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Exosome secretion of dendritic cells is regulated by Hrs, an ESCRT-0 protein

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ABSTRACT

Exosomes are nanovesicles derived from multivesicular bodies (MVBs) in antigen-presenting cells. The components of the ESCRT (endosomal sorting complex required for transport) pathway are critical for the formation of MVBs, however the relationship between the ESCRT pathway and the secretion of exosomes remains unclear. We here demonstrate that Hrs, an ESCRT-0 protein, is required for fascilitating the secretion of exosomes in dendritic cells (DCs). Ultrastructural analyses showed typical saucer-shaped exosomes in the culture supernatant from both the control and Hrs-depleted DCs. However, the amount of exosome secretion was significantly decreased in Hrs-depleted DCs following stimulations with ovalbumin (OVA) as well as calcium ionophore. Antigen-presentation activity was also suppressed in exsosomes purified from Hrs-depleted DCs, while no alteration in OVA degradation was seen in Hrs-depleted DCs. These data indicated that Hrs is involved in the regulation of antigen presentation activity through the exosome secretion.

1. Introduction

Exosomes are nanovesicles (60–90 nm in diameter) surrounded by a lipid bilayer. Exosomes are secreted from a variety of cells, including antigen-presenting cells (APCs), B cells, monocytes, and dendritic cells (DCs) [1], in physiological situations. They are generated as the intraluminal vesicles (ILVs) of a sorting endosome called a multivesicular body (MVB), by the inward budding of the MVB's limiting membrane. The release of exosomes into the extracellular milieu is achieved by the direct fusion of the MVB with the plasma membrane. Exosomes possess selected cargo proteins originating from the MVB membrane, including the major histocompatibility complex (MHC), costimulatory molecules, tetraspanins, adhesion molecules, and cytosolic proteins, and the biological functions of exosomes depend mainly on the types of cargo proteins they contain. For instance, APC-derived exosomes are capable of directly sensitizing naïve T cells via the MHC/peptide complex and the costimulatory molecules on their surface [2]. On the other hand, exosome like vesicles secreted from intestinal epithelial cells can induce tolerance in an antigen peptide-specific manner [3,4]. These findings suggest that exosomes carrying MHC proteins can regulate immune responses positively or negatively in vivo.

Exosomes also contain ubiquitinated proteins, suggesting that a subset of ubiquitinated cytoplasmic proteins is actively incorporated into the MVB pathway [5]. The sorting of ubiquitinated proteins on MVBs is mediated by a series of proteins involved in vacuolar protein sorting (VPS), called endosomal sorting complex required for transport (ESCRT). The first complex that binds the cargo on endosomes is ESCRT-0 (it includes Hrs [6,7] and STAM), and with the help of the ESCRTs-I, -II, and –III, the cargo accumulates on the endosomal membrane. At the end of the sorting, an AAA-type ATPase, VPS4, disrupts the ESCRT complexes, and the membrane with its accumulated cargo is invaginated into the maturing endosome to produce an intraluminal vesicle, called an MVB. Most of the ubiquitinated cargo, which includes epithelial growth factor (EGF) receptors, c-Met, and gp130, is degraded by lysosomal proteases. A deficiency of Hrs results in abnormally enlarged endosomes

Abbreviations: APC, antigen-presenting; ESCRT, endosomal sorting complex required for transport; ILV, intraluminal vesicle; MVB, multivesicular body; MHC, major histocompatibility complex; N-Rh-PE, N-(lissamine rhodamine B sulfonyl) phosphatidyl ethanolamine; OVA, ovalbumin; TEM, transmission electron microscopy; VPS, vacuolar protein sorting.

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and a marked reduction in cargo sorting to the MVBs, which accumulate ligand-activated membrane-bound growth factor receptors [8]. While most of the cargo is destined for degradation, some MVBs direct their ILVs to be secreted as exosomes by direct fusion with the plasma membrane [9]. In this context, the intracellular membrane traffic system seems to play key role in the formation and release of exosomes. Previous studies suggested that at least four Rab GTPase members, Rab5, Rab11, Rab27a and Rab27b are involved in the secretion of exosomes [10–12]. Nevertheless, the precise mechanisms of the exosome pathway are still unclear.

