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Original Article

TLR3 stimulation induces melanosome endo/phagocytosis through RHOA and CDC42 in human epidermal keratinocyte

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ABSTRACT

Background: Keratinocytes and melanocytes in human epidermis express Toll-like receptors (TLR) and induce immune responses. We previously reported that TLR3 stimulation increases melanosome transport from perinuclear to cell membrane in melanocytes and enhanced release of melanosome from melanocytes, which were followed by increase in melanosome uptake into keratinocytes.
Objective: In this study, we investigated whether TLR3 stimuli directly affect keratinocytes to enhance melanosome uptake.
Methods: To observe keratinocyte's melanosome uptake ability precisely without melanocytes influences, we isolated melanosomes from human melanocytes and applied isolated melanosomes to keratinocytes stimulated by Poly(I:C).
Results: Poly(I:C)-stimulated keratinocytes enhanced uptake of isolated melanosome-rich globules five-times as much as control. Poly(I:C) increases the RNA and protein expressions of RHOA and CDC42, which are small GTP-binding proteins inducing the endocytosis. Pull-down assay showed that Poly(I:C) increased the GTP-binding RHOA and CDC42, suggesting TLR3 stimulation activated RHOA and CDC42. The knockdown of TLR3 suppressed *RHOA* and *CDC42* induction by Poly(I:C). Consistently, the knockdown of RHOA and CDC42 significantly suppressed the melanosome-rich globules uptake by Poly (I:C)-stimulated keratinocytes.
Conclusion: Because RHOA and CDC42 activation induces endocytosis by modification of actin stress fiber and filopodia formation, respectively, these results suggested that TLR3 stimulation enhances melanosome uptake into keratinocytes through endocytosis mechanisms. Combining with the data of our previous publications, TLR3, which signal is activated by sensing viral molecules, enhance pigmentation by controlling both melanin transport system by RAB GTPases induction in melanocytes and uptake system by RHOA and CDC42 in keratinocytes.

1. Introduction

Toll-like receptors (TLRs) are part of the innate immune system involved in the response to microbial pathogenic molecules. TLR2 recognizes bacterial lipoprotein, lipopeptide, and peptidoglycan expressed by Gram-positive bacteria, while TLR3 responds to virus double stranded RNA [1–3]. Epidermal keratinocytes express TLR1-6 and 9 and respond to their respective ligands to produce cytokines, and chemokines, and to activate NF-κB [4–9]. Human melanocytes also express TLRs. We previously showed that TLR2 agonist HKLM increases melanogenetic genes tyrosinase (*TYR*) and dopachrome tautomerase (*DCT*) and increase melanin contents and release from melanocytes through RAB11A-dependent manner [10,11]. While, TLR3 agonists Poly(I:C) enhance melanosome

Abbreviations: MRG, melanosome-rich globules; TLR, Toll-like receptor; HKLM, heat-killed preparation of Listeria monocytogenes; UVB, ultraviolet B; TYR, tyrosinase; DCT, dopachrome tautomerase; PMEL, pre-melanosome protein; DAPI, 4'6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GTP, guanosine triphosphate; GDP, guanosine diphosphate; PAR2, protease activated receptor 2; RHO, Ras homologous; CDC42, cell division cycle42; RAC1, RAS-related C3 botulinus toxin substrate 1; GEFs, guanine nucleotide exchange factors; GAPs, GTPase-activating proteins; ROCK, Rho-associated coiled-coil containing protein kinase.

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transportation by RAB27A induction through TLR3 and intracellular signaling molecules TRIF and MAVS [10,12]. The low molecular weight G protein RAB27A and RAB11A are RAB family members, and RAB GTPases are regulatable switches to recruit effector molecules and control intracellular vesicle trafficking [13]. Thus, melanocytes enhance melanin production and transfer by TLR2 and TLR3 innate immune system.

Keratinocytes also play roles in skin pigmentation. To protect the human body from UVB, synthesized melanosome and melanin are transferred to neighboring keratinocytes from melanocytes, which results in the pigmentation of skin [14]. Regarding melanosome transfer from melanocyte to keratinocytes, there are four proposed mechanisms; cyto-phagocytosis, membrane fusion, shedding-phagocytosis, and exocytosis-endocytosis [15]. Except the membrane fusion model, melanin uptake requires endocytosis process of keratinocytes. The endocytic and phagocytic uptake is actin-dependent. Actin polymerization drives a zipper-like capture of phagocytic targets by phagocytes and a triggered intake of invasive bacteria by non-professional phagocytes [16]. These actin-dependent processes requires RHO GTP-binding proteins, which are evolutionarily conserved proteins and control cytoskeletal dynamics [17]. In human, 22 distinct genes encode at least 25 Rho-family GTPases [18]. Among them, the functions of RHO (Ras homologous) and, RAC (RAS-related C3 botulinus toxin substrate), and CDC42 (cell division cycle42) have been extensively characterized. The activation of RAC and CDC42 induces formation of lamellipodia and filopodia, respectively, which are essential machineries of endocytosis [19,20].

Since we observed TLR3 agonist Poly(I:C) enhanced melanosome transfer to keratinocytes in the co-culture of human melanocytes and keratinocytes, we sought in this study whether TLR3 agonists directly affect keratinocytes to enhance melanosome uptake. For this aim, we isolated melanosome-rich globules (MRGs) from human epidermal melanocytes and applied them to human keratinocytes stimulated by TLR3 agonist Poly(I:C). We also observed the dynamics of Rho-family GTPases RHOA and CDC42 in Poly(I:C)-stimulated keratinocytes and examined whether RHOA and CDC42 are involved in TLR3 agonist-dependent MRGs uptake to keratinocytes.

2. Materials and methods

2.1. Isolation of MRGs released from normal human epidermal melanocytes

The isolation of MRGs was performed as previously described by Ando et al. [21]. Briefly, normal human epidermal melanocytes (Kurabo, Osaka, Japan) were seeded at 2×10^6 cells per 10 ml DermaLife® Basal Medium supplemented with StiMel8™ Life Factor containing fetal bovine serum (FBS) (1 % V/V), L-glutamine (6 mM), epinephrine (1 μM), insulin (5 μg ml^{-1}), ascorbic acid (50 μg ml^{-1}) and calcium chloride solution (0.2 mM) (Lifeline Cell Technology, Walkersville, MD). The culture media of normal human melanocytes were collected and centrifuged to precipitate MRGs and floating melanocytes. The pellets were resuspended with HuMedia-KG2 (Kurabo, Osaka, Japan), and the MRGs were purified by passing through the filter of six-well Millicell 8 μm-pore PET (PIEP30R48; Millipore, Billerica, MA) to remove floating melanocytes and the MRGs were finally precipitated by centrifugation. The pellets of MRGs were covered with a small amount of culture medium to prevent desiccation and were stored at −20 °C until use. The pellets of MRGs were resuspended by 100 μl of HuMedia-KG2 with supplement and equal volume of solution were added to the keratinocyte culture medium after each stimulation.

