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## Supplemental Information

# $Ap_4A$ Regulates Directional Mobility and Antigen Presentation in Dendritic Cells

**Shin La Shu, Lakshmi Bhargavi Paruchuru, Neil Quanwei Tay, Yen Leong Chua, Adeline Shen Yun Foo, Chris Maolin Yang, Ka Hang Liong, Esther Geok Liang Koh, Angeline Lee, Hovav Nechushtan, Ehud Razin, and David Michael Kemeny**

**Supplemental Figure Legends**

**Figure S1.** FITC-dextran phagocytosis assay. *Nudt2*$^{+/+}$ and *Nudt2*$^{fl/fl}$ DCs do not differ in phagocytic capacity, a defining attribute in DC maturation status, indicating that in terms of DC maturation status based on phagocytic capacity they are identical.

**Figure S2.** MITF association with LysRS. Pull-down of MITF using LysRS antibody in a co-immunoprecipitation experiment was performed in BMDC.

**Figure S1.**

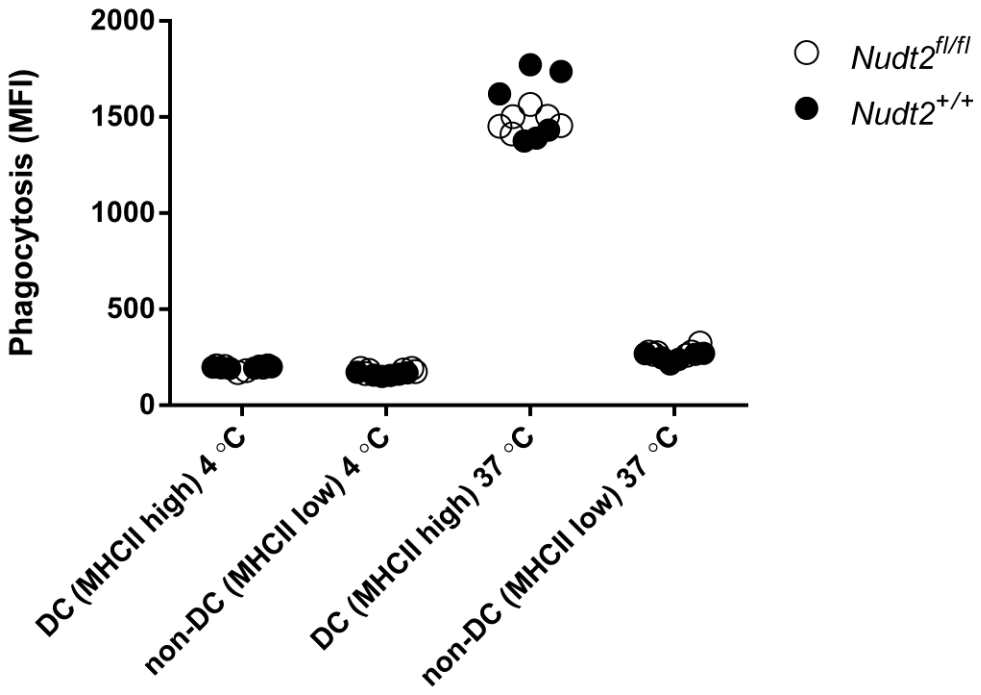
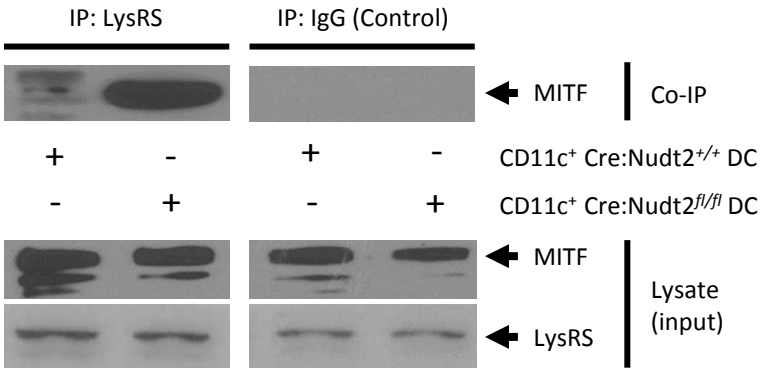

Phagocytosis (MFI)
2000
1500
1000
500
0
DC (MHCII high) 4 ◦C
non-DC (MHCII low) 4 ◦C
DC (MHCII high) 37 ◦C
non-DC (MHCII low) 37 ◦C
Nudt2fl/fl
Nudt2+/+

