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ORIGINAL ARTICLE

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Effects of platinum and palladium nanocolloid on macrophage polarization in relevance to repigmentation of vitiligo

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Abstract

Background: Elevated oxidative stress is accepted to be the initial event in vitiligo leading to the final pathological regulation of immune systems known as autoimmune reaction, which destroys melanin-forming cells, melanocytes. Recently, we reported an efficient topical use of PAPLAL, nanocolloid of platinum and palladium, having intense catalase-like activity to vitiligo patients. In addition, we found that PAPLAL has dual effects on the AhR and Nrf-2 pathways in keratinocytes, and suggested its contribution to the recovery of immune state in vitiligo. The precise mechanism developing autoimmune reaction in vitiligo, however, remains to be clarified. It is important to clarify what kinds of cells play an essential role in the development of vitiligo.

Objective: To further understand the effective mechanisms of PAPLAL on immunity of skin, and to confirm a role of autoimmunity in vitiligo development, we studied the effect of PAPLAL on macrophage polarization and its activities which are recognized to play a pivotal role in immune and inflammatory reactions in many organs.

Methods: Rat and human macrophages were cultured and stimulated in vitro with both LPS and IFN-γ for M1 polarization and IL-4 and IL-13 for M2 polarization with or without PAPLAL. Expression of typical M1 and M2 markers was determined at mRNA and protein levels.

Results: Simultaneous treatment with PAPLAL suppressed remarkably the production of M1 markers, iNOS, and TNF-α; further, PAPLAL also suppressed M2 markers, mannose receptor (Man R), Chitinase 3-like 3 (YM-1), and iron regulatory protein-1 (IRP-1), at mRNA and protein levels, but less effectively compared to those of M1. PAPLAL, however, did not suppress phagocytic activity of M0, M1, and M2 cells.

Conclusion: These results indicate that macrophages may be involved in the therapeutic potential of PAPAL by altering immunological environment disturbed in skin, with the delicate shift of the M1-M2 polarization, but without affecting on phagocytic activity.

KEYWORDS
macrophages, palladium, PAPLAL, platinum, vitiligo

1 | INTRODUCTION

The pathogenesis of vitiligo, an acquired cutaneous depigmenting disease, involves oxidative stress caused by increased H_2O_2 production and decreased level of catalase in epidermis, leading to an altered redox status of epidermal melanocytes.[1] The most favored hypothesis of vitiligo development is, however, autoimmune mechanisms, which may be induced by promoting activation of antigen-presenting dendritic cells with the subsequent activation and recruitment of anti-melanocyte autoreactive cytotoxic T lymphocytes to the skin.[2,3]

Several studies have shown a crucial role of CD8+ T cells specific for melanocyte killing and disturbed function of regulatory T cells (Tregs), which maintain peripheral tolerance of self-reactive T-cell activation and expansion.[4,5] Tregs have key roles in the immune response by suppressing the differentiation and proliferation of active immunogenic cells. Decreased number of circulating Tregs in active vitiligo patients and reduction in the number of Tregs in the marginal and lesional skin of the vitiligo patients have been reported.[5-7] A number of studies showed that decreased transforming growth factor beta (TGF-β) in serum and tissue levels[8-10] are found in vitiligo patients, suggesting a role of TGF-β on the prevention of deleterious effect of hyperreactive immunity of CD4 and CD8 cells by activation of Treg function in vitiligo patients.[11-13]

Further, in an inflammatory vitiligo case, the presence of more numerous CD68+OKM5- macrophages is shown in the perilesional skin compared to that in the lesional and non-lesional skin.[14] However, until recently, a role of macrophages in vitiligo skin relevant to immune regulation is not well studied yet, whereas macrophages are reported in recent years to control not only tissue repair and fibrosis, but also immune states in many tissues via the regulation of M1/M2 polarization as shown in several review articles.[15,16] Macrophages comprise a heterogeneous population of cells with diverse functions and phenotypic plasticity, and can switch from one functional phenotype to another.

Macrophages can generally be divided into several groups: mainly, M1 (classically activated) and M2 (alternatively activated) phenotypes.[17] M1 phenotype exhibits pro-inflammatory properties, high levels of reactive nitrogen and oxygen intermediates, promotion of Th1 response, whereas M2 phenotype has anti-inflammatory and regulatory properties resulting in fibrogenesis and tissue remodeling.[17] Further, recently docosahexaenoic acid was reported to alleviate atopic dermatitis, one of the typical inflammatory skin diseases, by activating Tregs by IL10/TGF-β-modified macrophages.[18]