2.2. Isolation of melanosomes and melanocores

The isolation of melanosomes and melanocores were performed as previously described by Ishida et al. [22]. Briefly, normal human melanocytes were cultured as described above. Then, confluent melanocytes were scraped off and homogenized in a homogenization buffer by passing through a 25-G needle 15 times. The precipitates were washed twice with the homogenization buffer and centrifugation. The pellets were resuspended with the homogenization buffer and used as a melanosome-rich fraction (melanosomes). To isolate melanocores, the supernatants from melanocytes homogenates were centrifuged, and the precipitates were lysed with a lysis buffer and incubated at 4 °C for 20 min on a rotary machine. After centrifugation, the precipitates were washed twice with the homogenization buffer and recentrifuged. The pellets were resuspended with the homogenization buffer by passing through a 27-G needle 10 times and used as a melanocore-rich fraction (melanocores). The equal volume of each fraction was added to the keratinocyte culture medium.

2.3. Keratinocyte culture and incubation with melanin fractions

Normal human epidermal keratinocytes (Kurabo, Osaka, Japan) were cultured in HuMedia-KG2 (Kurabo, Osaka, Japan) supplemented insulin (10 μg ml^{-1}), hEGF (0.1 ng ml^{-1}), hydrocortisone (0.5 μg ml^{-1}), antibiotics containing gentamicin (50 μg ml^{-1}) and amphotericin B (50 ng ml^{-1}), and FBS (0.4 % V/V) (Kurabo). Keratinocytes were seeded in 6-well plates or 4-well Lab-Tek™ chamber slide at a cell density of 1×10^5 cells/well or 2×10^4 cells/well, respectively. Cells at 80 % confluence were stimulated with TLR2/2 agonist heat-killed preparation of *Listeria monocytogenes* (HKLM) (10^8 cells ml^{-1}) or TLR3 agonist Poly(I:C) (1 μg ml^{-1}), and cultured with each melanin fraction for 24 h.

2.4. Immunofluorescence staining

To analyze melanosomes and MRGs in keratinocytes, we used a model previously reported [10,23]. Keratinocytes were incubated with melanin fractions in 4-well Lab-Tek™ chamber slides (Nunc, Rochester, NY, USA) for 24 h, and then PMEL/Gp100 and keratin were visualized. The detail of the immunofluorescence staining procedure is described in the supplementary information. We get representative images in 4 independent experiments.

2.5. Quantitative analysis of melanosome and melanocore transfer

Quantitative analysis of melanosome transfer was performed as previously described [10,23]. Briefly, the blinded person counted the PMEL/Gp100-positive dots (red dots) in the keratin-positive cells (green cells) in 25 randomly selected fields under confocal microscopy with a 40-fold objective lens. The keratinocyte numbers were determined by the DAPI-positive nucleus in the keratin-positive cells, and each field contained about 15–20 keratinocytes. Thus 375–625 cells were counted in each well. Then we calculated the mean of the Gp100-positive dots per keratinocytes. The quantification of melanocores in keratinocytes were done by counting black spots in the bright field images. We repeated this 4 times as independent experiments and the mean and the standard deviation of the 4 independent experiments are plotted on the graph.

2.6. Western blotting

Keratinocytes were seeded into 6-well plates at a cell density of 1×10^5 cells/well, cultured to 80 % confluence, and then stimulated with or without Poly(I:C) for 24 h. The datil of sample collection and the Western blotting procedures are described in the

supplementary information. The primary antibodies used are: mouse anti-human actin antibody (Sigma Aldrich, 1:1000), rabbit anti-human PAR2 antibody (Abcam, 1:1000), rabbit anti-human RHOA antibody (Abcam, 1:1000) or rabbit anti-human CDC42 antibody (Abcam, 1:500). Intensities of bands were semi-quantified using the ImageJ program (https://imagej.nih.gov). These results were confirmed in 2 independent experiments.

2.7. SiRNA transfection

Keratinocytes were seeded prior to transfection. The cells were transfected with stealth RNA of TLR3 (HSS110815) (Invitrogen, Carlsbad, CA, USA), PAR2 (HSS103471, HSS103472, HSS103473), RHOA (s758) and CDC42 (s227090, s55524) (ThermoFisher Scientific, USA) using Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's instructions. After 24 h incubation at 37 °C, cells were stimulated with TLR agonists. Silencing effects by specific siRNA were confirmed by quantitative RT-PCR. We compared cells treated with transfection reagent with siRNA and cells treated with the negative control siRNA; Stealth™ RNAi Negative Control Medium GC Duplex (ThermoFisher Scientific, USA) to eliminate possibilities of off-target effects by a negative control siRNA.

2.8. Quantitative reverse transcription polymerase chain reaction (RT-PCR)

Twenty-four hours after stimulation by the TLR agonists, total RNA was extracted using RNeasy Mini Kit (Qiagen) following manufacturer's instructions. The cDNA synthesis and quantitative PCR were performed as previously described [24]. *GAPDH* was amplified with the sense primer 5'- GAA GGT GAA GGT CGG AGT C -3' and the antisense primer 5'- GAA GAT GGT GAT GGG ATT TC -3'. We used TaqMan Gene Expression Assay sets to detect *TLR3* (Hs00706140_s1), PAR2*(F2RL1)* (Hs00608346_m1), *RHOA* (Hs00357608_m1) and *CDC42* (Hs00918044_g1). These Results were confirmed in 3 independent experiments.

2.9. RHOA and CDC42 activation assay

The activation of RHOA and CDC42 were determined by RhoA/Rac1/Cdc42 Activation Assay Combo Biochem Kit™ (Cytoskeleton Inc., York, UK) following the manufacturer's instructions. Briefly, rhotekin-RBD effector domain affinity beads were used to bind active RHOA, and PAK-PBD effector domain affinity beads were used to bind active CDC42. After 2 h incubation, the proteins were eluted with Laemmli buffer and separated on 12 % acrylamide/bis-acrylamide gels. The gels were transferred to nitrocellulose membranes, which were incubated overnight with antibodies against RHOA and CDC42 with gentle agitation. The proteins were detected by Western blotting.

