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ARTICLE

Expression of UNC93A Induced by CpG-DNA-liposome Complex in Mice

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Abstract UNC93B1 is involved in the delivery of nucleotide-sensing Toll-like receptors (TLR) including TLR9 to endolysosomes. However, possible functions of UNC93A, another member of the UNC-93 superfamily, have not been studied in the context of TLR signaling. Here, we injected naked CpG-ODNs or liposome-encapsulated CpG-ODN (Lipoplex(O)) into the BALB/c mouse peritoneal cavity and investigated expression of mouse UNC93B1 and UNC93A genes in the peritoneal cells. UNC93A mRNA expression was increased by Lipoplex(O) in a time-dependent manner, whereas the expression level of UNC93B1 was not changed. To evaluate whether the expression of UNC93A involves TLR9, TLR9 knock out (TLR-/-) mice were injected with Lipoplex(O) or LipoplexGC(O), and the peritoneal cells were analyzed. The expression of UNC93A was not induced by Lipoplex(O) in TLR9-/- mice. These results suggest that UNC93A is closely associated with TLR9 signaling induced by Lipoplex(O) in the peritoneal cells *in vivo*. Primary cells including peritoneal cells stimulated with CpG-ODNs, Lipoplex(O), and several cytokines *in vitro* were prepared. However, UNC93A expression was not induced by any stimulation. To identify cellular localization of UNC93A, human embryonic kidney 293 cells stably expressing UNC93A were established and analyzed by confocal microscopy. The human and mouse UNC93A proteins were detected in the cytoplasm. Further investigation of the UNC93A function related with the CpG-DNA-mediated immune response may provide information to support efficient application of CpG-DNA immunotherapeutics.

Keywords CpG-DNA · CpG-DNA-liposome complex · innate immunity · TLR9 · UNC93A

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Introduction

The current understanding of immunology is that the defense mechanisms involve two immune systems: ‘innate’ and ‘adaptive’. Antigen receptors such as B-cell receptor and T-cell receptor are generated through gene rearrangement or somatic hypermutation in the adaptive immune system. In contrast, the pathogen-specific receptors involved in the innate immune system are encoded in the germ line and recognize pathogen-associated molecular patterns. Although the two systems have different features, they are closely related to each other in some ways to efficiently defend against pathogens (Aderem and Ulevitch, 2000; Medzhitov et al., 2000; Krieg et al., 2006; Kanzler et al., 2007).

The innate immune system has an ability to recognize a variety of pathogen components as non-self to discriminate from self constituents. The innate immune system detects pathogens via a limited number of pattern-recognition receptors (PRRs). One of the well-known PRRs is the Toll-like receptors (TLRs) family. TLRs are divided into two types according to the recognizable pathogen types. Some TLRs are expressed at the membrane surface and detect extracellular pathogens by specific molecules that are found in pathogens, such as lipopeptides and lipopoly-saccharides. The other TLRs are expressed within immune cells and are specific for nucleic acids of extracellular pathogens. The DNA sequences that contain unmethylated CpG dinucleotides in

a specific context (CpG-DNA), which are included in most bacteria and DNA viruses, are recognized by TLR9, and an immune response is induced by the interaction (Akira et al., 2006; Krieg et al., 2006).

In addition to the bacterial CpG-DNA, synthetic oligodeoxynucleotides (ODNs) induce important immunomodulatory effects on several types of immune cells, such as B lymphocytes, macrophages, and natural killer cells (Messina et al., 1991; Krieg et al., 1995; Ballas et al., 1996). CpG-DNA stimulates some immune cells to secrete cytokines that are related with the regulation of Th1/Th2 response (Klinman et al., 1996; Kim et al., 2013), and regulates a variety of immune responses including the activity of antigen-presenting cells (Carson and Raz, 1997; Chu et al., 1997). The expression of accessory molecules, such as CD80, CD83, and CD86, is induced by TLR9-mediated recognition of microbial components in immune cells (Krieg et al., 2006; Park et al., 2013). CpG-DNA has consequently gained attention as an immune adjuvant and as a therapeutic for autoimmune diseases for decades (Akira et al., 2001; Iwasaki and Medzhitov, 2004; Bode et al., 2011).