**Figure S2.**


IP: LysRS
IP: IgG (Control)
MITF
Co-IP
+ - + -
CD11c+ Cre:Nudt2+/+ DC
- + - +
CD11c+ Cre:Nudt2fl/fl DC
MITF
LysRS
Lysate
(input)

**Transparent Methods**

## 1. Materials and methods

### *1.1 Mice*

The C57BL/6 mice (8-10 weeks old) and OT-1 mice were purchased from National University of Singapore CARE and Charles River Laboratories, respectively, and were bred at NUS CARE. *Nudt2*$^{fl/fl}$ mice were generated by OZ-gene. CD11c-cre transgenic (C57BL/6$^{CD11c\text{-}cre}$) mice were gifted from Florent Ginhoux (SIgN, A*STAR, Singapore). The *Nudt2*$^{fl/fl}$ mice were backcrossed to c57bl/6 mice for nine generations and crossbred with c57bl/6$^{CD11c\text{-}cre}$ for 3 generations to generate *Nudt2*$^{fl/fl}$/CD11c-cre mice. Both age and sex-matched littermate control mice were used in all experiments. All mice were maintained under pathogen-free conditions in the satellite animal house unit. All experiments were performed in accordance with the strict guidelines of the National Advisory Committee for Laboratory Animal Research (NACLAR), Singapore. The Institutional Animal Care and Use Committee of the National University of Singapore have approved the protocols (Protocol number: 102/10).

### *1.2 Diadenosine nucleotide assay*

The nucleotide assay detects the amount of $Ap_4A$ present in extracts of mammalian cells. For each determination, bone marrow cells were seeded at a density of 2 x $10^6$ cells per well and cultured for 7 days in accordance to

the method outlined in methods section 1.6 (generation of BMDCs). For the generation of BMDCs, cells in one well of a 6-well plate was grown for 3 to 8 days as specified. The cell layer was washed with warm serum-free medium and lysed with 0.4 M trichloroacetic acid. The extraction cum measurement of the nucleotides using luminometry was performed as described previously (Murphy *et al.*, 2000).

### *1.3 Quantitative polymerase chain reaction (qPCR)*

Total RNA was isolated from sorted splenic DCs using the RNeasy kit (Qiagen, CA, USA), followed by cDNA synthesis using QuantiTect Reverse Transcription kit (Qiagen). Both kits were used according to manufacturer's instructions. Real-time PCR was performed on an ABI7500 real-time PCR system using SYBR Green (Applied Biosystems Pte Ltd, Singapore). Primers used for RT-PCR are as follows: TAP1 forward, 5′-GGAGGCCTTGGCTTACGTCGC-3′; and reverse, 5′-GGAGCCCACAGCCTTCTGCA-3′; TAP2 forward, 5′-CCGGACCTGCCTTTCCTCATAGC-3′ and reverse, 5′-CCGCAGGTTGATCCTGGACATGG-3′; TAPASIN forward, 5′-CCAGCACTCTCTTCAGCCTCTCC-3′ and reverse, 5′-CCACTGTTGCCATGGTGATGAC-3′; GAPDH forward, 5'-CATCACTGCCACCCAGAAGACTG-3', reverse, 5'-ATGCCAGTGAGCTTCCCGTTCAG-3'. TRACP5 forward, 5'-GACCACAACCTGCAGTATCTT-3', and reverse, 5'-

GGGAGTCCTCAGATCCATAGT-3'; RPL12 forward, 5'-GGAAGGCATAGTGCTGGAGGT-3'; and reverse, 5'-CGATGACATCCTTGGCCTGA-3'.

### *1.4 Western Blotting*

Following experimental treatment, cells were washed with ice-cold PBS, pelleted and re-suspended in RIPA lysis buffer (Sigma Aldrich, Singapore) supplemented with 1X complete protease inhibitor cocktail (Roche Ltd, Singapore). The supernatant was collected for SDS-PAGE analysis. Laemmli sample buffer (Bio-Rad, USA) was used to load proteins onto SDS PAGE gel. Equal amounts of protein from each sample were subjected to 15% SDS-PAGE at a constant voltage (125V) using mini-PROTEAN system (Bio-Rad Laboratories, Singapore). The proteins on SDS-PAGE gels were transferred onto Immun-Blot PVDF membrane (Bio-Rad, USA) using Trans-blot Turbo (Bio-Rad Laboratories, Singapore) according to manufacturer's protocol. Antibodies from Cell Signaling Technologies (USA) for western blot were: LysRS, MITF, Hint1, Histone H2A.X and α-tubulin. $Ap_4A$ Hydrolase antibody was from Santa Cruz Biotechnology, USA.

