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Production of a Monoclonal Antibody Targeting the M Protein of MERS-CoV for Detection of MERS-CoV Using a Synthetic Peptide Epitope Formulated with a CpG–DNA–Liposome Complex

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Abstract
The Middle East respiratory syndrome-related coronavirus (MERS-CoV) contains four major structural proteins, the spike glycoprotein, nucleocapsid phosphoprotein, membrane (M) glycoprotein and small envelope glycoprotein. The M protein of MERS-CoV has a role in the morphogenesis or assembly of the virus and inhibits type I interferon expression in infected cells. Here, we produced a monoclonal antibody specific against the M protein of MERS-CoV by injecting BALB/c mice with a complex containing the epitope peptide and CpG–DNA encapsulated with a phosphatidyl-β-oleoyl-γ-palmitoyl ethanolamine (DOPE):cholesterol hemisuccinate (CHEMS). The monoclonal antibody was reactive to the epitope peptide of the M protein of MERS-CoV which was confirmed by western blotting and immunoprecipitations. Indirect immunofluorescence assay and confocal image analysis showed that the monoclonal antibody binds specifically to the M protein of MERS-CoV in the virus-infected cells. Further studies using this monoclonal antibody may provide important information on the function of the M protein and its future application in diagnostics.

Keywords B cell epitope · Monoclonal antibody · MERS-CoV · M protein

Introduction

Coronaviruses (CoVs) are well-known as a major cause of the common cold in humans by mild infection in the upper respiratory tract. CoVs have gained attention since the outbreak of severe acute respiratory syndrome (SARS) in 2002–2003 when SARS-related CoV (SARS-CoV) was identified (Anderson et al. 2004). SARS-CoV is the cause of human lower respiratory tract diseases such as bronchitis and pneumoniae (Holmes 2003; Girard et al. 2005; Peiris et al. 2004). In 2012, one more CoV was isolated in Saudi Arabia and named MERS-CoV. This virus belongs to lineage C of beta-coronaviruses (Zaki et al. 2012). According to a World Health Organization (WHO) survey, 2143 cases of MERS-CoV infections were reported, and 750 people were killed in 27 countries by March 2018. In South Korea, 186 people were infected by MERS-CoV, and 36 people died in 2015. The symptoms of the MERS-CoV infection in South Korea included fever, cough, upper respiratory tract signs, lower respiratory tract distress with lymphopenia and elevated liver enzymes, acute respiratory distress syndrome, and multiple organ system failure (Zhang et al. 2016). MERS-CoV is reportedly transmitted to humans generally from bats or dromedary camels, but the exact route of infection has not yet been identified (Who Mers-Cov Research Group 2013; Yin and Wunderink 2018; Mackay and Arden 2017).

MERS-CoV consists of four major protein complexes: spike (S) glycoprotein, nucleocapsid (N) phosphoprotein, Membrane (M) glycoprotein and small envelope (E) glycoprotein (Mustafa et al. 2018; Boheemen et al. 2012; Almazán et al. 2013; Scobey et al. 2013). The S protein is a major protein and consists of the S1 subunit with an RBD domain and the S2 subunit with the HR 1 and HR 2

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domains. The RBD domain on the S1 subunit was reported to bind with human dipeptidyl peptidase 4 (hDPP4, CD26) during the attachment of the virus to the target cell (Li 2015a; Lu et al. 2013; Raj et al. 2013). On the other hand, membrane fusion between the virus and the target cell was reported to be done by the HR 1 and HR 2 domains of the S2 subunit after the RBD domain interacts with hDPP4 (Gao et al. 2013; Lu et al. 2014). The M protein is a transmembrane protein of the viral envelope and functions in the morphogenesis or assembly of MERS-CoV in the Golgi complex interacting with other viral proteins (Yang et al. 2013). MERS-CoV M protein inhibits type I interferon expression by interacting with TNF receptor-associated factor 3 (TRAF3) which leads to the inhibition of interferon regulatory factor 3 (IRF3) activation (Lui et al. 2016). Many research groups have developed antibodies against MERS-CoV using a synthetic protein of the S1 or S2 subunit as an antigen while some other research groups have focused on hDPP4 as a new target antigen (Lu et al. 2014; Wang et al. 2015; Du et al. 2014; Li et al. 2015b; Tao et al. 2015; Ohnuma et al. 2013; Raj et al. 2014). However, production of an antibody against the M protein has not been well studied yet.