In a previous study, phosphodiester bond CpG-DNA (PO-ODN) with immunomodulatory activity from *Mycobacterium bovis*, a natural counterpart of phosphorothioate-modified types of CpG-DNA (PS-ODN) was screened. The screened potent PO-ODN was named MB-ODN 4531(O), which induces an immune response with less severe side effects than PS-ODNs (Lee et al., 2006; Kim et al., 2009). We evaluated the ability of MB-ODN 4531(O) encapsulated in several different liposomes to stimulate immune responses. As a result, we found that MB-ODN 4531(O) encapsulated in a phosphatidyl-β-oleoyl-γ-palmitoyl ethanolamine (DOPE): cholesterol hemisuccinate (CHEMS) complex (Lipoplex(O)) was the most potent on human and mouse cells (Kim et al., 2011a; Kim et al., 2011b).

Previously, the function of the UNC93B1 gene was identified in association with CpG-DNA (Brinkmann et al., 2007; Kim et al., 2008). Their study results suggested that nucleotide-sensing TLRs, such as TLR7 and TLR9, are delivered from the endoplasmic reticulum (ER) to endolysosomes by UNC93B1 in dendritic cells.

In the present study, we found that expression of UNC93A, another member of the UNC-93 superfamily (Liu et al., 2002), was specifically induced in the peritoneal cells of mice injected with Lipoplex(O) and that over-expressed UNC93A is localized in the cytoplasm of host cells.

Materials and Methods

Oligodeoxynucleotides. ODNs were purchased from GenoTech (Korea). The ODN sequences used in this study were either phosphodiester (O) or phosphorothioate-modified (S). We used several CpG-DNA sequences: CpG-DNA 1826(S), 5'-TCCAT GACGTTCCTGACGTT-3', Non-CpG-DNA 2041(S), 5'-CTGGT CTTTCTGGTTTTTTTCTGG-3', MB-ODN 4531(O), 5'-AGCA GCGTTCGTGTCGGCCT-3'. The phosphorothioate version of MB-ODN 4531(O) is MB-ODN 4531(S). MB-ODN 4531GC(O) is a derivative of MB-ODN 4531(O) with one of the CG sequences reversed to GC. CpG motifs are underlined. The non-CpG-DNA 2041(S) served as the negative control. The endotoxin content of the ODNs was less than 1 ng/mg of ODN as measured by a *Limulus amebocyte* assay (Whittaker Bioproducts, USA).

Preparation of CpG-DNA encapsulated in liposome complexes. The liposomes CHEMS and DOPE were purchased from Sigma-Aldrich (USA). Briefly, DOPE and CHEMS were mixed in ethanol at a molar ratio of 1:1, evaporated with nitrogen gas to make a solvent-free lipid film and resuspended in a mixture containing equal volumes of water-soluble MB-ODN 4531(O) or MB-ODN 4531GC(O), followed by vigorous stirring at room temperature for 30 min. After adjusting the pH to 7.0, the Lipoplex solution was sonicated for 30 s with a sonicator. The solution was then filtered with a 0.22-μm filter and freeze-thawed three times with liquid nitrogen (Kim et al., 2011b). The resulting liposome-encapsulated MB-ODN 4531(O) and MB-ODN 4531GC(O) are abbreviated as Lipoplex(O) and LipoplexGC(O), respectively.

Mice and ODN treatment. Mice were maintained under specific-pathogen-free conditions. Four-week-old male BALB/c mice were purchased from Central Lab. Animal Inc. (Korea). Mice were maintained under specific-pathogen-free conditions in a controlled environment (20–5°C, 32–7% humidity). All animal procedures performed in the present study are in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research & Quarantine Service of Korea. The protocol was approved by the Institutional Animal Care and Use Committee of Hallym University (Hallym 2012-35). The mice were sacrificed under Zoletil 50+Rompun (1:1 in v/v, 200 μL/mouse) anesthesia, and efforts were made to minimize suffering. The BALB/c mice were injected intraperitoneally (IP) with 50 μg of naked or liposome-encapsulated ODNs.