Considering that the importance of MVB formation in exosomal pathway, as well as the presence of ubiquitinated proteins in exosomes, we suspected that Hrs might be involved in exosomal pathway. We report here that Hrs is required for secretion of exosomes in DCs.

2. Materials and Methods

2.1. Ethics Statements

This study was conducted according to the principles expressed in the Declaration of Helsinki and Fundamental Guidelines for Animal Experiments and Related Activities. The study was approved by the research committees of the Miyagi Cancer Center and Tohoku University. All the animal experiments were conducted under the approval of the Institutional Animal Care and Use Committees of Miyagi Cancer Center and Tohoku University.

2.2. Cells

DC2.4 cells (murine DC line) were maintained in RPMI medium containing 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 10 mM hepes buffer, and antibiotics. We also generated primary DCs from mouse bone marrow using a standard method, as previously described [13]. To express Hrs-specific short hairpin RNA (shRNA), a retroviral vector (pSIREN-RetroQ, BD Biosciences) was generated as described previously [14]. The target sequence consisted of nucleotide residues 302–320 (5′-AGG TAA ACG TCC GTA ACA A-3′) in the human hrs cDNA. A control plasmid, pSIREN-RetroQ-Luc, targeted bp 413–434 of firefly luciferase (5′-GCA ATA GTT CAC GCT GAA AAG-3′). The retrovirus was prepared as previously described [14].

2.3. Mice

We generated a conditional knock-out of Hrs as described previously (Acc. No. CDB0476 K, Center for Developmental Biology, Kobe, Japan) [15]. To generate a dendritic-cell-specific conditional knockout of Hrs, We crossed this mouse with a LysM-cre transgenic mouse (a gift from Dr. I. Foerster) [16]. The OT-I TCR-transgenic mice were a gift from Dr. W. Heath (Walter and Eliza Hall Institute, Melbourne, Australia) and were used as the source of $CD8^+$ T cells that were specifically responsive to the OVA257–264 peptide [17].

2.4. Genotype analysis

The *hrs flox* allele was genotyped as described previously [15]. Genotyping for the presence of the LysM-Cre allele was performed using the following primer pair: forward (5′-TTA CCG GTC GAT GCA ACG AGT GAT G) and reverse (5′-TTC CAT GAG TGA ACG AAC CTG GTC G).

2.5. Isolation and purification of exosomes

Exosomes were purified as previously described [2,18]. In brief, the cell culture medium was centrifuged for 10 min at 300 g, 10 min at 1200 g, and 30 min at 10,000 g to remove the cells and debris. The supernatant obtained from the last spin was then centrifuged for 60 min at 100,000 g, and the pellet was solubilized in SDS sample buffer for analyses by Western blotting.

2.6. Fluorescent N-Rh-PE measurement

To measure exosome secretion, the fluorescent phospholipid analog N-(lissamine rhodamine B sulfonyl) phosphatidyl ethanolamine (N-Rh-PE) was inserted into the plasma membrane as described previously [19], and eventually secreted into the extracellular medium [10]. Briefly, the lipid was solubilized in absolute ethanol and injected into serum-free RPMI (<1% v/v) during vigorous vortexing. The mixture was then added to the cells, which were incubated for 60 min at 4 °C. After this incubation, the medium was removed, and the cells were extensively washed with cold PBS. The labeled cells were cultured in complete RPMI medium to collect the exosomes. To measure the exosome secretion, 50 μl of the exosomal fraction was solubilized in 1.5 ml PBS containing 0.1% Triton X-100, and the N-Rh-PE was measured at 560 nm and 590 nm excitation and emission wavelengths, respectively.

2.7. OVA protein degradation

Control and Hrs-depleted dendritic cell lines were treated with a lysosomal inhibitor (50 mM NH_4Cl) and/or proteasomal inhibitor (10 μM epoxomicin) for up to 2 h. The cells were pulsed with 300 μg/ml ovalbumin (OVA) protein for 1 h, and incubated with fresh medium for the indicated times. For western blot analysis, an anti-OVA antibody (rabbit polyclonal antibody, Abnova, Taiwan) was used for the first antibody.