2.10. Statistical analysis

Statistical analysis was performed using one-way ANOVA and Graph Pad Prism 5 (Graph Pad Software, La Jolla, CA). Statistically significant differences are denoted with asterisks: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All experiments were repeated at least three times, and representative data of triplicated samples are shown as the mean ± SD in the figures, unless otherwise specified.

3. Results

3.1. TLR3 agonist Poly(I:C) increases the MRGs uptake by keratinocytes

To examine if TLRs stimuli directly affect keratinocytes to enhance melanosome uptake, we stimulated normal human epidermal keratinocytes with TLR2 agonist HKLM (heat-killed preparation of *Listeria monocytogenes*) or TLR3 agonist Poly(I:C), and then incubate with the equal amount of isolated MRGs. After 24 h incubation, melanosomes and keratin were visualized by immunofluorescence staining of PMEL/Gp100 and keratin, respectively, and the number of melanosomes localizes in keratinocytes were detected. Keratinocytes stimulated by Poly(I:C), but not TLR2 agonist HKLM, increased uptake of isolated MRGs about four to five times as much as no stimuli control (Fig. 1A, B). These results suggested that TLR3 stimuli enhanced keratinocytes to increase MRGs uptake through endocytosis without co-culturing of melanocytes.

3.2. Poly(I:C) increases keratinocyte's uptake of melanosomes and melanocores as well as MRGs

It is observed that melanin release and uptake are performed in the state of MRGs, melanosomes and melanocores [15]. To examine if Poly(I:C) increase only MRGs uptake, we prepared isolated melanosomes and melanocores and added to Poly(I:C)-stimulated keratinocytes. The melanosomes uptake was significantly increased by Poly(I:C), which was quantitatively confirmed by Gp100-positive particles in keratinocytes (Fig. 2). Since melanocores were hardly detectable by Gp100 staining, we observed the melanocore uptake in the bright field images. In the images of controls without Poly(I:C) stimulation, we observed diffuse darker background on the slide treated with melanosomes and melanocores because melanosomes and melanocores are sticky to the glass slide (Fig. 2). We observed the accumulation of melanosomes and melanocores as well as MRGs in keratinocytes stimulated with Poly(I:C) in the bright field images. These suggested that TLR3 agonist Poly(I:C) enhanced all currently proposed models of melanin particle uptake by keratinocytes.

To further examine if Poly(I:C) enhances keratinocyte endo/phagocytosis in general for any particles, we added fluorescence-labeled microspheres (0.5 and 1 μm) to Poly(I:C)-stimulated keratinocytes. Contrary to the increase in melanin uptake, Poly (I:C) decreased uptake of both 0.5 and 1 μm microspheres in a half of the control keratinocytes (Supplementary Figure). These data suggested that endo/phagocytosis of keratinocytes induced by Poly (I:C) is relatively specific for uptake melanin-relating particles.

3.3. Poly(I:C) increases protease-activated receptor-2 (PAR2) expression in keratinocytes through TLR3

Since the protease-activated receptor-2 (PAR2) enhances keratinocyte endocytosis ability and uptake of melanosome [25,26], we examined if TLR3 stimuli enhance PAR2 expression in keratinocytes. Poly(I:C) increased the expression of PAR2 protein (Fig. 3A). Poly(I:C) also enhanced *TLR3* and *F2RL1*(*PAR2*) mRNA expression in keratinocytes (Fig. 3B, C). Presence of TLR3 siRNA suppressed *F2RL1*(*PAR2*) induction along with the suppression of *TLR3* (Fig. 3B, C). These results indicated that Poly(I:C) enhanced PAR2 expression through TLR3 in keratinocyte.

3.4. The knockdown of PAR2 decreases over all uptake of MRGs but has little effect on Poly(I:C)-dependent increase in MRGs uptake

To examine whether Poly(I:C) enhances melanin uptake through PAR2, we used siPAR2 and confirmed *F2RL1(PAR2)* suppression in keratinocytes (Fig. 3D). All three siPAR2 (siPAR2-a, b, c) significantly reduced MRGs uptake in unstimulated control and Poly(I:C) stimulated keratinocytes compared to keratinocytes treated with the mock siRNA (NC) (Fig. 3E, F). However, very interestingly, when we compared between no stimulation and the Poly(I:C)-stimulation in the siPAR2-pretreated keratinocytes, we observed the nearly twice increase in melanin uptake by Poly(I:C)