Preparation of peritoneal cells, splenocytes, and bone marrow cells. The mice were sacrificed 12 h after the injection with indicated reagents. The peritoneal cells were collected from a mouse body with 10 mL RPMI 1640 culture medium. The spleen was removed aseptically and immediately minced in the RPMI 1640 culture medium and passed through a mesh to obtain a single cell suspension. The bone marrow cells were obtained from femurs and tibiae of BALB/c mice. After the cells were washed twice with phosphate-buffered saline (PBS), erythrocytes were removed by treating the cells with 5 mL of a red blood cell lysis buffer containing 20 mM Tris-HCl (pH 7.2) and 140 mM ammonium chloride at 37°C for 5 min. The cells were counted with the aid of trypan blue dye exclusion.

Stimulation of primary cells with cytokines. Prepared BALB/c peritoneal cells, splenocytes, and bone marrow cells were cultured in 6-well plates with RPMI 1640 culture medium, and stimulated with each cytokine: TNF-α (R&D Systems, USA), IFN-γ, IL-4, Flt-3-Ligand, GM-CSF (Pepro Tech, USA) and lipopysaccharide (LPS) (Sigma). After 24 h, the RNA samples were prepared and

analyzed by reverse transcriptional-polymerase chain reaction (RT-PCR).

RNA isolation and reverse-transcription PCR analysis. After cells were treated with CpG-DNA or liposome-encapsulated CpG-DNA for the indicated time periods, and total RNA was extracted using an RNeasy RNA isolation kit (Qiagen, USA). Five micrograms of total RNA were reverse-transcribed in a first-strand synthesis buffer containing 6 μg/mL of oligo (dT) primer, 50 U of reverse transcriptase, 4 mM of dNTP, and 40 U of RNase inhibitor. To examine the tissue distribution of human UNC93A (hUNC93A) and human UNC93B1 (hUNC93B1), Human Total RNA Master Panel II purchased from Clontech (USA) was used. We performed a standard PCR reaction for 30 or 35 cycles using a cDNA mixture as a template with the following primer sets: hUNC93A, 5'-CTC TAT GGA GGC ATG CTC CT-3' (sense), 5'-GCT GCT CTT CTG GAA GGG TC-3' (anti-sense); hUNC93B1, 5'-CGG AGC GGA AAC CTC ATT GT-3' (sense), 5'-GGG CCC AGA AAA AGA GGA TG-3' (anti-sense); mouse UNC93A (mUNC93A), 5'-GGA GCT GGA CGG GTA CCC TCT TT-3' (sense), 5'-GGT AGG TAC CCT GAG CCG ACC AC-3' (anti-sense); mouse UNC93B1 (mUNC93B1), 5'-GCA AGC GCC TCG GAG TGG TC-3' (sense), 5'-GGG TGC GTG GGA ACC TCG TG-3' (anti-sense); human actin, 5'-GGG TCA GAA GGA TTC CTA TG-3' (sense), 5'-CCT TAA TGT CAC GCA CGA TTT-3' (anti-sense); mouse GAPDH, 5v-ATG GTG AAG GTC GGT GTG AAC G-3' (sense), 5'-GTT GTC ATG GAT GAT CTT GGC C-3' (anti-sense). The PCR products were detected by UV light after being resolved on a 1.2% agarose gel.

Cell lines. Human embryonic kidney (HEK) cells (CRL-1573; ATCC) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 25 mM HEPES, 100 U/mL, penicillin, and 100 g/mL streptomycin. Cells were cultured at 37°C in an atmosphere of 95% air and 5% CO_2.