We have found that PAPLAL, nanocolloid of platinum (Pt) and palladium (Pd),[19] effectively induced repigmentation of vitiligo skin of the patients who were resistant to the conventional treatments for vitiligo for more than 1 year.[20] We hypothesized that imbalance and/or dysfunction of macrophages, M1 and M2, may contribute to the inflammatory environment in the skin and the disturbance of immunological homeostasis, which may be induced by H_2O_2 insult in the melanocyte environment at the early stage of vitiligo development, and may deprive of the function of and kill melanocytes by CD8+ T cells which are homed easily by possible reduced migration of functional Tregs.[6] In the present study, we evaluated the effects of PAPLAL on the functional activation of macrophages obtained from rat spinal fluids and THP-1-derived human macrophage, and suggested a role of macrophage polarization regulation without suppressive effect on phagocytic activity which may contribute to recovery of healthy immunological environment in the skin from disturbed immunological and inflammatory states leading to autoimmune skin diseases, such as vitiligo. Further, it is important to show macrophage involvement, by analyzing histopathological features in vitiligo skin, focusing on macrophage phenotypes.

2 | METHODS

2.1 | Reagents

Pt and Pd nanocolloid (PAPLAL) was purchased from Musashino Pharmaceutical Co., Ltd. (Tokyo). PAPLAL solution contains Pt (0.2 mg/mL) and Pd (0.3 mg/mL), and the solution was diluted in phosphate buffer (pH 7.4).

The following reagents were purchased from the indicated sources: Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan); FBS, fetal bovine serum (Equitech-Bio, Inc., Kerrville, TX, USA); penicillin and streptomycin (Gibco, Waltham, MA, USA); LPS, WE coli serotype 055:B5, (Difco Laboratories, Franklin Lakes, NJ, USA); IFN-γ (ITSI-Biosciences, Johnstown, PA, USA); IL4 and IL-13 (PeproTech, Rocky Hill, NJ, USA); anti-iNOS mouse monoclonal antibody (BD Bioscience, Franklin Lakes, NJ, USA); anti-mannose receptor (CD206) rabbit polyclonal antibody (Abcam, Cambridge, UK); anti-chitinase 3-like protein 3+ chitinase 3-like protein 4 (Ym-1) rabbit polyclonal antibody (Abcam); anti-TNF-α (PEPROTECH), polyclonal goat anti-rabbit or anti-mouse or anti-goat secondary antibodies (Dako, Glostrup, Denmark); and ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, Buckinghamshire UK).

2.2 | Cell isolation

Normal young adult male Wistar rats weighing 200-250 g were killed by Urethane anesthesia (1 g/kg, ip), and intact femurs were aseptically dislocated from the hind legs. The bone marrow cells (BMCs) were flushed from the femurs with 5-10 mL of cold DMEM using 23G needle.

The cells were centrifuged for 5 minutes at 150 *g*, and the pellet was resuspended into 0.015 mol/L Tris-NH_4Cl (pH 7.4) and incubated for 10 minutes at 4°C in order to lyse erythrocytes. Approximately 8-12 × 10^7 cells were usually obtained from each femur. The cell viability as measured by trypan blue exclusion was >90%.

2.3 | Cell culture

(a) Rat bone marrow-derived macrophages (BMDMs) were washed twice in medium. BMCs were resuspended and differentiated in DMEM with 15% fetal bovine serum (FBS) at 37°C in a humidified atmosphere (5% CO_2) containing 15 ng/mL rGM-CSF (Wako) for 7 days. For the experiments, BMDM cells were suspended in the culture medium at a density of 1 × 10^6 cell/mL and plated at a density of 1 × 10^6 cells/well into 6-well tissue culture plates.

(b) The human monocytic cell line, THP-1, was obtained from RIKEN Bioresource Center (RBC; Tsukuba, Japan) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher, Waltham, MA, USA), 3% L-glutamine, and 10% sodium hydrogen carbonate. To induce differentiation into macrophage-like cells, THP-1 cells were seeded onto 35-mm culture dishes (2.5 × 10^5 cells/dish) and incubated for 48 hours with 200 ng/mL 12-o-tetradecanoyl-13-acetate (TPA; Sigma-Aldrich, St. Louis, MO, USA).

2.4 | PAPLAL treatment

Twenty-four hours before the experiment, BMDMs grown on six-well cell culture plates were supplemented with fresh DMEM with 15% FBS and stimulated with LPS (5 ng/mL) and IFN-γ (10 U/mL), or IL-4 (10 ng/mL) and IL-13 (10 ng/mL), with immediate addition of various dose of PAPLAL solution (0.1%-10%). Cells were incubated for various periods of time (24 and 48 hours). Subsequently, the cells were washed twice with PBS and collected cells were solubilized in 150 μL REPA buffer (HEPES [pH 7.5] 50 mmol/L, NaCl 200 mmol/L, EDTA2Na 1 mmol/L, EGTA 2.5 mmol/L, Tween-20 0.1%, glycerol 10%, sodium orthovanadate 0.1 mmol/L, sodium fluoride 1 mmol/L), containing a protease inhibitor cocktail (Roche Diagnostic GmbH, Mannheim, Germany), and stored at −80°C until analysis.