2.8. Western blotting

Immunoblotting was carried out as described previously [20]. For dot-blot analysis, each cell lysate was spotted onto PVDF membranes.

2.9. Phenotypic analysis of cells by flow cytometry

Cells were assessed for surface marker expression by fluorescent multicolor flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA). The immunophenotypic profile of DCs was evaluated by staining with anti-CD40, anti-CD80, anti-I-A^β, and anti-H-2 K^β antibodies (Pharmingen).

2.10. Antigen presentation assays

To assess the function of DC-derived exosomes, we performed an in vitro $CD8^+$ T-cell proliferation assay as described previously [21]. The spleen was harvested from OT-I mice, and the $CD8^+$ T cells were positively selected using MACS magnetic beads (Miltenyi Biotec). Next, 4×10^6 BMDCs from $Hrs^{+/+}$; LysM-Cre and $Hrs^{flox/flox}$; LysM-Cre mice were incubated with 300 mg/ml OVA for 24 h. Exosomes in 10 ml of supernatant were purified as described above. The purified OT-I $CD8^+$ T cells were cocultured with the purified exosomes in 96-well plates for 5 days. The exosome induced proliferation of the OT-I T cells was measured after 5 days by adding [^{3}H]thymidine (1 μCi/well; ICN Pharmaceuticals) during the last 8 h of each culture.

2.11. Negative staining in electron microscopy

Samples of exosomes pelletted by ultracentrifugation as described above were resuspended in 0.1% glutaraldehyde, and a

drop of each resuspension was mounted on an ion-coated copper grid supported by a carbon-coated collodion film. The grid was stained with 1% uranyl acetate for 1 min and observed under an electron microscope (H-7650, Hitachi, Tokyo, Japan).

3. Results

3.1. Establishment of Hrs-depleted DCs and purification of exosomes

To examine the role of Hrs in exosome secretion, we established an Hrs-depleted dendritic cell (DC) line through retrovirus-mediated shRNA expression (Fig. 1A). The exosome-containing fractions were purified from the DCs by sucrose gradient centrifugation, using an anti-MHC-class II (I-A^{β}) antibody to detect the exosomes. The fraction containing the peak I-A^{β}-binding activity occurred at 1.15 g/ml sucrose in the sample from control DCs, and 1.14 g/ml from the Hrs-depleted DCs, indicating a similar distribution irrespective of the Hrs expression (Fig. 1B). Although these values were slightly different, they were both within the normal density profile for DC-derived exosomes in sucrose gradients [22]. Ultrastructural analysis of the ultracentrifuged exosome pellets by negative stain method of transmission electron microscopy (TEM) showed them to be markedly enriched in typical saucer-shaped exosomes, 40–100 nm in diameter, from both the control and knock-out DCs (Fig. 1C).

We further examined the exosome-containing fractions by Western blot analysis. Exosomes from the Hrs-depleted DCs under steady-state conditions contained less ubiquitinated protein than exosomes from control DCs, although the total ubiquitinated protein in the whole-cell lysates of both types of DCs was the same (Fig. 1D). MHC-I and -II, two marker proteins for DC-derived exosomes, were included at similar levels in the two exosome fractions (Fig. 1E). Although Hrs was not detected in the exosome fractions, TSG101 and VPS4B, two downstream ESCRT proteins, were clearly identified in the exosomes, and at lower amounts in the Hrs-depleted DCs. These data suggested that Hrs and another ESCRT trafficking pathway are involved in exosome secretion.