Construction of recombinant hUNC93A expression vector. The hUNC93A gene was amplified by PCR using fetal liver cDNA in Human Total RNA Master Panel II (Clontech) as a template with the following primer sets: 5' primer, hUNC93A 5'-CTC GAG CGA TGG ACA GAA GTC TAA GGA A-3'; 3' primer, hUNC93A 5'-GAA TTC CAT TTT TGT TTG TAT TTC TTC ATC-3'; the engineered *Xho*I and *EcoR*I restriction enzyme sites are underlined. The amplified PCR product was inserted into pGEM-T easy vector I (Promega, USA). The cDNA for hUNC93A was subcloned into the mammalian expression vector pcDNA-3.1/myc-His(-)B (Invitrogen, USA) using *Xho*I and *EcoR*I enzyme sites. The resulting vector construct was verified by DNA sequencing.

Construction of recombinant mUNC93A expression vector. The mUNC93A gene was amplified by PCR using the cDNA of Lipoplex(O) stimulated BALB/c peritoneal cells as a template with the following primer sets: 5' primer, mUNC93A 5'-CTC GAG CGA TGG AGA GGA GTC TGA AG-3'; 3' primer, mUNC93A 5'-GAA TTC TAT TTT TGT TTT CAT TTC TTC CT-3', in which the engineered *Xho*I and *EcoR*I restriction enzyme sites are underlined. A mammalian expression vector for expression of the mUNC93A gene was constructed as described for the hUNC93A expression vector.

Selection and characterization of stable cell line expressing UNC93A. For the generation of UNC93A, over-expressing stable cell lines, the UNC93A constructs were transfected with FuGENE 6 Transfection Reagent (Roche, USA) following the manufacturer's instructions. The cells were selected in the presence of 500 mg/mL of G418 (Calbiochem, Germany) for 14 days. The G418-resistant clones were expanded and assayed for UNC93A expression by immunoblotting with an anti-Myc-Tag mouse antibody (Cell Signaling Technology, USA).

Indirect immunofluorescence and confocal microscopy. Cellular localization of UNC93A was detected by indirect immunofluorescene assays using confocal microscopy. Briefly, HEK293 stable cell lines expressing UNC93A were cultured directly on poly-L-lysine treated glass cover slips in 12-well plates for 48 h. Cells were washed with PBS and fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS (PBST), and blocked with 1.5% normal donkey serum (Sigma) with PBST. The anti-Myc-Tag mouse antibody was applied to the fixed cells for 30 min followed by additional 1 h incubation with Alexa Flour 488 conjugated rabbit anti-mouse IgG (Molecular Probes, USA). DNA staining (Hoechst No. 33258; Sigma) was used to identify cell nuclei. The mounted samples were scanned with a LSM 710 laser scanning microscope (Carl Zeiss, Germany).

Results and Discussion

TLR9-dependent expression of UNC93A induced by Lipoplex(O) in BALB/c peritoneal cells. UNC93B1 is reported to be essential for proper CpG-DNA-TLR9 signaling in the ER (Brinkmann et al., 2007). Nevertheless, almost no information is available on UNC93A in the context of TLR signaling. Therefore, in the present study the expression and possible function of UNC93A were studied. First, we investigated expression of the mUNC93A and mUNC93B genes in peritoneal cells from BALB/c mice treated with naked or liposome-encapsulated ODNs. BALB/c mice were injected IP with CpG-DNA 1826(S), non-CpG-DNA 2041(S), Lipoplex(O), or LipoplexGC(O). After 12 h, the peritoneal cells were harvested and analyzed by RT-PCR. As a result, mUNC93A mRNA was induced in Lipoplex(O) stimulated peritoneal cells but not in the cells stimulated with 1826(S), non-CpG-DNA 2041(S) or LipoplexGC(O) (Fig. 1A). To confirm the optimal time for detecting mUNC93A mRNA expression, we prepared Lipoplex(O)-stimulated peritoneal cells for RT-PCR at the indicated time points. Expression of mUNC93A was slightly detected 6 h after IP injection, and more mUNC93A mRNA was expressed after 12 h (Fig. 1B). In order to determine whether the expression of mUNC93A induced by Lipoplex(O) is TLR9-dependent, TLR9 knock out (TLR9-/-) mice were used. The mice were injected IP with Lipoplex(O) or LipoplexGC(O), and the