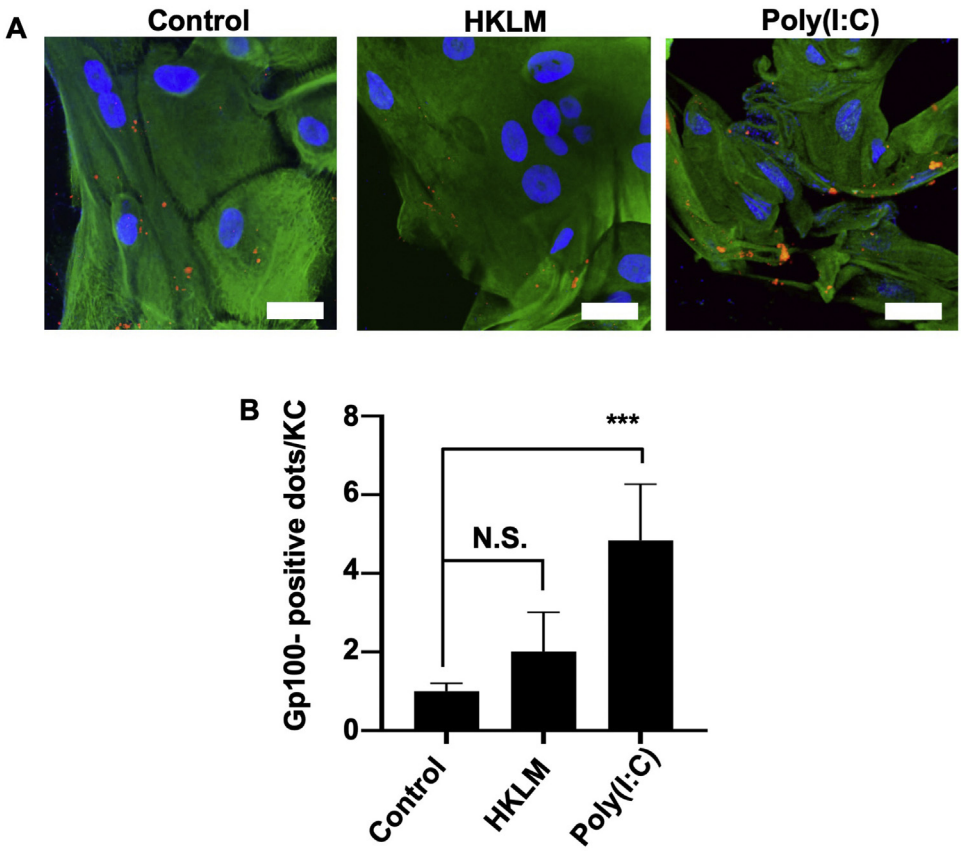


Fig. 1. Keratinocytes stimulated by Poly(I:C) increase the uptake of isolated MRGs.
Keratinocytes were treated with HKLM (10^8 cells ml^{-1}) or Poly(I:C) (1 μg ml^{-1}) for 24 h, and then added the equal amount of isolated MRGs. (A) After 24 h incubation, PMEL/Gp100 (red) and keratin (green) were visualized by immunofluorescence staining and the level of MRGs transfer to keratinocytes was examined. Representative images of 3 independent experiments are shown. Scale bars = 50 μm. (B) The number of PMEL/Gp100-positive dots per keratinocyte were counted as described in the methods and shown as mean ± SD in the graph. $^{***}P < 0.001$.

stimulation (Fig. 3E) The twice increase was also observed in Poly (I:C) stimulation compared to no stimulation in the mock siRNA-treated keratinocytes (NC in Fig. 3E, F). These results suggested that PAR2 influences over all melanin uptake, but Poly(I:C) likely controls melanin uptake in a PAR2-independent manner.

3.5. Poly(I:C) increases the expressions and activation of Rho-family member RHOA and CDC42

It is known that keratinocyte phagocytosis occurs in RHO-dependent manner [27]. Among RHO GTPase family, we examined if TLR3 stimuli affect RHOA and CDC42 expression in keratinocytes because RHOA and CDC42 are involved in endocytosis as well as in melanosome transfer from melanocytes through filopodia formation in melanocytes [28,29]. In western blotting analysis, Poly(I:C) increased protein expressions of RHOA and CDC42 in keratinocytes (Fig. 4A, B; upper row). We also confirmed that Poly(I:C) also increased the GTP-binding forms of RHOA and CDC42 (Fig. 4A, B; middle rows). In RNA level, Poly(I:C) enhanced *RHOA* and *CDC42* expressions, and siTLR3 suppressed *RHOA* and *CDC42* induction by Poly(I:C) (Fig. 4C, D). These results indicated that TLR3 stimuli increased RHOA and CDC42 mRNA and protein expression and activated RHOA and CDC42 shown as an increase of GTP-binding forms.

3.6. The knockdown of RHOA and CDC42 suppress the MRGs uptake by Poly(I:C)-stimulated keratinocytes

To examine if RHOA and CDC42 are involved in TLR3-dependent MRGs uptake, we pretreated keratinocytes with siRNA for *RHOA* and *CDC42* and stimulated by Poly(I:C). SiRHOA and siCDC42 suppressed *RHOA* and *CDC42* increase by Poly(I:C), respectively (Fig. 5A, B). Both siRHOA and siCDC42 suppressed uptake of isolated MRGs into keratinocytes induced by Poly(I:C) (Fig. 5C, D). These data indicated that TLR3 stimuli enhanced MRGs uptake by keratinocytes in the RHOA and CDC42-dependent manner.

4. Discussion

We have reported that TLR3 signaling increase melanosome release from human melanocytes by induction of RAB27, a member of Rab GTPase family [10,12]. In this study, we examined if TLR3 stimuli affect MRGs uptake into keratinocytes. Among the four proposed mechanisms of melanin transfer from melanocytes to keratinocytes; cyto-phagocytosis, membrane fusion, shedding-phagocytosis and exocytosis-endocytosis [15], the isolated MRGs method were performed to prove the shedding-phagocytosis model [21]. In our previous report, when melanocytes and keratinocytes were co-cultured, melanin transferred to keratinocytes under Poly(I: C) stimulation was mostly aggregated [30]. Therefore, we considered that melanin secretion induced by Poly(I: C) is mainly MRGs. Then we isolated MRGs from normal human melanocytes using the method developed by Ando et al. [21], and applied MRGs directly to keratinocytes to eliminate juxtacrine effects of co-cultured melanocytes. In this study, we observed that TLR3 agonist Poly(I:C) enhanced uptake of MRGs by keratinocytes (Fig. 1). We also observed TLR3 stimuli activated RHOA and CDC42 (Fig. 4), and suppression of RHOA and CDC42 reduced MRGs uptake by keratinocytes (Fig. 5). These observations indicated that TLR3