Six hours after TPA treatment was started, differentiating THP-1 macrophages were treated with LPS (100 pg/mL) and IFN-γ (20 ng/mL), or IL-4 (20 ng/mL) and IL-13 (20 ng/mL) for M1 or M2 polarization, respectively, with TPA for 42 hours. The treatment with 10% PAPLAL solution was carried out during the last 24 hours simultaneously with these factors for M1 and M2 polarization.

2.5 | Measurements of apoptosis and necrosis

Cell viability was assessed by the "Apoptotic/Necrotic/Healthy cells Detection" kit (PromoCell GmbH, Heidelberg, Germany) in accordance with the manufacturer's instructions. Briefly, BMDMs were plated at a density of 2 × 10^4 cell/well into 96-well tissue culture plates. Cell staining was performed 48 hours after treatment with 10% PAPLAL or hydrogen peroxide (1 mmol/L) as a positive control. Cells were analyzed for apoptosis (Annexin V, PromoCell GmbH, Heidelberg, Germany; green fluorescence), necrosis (ethidium homodimer III, PromoCell GmbH, Heidelberg, Germany; red fluorescence) and healthy cells (Hoechst 33342, PromoCell GmbH, Heidelberg, Germany; blue fluorescence) within the same cell population by fluorescence microscopy (Nikon ECLIPSE Ti2, Tokyo, Japan). Images were obtained using Nikon Digital Camera System.

2.6 | Effect of PAPLAL on the phagocytic activity of human macrophage

Polarization of M1 and M2 and PAPLAL treatment were carried out as described above. Phagocytic activity was evaluated by internalized beads ratio measured by fluorescence (IBRf) developed by Ishikawa et al.[21]

2.7 | Western blot analysis

Protein concentration in the samples was assessed by BCA protein assay (Thermo Fisher Scientific). Equal amounts of protein (5 μg) were subjected to 10% SDS-PAGE and subsequent transfer onto polyvinylidene difluoride (PVDF) microporous membranes (Merck Millipore, Burlington, MA, USA). Membranes were blocked for 30 minutes with 4% block ace (DS Pharma Biomedical Co., Ltd, Osaka, Japan) and incubated overnight at 4°C with antibody directed against either iNOS (1:1000 dilution), TNF-α (1:1000 dilution), YM-1(1:500 dilution), Man R:CD206 (1:500 dilution), or iron regulatory protein-1 (IRP-1; 1:500 dilution). Then, the membranes were washed in TBST buffer solution and incubated with goat anti-rabbit or anti-mouse alkaline phosphatase-conjugated antibody (1:20 000 dilution) for 1 hour. Protein bands were detected using enhanced chemiluminescence immunoblot assay kit (ECL prime kit; GE Healthcare) and chemiluminescence detection system/equipment (Fujifilm, Tokyo, Japan). The protein bands were quantified using ImageJ software densitometer, National Institutes of Health, Bethesda, Maryland, USA.

2.8 | Real-time PCR

The total RNAs of cultured cells were extracted with RNeasy (Qiagen, Tokyo, Japan), and cDNAs were obtained with reverse transcription (SuperScript IV; Thermo Fisher Scientific). Real-time PCR was carried out with following primer sets for human genes.

GAPDH-F; TCAAGGCTGAGAACGGGAAG
GAPDH-R; CATCGCCCCACTTGATTTTG
CXCL10-F; TTCCTGCAAGCCAATTTTGT
CXCL10-R; TGATGGCCTTCGATTCTGG
SOCS3-F; ACACTTCGGGAATGCTGAAC
SOCS3-R; GAGCAAACAAGTTCCGTTGG
IRF5-F; TTATATTGTCCCCCGAGGTG
IRF5-R; ATGGCTGAAGGCAGAAAGTG
CCL22-F; AAACTAATGTCCCTCCCCTCTC
CCL22-R; TTTGGGGCTTCACATTGACC