Previously, we isolated the natural phosphodiester bond CpG–DNA (MB-ODN 4531(O)) from *Mycobacterium bovis* chromosomal DNA. CpG–DNA has an adjuvant activity and the ability to modulate the innate immune response (Lee et al. 2006). When CpG–DNA was encapsulated with phosphatidyl-β-oleoyl-γ-palmitoyl ethanolamine (DOPE):cholesterol hemisuccinate (CHEMS) complex, CpG–DNA showed a significant adjuvant effect increasing antibody production against protein antigens such as ovalbumin and hen egg lysozyme (Kim et al. 2011a). We also developed CpG–DNA encapsulated with the DOPE:CHEMS complex as an adjuvant to produce a synthetic peptide epitope-specific antibody without conventional carriers. We reported that the CpG–DNA encapsulated with the DOPE:CHEMS induced the production of antibodies against epitope peptides from viruses such as hepatitis C virus (Kim et al. 2011b), influenza virus (Rhee et al. 2012), and respiratory syncytial virus (Park et al. 2015). Furthermore, we validated the adjuvant effects of the CpG–DNA encapsulated with the DOPE:CHEMS on the synthetic epitope peptide of TM4SF5 as a hepatocellular carcinoma antigen (Kwon et al. 2012).

Here, we produced a monoclonal antibody that recognizes the M protein of MERS-CoV by immunizing mice with a complex of the B-cell epitope of the M protein and the CpG–DNA–liposome. We found that the monoclonal antibody specifically recognizes the recombinant M protein as well as native M protein in MERS-CoV-infected cells.

Materials and Methods

Cell and Virus

The African green monkey kidney cells, Vero cells, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific), 25 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were incubated at 37 °C in an atmosphere of 95% air and 5% CO_2. MERS-CoV/KOR/KNIH/002_05_2015 was obtained from the Korea Centers for Disease Control and Prevention (Permission No. 1-001-MER-IS-2015001).

Preparation of the B Cell Epitope Peptides

To produce a specific antibody against the M protein of MERS-CoV, we selected a B cell epitope by analyzing the M protein based on epitope prediction, surface accessibility and antigenicity index (http://tools.iedb.org/bcell). The B cell epitope sequence for the M protein of MERS-CoV (MERS-M158, ^{158}CDYDRLPNEVTVAKPNVLIALKMVK^{182}) was synthesized with an automated peptide synthesizer (Peptron III-R24, Peptron, Daejeon, Korea). The peptides were purified by reverse-phase HPLC (Prominence HPLC, Shimadzu Corp., Kyoto, Japan) to a purity > 90%.

Mice Immunization

BALB/c (4-week-old, female, H-2^b) mice were obtained from Nara-Biotec (Seoul, Korea). The peptide corresponding to the B-cell epitope of the M protein of MERS-CoV (MERS-M158) and the CpG–DNA (MB-ODN 4531(O), AGCAGCGTTCGTGTCGGCCT) were co-encapsulated in the DOPE:CHEMS complex (molar ratio of 1:1) as reported previously (Kim et al. 2011a; Park et al. 2015; Wu et al. 2018). The mice were injected intraperitoneally (i.p.) three times at 10-day intervals with the liposome complex containing 50 µg of the MERS-M158 peptide and 50 µg of the CpG–DNA as described previously (Wu et al. 2018). Animal experiments were approved by the Institutional Animal Care and Use Committee of Hallym University (Hallym2016-51).

Production of the Mouse Monoclonal Antibody Against the M Protein of MERS-CoV

Splenocytes were isolated from mice immunized with the epitope peptide of the M protein of MERS-CoV,

MERS-M158, and fused with mouse SP2/0 myeloma cells in a polyethylene glycol solution (PEG, Sigma Aldrich, St. Louis, MO, USA). After the fusion, hybridoma cells were selected to obtain clones producing the anti-MERS-M158 peptide monoclonal antibody in HAT medium (Sigma Aldrich) and HT medium (Sigma Aldrich) according to the standard hybridoma production method as described previously (Wu et al. 2018; Yokoyama et al. 2006). Ascites containing the monoclonal antibody was generated by injecting the selected hybridoma cells into the peritoneal cavity of BALB/c mice. The monoclonal antibody against the M protein was isolated from the ascitic fluid by protein-A column chromatography as described previously (Park et al. 2015).