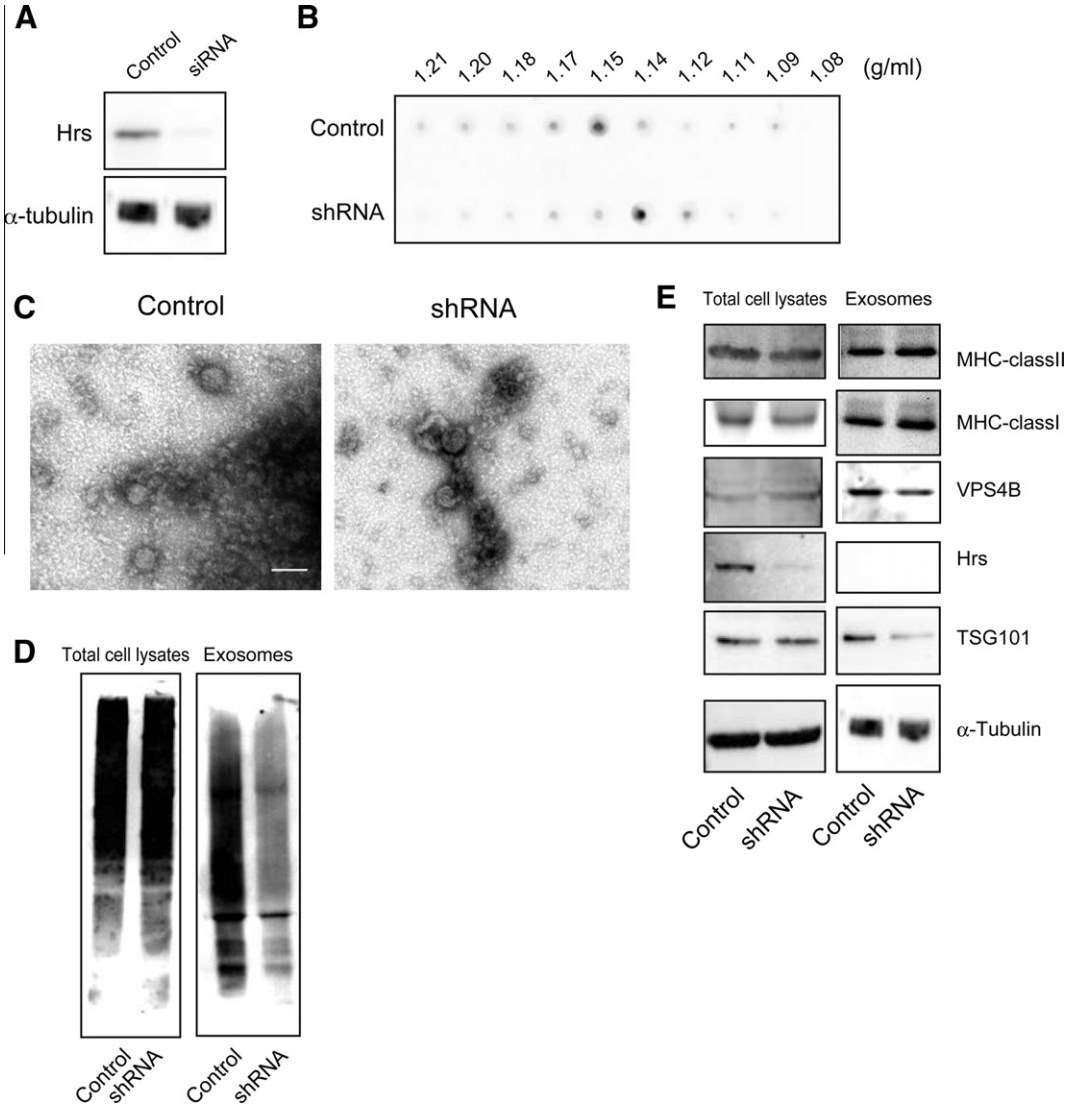

Fig. 1. Hrs-knock-down dendritic cells (DCs), Hrs knock-out bone marrow DCs, and characterization of their exosomes. (A) Western blot analysis of total cell lysates from control and Hrs knock-down DCs. (B) Sucrose gradient fractionation and dot-blot analysis of exosomes produced from DCs. The same amount of protein was fractionated and blotted on the membranes. (C) Negative staining TEM of exosomes from murine DCs. DCs were grown from monocytic precursors and cultured in GM-CSF for 10 days. The cell culture supernatants were sequentially centrifuged to obtain a pellet containing exosomes. The 100,000 x g pellet was washed and analyzed by TEM. Bar, 100 nm. (D) Western blot analysis of total cell lysates (left panel) and exosome fraction (right panel) using an antibody against ubiquitinated proteins (FK2). (E) Western blot analysis of total cell lysates (left panel) and exosomes (right panel) using the indicated antibodies.

