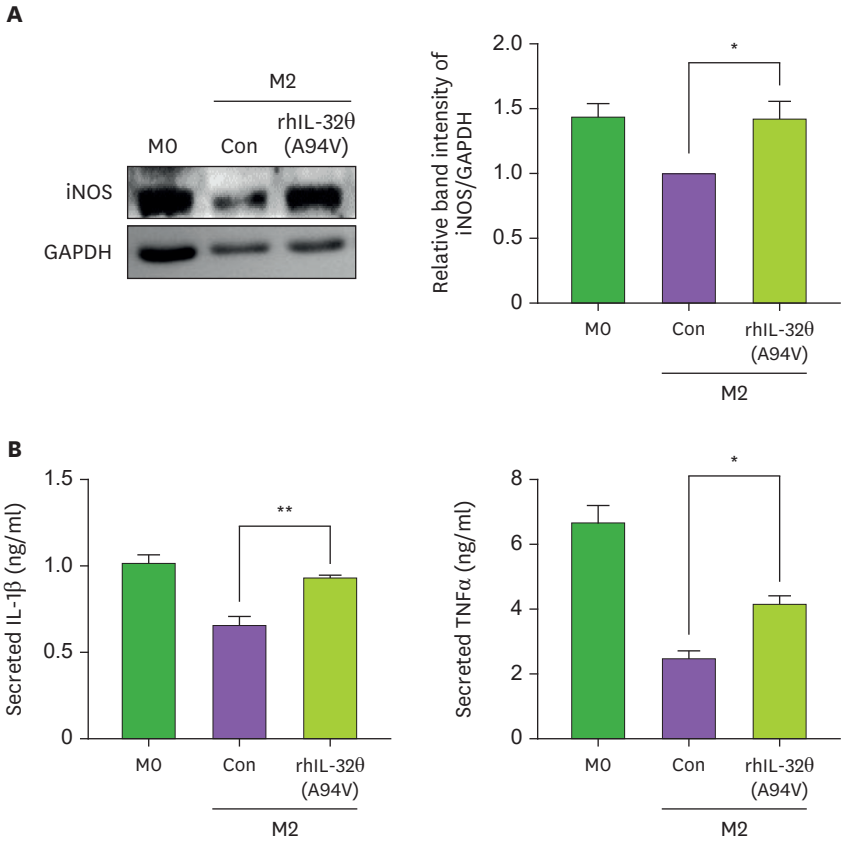

Figure 5. Modulatory effects of rhIL-32θ on the re-polarization of macrophages. THP-1 cells were polarized using IL-4/IL-13 (20 ng/ml). After inducing M2 polarization, the cells were treated with rhIL-32θ for 72 h. The supernatant was collected before harvest. (A) Protein levels of iNOS in M2 macrophages derived from THP-1 cells were analyzed by western blot. The graph shows the intensity of the iNOS bands normalized with the intensity of the GAPDH bands for each sample. Intensities were measured using the ImageJ software. (B) The secreted protein levels of IL-1β and TNFα were measured by ELISA. The results are expressed as the mean ± SD of 3 experiments. $^{*}p<0.05$, $^{**}p<0.01$ by one-way ANOVA with Tukey's honest test, n=3.

Previous studies revealed that immune cells exhibit high plasticity in response to foreign substances (32). Macrophages modify their functions according to the immune response environment (33). For example, M1 macrophages induce inflammation to eliminate infectious agents and neoplasms formed in tissues (34). Because excessive inflammation can lead to tissue damage, after an infection or inflammatory stimulus has been removed, M1 macrophages polarize to the M2 phenotype, which promotes tissue regeneration and anti-inflammatory responses (35,36). However, cancer promotes polarization into M2 macrophages to evade immune surveillance and to induce cancer progression (37-40). M2 macrophages attract anti-inflammatory immune cells, such as regulatory T cells, and secrete anti-inflammatory cytokines including TGFβ and IL-10, which inactivate cytotoxic T cells and neutrophils (41). Therefore, inhibiting M2 macrophages and promoting their repolarization to the M1 phenotype are crucial for cancer immunotherapy. In this study, RT-qPCR analysis revealed that the mRNA levels of pro-inflammatory genes (IL-1β, TNFα, and iNOS) were increased by rhIL-32θ (**Fig. 4B**). Moreover, the expression of M2 macrophage-related genes (CD206, TGFβ, CCL17, CCL22) was decreased by rhIL-32θ in M2 macrophages (**Fig. 4B**). Western blot analysis and ELISA revealed that the expression levels of TNFα, IL-1β and iNOS was induced by rhIL-32θ (**Fig. 5**). Additionally, the level of phosphorylated p38 MAPK increased, whereas that of phosphorylated AKT decreased after the rhIL-32θ treatment (**Fig. 6A**). Nuclear translocation of NF-κB (p65 and