Antigen-Specific Ig ELISA Assay

The MERS-M158 peptide was coated at 5 µg/well onto 96-well immunoplates (Thermo Fisher Scientific) and then blocked with PBST (PBS supplemented with 0.05% Tween-20) containing 1% BSA. Mouse sera were collected by orbital bleeding to measure the epitope peptide-specific antibody production. The epitope peptide-specific antibody levels in the mouse sera, hybridoma culture supernatants and ascites as well as the purified monoclonal antibody were quantified as previously described (Park et al. 2015). The isotype of the monoclonal antibody was identified with HRP-conjugated anti-mouse IgG (each isotype) antibody (Southern Biotech, Birmingham, AL, USA).

Affinity Constant Measurement by ELISA

To measure the binding affinity of the MERS-M158-specific monoclonal antibody, 5 µg/well of MERS-M158 peptide was coated onto 96-well immunoplates and then blocked with PBST containing 1% BSA. The monoclonal antibody was added to each plate with a serial dilution of 1:5 in PBST and then incubated for 2 h at room temperature. After washing with PBST, anti-IgG antibody conjugated with horseradish peroxidase was added to each plate. The amounts of antibody in the plates were determined by developing with the tetramethylbenzidine (TMB) peroxidase substrate (KPL, SeraCare, Milford, MA, USA). The absorbance was evaluated with the Spectra Max 250 microplate reader (Molecular Devices, San Jose, CA, USA) at 405 nm and then calculated with the SigmaPlot program to determine the EC50 value as described previously (Park et al. 2015).

Western Blotting and Immunoprecipitation

To determine whether the MERS-M158 peptide-specific monoclonal antibody detects the M protein of MERS-CoV, western blot analysis was performed. Briefly, MERS-CoV-infected Vero cells were lysed with cell lysis buffer (10 mM HEPES, 150 mM NaCl, 5 mM EDTA, 100 mM NaF, 2 mM Na_3VO_4, protease inhibitor cocktail and 10% NP-40) and then loaded onto a 15% SDS-PAGE. The separated proteins were transferred onto a nitrocellulose membrane, and the membrane was blocked by PBST containing 3% BSA. The membrane was subsequently treated with the MERS-M158 peptide-specific monoclonal antibody and incubated at room temperature for 3 h. After incubation, the membrane was rinsed with PBST three times and treated with HRP-conjugated goat anti-mouse IgG antibody (1:5000, Jackson ImmunoResearch Laboratories, PA, USA) in PBST containing 5% skim milk. The membrane was developed with ECL solution (SeraCare). To perform immunoprecipitation, the MERS-CoV-infected Vero cell lysates were incubated with the MERS-M158 peptide-specific monoclonal antibody at 4 °C overnight. The samples were incubated with protein-A beads (Repligen, Waltham, MA, USA) for 1 h, and the immunoprecipitated proteins were analyzed by SDS-PAGE and western blotting with the MERS-M158 peptide-specific monoclonal antibody.

Deglycosylation Assay

Cell lysates were prepared from the Vero cells and MERS-CoV-infected Vero cells with a lysis buffer (0.5% SDS, 1% β-mercaptoethanol) and boiled at 100 °C for 10 min. The lysates were treated with peptide-*N*-glycosidase (PNGase) F (Elpis Biotech, Daejeon, Korea) at 37 °C for 2 h and then boiled at 100 °C for 10 min. The lysates were analyzed by SDS-PAGE and western blotting with the MERS-M158 peptide-specific monoclonal antibody. After dilution of the lysates with lysis buffer, immunoprecipitation was performed with the MERS-M158 peptide-specific monoclonal antibody. The immune complex was analyzed by SDS-PAGE and western blotting with the MERS-M158 peptide-specific monoclonal antibody.