3.2. Impairment of exosome secretion in Hrs-depleted DCs

In addition to steady-state secretion, DCs release additional exosomes upon stimulation. To examine the effect of Hrs on activation-dependent exosome secretion, the amount of purified exosomes released under several types of stimulation was measured. Ovalbumin (OVA) is known to induce exosome secretion from DCs [18,21]. Exosome secretion by the control DCs was clearly increased by 24 h of stimulation with OVA, whereas no significant increase was found in the Hrs-depleted DCs (Fig. 2A). To evaluate the possible effect of lipopolysaccharide (LPS) contamination in the OVA, we examined the amount of exosomes released under stimulation with LPS alone. Exosome secretion was not enhanced by the LPS stimulation, irrespective of the Hrs level (Fig. 2A). We also

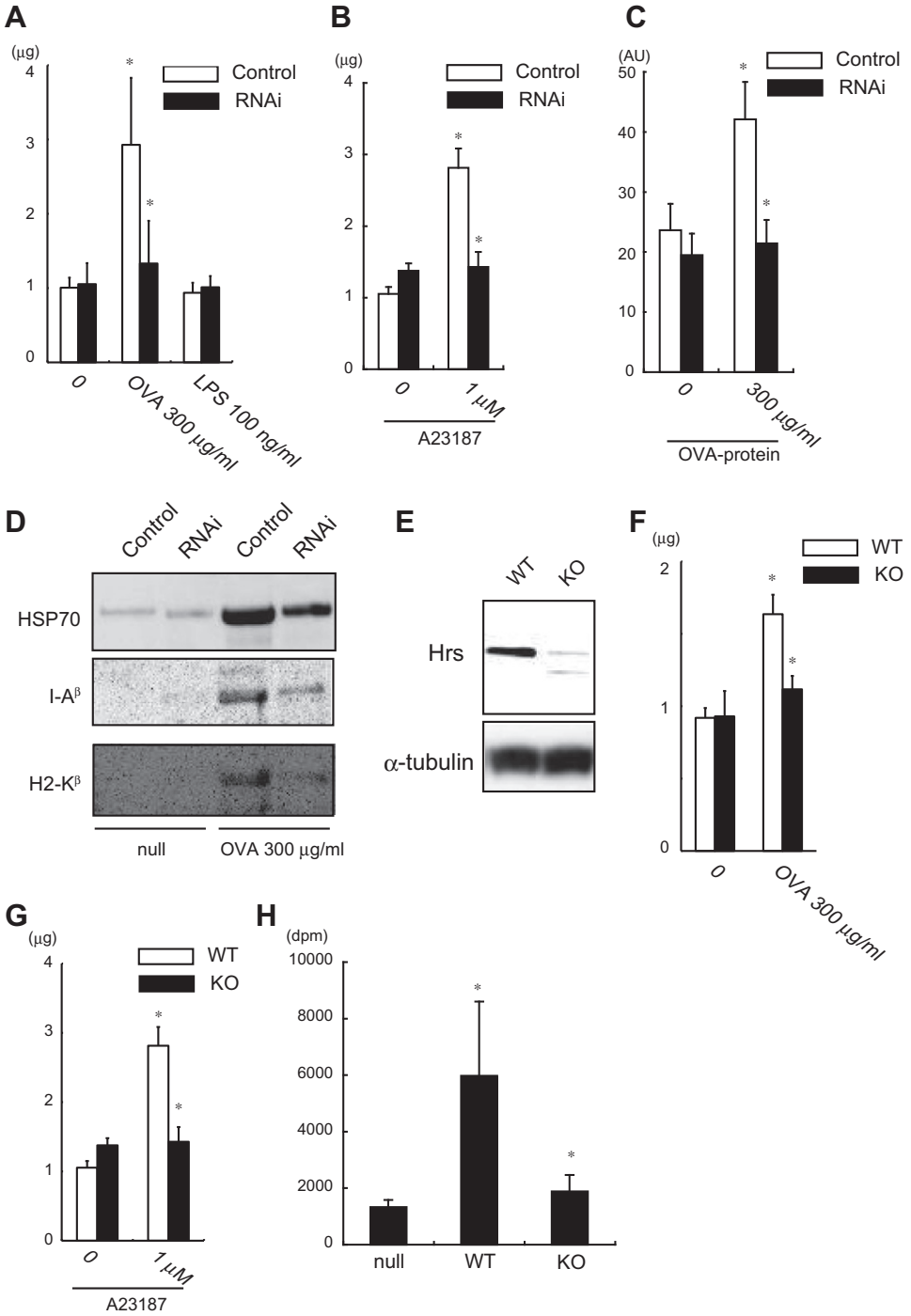

Fig. 2. Hrs is required for the secretion of exosomes. (A) Measurement of the exosome secretion from DCs under stimulation with OVA or LPS, using a protein assay. $^*p < 0.007$. (B) Measurement of exosomes under stimulation with a Ca^{2+} ionophore (1 μM A23187), using a protein assay. $^*p < 0.005$. (C) Measurement of exosome production under simulation with OVA, using N-Rh-PE release. $^*p < 0.05$. (D) Western blot analysis of exosomes under stimulation with OVA. (E) Western Blot analysis of total lysates from control and Hrs-knock out BMDCs. (F) Measurement of the exosome secretion from BMDCs under stimulation with OVA in BMDCs, using a protein assay. $^*p < 0.006$. WT, $hrs^{+/+}$, LysM-Cre; KO, $hrs^{flox/flox}$, LysM-Cre. (G) Measurement of exosomes under stimulation with a Ca^{2+} ionophore (1 μM A23187) in BMDCs, using a protein assay. $^*p < 0.005$. (H) [3H]-thymidine incorporation of CD8 T cells from OVA-specific TCR transgenic mice (OT-I mice). CD8 T cells were stimulated with purified exosomes from control and Hrs-knock out dendritic cells for 5 days. $^*p < 0.05$.

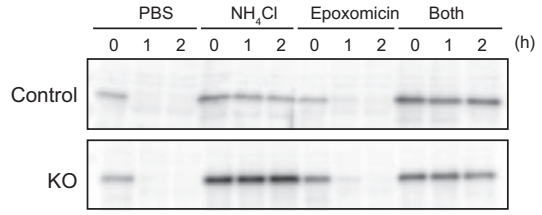

Fig. 3. Characteristics of Hrs-knock-out DCs under antigen stimulation. The degradation rate of OVA in control and Hrs-knock-down DCs was shown by western blot. A lysosomal inhibitor (NH_4Cl) and/or protease inhibitor (Epoxomicin) were included in the culture medium in some cases.