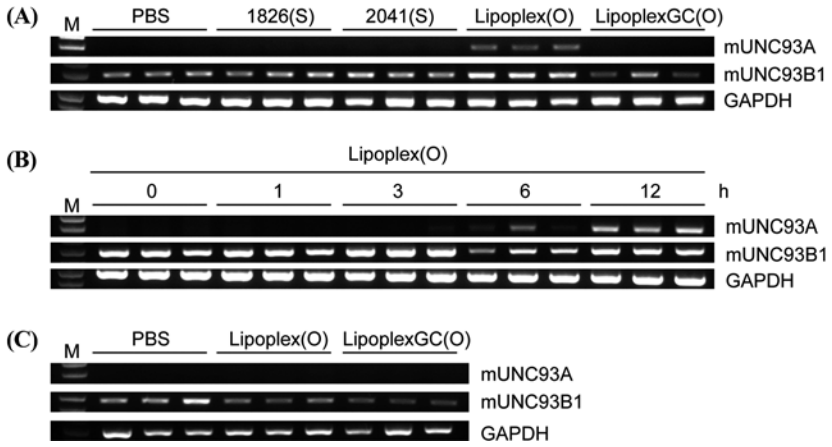

Fig. 1 TLR9-dependent expression of mUNC93A in BALB/c peritoneal cells. BALB/c mice (n=3/group) were injected IP with indicated reagents and the peritoneal cells of the mice were harvested. The expression levels of mUNC93A and mUNC93B1 mRNA were analyzed by RT-PCR. (A) The mice were injected with CpG-DNA 1826(S) (1826(S)), non-CpG-DNA 2041(S) (2041(S)), Lipoplex(O), or LipoplexGC(O) and the samples were analyzed 12 h after injection. (B) The mice were injected with Lipoplex(O), and the samples were analyzed at the indicated time points. (C) TLR9 knock out (TLR9-/-) mice (n=3/group) were injected IP with Lipoplex(O) or LipoplexGC(O), and the samples were analyzed 12 h after injection. M, molecular weight marker DNA.

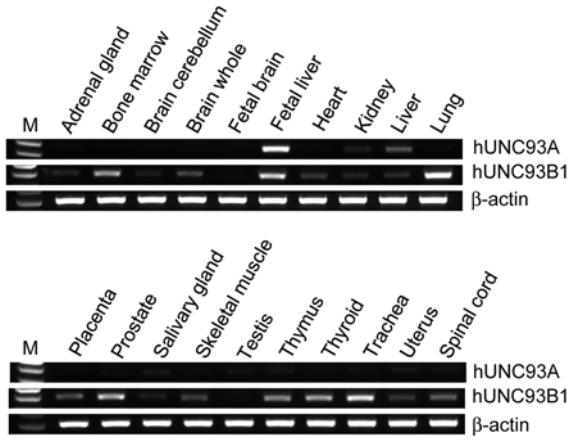

Fig. 2 Expression of hUNC93A in total tissues of human. To investigate where hUNC93A mRNA is expressed in human tissues, we used Human Total RNA Master Panel II as templates for RT-PCR. Expression of mUNC93A and mUNC93B1 mRNA was assessed.

peritoneal cells were harvested 12 h after injection. Through the analysis of RT-PCR, we confirmed that the expression of mUNC93A in peritoneal cells is TLR9-dependent (Fig. 1C). In addition, we examined the expression of the mUNC93B1 mRNA in all samples and found that the mUNC93B1 gene is consistently expressed under all conditions (Figs. 1A, B, and C). Therefore, we can conclude that expression of the mUNC93A gene is regulated specifically in response to Lipoplex(O) in a TLR9-dependent manner.