Indirect Immunofluorescence Assay and Confocal Images

To perform the indirect immunofluorescence assay (IFA), Vero cells were mixed with MERS-CoV infected Vero cells (ratio 1:3) and plated onto slide glasses. The mixed cells were fixed with acetone, washed with distilled water (DW), and incubated with normal mouse IgG or the MERS-M158 peptide-specific monoclonal antibody at 37 °C. After a 2 h incubation, the slides were washed with PBS and DW, and then incubated with Alexa Flour 488-conjugated goat anti-mouse IgG antibody (Thermo Fisher Scientific, Waltham, MA, USA). The samples were mounted and then observed using a fluorescence microscope (IX70, Olympus, Tokyo, Japan). To obtain confocal images, Vero cells (5×10^4 cells) were plated onto cover glass in 12-well culture plates and infected with MERS-CoV

(0.1 MOI) for 2 days. The cells were treated with 4% paraformaldehyde to fix the cells and incubated with PBS containing 1% BSA and 0.1% triton X-100 for 30 min. to block the non-specific binding. The MERS-M158 peptide-specific monoclonal antibody was loaded into the wells and incubated for 2 h and then incubated with Alexa Flour 488-conjugated goat anti-mouse IgG for 1 h. The nuclei were observed by Hoechst 33258 (Thermo Fisher Scientific) staining. The cells were examined by Carl Zeiss LSM710 (Carl Zeiss, Oberkochen, DE).

Results

Epitope Selection and Production of Antibody Specific to the M Protein of MERS-CoV

To select the B cell epitope of the M protein of MERS-CoV, we analyzed the amino acid sequence of the M protein in terms of surface accessibility, antigenicity and epitope probability, and consequently, we selected the B cell epitope amino acid sequence MERS-M158 (158–182 amino acids, Fig. 1a). To determine whether the MERS-M158 peptide can work as a B cell epitope, we synthesized the MERS-M158 peptide and formulated a complex of the MERS-M158 peptide and CpG–DNA co-encapsulated in a liposome (DOPE:CHEMS) which was subsequently used to immunize BALB/c mice. The production of MERS-M158 peptide-specific antibody in the immunized mice was confirmed by ELISA shown in Fig. 1b.

