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Letter to the Editor

Toll-like receptor 2 utilizes RAB11A for melanosome transfer from melanocytes to keratinocytes

To the Editor

Epidermal cells including keratinocytes express TLRs to sense the signals from the outer world and induce cytokines and antimicrobial peptides to protect the human body. TLR2 recognizes bacterial lipoprotein, lipopeptide, and peptidoglycan, and TLR2 agonists activate the MyD88-dependent pathway. We previously reported that stimulation of TLR2 and TLR3 enhances melanogenesis in melanocytes and melanosome transfer to keratinocytes [1]. TLR2 agonist HKLM increases melanin contents and melanosome release from melanocytes by enhancing expression of melanogenic genes, tyrosinase (*TYR*) and dopachrome tautomerase (*DCT*) [1]. However, the molecular mechanism of melanosome transfer by TLR2 stimuli has not been elucidated. TLRs signaling affect recycling endosome (RE) and RE-associated RAB11 in dendritic cells [2]. In melanocytes, Tarafder et al., demonstrated peripheral RAB11-positive RE remodel mature melanosomes [3]. RAB17-dependent filopodia formation serves as conduits for melanosome transfer and the knockdown of RAB17 or RAB11 induces melanosome accumulation in melanocytes and decreased melanosome release [4]. Based on these knowledges we sought in this study whether RE-associated RABs RAB11A, RAB11B and RAB17 are involved in TLR2-dependent melanosome transfer.

Normal human epidermal melanocytes (Kurabo, Osaka, Japan) 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 $\mu g\, ml^{-1}$) and were irradiated UVB at 15 mJ cm^{-2} as described in the Supplementary information. Among the three RABs, *RAB11A* showed higher expression than *RAB11B* and *RAB17* in melanocytes (Fig. 1A). HKLM increased the *RAB11A*, did not affect *RAB11B*, and suppressed *RAB17* expression (Fig. 1A). Poly (I:C) increased *RAB11A* and *RAB11B* but suppressed *RAB17* expression. UVB irradiation increased *RAB11B* and *RAB17* but did not affect *RAB11A* expression. In the protein levels, HKLM increased RAB11A protein expression 2-fold, and Poly(I:C) and UVB barely increased RAB11A protein (Fig. 1B). RAB11B and RAB17 expression did not change dramatically (Fig. 1C, 1D). Knockdown of TLR2 and TLR3 by siRNA confirmed TLR2 and TLR3 are involved in these RABs induction by HKLM and Poly(I:C), respectively (Supplementary Fig. S1). We also examined the localization of RAB11A, RAB11B and RAB17 in melanocytes. Without stimulation, RAB11A localized perinuclearly (Supplementary Fig. S2, control). HKLM, Poly(I:C) and UVB distributed RAB11A diffusely in cytosol along with melanosome marker Gp100 (Supplementary Fig. S2). In contrast, HKLM, Poly(I:C) and UVB did not alter the localization of RAB11B and RAB17 (Supplementary Figs. S3 and S4). These data indicate that RAB11A is most abundant among RE-associated RABs, and TLR2 agonist HKLM enhances RAB11A expression and localization in normal human epidermal melanocytes.

We next employed siRNA for RABs. Pretreatment of siRNA for RAB11A (siRab11A), RAB11B (siRab11B) and RAB17 (siRab17) suppressed each mRNA and protein expression in melanocytes (Fig. 2A-F). We cocultured siRABs-pretreated melanocytes with human keratinocytes, then examined the Gp100-positive particles transferred in keratinocytes. We observed siRab11A suppressed melanosome transfer to keratinocytes by HKLM and Poly(I:C) stimuli but not by UVB irradiation (Fig. 2G, H). SiRab11B and siRab17 did not affect melanosome transfer by HKLM, Poly(I:C) and UVB irradiation. Following our previous report showing that RAB27A is involved in TLR3-dependent but not TLR2-dependent melanosome transfer [1], we confirmed that siRab11A but not siRab27A alone suppressed melanosome transfer by HKLM. The combination of siRab11A and siRab27A did not enhance the suppression of melanosome transfer by HKLM more than siRab11A alone (Supplementary Fig. S5A and B). SiRab27A alone suppressed melanosome transfer by Poly(I:C) and UVB more than siRab11A alone, and the combination of siRab11A and siRab27A did not enhance the suppression of melanosome transfer by Poly(I:C) and UVB more than siRab27A alone. Thus, TLR2 agonist HKLM preferentially utilize RAB11A, and RAB27A dominantly affect melanosome transfer by TLR3 agonists Poly(I:C) and UVB.