Expression of UNC93A in several human tissues. In order to determine the expression of human UNC93A (hUNC93A) in the human body, we used Human Total RNA Master Panel II as templates for RT-PCR. Slight expression of hUNC93A in the kidney and liver and significant expression in fetal liver were detected (Fig. 2). In contrast, hUNC93B1 was expressed in most tissues except fetal brain. Therefore, we can assume that the expression of hUNC93A is regulated in a tissue-specific manner and possibly in a stimulus-dependent manner.

mUNC93A was not induced in cultured BALB/c peritoneal cells stimulated with ODNs and Lipoplex(O) *in vitro*. In the present study, mUNC93A was confirmed to be induced in Lipoplex(O)-injected BALB/c peritoneal cells *in vivo*, thus *in vitro* effects of Lipoplex(O) using primary peritoneal cells were evaluated. The peritoneal cells were collected from mice with 10 mL RPMI 1640 culture medium, washed, and cultured in a 6-well plate. The cells were treated with the indicated ODNs or Lipoplex(O) for the indicated periods and the total RNA level was analyzed by RT-PCR. The expression of mUNC93B mRNA was detected in all samples regardless of the treatment, but the expression of mUNC93A mRNA was not detected (Fig. 3). Therefore, it is likely that the induction of mUNC93A mRNA requires the presence of other cells or factors than the peritoneal cells.

Several cytokines had no effect on UNC93A expression in the cultured BALB/c peritoneal cells, splenocytes, and bone marrow cells. TLR9-dependent expression of mUNC93A induced by injection of Lipoplex(O*) in vivo* indicates that the stimulation of TLR9 with Lipoplex(O) stimulates some signal to induce mUNC93A. As Lipoplex(O) induces expression of many cytokines, we questioned whether the cytokines can induce mUNC93A as well. The cultured BALB/c peritoneal cells, splenocytes, and bone marrow cells were treated with several cytokines and the expression levels of mUNC93A mRNA were examined by RT-PCR. The cytokines include TNF-α, IFN-γ, IL-4, Flt-3-Ligand, GM-CSF, and LPS. Although expression of mUNC93B1 was detected, no change was observed in any