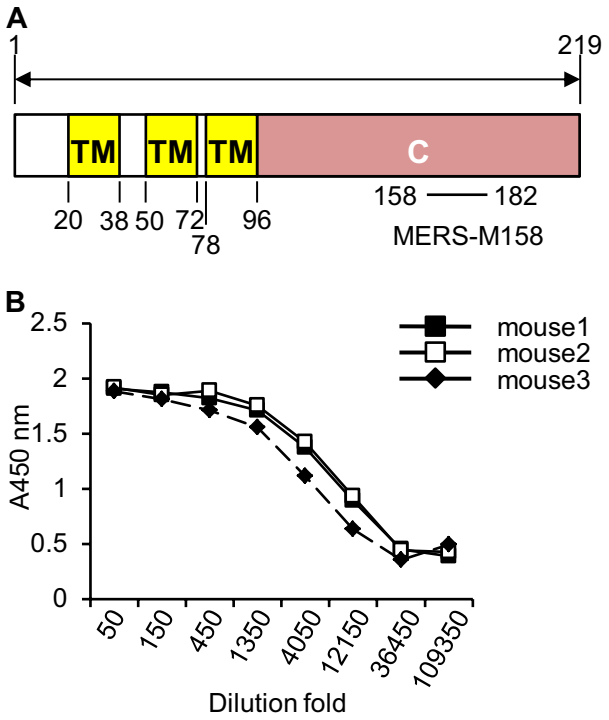


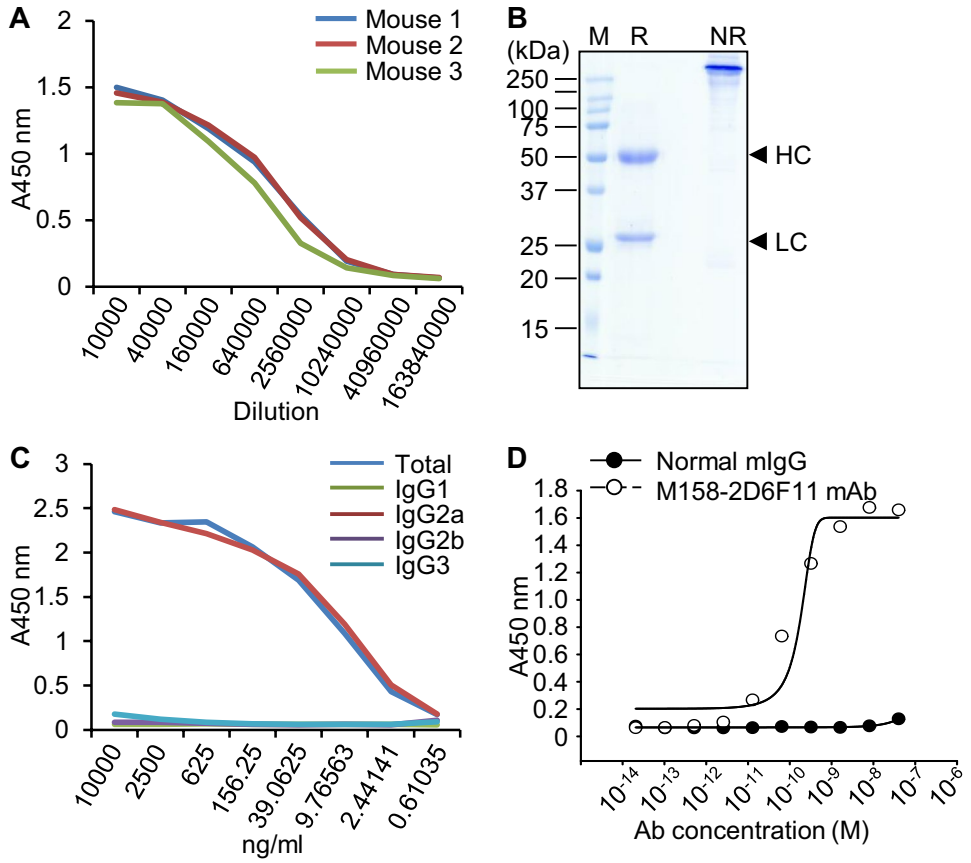
Fig. 1 B cell epitope of the M protein of MERS-CoV and production of the epitope-specific antibody. **a** Linear structure of the M protein of MERS-CoV. MERS-M158 denotes the B cell epitope sequence of M protein. The B cell epitope was predicted by analysis based on epitope prediction, surface accessibility and antigenicity index. *C* cytoplasmic domain, *TM* transmembrane domain. **b** Production of the MERS-M158-specific antibody in mice. BALB/c mice were immunized with the B-cell epitope peptide (MERS-M158) and CpG–DNA co-encapsulated in a DOPE:CHEMS complex. After three immunizations, sera were analyzed for the production of the MERS-M158-specific antibody by ELISA

Production of Monoclonal Antibody Specific to an Epitope of the M Protein of MERS-CoV

To produce a monoclonal antibody (mAb) against a specific epitope (MERS-M158) of the M protein of MERS-CoV, splenocytes were collected from mice immunized with the epitope peptide–CpG–DNA–liposome complex. The mouse splenocytes were fused with SP2/0, and the M158-2D6F11 clone producing the MERS-M158 peptide-specific antibody was selected. The M158-2D6F11 clone was injected into the mouse peritoneal cavity to produce ascites (Fig. 2a), and the MERS-M158 peptide-specific mAb (named M158-2D6F11) was purified (Fig. 2b). The isotype of the mAb was confirmed by ELISA as IgG2a (Fig. 2c). The analysis of the antibody binding with the MERS-M158 peptide by ELISA showed that the mAb M158-2D6F11 had an EC50 value of ~56 pM (Fig. 2d).