examined the exosome secretion induced by intracytoplasmic Ca^{2+} release [23]. The incubation of DCs with the Ca^{2+} ionophore A23187 induced a 2.8-fold increase in exosome release from the control DCs; however, the Hrs-depleted DCs showed very low Ca^{2+} responsiveness (Fig. 2B). We also measured the exosome secretion using N-(lissamine rhodamine B sulfonyl) phosphatidyl ethanolamine (N-Rh-PE) [10], and obtained similar results (Fig. 2C). Western blot analysis revealed that the levels of three exosome markers, HSP70, I-A^{β}, and H-2 K^{β}, were reduced in the Hrs-depleted DCs compared with the control DCs, after incubation with OVA (Fig. 2D).

To verify the essential role of Hrs in exosomal secretion, Hrs-depleted bone marrow-derived DCs (BMDCs) were successfully generated from $hrs^{flox/flox}$; LysM-Cre mice, and they expressed dramatically less Hrs than DCs derived from the $hrs^{+/+}$; LysM-Cre mice (Fig. 2E). No significant difference was seen between the population of $CD11c^{+}$ cells in BMDCs from $hrs^{+/+}$; LysM-Cre and $hrs^{flox/flox}$; LysM-Cre mice (data not shown). First, we examined the amount of exosome secretion under stimulation of OVA as well as Ca^{2+} ionophore A23187. Similar to the results obtained from Hrs-knock-down DCs, BMDCs from $hrs^{flox/flox}$; LysM-cre secreted lesser amount of exosome than those from $hrs^{+/+}$; LysM-Cre mice (Fig. 2F and G). We further investigated whether the Hrs depletion affected the amount of exosomes secreted from these cells, by measuring the antigen-presenting activity of the exosomes for T cells. DCs derived from the $hrs^{+/+}$; LysM-cre and $hrs^{flox/flox}$; LysM-Cre mice were incubated with OVA for 48 h, and the exosomes secreted from the DCs were purified. The exosomes were co-cultured with splenic $CD8^{+}$ T cells derived from OT-I transgenic mice. We found a significant increase in the proliferation of OVA-peptide restricted $CD8^{+}$ T cells cultured with the exosomes from the $hrs^{+/+}$; LysM-cre DCs, but not with those from the $hrs^{flox/flox}$; LysM-Cre DCs (Fig. 2H). Collectively, these data suggested that Hrs is required for exosome secretion.