For co-immunoprecipitation (pull down assay), Thermo Scientific Pierce IP lysis Buffer was used to extract proteins from cell lysate according to manufacturer's protocol, and was loaded onto SureBeads Protein G (Bio-Rad, USA) according to manufacturer's protocol. Briefly, 10 μg of LysRS antibody or 10 μg of IgG1 antibody (ThermoFisher Scientific, USA) was

added to 200 µl of SureBeads and the suspension was rotated for 10 mins at room temperature. The bead-antibody complex was then washed three times with 1 mL of PBS + 0.1% Tween 20 (PBS-T) by magnetization of beads to discard supernatant. 1 mg of protein lysate extracted using IP lysis buffer was added to the bead-antibody complex (per sample) and was rotated for 1 hr at room temperature. Beads were washed with 1 mL of PBS-T three times by magnetization. Laemmli sample buffer was used to load bead-antibody-protein complex onto 15% SDS-PAGE at a constant voltage (125V) using mini-PROTEAN system (Bio-Rad Laboratories, Singapore). The proteins on SDS-PAGE gels were transferred onto Immun-Blot PVDF membrane using Trans-blot Turbo (Bio-Rad Laboratories, Singapore) according to manufacturer's protocol. The resultant blot was probed with MITF antibody to detect MITF pull-down. Input lanes represent 5% of total protein lysate sample mixed with bead-antibody complex as a positive control.

### *1.5 Cell motility assay*

To create a video recording of cells moving in a controlled environment, Olympus IX81 (Olympus, USA) inverted fluorescence microscope with stage-top live-cell imaging chamber with integrated 37˚C incubation chamber (model number TC-L-10. 96S106-O3, Chamlide, South Korea) maintained with 5.0% $CO_2$ for live capturing cells using a dynamically controlled heating stage that maintains the temperature during the time-lapse video recording (model number TC-L-10. 96S106-O3, Chamlide, South Korea).

MetaMorph NX (version 2.5) was used to capture the image and process for image analysis and post-processing of image was done with Fiji ImageJ (Schindelin *et al.*, 2012). To determine the motility of each cell, a specific position within the centre of nucleus using Manual Track plug-in for ImageJ (National Institute of Health, USA) analysis by frame-for-frame assigning tracker with tracer on an exact point in the nucleus over at least 20 frames was accomplished.

### *1.6 Generation of BMDCs*

To generate BMDCs, the tibia and femur of mice were excised and the cells were suspended in RPMI containing 1% Hyclone Fetal Calf Serum (GE Healthcare, Singapore). After passing the cell suspension through 0.70 µm cell strainer (Thermo Fisher, Singapore) cells were pelleted (centrifugation at 300g for 7 min) and allowed for red blood cell lysis. The bone marrow cells were cultured at a concentration of 1 x $10^6$ cells/ml in RPMI with 10% FCS, 1% non-essential amino acid (Sigma Aldrich), 1mM Sodium pyruvate (Sigma Aldrich), 5µM β-Mercaptoethanol (Sigma Aldrich), 100 IU/ml Penicillin (Sigma Aldrich), 0.1 mg/ml Streptomycin (Sigma Aldrich), and 5 ng/ml GM-CSF in 6-well plates. Cells were incubated in a humidified incubator at 37˚C, 5% $CO_2$. On day 2, 4 and 6, 75% of volume in medium was replaced with fresh medium supplemented with GM-CSF (5 ng/ml). After 7 days, the non- and loosely- adherent cells were harvested, washed, and magnetically isolated for CD11c positivity using anti-CD11c-conjugated MACS beads

(Miltenyl Biotec, USA). Flow-through or non-$CD11c^+$ cells were also collected as $CD11c^-$ cells as a control. The BMDCs were routinely examined and was $CD11c^+$ (high) MHC class $II^+$ (>90%).