There are several models of melanosome transfer from melanocyte to keratinocytes. One is the exocytosis-endocytosis model, in which melanocores are exocytosed from melanocytes and endocytosed into keratinocytes via phagocytosis [3]. RAB11 locates in RE, remodels the protein and lipid composition of the plasma membrane, and regulates membranes recycling back to the plasma membrane and constitutive exocytosis as a post-Goldi exocytotic carrier [5]. RAB11A binds to the Rab11 family interacting protein 2 and the globular tail domain of myosin-5b, and the myosin-5b complex binding to actin filaments facilitate RAB11A-coated vesicle transportation [6]. We observed RAB11A localized in perinuclear area in non-stimulated melanocytes accordingly to previous

Abbreviations: TLR, Toll-like receptor; HKLM, heat-killed preparation of Listeria monocytogenes; RE, recycling endosome; UVB, ultraviolet B; MyD88, myeloid differentiation primary response gene 88; TYR, tyrosinase; DCT, dopachrome tautomerase; AP, Adaptor Protein; BLOC, biogenesis of lysosome-related organelle complex; PMEL, pre-melanosome protein; DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GTP, guanosine triphosphate.

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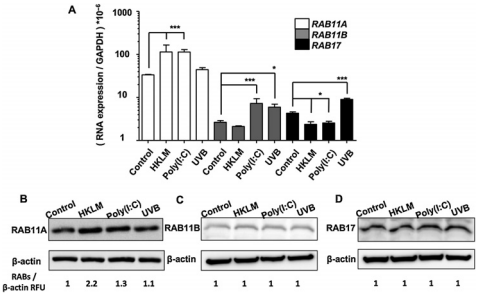

Fig. 1. TLR2 and/or TLR3 stimuli induce the alternation of RABs mRNA and protein expressions in normal human melanocytes.
(A) Melanocytes were treated with HKLM (10^8 cells ml^{-1}), Poly(I:C) (1 μg ml^{-1}) or UVB irradiation (15 mJ cm^{-2}) for 24 h. After 24 h incubation, the expressions of *RAB11A*, *RAB11B* and *RAB17* mRNA were examined. The results are shown as r as the relative value to *GAPDH*. $^*P < 0.05$, $^{***}P < 0.001$. (B–D) RAB11A, RAB11B, RAB17 and β-actin protein expressions were examined in melanocytes treated with HKLM, Poly (I:C) or UVB irradiation for 24 h. Representative images of 3 independent experiments are shown.

observations on perinuclear distribution of RE. TLR2 agonist HKLM distributed RAB11A diffusely in cytosol toward cell periphery (Supplementary Fig. S2). Involvement of TLR2 and RAB11A in HKLM-dependent melanosome transfer was confirmed by siTLR2 and siRab11A. Although Poly(I:C) also increased *RAB11A*, siRab27A