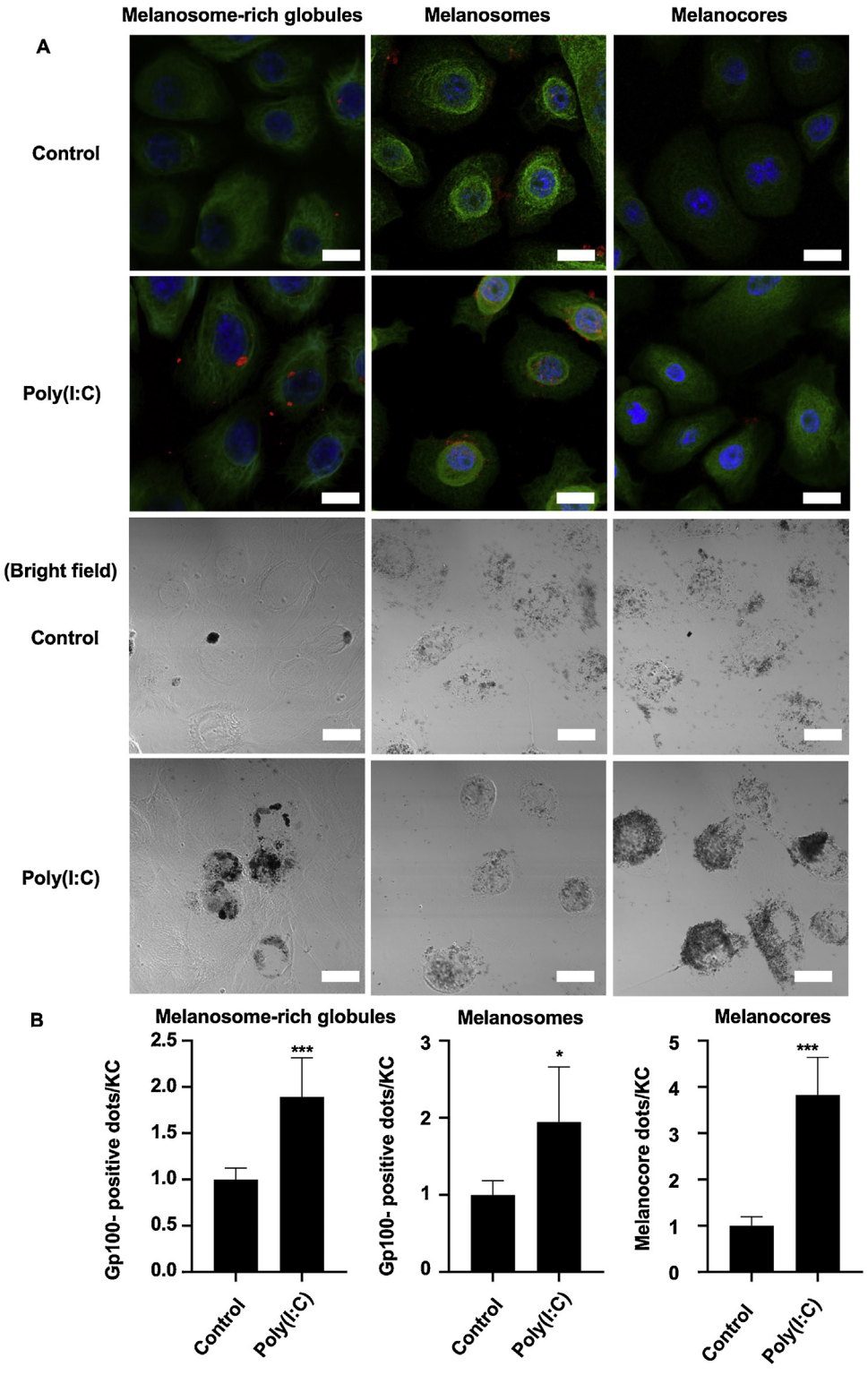


Fig. 2. Poly(I:C) induced uptake of MRGs, melanosomes and melanocores in keratinocytes.
Keratinocytes were treated with Poly(I:C) (1 μg ml^{-1}) for 24 h, and then added the equal amount of isolated MRGs, melanosomes, and melanocores. After 24 h incubation, PMEL/Gp100 (red) and keratin (green) were visualized by immunofluorescence staining and the level of melanin transfer to keratinocytes was examined. The bright-field images are also shown in the lower panels. Representative images of 3 independent experiments are shown. Scale bars = 30 μm. (B) The number of PMEL/Gp100-positive dots (which labeled melanosome-rich globules and melanosomes, but not melanocores) per keratinocyte were counted. The quantification of melanocores in keratinocytes were done by counting black spots in the bright field images. Mean ± SD are shown in the graph. $^{*}P < 0.05$, $^{***}P < 0.001$.

stimulation enhances melanin uptake by keratinocytes through RHOA and CDC42 activation.

We observed that TLR3 stimuli to keratinocytes enhanced PAR2 expression in keratinocytes (Fig. 3A–C). PAR2 regulates melanosome transfer in co-culture of melanocytes and keratinocytes [25]. PAR2 is expressed on keratinocytes but not on melanocytes, and PAR2 expression is enhanced by UV irradiation in human skin and cultured keratinocytes [26]. TLR3 not only