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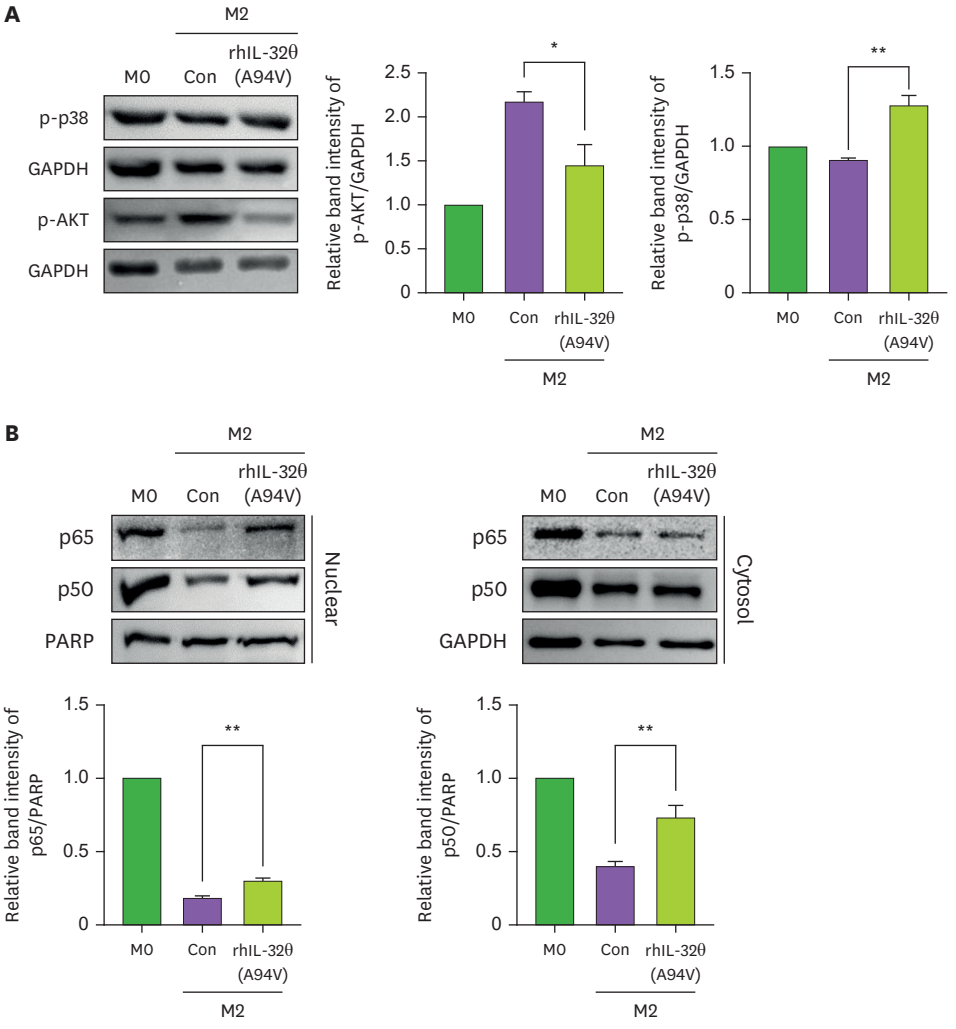

Figure 6. Modulatory effects of rhIL-32θ on the intracellular signaling pathways in M2 macrophage. (A) Western blot analysis of AKT and p38 MAPK pathways in M2 macrophages. The graph shows the intensity of the phosphorylated AKT and p38 MAPK bands normalized with the intensity of the GAPDH bands for each sample. (B) Nuclear translocation of NF-κB was detected by western blot with nuclear-cytosol fractionation. The graph shows the intensity of the NF-κB bands in nucleus normalized with the intensity of the PARP bands for each sample. Intensities were quantified using the ImageJ software. The results are expressed as the mean ± SD of 3 experiments. *p<0.05, **p<0.01 by one-way ANOVA with Tukey's honest test, n=3.

p50) into the nucleus was increased by rhIL-32θ in M2 macrophages (**Fig. 6B**). Furthermore, re-polarizing M2 to M1 macrophages with rhIL-32θ inhibited the migration of the breast cancer cell line MCF7 and regulated migration-related factors (N-cadherin and E-cadherin) (**Supplementary Fig. 3**). These results demonstrated that rhIL-32θ promoted the polarization of M2 to the M1-like phenotype (**Figs. 4-6**, **Supplementary Fig. 3**).

Cancer is a complex disease requiring multiple treatment modalities, with immunotherapy having gained significant attention in recent years (42). Adoptive cell therapy, which involves the activation and expansion of immune cells from a patient's own cells, has shown great promise for cancer treatment (43). However, the efficacy of adoptive cell therapy can be hampered by tumor-associated immune cells in the TME that suppress other immune cells (44). In our study, we demonstrated that rhIL-32θ enhanced the inflammatory response of macrophages and promoted their polarization into M1-like macrophages. These properties of