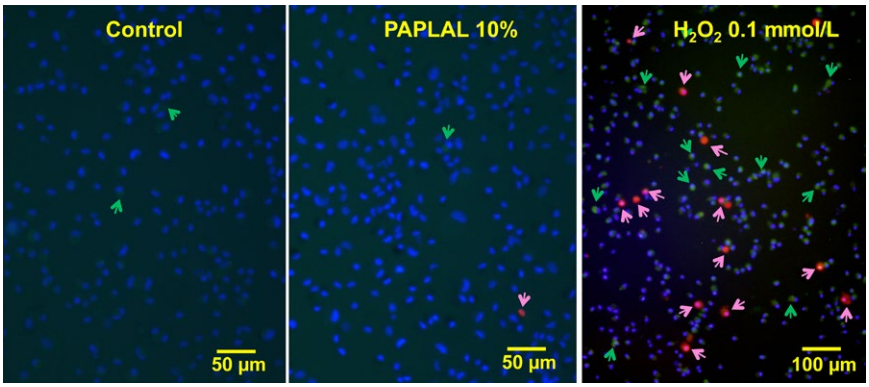

FIGURE 1 Effect of Pt and Pd nanocolloid (PAPLAL) on bone marrow-derived macrophage (BMDM) cytotoxicity. Cell viability of BMDM treated with PAPLAL (10%) or hydrogen peroxide (0.1 mmol/L) for 48 h was assessed by the "Apoptotic/Necrotic/Healthy cells Detectio Kit." Cells were analyzed for apoptosis (Annexin V; green fluorescence, green arrows), necrosis (ethidium homodimer III; red fluorescence, red arrows), and healthy cells (Hoechst 33342; blue fluorescence) within the same cell population by fluorescence microscopy. Scale bar, 100 μm

CD209-F; GTCCCATTCTGTCCTTCTTGTC
CD209-R; CCACACCAGCTCACTCATAAA
FGL2-F; TCTGCCCAGTGAGACTAGAA
FGL2-R; TTGCTTCGGGAGCTGAATAG
PPARG-F; GCCTGCATCTCCACCTTATTA
PPARG-R; ATCTCCACAGACACGACATTC

2.9 | Statistical analysis

All values are presented as mean ± SE. Statistical analysis was performed using one-way ANOVA. *P* values of <0.05 were considered to be statistically significant. Statistical analysis was performed with the MAC statistics software ver. 2.0 (Esumi Co., Ltd., Tokyo, Japan).

3 | RESULTS

3.1 | Effect of PAPLAL on cell viability

M0 cells prepared from bone marrow of rat and cultured for 1 day with 15% FCS were treated with PAPLAL at the concentration of 10% for 48 hours, and analyzed with Annexin V for apoptotic cells, ethidium homodimer III for necrosis, and Hoechst 33342 for healthy cells. PAPLAL did not show cell toxicity at these concentrations (Figure 1).

3.2 | Effect of PAPLAL on iNOS, TNF-α, and YM-1 levels of M0 macrophages

PAPLAL from 0.1% to 10% did not show any effects on the induction of the protein levels of iNOS, TNF-α, and YM-1 in M0 macrophage derived from rat bone marrow (Figure 2).

3.3 | Effect of PAPLAL on M1-skewing macrophage

Rat BMDMs were cultured for 24 hours with the medium containing PAPLAL simultaneous treatment with LPS and IFN-γ,

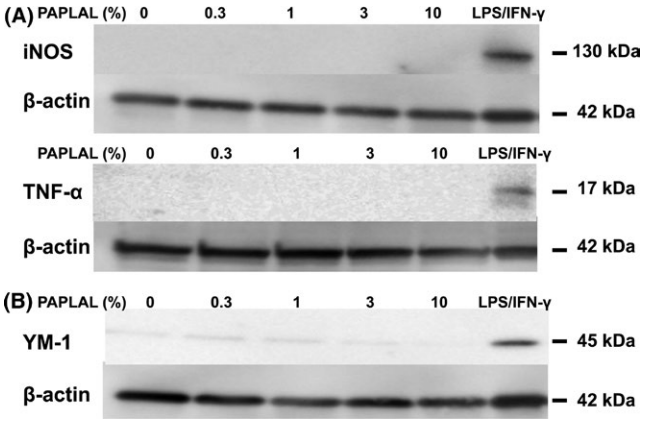

FIGURE 2 Effect of Pt and Pd nanocolloid (PAPLAL) on naïve macrophages. Bone marrow-derived macrophages (BMDMs) were supplemented with fresh Dulbecco's modified Eagle's medium with 15% fetal bovine serum with addition of various dose of PAPLAL solution (0.1%-10%) and cultured for 24 h. LPS + INF-γ and IL-4 + IL-13 represent positive control of M1 and M2 skewing with the treatment of LPS (5 ng/mL) + IFN-γ (1 ng/mL) and IL-4 (10 ng/mL) + IL-13 (10 ng/mL), respectively. A, Representative Western blot images showing M1 marker (iNOS and TNF-α) levels. B, Representative Western blot image showing M2 marker, YM-1 level

suppressed TNF-α induction at the dose of 3% and 10%, and suppressed iNOS induction at the dose of 1%, 3%, and 10% by protein level. Relative intensity of TNF-α and iNOS protein level induced by LPS and IFN-γ with simultaneous treatment with PAPLAL are shown in Figure 3A,B, respectively. Results are means ± SE of 6-8 experiments.