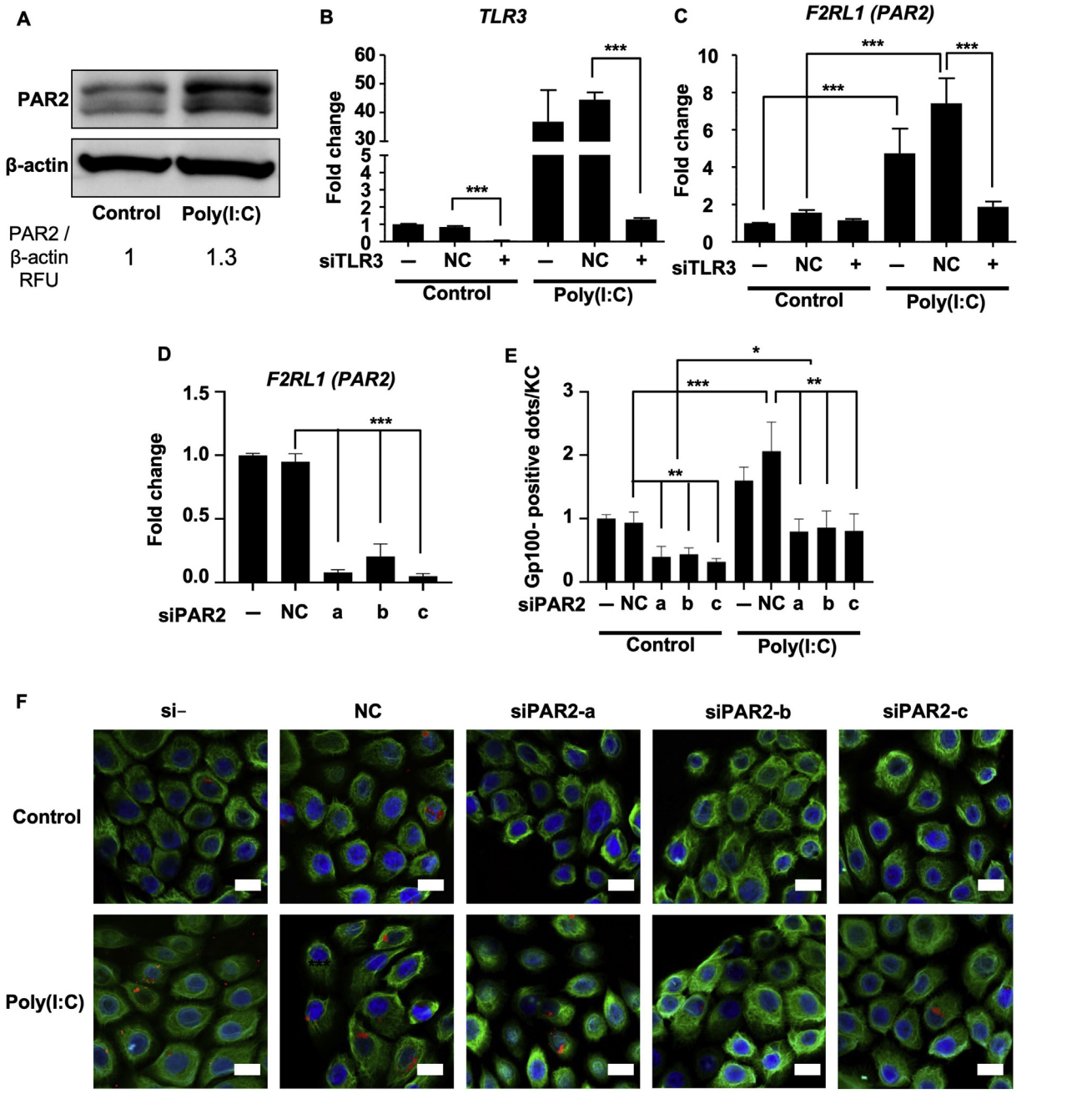
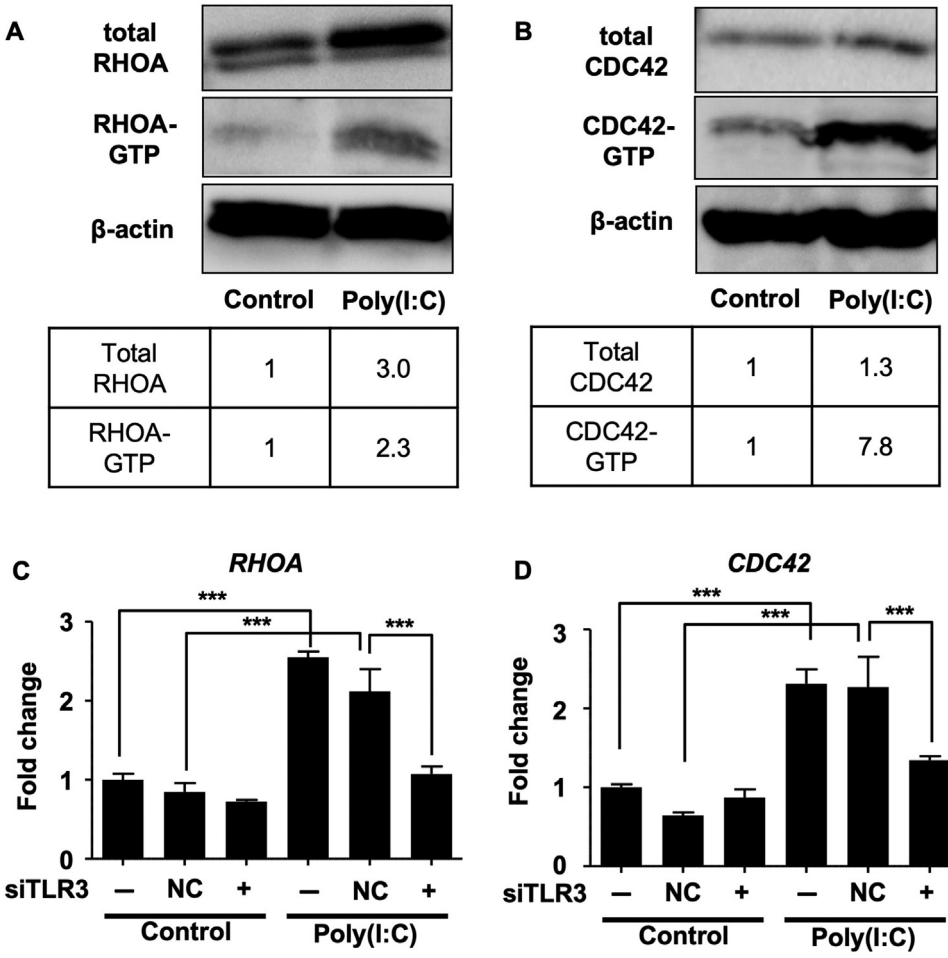


Fig. 3. PAR2 induction by Poly(I:C) and PAR2 involvement in Poly(I:C)-dependent MRGs uptake.
(A) Keratinocytes were incubated with Poly(I:C) (1 μg ml^{-1}) for 24 h, and the PAR2 protein levels were examined. β-actin served as the loading control. (B, C) Twenty-four hours after siTLR3 transfection, *TLR3* and *F2RL1* (*PAR2*) expressions in keratinocytes were confirmed by real-time PCR.
'–' and '+' mean samples treated without and with siTLR3, respectively. 'NC' indicates 'negative control siRNA. (D) Twenty-four hours siPAR2 transfection, *F2RL1* (*PAR2*) expressions in keratinocytes were confirmed by real-time PCR. '–' and 'NC' means samples treated without siPAR2 and 'negative control siRNA respectively. 'a', 'b', 'c' indicates individual siRNA of PAR2 (HSS103471, HSS103472, HSS103473). (E) Keratinocytes treated with siRNA of PAR2 for 24 h were stimulated by Poly(I:C) (1 μg ml^{-1}) and added an equal amount of isolated MRGs. After 24 h incubation, PMEL/Gp100 (red) and keratin (green) were visualized by immunofluorescence staining. Scale bars = 50 μm. (F) The number of PMEL/Gp100-positive dots per keratinocyte were counted as described in the methods and shown as mean ± SD in the graph. $^{*}P < 0.05$, $^{***}P < 0.001$.

respond to viral RNA represented by Poly(I:C), but also sense fragmenting noncoding RNA induced by UVB-irradiation in epidermal keratinocytes [10,31]. Stimuli for PAR2 and TLR3 cooperatively activate NFκB signaling [32]. In line with these previous observations, PAR2 knockdown decreased uptake of MRGs in keratinocytes (Fig. 3). Interestingly, when we compared PAR2 siRNA-pretreated keratinocytes between Poly (I:C) stimulation and unstimulated control, we observed that Poly(I:C) stimulation significantly increased melanin uptake about 2 folds more than unstimulated control though Poly(I:C)-induced melanin uptake was reduced in a half in the presence of siRNA for PAR2 (Fig. 3E). This observation indicated that knockdown of PAR2 did not completely suppress the increase in MRGs uptake by Poly(I:C). The results suggested that keratinocytes uptake MRGs majorly through PAR2-depdendnt mechanisms and that Poly(I:C) increased MRGs uptake partially through PAR2-independent mechanism. Furthermore, contrary to results of increased MRG, melanosome, and melanocore uptake (Fig. 2), we observed that Poly(I:C)-stimulated keratinocytes showed less microspheres uptake compared to unstimulated keratinocytes (Supplementary