Western Blot and Immunoprecipitation Analyses with the mAb M158-2D6F11

To characterize the mAb M158-2D6F11, we performed western blot and immunoprecipitation analyses (Fig. 3). In a preliminary experiment, the mAb M158-2D6F11 recognized two types of protein bands with molecular weights of about 20 and 25 kDa in MERS-CoV infected Vero cells but not in the control Vero cells. The larger protein band of 25 kDa was the major form seen in lane 3 of Fig. 3a, b. Because the M protein of MERS-CoV is a glycoprotein, there could exist a fully glycosylated mature form and a not-yet-glycosylated or de-glycosylated form of the M protein. To confirm whether the two types of protein bands are the same protein produced by a protein modification, we repeated the same experiments but treated the cell lysates with a PBS control or PNGase F. As seen in Fig. 3a, b, the band intensity of the smaller protein increased in response to the PNGase F treatment suggesting that the protein bands of 25 and 20 kDa represent the glycosylated and de-glycosylated forms of the M protein, respectively. Because both protein bands were detected by the mAb M158-2D6F11, we conclude that the mAb M158-2D6F11 can recognize the M protein



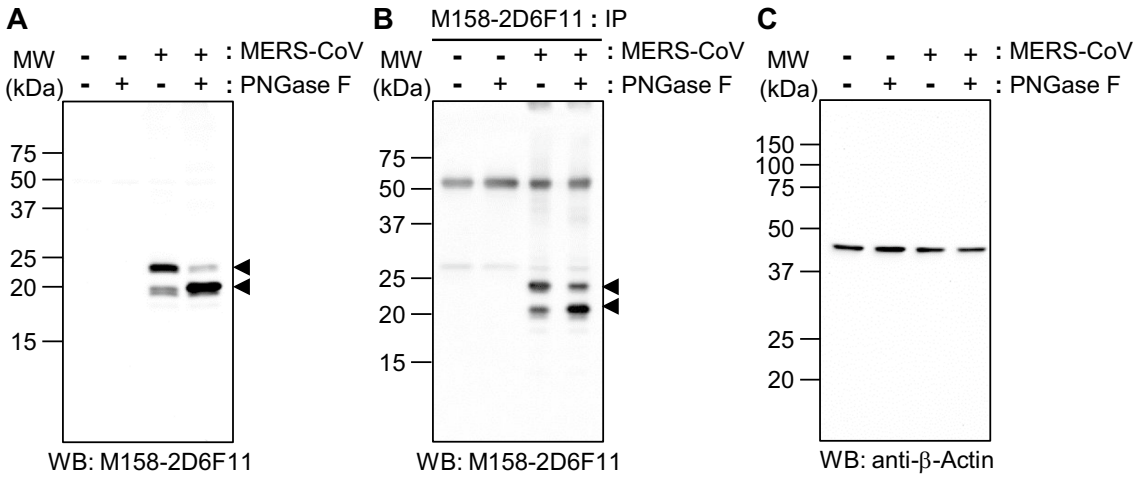


Fig. 2 Purification and characterization of the MERS-M158-specific monoclonal antibody. **a** Titration curves of the ascites. The M158-2D6F11 clone was injected into the pristine-primed mice to produce ascites. Production of the MERS-M158-specific monoclonal antibody from the M158-2D6F11 clone (mAb M158-2D6F11) was confirmed by ELISA using MERS-M158 peptide-coated plates. **b** Purification of the monoclonal antibody. The mAb M158-2D6F11 was purified from the ascites by protein A agarose column chromatography and analyzed by SDS-PAGE. *R* reducing condition, *NR* non-reducing condition. **c** Determination of the isotype. ELISA was performed to determine the isotype of the mAb M158-2D6F11. **d** Evaluation of the EC50 value. The ability of the mAb M158-2D6F11 to bind with the MERS-M158 peptide was estimated by ELISA

Fig. 3 Western blot and immunoprecipitation analyses for the characterization of the mAb M158-2D6F11. Vero cells and MERS-CoV-infected Vero cells were lysed with lysis buffer, and then, the lysates were treated with PBS (−) or PNGase F (+). **a** The lysates were analyzed by western blotting using the mAb M158-2D6F11 as a probe. **b** The lysates were immunoprecipitated with the mAb M158-2D6F11, and a western blot analysis was performed using the mAb M158-2D6F11 as a probe. **c** A western blot of the lysates probed by an anti-β-actin antibody as a control

irrespective of the *N*-glycosylation probably because the two asparagine residues in the epitope region are known not to be glycosylated. When we analyzed the protein band of 20 kDa obtained by immunoprecipitation with the mAb M158-2D6F11 (Fig. 3b) using mass spectroscopy, the protein was proved to be M protein of MERS-CoV (data not shown).