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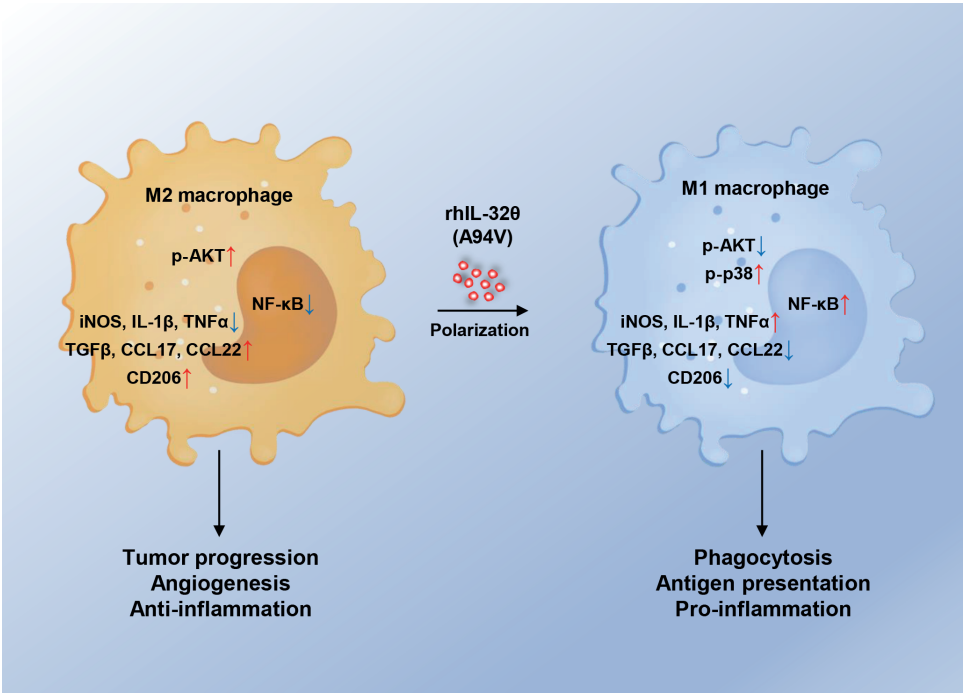

Figure 7. Schematic diagram of the effect of rhIL-32θ on the macrophage polarization. rhIL-32θ induced the expression of IL-1β, TNFα, iNOS and attenuated the expression of CD206, TGFβ, CCL17, CCL22 via modulate phosphorylation of p38 MAPK and nuclear translocation of NF-κB in M2 macrophages.

rhIL-32θ have the potential to synergize with adoptive cell therapies. However, to understand the effects of rhIL-32θ, it is crucial to investigate its relationship with other cell types in the TME, such as dendritic cells, cancer-associated fibroblasts, and tumor-associated neutrophils. Further studies are required to elucidate the precise interactions and effects of rhIL-32θ on these cells in the TME.

In conclusion, the newly discovered mutated rhIL-32θ variant induced M1 macrophage markers (IL-1β, TNFα, iNOS) via increased phosphorylation of p38 MAPK and nuclear translocation of NF-κB (p65, p50). On the other hand, rhIL-32θ decreased the expression of M2 macrophage-related factors (CD206, TGFβ, CCL17, and CCL22) in M2 macrophages. rhIL-32θ induced the phosphorylation of p38 MAPK and the nuclear translocation of NF-κB (p65 and p50) in M2 macrophages (**Fig. 7**). These properties suggest that rhIL-32θ has the potential to enhance the pro-inflammatory response and promote the polarization of TAMs towards an antitumor phenotype. This may be useful in the context of adoptive cell therapy by potentiating the effects of the immune cells used in the therapy.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1
List of primer sequences

Supplementary Table 2
List of antibodies

Supplementary Figure 1
Endotoxin assay using endotoxin removal reagent polymyxin B. (A) The mRNA levels of IL-6 were measured using RT-qPCR. M0 macrophage treated with or without polymyxin B (50 μg/ml), LPS (100 ng/ml) and rhIL-32θ (A94V) (100 ng/ml) for 24 h. The results are expressed as the mean ± SD of 3 experiments.

Supplementary Figure 2
Time and dose-dependent effects of rhIL-32θ (A94V) on the expression of M1 macrophage related genes. (A) Does-dependent effects of rhIL-32θ (A94V) on the expression levels of M1 macrophage related genes. After 24 h of the PMA treatment, the cells were treated with rhIL-32θ (A94V) for another 24 h. (B) Treated time-dependent effects of rhIL-32θ (A94V) (50 ng/ml) on the expression levels of M1 macrophage related genes. After 24 h of the PMA treatment, the cells were treated with rhIL-32θ (A94V) for time-dependent manner. The mRNA levels were measured using RT-qPCR. The results are expressed as the mean ± SD of 3 experiments.

Supplementary Figure 3
Repolarized macrophage effects on migration of breast cancer cells. (A) For the wound healing assay, MCF7 cells (1.5×10^5 cells/well) were seeded in 24-well plates. The plates were scratched using a sterile 200 μl pipette tip, washed with PBS, and a serum-free medium with CM was added for 24 h. Scale bar, 200 μm. (B) The western blot analysis representative protein levels of migration related factors (N-cadherin and E-cadherin). The wound areas and western blot analysis were quantified using ImageJ. The results represent the mean ± SD of 3 experiments.

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