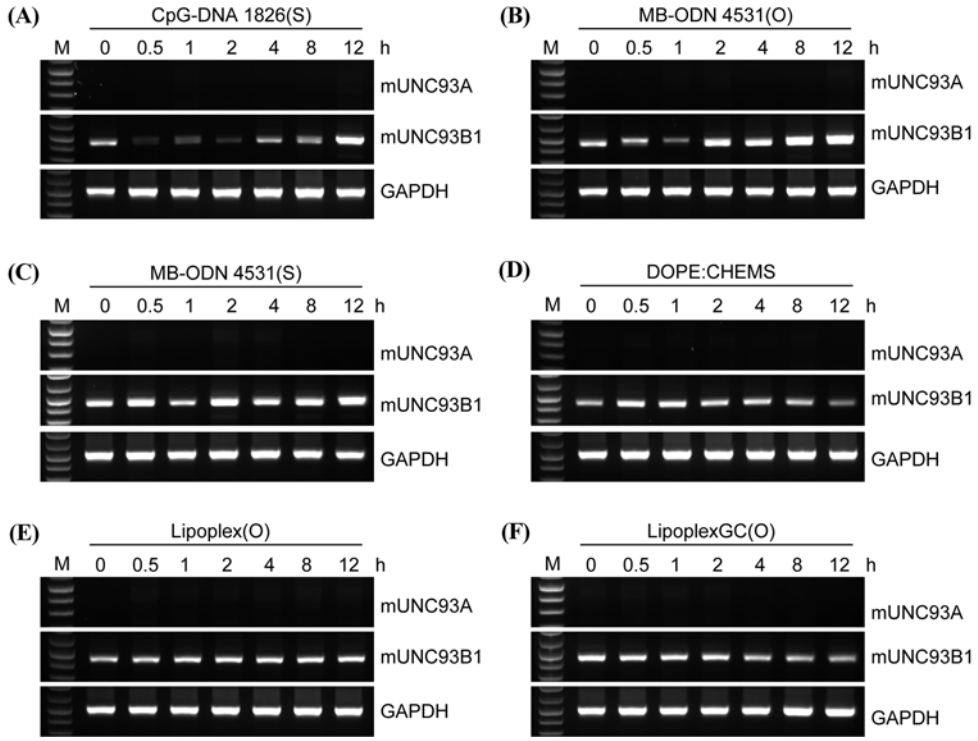

Fig. 3 Effects of CpG-DNA and Lipoplex(O) on cultured BALB/c peritoneal cells. Peritoneal cells collected from mice were cultured in 6-well plates and treated with the indicated CpG-DNA or Lipopex(O) for the indicated periods (A~F). Total RNA was extracted, and the expressions of mUNC93A and mUNC93B1 was analyzed by RT-PCR.

cytokine. Furthermore, no expression of mUNC93A mRNA was detected (data not shown). Therefore, we can conclude that addition of a single cytokine cannot induce expression of mUNC93A in the primary cells.

Expression of UNC93A in HEK293 cells. The sequence homology between hUNC93A and mUNC93A were analyzed and found that the sequence identities between them are about 66%. Two mammalian expression vectors were constructed in which either hUNC93A or mUNC93A gene was inserted. The parental vector was used as a negative control. The constructed plasmids were transfected to HEK293 cells and cultured in the presence G418, as a selective marker, for 14 days. The selected clones were assayed for UNC93A expression by immunoblotting with an anti-Myc-Tag mouse antibody. Potential hUNC93A proteins of about 90 kDa and over 200 kDa were detected in hUNC93A-transfected cells, but not in the control vector-transfected cells (Fig. 4A). In the case of mUNC93A-transfected cells, potential mUNC93A proteins of about 70 kDa were detected (Fig. 4B). As the apparent molecular weights of the recombinant UNC93 proteins are much larger than those predicted from the amino-acid-sequences, it is likely that the proteins undergo post-translational modification such as glycosylation. The presence of bands with size of greater than 200 kDa in the hUNC93A-transfected cells suggests the possibility of polymers that are resistant to general denaturation and reduction.

Localization of UNC93A in HEK293 cells. To determine the cellular localization of the hUNC93A and mUNC93A in HEK293 cells, hUNC93A and mUNC93A-transfected HEK293 cells were analyzed by immunofluorescence and confocal microscopy. The hUNC93A and mUNC93A proteins were detected in the cytoplasm; no signal was detected in the HEK 293 cells transfected with the control vector (Fig. 5). Previously, mUNC93B1 was reported to be a multi-membrane spanning protein involved in the transport of nucleotide-sensing TLRs such as TLR3, TLR7, and TLR9 to ER, where the TLRs activate the innate immunity (Brinkmann et al., 2007). Considering that UNC93A is also a multi-pass membrane protein, the specific location and the function of this protein should be examined in detail.

Because CpG-DNA-triggered TLR9 signaling contributes to activation of innate immune responses as well as acquired responses, the application of CpG-DNA has been widely explored (Krieg et al., 2006). Excess TLR signaling is also known to be involved in some autoimmune diseases, such as asthma and other allergic diseases (Kumagai et al., 2008). Therefore, fine regulation of TLR signaling is necessary to use CpG-DNA as a therapeutic agent (Okabe et al., 2005; Ishii et al., 2006; Kawane et al., 2006).