For better understanding of the effect of PAPLAL on inflammatory and immunologically disturbed skin, in relevance to macrophage, one of the human macrophages, THP-1, was used. In the study of THP-1, almost similar results to those of rat macrophages were obtained (Figure 4). Simultaneous PAPLAL treatment with M1 polarization factors, LPS and IFN-γ, for 24 hours, clearly and

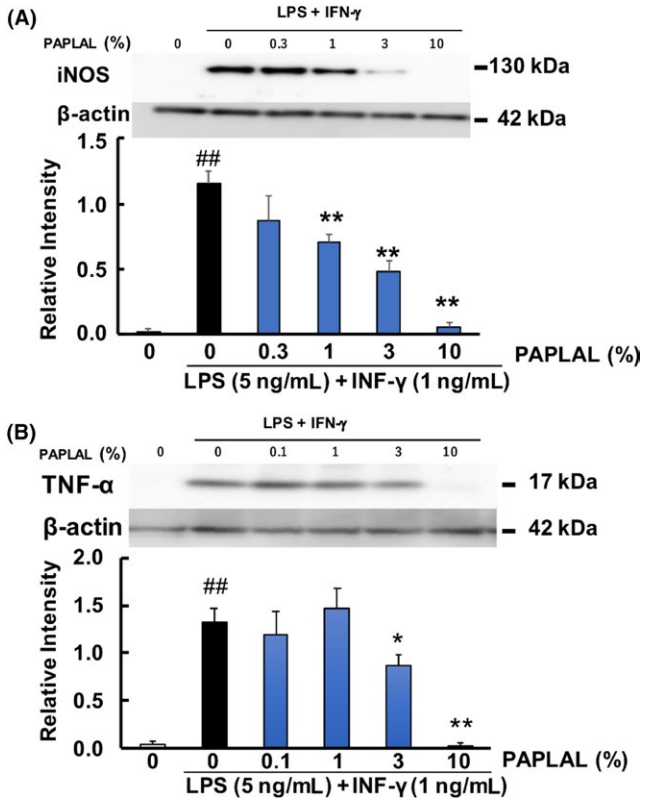

FIGURE 3 Effect of Pt and Pd nanocolloid (PAPLAL) on M1 marker protein expression. Bone marrow-derived macrophages (BMDMs) were supplemented with fresh Dulbecco's modified Eagle's medium with 15% fetal bovine serum and stimulated with LPS (5 ng/mL) + IFN-γ (1 ng/mL) with immediate addition of various dose of PAPLAL solution (0.1%-10%). Cells were incubated for 24 h and then washed twice with PBS, and collected cells were solubilized in 150 μL REPA buffer. The levels of iNOS protein expression were assessed by Western blotting. A, Representative Western blot image showing iNOS levels at 24 h following stimulation with LPS + IFN-γ and densitometric analysis of iNOS blots. B, Representative Western blot image showing TNF-α levels at 24 h following stimulation with LPS + IFN-γ and densitometric analysis of TNF-α blots. Results are means ± SE of 6-8 experiments. ##: $P < 0.01$ as compared to control group (naïve BMDM). *: $P < 0.05$, **: $P < 0.01$ as compared to PAPLAL non-treated group

obviously suppressed the expression of M1 marker genes, CXCL10, SOCS3, and IRF5. These results obtained from both rat and human macrophage studies indicate that PAPLAL may contribute to recovery of normal immune state by attenuation of causal inflammatory environment developed in vitiligo skin.

3.4 | Effect of PAPLAL on M2-skewing macrophage

Bone marrow-derived macrophages treated with IL-4 and IL-13 for 48 hours produced IRP-1, YM-1, and mannose receptor proteins, and production levels of these proteins were suppressed statistically significantly with simultaneous addition of PAPLAL at the dose of 10% (Figure 5). IRP-1 and YM-1 were suppressed significantly even at the dose of 3% PAPLAL.

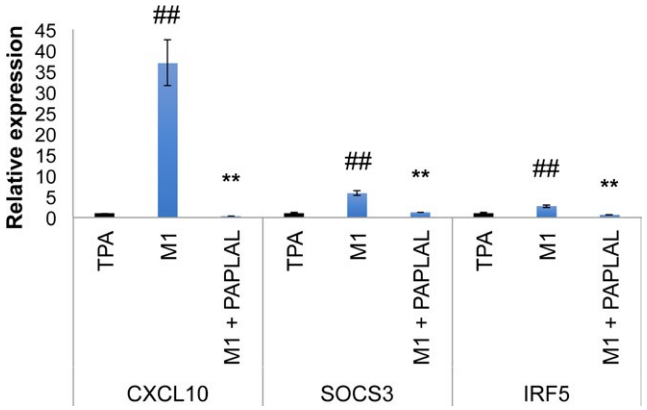

FIGURE 4 Effect of Pt and Pd nanocolloid (PAPLAL) on human M1-polarized macrophage. Human monocytic cell line, THP-1, was differentiated into macrophage with 200 ng/mL 12-O-tetradecanoylphorbol-13-acetate (TPA). M1 polarization was carried out with LPS (100 pg/mL) + IFN-γ (20 ng/mL) and simultaneously incubated with (M1 + PAPLAL) or without (M1) the 10% PAPLAL solution during the last 24 h of polarization. The expression of indicated M1 marker genes (CXCL10, SOCS3, IRF5) was analyzed by real-time PCR. Results are means ± SE of three experiments. ##: $P < 0.01$ as compared to non-activated control (TPA). **: $P < 0.01$ as compared to PAPLAL non-treated macrophage