Detection of the M Protein in the MERS-CoV-Infected Cells with the mAb M158-2D6F11

To investigate whether the mAb M158-2D6F11 recognizes the M protein in the MERS-CoV-infected cells, we performed an IFA. The slide containing a mixture of Vero cells and MERS-CoV-infected Vero cells was treated with normal mouse IgG as a control or the mAb M158-2D6F11. While there was no significant signal detected by the normal mouse IgG, a distinct fluorescence signal was detected by the mAb M158-2D6F11 in the virus infected cells (Fig. 4a). For a more definitive detection of the MERS-CoV M protein in the infected cells, we examined the binding of the mAb M158-2D6F11 to the MERS-CoV infected cells by confocal microscopy. Vero cells or MERS-CoV-infected Vero cells were cultured on cover glasses and then analyzed. The mAb

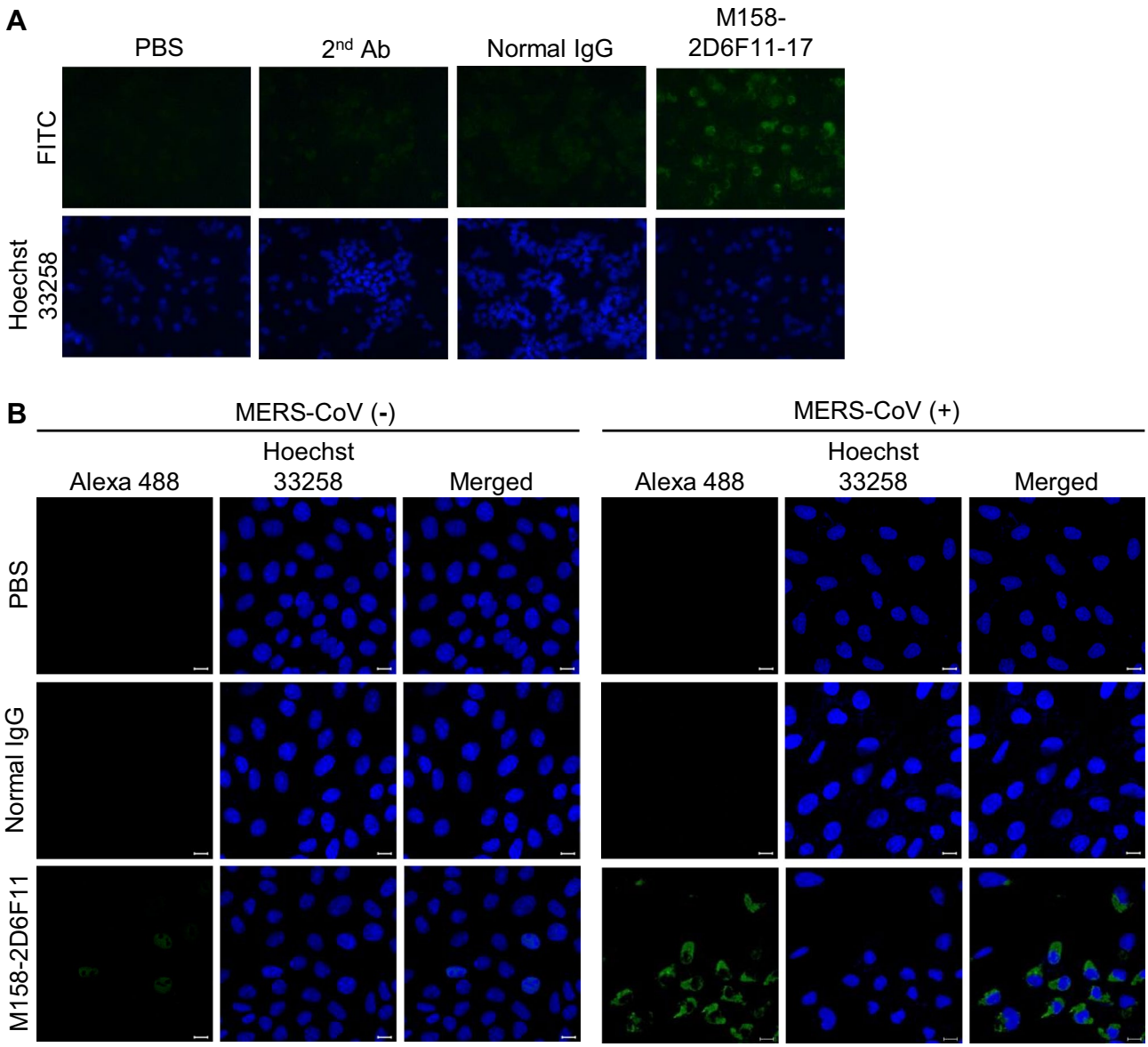


Fig. 4 Detection of MERS-CoV-infected cells with the mAb M158-2D6F11. **a** Indirect immunofluorescence assay. Vero cells were mixed with the MERS-CoV-infected Vero cells and plated onto slide glasses. The mixed cells were fixed and then incubated with normal mouse IgG or the mAb M158-2D6F11. The mixed cells were incubated with Alexa488-conjugated goat anti-mouse IgG antibody. The nuclei were stained with Hoechst 33258. Images were obtained using a fluorescence microscope. **b** Localization of the M protein of MERS-CoV in Vero cells. Vero cells were cultured on a cover glass and infected with MERS-CoV (0.1 MOI) for 2 days. The MERS-CoV-infected Vero cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% triton X-100. The cells were incubated with the mAb M158-2D6F11 and then incubated with Alexa488-conjugated goat anti-mouse IgG. The nuclei were stained with Hoechst 33258. Images were obtained using a confocal microscope. Scale bar, 10 μm

M158-2D6F11 specifically recognized the cytosol region of the infected cells where the M protein is known to be localized (Yang et al. 2013). However, normal mouse IgG did not recognize the region (Fig. 4b). From these results, we suggest that the mAb M158-2D6F11 can recognize the M protein of MERS-CoV in MERS-CoV-infected cells.

Discussion

Previously, we established a protocol for epitope peptide-based antibody production using CpG–DNA encapsulated with a DOPE:CHEMS complex as an adjuvant without conventional carriers (Kim et al. 2011a). Our protocol has been proven to be useful for prompt epitope screening and antibody production; therefore, it has great potential in various applications especially in an urgent situation such as a pandemic (Rhee et al. 2012). Here, we applied our strategy to produce a mAb targeting MERS-CoV which has emerged as a life-threatening virus with pandemic potential.