In the present study, we found that the expression of mUNC93A is induced specifically by Lipoplex(O) but not by CpG-DNA only or LipoplexGC(O). In contrast, expression of mUNC93B1 was not changed by Lipoplex(O). Previously, we found that Lipoplex(O)

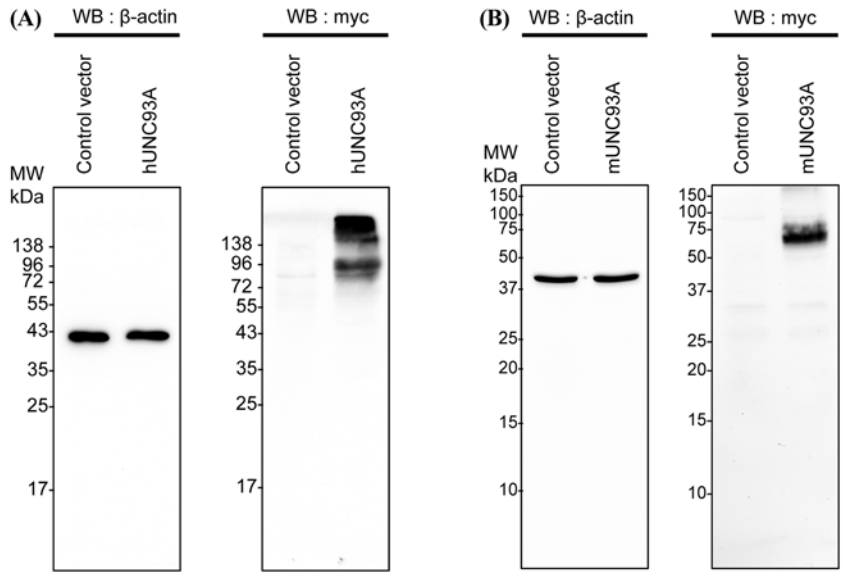

Fig. 4 Expression of hUNC93A and mUNC93A in HEK293 cells. (A) Cell lysates from HEK293 cells transfected with control vector or hUNC93A-pcDNA were immunoblotted with Myc-Tag mouse antibody. (B) Cell lysates from HEK293 cells transfected with control vector or mUNC93A-pcDNA were detected with Myc-Tag mouse antibody. b-actin is a loading control.

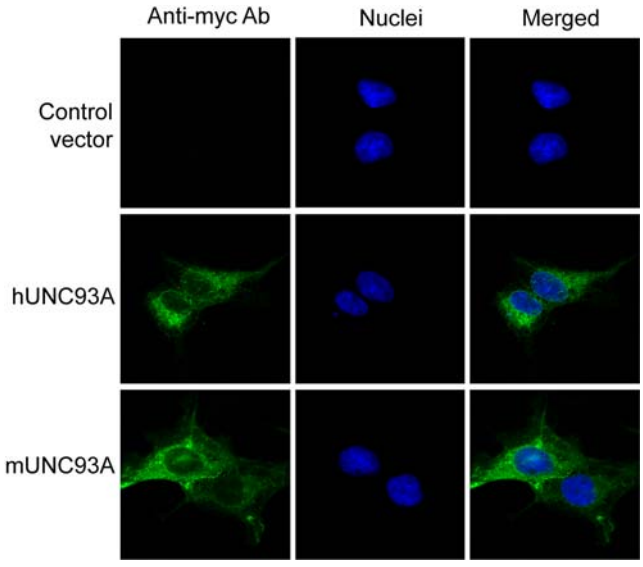

Fig. 5 Cellular localization of hUNC93A and mUNC93A in HEK293 cells. Localization of Myc-tagged hUNC93A and mUNC93A was visualized with a confocal laser scanning microscope by indirect immunofluorescence. Myc-tagged hUNC93A and mUNC93A were stained with Alexa Flour 488 conjugated rabbit anti-mouse IgG (green). Cell nuclei were stained with Hoechst No. 33258 (blue).

has potent immunostimulatory activity triggering TLR9-mediated signaling in immune cells such as B cells and macrophage cells (Kim et al., 2011a; Kim et al., 2011b). Therefore, concurrent induction of mUNC93A by Lipoplex(O) in the peritoneal cells suggests the possibility that mUNC93A can be a regulator of TLR9 signaling in specific locations. This should be clarified in future studies.

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