In the THP-1-derived human macrophage polarization study, we observed almost similar results from those of rat macrophages (Figure 6). Simultaneous PAPLAL treatment for 24 hours with M2 polarization factors, IL-4 and IL-13, suppressed the expression of M2 marker genes, CD209, FGL2, and PPARG. While the expression of one of the M2 markers, CCL22, was slightly enhanced in human macrophage, the expression of CCL22 protein was obviously suppressed in BMDM by 10% PAPLAL treatment (data not shown). These results suggested that PAPLAL may play a role in the regulation of immunological environment via relatively weak downregulation of M2 compared to strong downregulation of M1, which contribute to the acquisition of the less or non-inflammatory environment of vitiligo skin, leading to the recovery of melanocyte function in vitiligo skin.

3.5 | Effect of PAPLAL on the phagocytic activity of human macrophage

Phagocytic activity of M0, M1, and M2 cells treated with and without simultaneous addition of PAPLAL with each polarization factors evaluated with IBRf was shown in Figure 7. PAPLAL did not alter phagocytic activity of these cells.

4 | DISCUSSION

The pathogenesis of vitiligo still remains as a challenging target among dermatologists and basic scientists. Loss of function or death of melanocytes in epidermis and hair follicles is the cause of whitening of the skin and hairs. The oxidative stress is proposed as an initial pathogenic factor for melanocyte degradation in vitiligo.[22,23] In acute