The MERS-CoV structural proteins, S, E, and M protein, associate in the membrane of the rough endoplasmic reticulum and interact with N proteins in the endoplasmic reticulum-Golgi region of the host cell for viral assembly (Durai et al. 2015). Yang et al. showed that the C-terminal domain of the M protein of MERS-CoV leads to the inhibition of type I interferon expression in response to virus infection. The M protein interacts with TRAF3 and disrupts the TRAF3–TBK1 interaction resulting in the inhibition of IRF3 activation (Lui et al. 2016). Therefore, the M protein of MERS-CoV has been investigated as a therapeutic target. In this context, we selected the M protein as a target for antibody production.

We obtained a hybridoma clone (M158-2D6F11) producing an antibody specifically reactive to the M protein of MERS-CoV using the immunization method of injecting a mouse with a complex of the M protein epitope peptide and the CpG–DNA–liposome. The mAb M158-2D6F11 showed specific binding with the M protein in western blot and immunoprecipitation analyses and detected MERS-CoV-infected cells in an immunofluorescence assay and confocal analysis. These results suggest that this antibody could be useful to detect MERS-CoV infection.

In conclusion, we generated, using a peptide epitope-based immunization method, a hybridoma clone producing a mAb specific to the M protein of MERS-CoV. Further studies on the efficacy of the mAb in detecting MERS-CoV in the sera of MERS patients may provide important information for future applications in diagnostics. We also expect that the mAb will be useful in investigating the function of the M protein of MERS-CoV including its possible effect on the innate immunity.

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Compliance with Ethical Standards

Conflict of interest All the authors declare that they have no conflict of interests.

Human and Animal Rights All institutional and national guidelines for the care and use of laboratory animals were followed. All procedures performed in studies involving animals were approved by the institutional animal ethical committee. This article does not include any studies using human subjects.

Informed Consent The article does not contain any studies in patients by any of the authors.

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