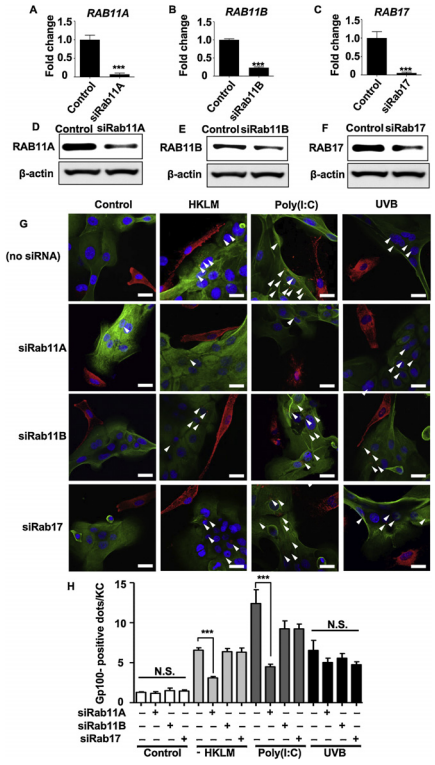

Fig. 2. Knockdown of RAB11A decreases melanosome transfer by HKLM and Poly(I:C).
Twenty-four hours after siRab11A, siRab11B and siRab17 transfections, the decrease in target molecules expression in melanocytes was confirmed by real-time PCR (A–C) and western-blotting (D–F). $^{***}P < 0.001$. (G) Melanocytes treated with siRNA of RAB11A, RAB11B, RAB17 for 24 h were co-cultured with keratinocytes, and stimulated by HKLM (10^8 cells ml^{-1}), Poly(I:C) (1 μg ml^{-1}) or UVB irradiation (15 mJ cm^{-2}). After 24 h incubation, PMEL/Gp100 (red), keratin (green) and nuclei (DAPI, blue) were visualized by immunofluorescence staining. The arrowheads indicate melanosomes transferred to keratinocytes. Scale bars = 50 μm. (H) The number of PMEL/Gp100-positive dots per keratinocyte were counted as described in the methods and shown as mean ± SD in the graph. The result was shown as relative values to the control. '–' and '+' mean samples treated without and with siRabs, respectively. 'N.S.' indicates 'not significant. $^{***}P < 0.001$.

suppressed Poly(I:C)-dependent melanosome transfer more than siRab11A did (Supplementary Fig. S5). These indicate that TLR2 utilizes RE-associated RAB11A in melanosome transfer to keratinocytes possibly through the exocytosis-endocytosis model (Supplementary Fig. S6).

Melanosome maturation is another factor affecting melanosome transfer. At least two cargo transport pathways are required for melanosome maturation to transport molecules from early endosomes to melanosomes through endocytic recycling pathway; adaptor Protein-1 (AP-1) and AP-3 pathway and Biogenesis of Lysosome-related Organelle Complex-1 (BLOC-1) and BLOC-2 pathway [7]. AP-3 has been linked to TLRs signaling. TLR2 ligands trafficking is controlled by AP-3, and TLR2 is distributed to the plasma membrane, early endosomes, and late endosomes/lysosomes through RAB11-positive compartments [8]. In plasmacytoid dendritic cell, AP-3 is essential to mobilize TLR7 and TLR9 from endosomes to lysosome-related organelle to produce type I interferon. RAB11A deficiency impacted TLR9 distribution to endoplasmic reticulum, fragmentation, and activation in mouse intestinal epithelial cells [9]. AP-3 also regulates TLR4 delivery to phagosomes and subsequent inflammatory signaling [10]. Thus TLRs signaling and melanosome maturation share similar trafficking molecules of endocytic recycling pathway and RE-associated RABs.

In conclusion, TLR2 stimuli augment RAB11A expression in human epidermal melanocytes and facilitate melanosome transfer to neighboring keratinocytes through RAB11A-associated melanosome transportation. TLR2 agonist HKLM induces de-novo melanin synthesis by increasing melanogenic genes *TYR* and *DCT* expression and melanosome maturation [1]. Because the membrane trafficking and vesicular transformation are the fundamental of melanosome maturation and transportation, our studies indicate molecular mechanisms how microenvironment including microbiota in epidermis would affect pigmentation process and post-inflammatory pigmentation through innate immune receptor TLRs.

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Conflicts of interest

The authors have no conflict of interest to declare.

Author contributions

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

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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.2019.04.005.

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