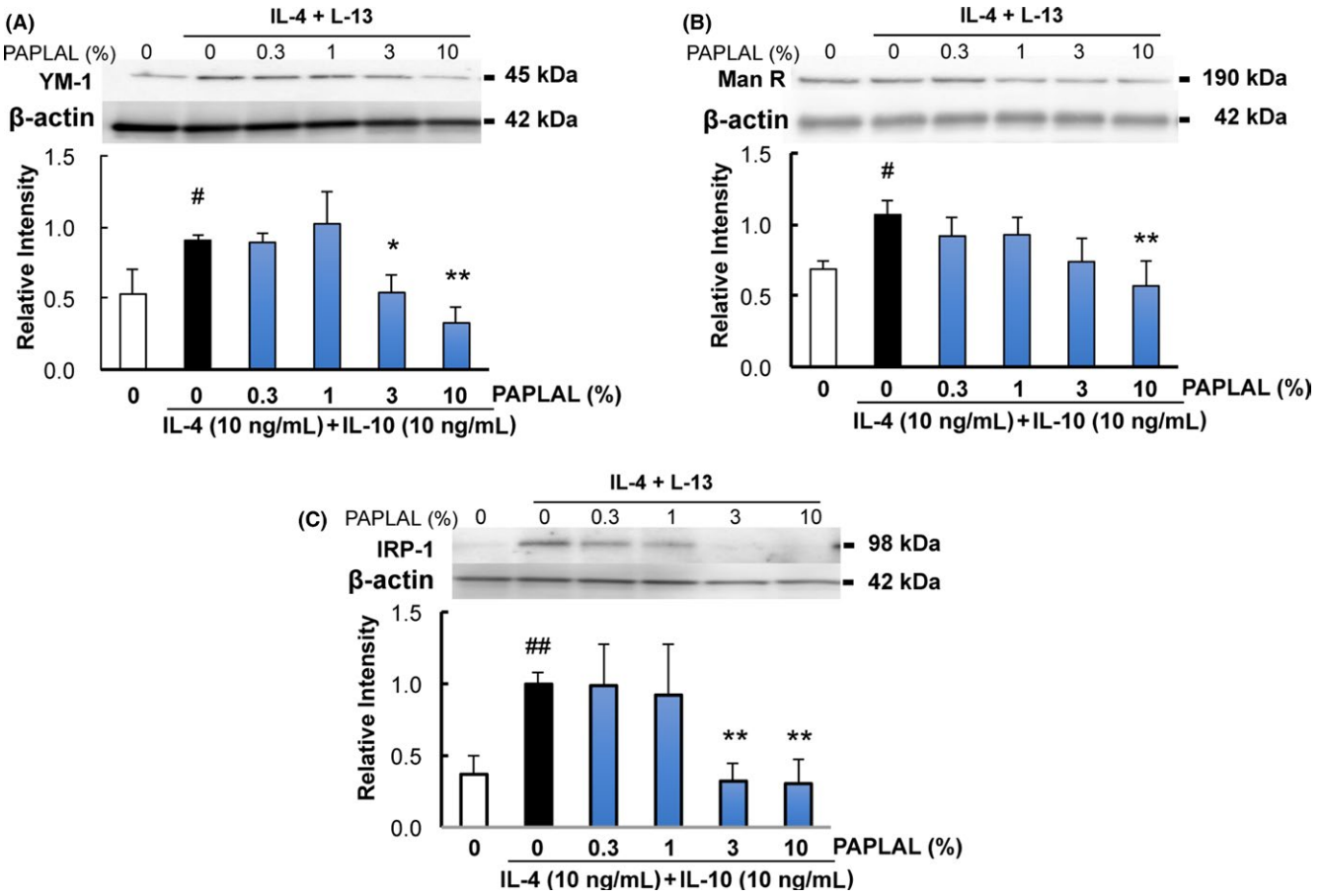

FIGURE 5 Effect of Pt and Pd nanocolloid (PAPLAL) on M2 marker protein expression. Bone marrow-derived macrophages (BMDMs) were supplemented with fresh Dulbecco's modified Eagle's medium with 15% fetal bovine serum and stimulated with IL-4 (10 ng/mL) + IL-13 (10 ng/mL) with immediate addition of various dose of PAPLAL solution (0.3%-10%). Cells were incubated for 48 h and then washed twice with PBS, and collected cells were solubilized in 150 μL RIPA buffer. The levels of M2 marker protein expression were assessed by Western blotting. A, Representative Western blot image showing YM-1 levels at 48 h following stimulation with IL-4 + IL-13 and densitometric analysis of YM-1 blots. B, Representative Western blot image showing Man R levels at 48 h following stimulation with IL-4 + IL-13 and densitometric analysis of Man R blots. C, Representative Western blot image showing iron regulatory protein-1 (IRP-1) levels at 48 h following stimulation with IL-4 + IL-13 and densitometric analysis of IRP-1 blots. Results are means ± SE of 6-8 experiments.#: $P < 0.05$, ##: $P < 0.01$ as compared to control group (naïve BMDM). *: $P < 0.05$, **: $P < 0.01$ as compared to PAPLAL non-treated group

vitiligo epidermis, increased production of H_2O_2 has been reported with additional lower activity of catalase, which degrades H_2O_2 into O_2 and H_2O.[1,24] Disturbed redox state may induce etiological inflammatory environment, leading to the development of vitiligo skin.

We have recently found that PAPLAL, platinum and palladium nanocolloid with strong catalase-like activity, stimulated the mRNA expression of both aryl hydrocarbon receptor (AhR) and nuclear factor erythroid 2-related factor 2 (Nrf-2), and induced the expression of target gene cytochrome p450 1A1 (CYP1A1) and NAD(P)H quinone dehydrogenase-1 (NQO-1), respectively, in cultured human keratinocytes. Further, we showed that CXCL10 expression, which may play a pivotal role in the development and exacerbation of vitiligo, was suppressed in keratinocytes by PAPLAL via AhR pathway.[25]

As far as we know, a role of macrophages in inflammatory vitiligo skin has been reported to scavenge the debris of melanocytes killed possibly by ROS activities and/or autoimmune response. However, there is no study reporting a role of macrophages in the regulation of vitiligo immunity in relevance to macrophage polarization, probably owing to lack of scientific methods to define clearly the resident and infiltrated macrophage characteristics in the skin. Here, we showed the strong and weak suppression of M1 and M2 macrophage activity, respectively, in vitro, by PAPLAL, suggesting a role of immune state alteration in vitiligo pathogenesis and the mechanisms of the therapeutic effects of PAPLAL in vitiligo skin. In addition, PAPLAL had no suppressive effect on the phagocytic activity of M0, M1, and M2 macrophages.

In the present study, we confirmed non-toxic effect of PAPLAL on macrophages by its effects on apoptosis, cell growth, and phagocytic activity; then, we evaluated a therapeutic effect of PAPLAL on vitiligo by analyzing the effect on macrophage activation which is recognized to be one of the key players controlling inflammatory immune

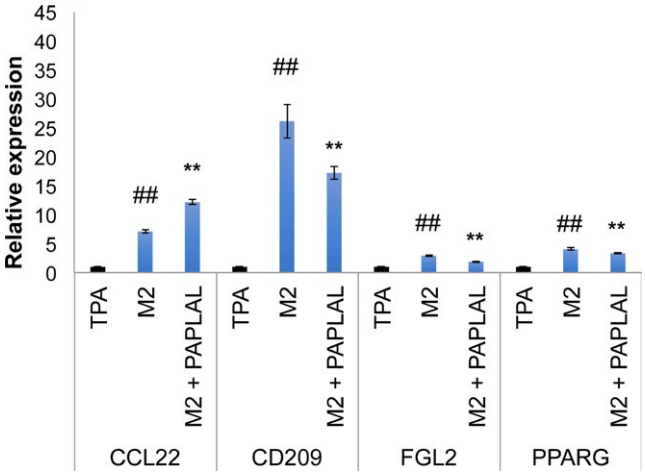

FIGURE 6 Effect of Pt and Pd nanocolloid on human M2-polarized macrophage. Human monocytic cell line, THP-1, was differentiated into macrophage Results are means ± SE of three experiments $^{\#\#}P < 0.01$ as compared to non-activated control (TPA). $^{**}P < 0.01$ as compared to PAPLAL non-treated macrophage