Total RHOA	1	3.0
RHOA-GTP	1	2.3

Total CDC42	1	1.3
CDC42-GTP	1	7.8

Fig. 4. Poly(I:C) induces RHOA and CDC42 mRNA and protein expressions and activations in keratinocytes.
(A, B) Keratinocytes were treated with Poly(I:C) (1 μg ml^{-1}) for 24 h. GTP-binding RHOA (RHOA-GTP) and total RHOA (A), and GTP-binding CDC42 (CDC42-GTP) and total CDC42 (B) were visualized. β-actin served as the loading control. (C, D) Keratinocytes were treated with Poly(I:C) (1 μg ml^{-1}) for 24 h in the presence of siTLR3. The mRNA expressions of *RHOA* (C) and *CDC42* (D) were examined. '–' and'+' mean samples treated without and with siTLR3, respectively. 'NC' indicates 'negative control siRNA'. The relative values to the non-siTLR3 transfected cells '–' are shown. ***$P < 0.001$.

Figure). Given that both melanosome and beads uptake is mediated by PAR2-dependent phagocytosis by growth factor stimuli [33], Poly(I:C) is likely to enhance MRG uptakes not solely through PAR2-dependent manner. Thus our data suggested that melanin uptake in keratinocytes are mostly depends on PAR2 functions, but TLR3 stimuli may also utilize PAR2-independent molecular action.

RHO, CDC42 and RAC are the three best-characterized members in Rho-family. We observed that TLR3 stimuli to keratinocytes enhanced RHOA and CDC42 expression in keratinocytes (Fig. 4A–D) and increased endo/phagocytosis of MRGs in RHOA and CDC42-dependent manner (Fig. 5C, D). RHOA and CDC42 are major factors controlling both endocytic traffics and phagocytosis [34–36], and RHO and RHO kinase are involved in phagocytosis of PAR2-stimulated keratinocytes [27]. In our experiments, RHOA and CDC42 seem not have a major contribution to the fundamental melanin uptake since siRHOA and siCDC42 did not alter the MRGs uptake in the unstimulated keratinocytes and the GTP forms of RHOA and CDC42 in the unstimulated keratinocytes were lower than in Poly(I:C)-stimulated keratinocytes (Fig. 4A, B). While, siRHOA and siCDC42 suppressed the Poly(I:C)-dependent MRGs uptake by keratinocytes (Fig. 5C, D). Thus, our data suggested that RHOA and CDC42 are more actively involved in melanin uptake by Poly(I:C)-stimulated keratinocyte, and that TLR3 agonist Poly(I:C) enhanced MRGs uptake through the RHOA and CDC42 induction in keratinocytes (Fig. 6).

Among the well characterized Rho-family members RHO, CDC42 and RAC, TLR3 stimuli slightly, but not significantly, suppressed RAC1 expression in keratinocytes (data not shown). CDC42 triggers the filopodia formation at the cell periphery [20]. RHO and RAC regulates the actin polymerization to produce stress fibers and lamellipodia, respectively [20]. Of note, RHO and RAC GTPases inhibit each other in cytoskeleton polarization [37,38]. RHOA activates Rho-associated coiled-coil containing protein kinase (ROCK) following RAC inhibition and restricts spontaneous lamellae formation [39,40]. On the other hand, RAC-mediated reactive oxygen spices (ROS) production inhibits RHOA through p190RhoGAP activation and inactivate Rho exchange factor NET1 [41,42]. Therefore, in Poly(I:C)-stimulated keratinocytes, no induction or slight decrease of RAC1 might be due to RHOA induction and activation because RHO and RAC GTPases compete each other.

Rho-family is also involved in pigmentation processes in melanocytes. The melanocyte dendrite formation depends on RHO, CDC42 and RAC functions, and melanocytes contact to keratinocytes through the dendrite formation to transfer melanosomes. UV irradiation and α-MSH increases dendrite formation via RAC activation [43]. In the melanocyte's dendrite formation,