3.3. No alteration in OVA degradation in Hrs-depleted DCs

It was possible that the decreased antigen-presentation activity of exosomes from the Hrs-depleted DCs was due to insufficient degradation of the OVA protein that was taken up. We therefore measured its degradation rate. The OVA protein was completely degraded one hour after the OVA pulse, and the degradation was the same, regardless of Hrs expression. The degradation of the OVA was efficiently blocked by a lysosomal acidification inhibitor, NH_4Cl, but not by a proteasomal inhibitor, epoxomicin, in both the control and the Hrs-depleted DCs (Fig. 3). These results suggested that Hrs does not affect the lysosome-dependent degradation of the OVA protein in DCs.

3.4. Characteristics of Hrs-depleted DCs under stimulation by OVA

We next investigated whether Hrs-depleted DCs were activated by stimulation with OVA. When stimulated with OVA proteins, DC activation markers increased more in Hrs-depleted DCs than in control cells (Fig. 4A to C). This was also true of the production of cytokines, including interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) (Fig. 4D and E). These findings indicated that exosome secretion and DC activation were regulated by different mechanisms.

4. Discussion

There is accumulating evidence that the ESCRT proteins play important roles in the formation of MVBs, which undergo lysosomal digestion or are released into the extracellular environment as exosomes [24]. The ESCRT machinery, glycosylphosphatidylinositol-