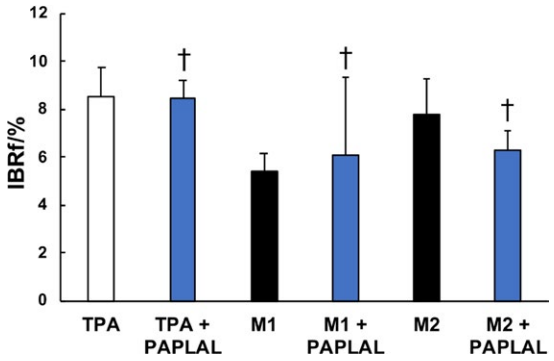

FIGURE 7 Effect of Pt and Pd nanocolloid (PAPLAL) on the phagocytic activity of human macrophage. Human monocytic cell line, THP-1, was differentiated into macrophage (M0) with 200 ng/mL 12-O-tetradecanoylphorbol-13-acetate (TPA). M1 or M2 polarization was carried out with LPS (100 pg/mL) + IFN-γ (20 ng/mL) or with IL-4 (20 ng/mL) + IL-13 (20 ng/mL). PAPLAL treatment (+ PAPLAL) was simultaneously incubated with 10% PAPLAL solution with polarization treatment. Phagocytic activity was evaluated by internalized beads ratio measured by fluorescence (IBRf). Details were described in Ishikawa et al.[21] Results are means ± SE of three experiments. †: $P > 0.9$ as compared to PAPLAL non-treated macrophage

reaction. We speculated before the present study that PAPLAL may suppress M1 macrophage polarization induced by LPS and IFN-γ treatment, and may enhance M2 macrophage activation by IL-4 and IL-13. However, PAPLAL did not enhance the polarization of M2, rather, slightly suppressed although apparently weakly compared to M1 suppression. PAPLAL suppressed M2 polarization at the protein levels of chitinase 3-like 3 (YM-1), IRP-1, and mannose receptor (Man R) in rat macrophage, and mRNA level of CD209, FGL2, and PPAR-γ in human macrophage by IL-4 and IL-13 activation. Further, the protein level of iNOS and TNF-α of rat M1 macrophages, and mRNA levels of CXCL10, SOCS3, and IRF5 of in human M1 macrophage were strongly suppressed by PAPLAL. In the present study, we found that M2 activation was weakly downregulated by PAPLAL, compared to those of M1. These suppressive effects of PAPLAL on M2 polarization were unexpected but observed in both rat and human macrophages. Interestingly, only in the human macrophage, PAPLAL increased the expression of CCL22, one of the M2 markers which is reported to increase the frequency and recruitment of Treg in autoimmune tissue,[26] suggesting that the therapeutic effect of PAPLAL might be exerted via Treg functions. The present study may suggest that anti-inflammatory skin conditions inducible by macrophage regulation with PAPLAL play a role in the recovery of normal functional immune state in vitiligo skin where melanocytes can survive and produce melanin.

Increased systemic and epidermal levels of IL-17A and IL-1β, and decreased level of TGF-β are proposed to be an immunological cause of vitiligo, although a role of TGF-β in vitiligo is not clear yet owing to a controversial publication showing increased level of TGF-β in vitiligo skin.[27]

Activation of AhR of keratinocytes by PAPLAL strongly indicates the induction of indoleamine dioxygenase (IDO)[28] and hence Tregs, and recovering of healthy environment for melanocytes in epidermis. In future, we are interested in the study of the effect of PAPLAL on IDO activation in epidermal keratinocytes and melanocytes. Further, it is crucial to study and confirm a dynamic involvement of skin-resident macrophages and possible migration of blood-derived macrophages in vitiligo skin relevant to disease phase.

During the past decade, the link between the redox imbalance caused by high toxic metabolites and free radicals leading to a defective antioxidant state of melanocytes and immune-mediated damage of the melanocytes has been proposed as the cause of vitiligo.[29,30]

Conclusively, our present finding suggests that PAPLAL with strong catalase-like activity is an efficient therapeutic agent of vitiligo by recovering healthy skin environment by skewing the antiinflammatory state of vitiligo environment with the delicate regulation of the M1 and M2 macrophage activity, in addition to our previous findings of the efficacy of PAPLAL showing potent antioxidant activity and a possible prevention of melanocytes against cytotoxic T-cell attack via AhR activation. According to these predicted mechanisms of anti-inflammatory and antioxidant activities, PAPLAL is also expected to have therapeutic effects on skin diseases with disturbed immunity such as atopic dermatitis and alopecia areata, and preventive effects on photo-aging of the skin.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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