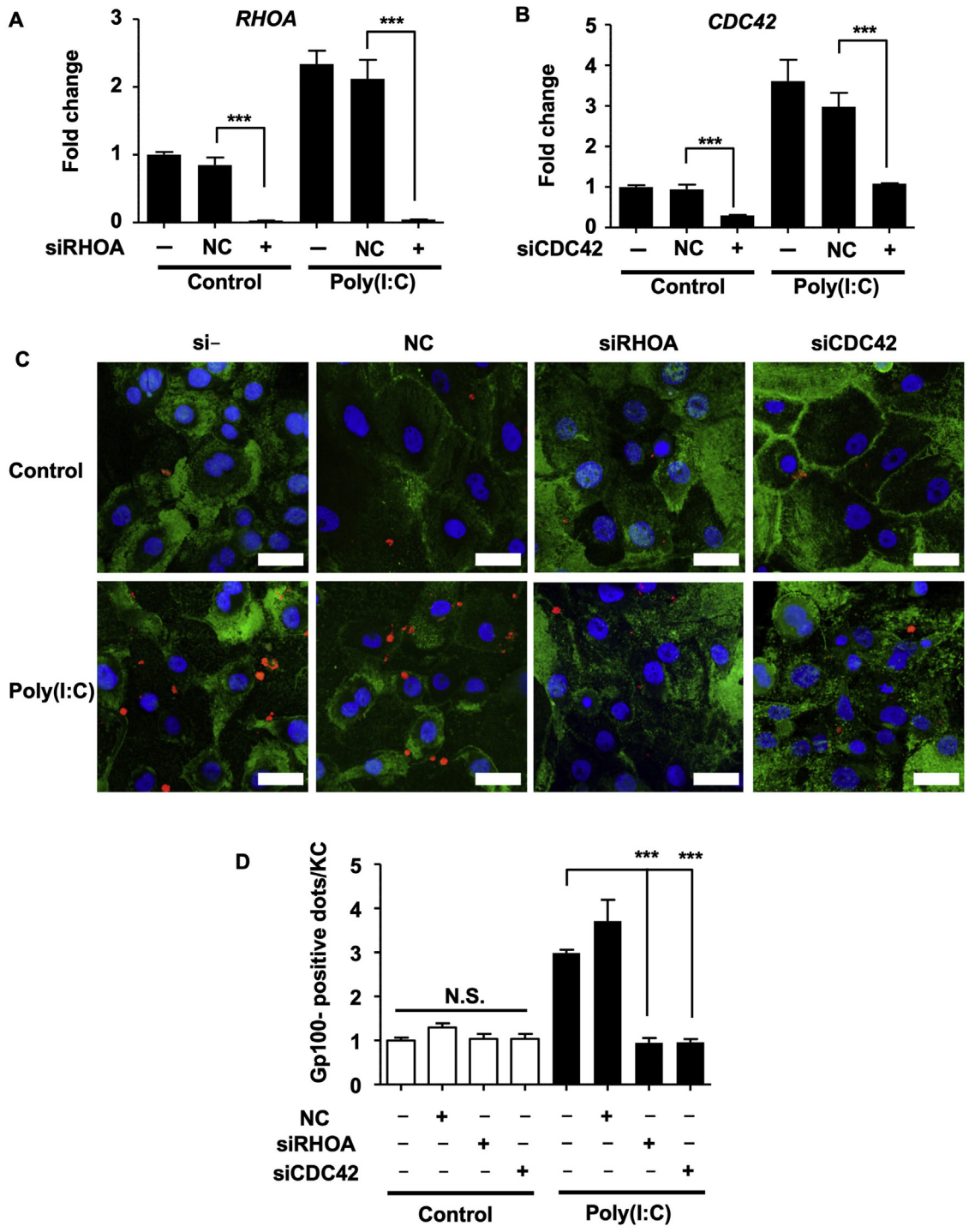


Fig. 5. Knock down of RHOA and CDC42 suppressed MRGs endo/phagocytosis into keratinocytes by Poly(I:C).
(A, B) Twenty-four hours after siRHOA or siCDC42 transfections, the decrease in target molecules expression in melanocytes was confirmed by real-time PCR. (C) Keratinocytes treated with siRNA of RHOA or CDC42 for 24 h were stimulated by Poly(I:C) (1 μg ml^{-1}) and added an equal amount of isolated MRGs. After 24 h incubation, PMEL/Gp100 (red) and keratin (green) were visualized by immunofluorescence staining. Scale bars = 50 μm. (D) The number of PMEL/Gp100-positive dots per keratinocyte were counted as described in the methods and shown as mean ± SD in the graph. '–' and'+' mean samples treated without and with siRNAs, respectively. 'NC' indicates 'negative control siRNA'. The relative values to the non-siRNA transfected cells '–' are shown. 'N.S.' indicates 'not significant'. ***$P < 0.001$.

cAMP mediates upregulation of RAC activity and downregulation of RHO activity [43]. RHOA activation is also critical for membrane blebbing and vesicle release from melanocytes [28]. In addition, Rho-family RHOA, CDC42, and RAC proteins enhance the transcriptional activity of NFκB [44]. We have shown that TLR3 agonist induced RAB27A and then increases melanosome release from melanocytes through NFκB signals [10]. Given that Rho-family is involved in NFκB signaling activation as well as dendricity and vesicle release from melanocytes, RHOA and CDC42 activation by TLR3 signaling suggest that Rho-family would be involved in RAB27A increase and melanosome release from TLR3-stimulated melanocytes via enhancement of NFκB activity. Further

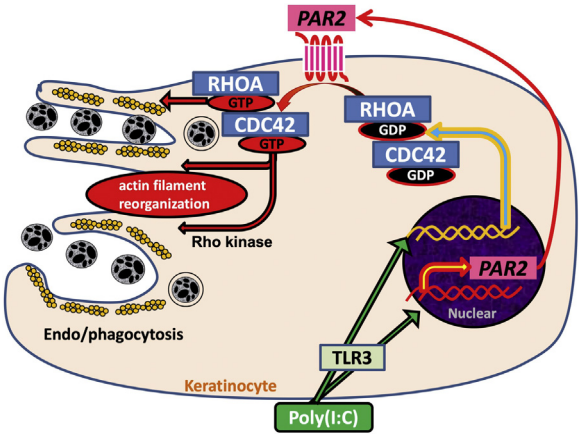


Fig. 6. The scheme of TLR3 functions in melanosome endocytosis in keratinocytes. TLR3 stimuli by Poly(I:C) enhances the expression of Rho-family molecules RHOA and CDC42. Poly(I:C) stimulation also increase the transcription of PAR2, which converts RHOA and CDC42 to active GTP-binding forms from inactive GDP-binding forms. Active RHOA and CDC42 in turn activate Rho kinase, modify actin stress fiber and filopodia formation, and enhance endocytosis/phagocytosis.

examinations would reveal new mechanisms and cell-specific roles of Rho-family for melanocytes as well as keratinocytes in pigmentation process by TLR3 stimuli.

An interesting observation is that, contrary to siPAR2 suppressing over all fundamental MRGs uptakes in keratinocytes (Fig. 3D–F), siRHOA or siCDC42 did not significant decrease the basal level of MRGs uptake in the absence of Poly(I:C) though siRHOA or siCDC42 suppressed Poly(I:C)-dependent MRGs uptake (Fig. 5). In the current study, we did not address the mechanisms of fundamental melanin uptake by keratinocytes without Poly(I:C) stimulation. We speculated that PAR2-independent and RHOA or CDC42-dependent mechanisms are dominantly act in the fundamental MRGs uptake by keratinocytes, and future studies will be conducted to elucidate the detail.

In this study, we showed the new mechanism that human keratinocytes enhanced uptake of MRGs via RHOA and CDC42 activation following TLR3 stimulation. In melanocytes, innate immune stimuli via TLR3 promote melanosome transfer and release through RAB27A induction [10]. Thus, in conclusion, TLR3 signaling pathway augments multiple functions in pigmentation process in both keratinocytes and melanocytes, particularly melanosome release and uptake by RAB and RHO molecules, respectively.

Author contributions

S.K. and K.Y. designed the project and wrote the manuscript. S. K., T.Y., R.S.O. and K.T. performed experiments. S.K. and K.Y. performed statistical data evaluation. H.A. supervised the melanosome isolation. S.A. contributed to the data interpretation. K.Y. conceived and supervised all aspects of this work.

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Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2019.11.005.

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