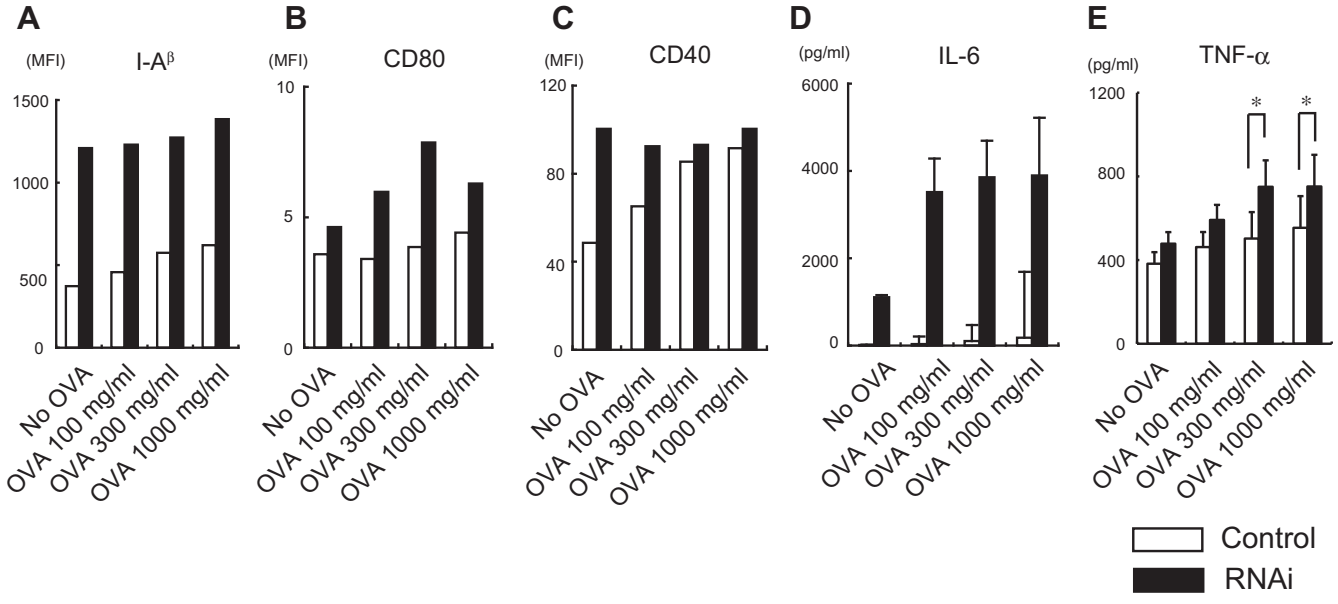

Fig. 4. Characteristics of Hrs-depleted DCs under stimulation of OVA (A to C) FACS analysis using anti-I-A^{β} (A), CD80 (B), and CD40 (C) antibodies. Control and Hrs-knock-down DCs were incubated with OVA for 24 h. Data are representative of at least three experiments. (D and E) Measurement of cytokine secretion from control and Hrs-knock-down DCs. Control and Hrs-knock-down DCs were incubated with OVA for 16 h. Data are representative of at least three experiments performed in triplicate. $^{*}p < 0.05$.

associated lipid affinity, and tetraspanin-associated protein affinity are involved in the mechanisms of cargo-protein sorting and intraluminal-vesicle formation, which are related to exosome maturation [25]. So far, two Ras family monomeric G protein Rab27a and Rab27b were reported to function in MVB docking at the plasma membrane, and to control the exosomal pathway [11], suggesting that MVBs contribute to exosome secretion through their direct fusion to the cell surface membranes. In this context, we demonstrated here that Hrs, one of the ESCRT proteins, is required for the production of exosomes within MVBs. Regarding the relationship between exosome production and the ESCRT machinery, there have been some contradictory reports. For example, the formation of proteolipid protein-containing exosomes is dependent on Rab5, but independent of the ESCRT machinery in oligodendrocytes under ceramide stimulation [12]. Since our present study on exosome production was limited to DCs stimulated by OVA or a calcium ionophore, there may be some cargo-dependent or stimulation-dependent pathways for MVB formation and exosome production.

In antigen-presenting cells, exosomes are secreted upon exposure to various stimulants, such as OVA or the calcium ionophore A23187 [18]. OVA is usually considered to act as an antigenic peptide source, and other report also utilized this agent alone to stimulate DCs [26], although we cannot fully exclude a possibility that a faint contamination of LPS as a stimulator is included in OVA. The exosome secretion in Hrs-depleted DCs was decreased in spite of their higher expression of the cell-surface-activation markers, MHC class I/II molecules as compared with the control DCs. Those markers are also known to be ubiquitinated and sequestered through MVBs [27,28]. Therefore, our findings suggested that Hrs is required for the efficient endocytosis and degradation of the MHC molecules through MVB formation as well as the exosome secretion. We previously demonstrated that the deficiency of Hrs suppresses degradation of gp130, a subunit of IL-6 receptor, in HeLa cells, which leads to a prolonged and amplified IL-6 signal [8]. Epidermal growth factor receptor (EGFR) degradation is impaired and the signaling is enhanced in Hrs-depleted mouse embryonic fibroblasts and *Drosophila* [29,30]. We suspect that Hrs-depleted DCs may be more sensitive to some stimulations than control DCs. This may be true of IL-6 secretion, because the Hrs-depleted cells secreted small but significantly more IL-6 even in the absence of OVA (Fig. 4D). It is possible that IL-6 secretion is somewhat enhanced by a factor(s) contained in FCS in the medium without OVA stimulation. We suspect that both the prolongation of cytokine signals and the deficiency of exosome production in Hrs-depleted DCs are caused by the impaired MVB formation.

Finally, our present study suggests a biological role of Hrs in immune regulation (Fig. 2H). DC-derived exosomes have been shown to have potent immunostimulatory potential, and MHC-I and B7.2 (CD86)-bearing exosomes generate $CD8^+$ T-cell responses against tumors in vivo [31]. Further study using DC-specific Hrs knockout mice will provide precise roles of the Hrs-dependent exosomal pathway in anti-tumor immune response.

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