### *1.7 Cell isolation*

To isolate naïve $CD8^+$ T cells from OT-1 mice, spleens were collected from euthanized mice. Single-cell suspensions were layered on Ficoll-Paque (GE Healthcare, Singapore) and centrifuged at 600 *g* for 20 min. Cells accumulating at the interface were collected, washed twice with MACS buffer and incubated with anti-CD8α-conjugated magnetic cell sorting (MACS) beads (Miltenyi Biotec Pte Ltd, Singapore). Then the cells were isolated by passing through a MACS column. To isolate splenic DCs from *Nudt2*$^{fl/fl}$ mice and *Nudt2*$^{+/+}$ c57bl/6 mice spleens were perfused and digested with Liberase Cl (Roche) for 30 min at 37˚C. Single-cell suspensions were reconstituted with Optiprep (Sigma Aldrich) diluted to 1.062 g/ml and subjected to density centrifugation at 1700g, 10 min at 4˚C. Low-density cells that accumulated at the interface were collected, washed, and selected using anti-CD11c-conjugated MACS beads.

### *1.8 Small GTPase activity assay*

The G-LISA small GTPase Activation Assay Biochemistry Combo Kit (RhoA, Rac1, cdc42) (Cytoskeleton, Colorado, USA) was used for the detection of

activated form of RhoA, Rac1 and cdc42 (i.e. GTP-bound form). The experiments were carried out in accordance to the manufacturer protocol.

### *1.9 FITC-dextran uptake assay*

To measure the phagocytic ability of DCs, the cells were incubated with FITC-dextran (70,000 MW, Invitrogen, Singapore). The BMDCs cultured with GM-CSF for 8 days were seeded on 12-well plates at a concentration of $1.0 \times 10^6$ cells/well. FITC-dextran was added to a final concentration of 0.5 mg/ml. The cells were incubated in either 4˚C or 37˚C for 30 min. After the incubation period, the cells were detached from plate by flushing with media and collected (centrifugation at 500g for 5 min), followed by washing in excess 1% BSA/PBS. After giving twice washing cells were immediately subjected for FACS analysis and the median florescence intensity (MFI) for FITC was measured.

### *1.10 Co-culture for antigen cross-presentation to $CD8^+$ T cells*

One µM of relevant peptide $OVA_{257–264}$ or whole OVA was added to DC (1 hr) and the excess unbound content was discarded. Subsequently, the OT-I $CD8^+$ T cells were co-cultured with peptide-pulsed DCs with or without LPS. Unless otherwise stated, cells were co-cultured in triplicate wells using U-bottomed 96-well plates with a total of $1\times10^5$ DCs / ml (100ul total volume) at a 10:1 $CD8^+$ T cell-to-DC ratio for 72 hr in a 37˚C/5% $CO_2$

incubator. CellTrace Violet (Invitrogen, USA) was used in accordance to manufacturer's protocol to label and measure proliferation of $CD8^+$ T cells.

### *1.11 Cytokine detection*

The levels of IL-12p40 and IL-12p70 in culture supernatants were measured using the mouse DuoSet ELISA development kit (R&D Systems, USA) according to the manufacturer instructions. Analysis was performed using a Luminex 100 plate reader (Qiagen, USA).

### *1.12 Flow cytometry*

After blocking the cells with anti-Fc antibody (anti-CD16/32; BD, United Kingdom), staining of cells was performed and are later re-suspended in flow buffer (PBS containing 2% BSA and 5mM EDTA). The following antibodies were purchased from (BD, USA), unless otherwise stated: anti-CD11c BV421, anti-CD8 APC, anti-CD80 PE, anti-CD86 APC, anti-CD40 Cy5.5 and anti-MHC class II (IA/IE) PB and a live/dead marker on APC-Cy7 (Invitrogen, USA). Cells were run on an LSR Fortessa or X-20 flow cytometer (BD, USA) and data were analysed by using the Flowjo analysis program (version 10.0.8).

### *1.13 Statistical analysis*

The experimental significance between the treatments was calculated by performing analysis of variance (ANOVA) followed by Student's t test

Mann-Whitney test as appropriate (GraphPad Prism 7.01, USA). The experiments for which the *'P'* value <0.05 in comparison to the control were considered as statistically significant.

**Supplemental References**


- Murphy, G.A., Halliday, D., and McLennan, A.G. (2000). The Fhit Tumor Suppressor protein regulates the intracellular concentration of diadenosine triphosphate but not diadenosine tetraphosphate. Cancer Res. *60*, 2342 LP-2344.
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., *et al.* (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods *9*, 676–682.