Brief Report

Binding of Divalent H-2K^d/IgG2aFc Fusion Protein to Murine Macrophage *via* Fc-FcR Interaction

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Peptide-MHC class I complex (pMHC) is a specific ligand for TCR recognition, and important for CD8$^+$ T cell activation. Here we described a genetically engineered divalent class I major histocompatibility complex (MHC) molecule, H-2K^d/IgG2aFc, a fusion protein consisting of the extracellular domains of H-2K^d, a murine MHC class I molecule, and the Fc region of IgG2a. This fusion protein is expected to attach the H-2K^d molecule to the surface of murine macrophage (Mϕ) through its Fc portion binding to Fc receptor (FcR) of Mϕ. cDNAs coding for the extracellular domains of H-2K^d and the Fc region of IgG2a were cloned respectively, and then recombined into plasmid pcDNA3.1(+). The H-2K^d/IgG2aFc protein was expressed by the plasmid-transfected cell line J558L, and purified from its supernatant with a Staphylococcal Protein A (SPA) column. The fusion protein showed a 58.4 kDa band as revealed by SDS-PAGE and Western blotting with murine IgG-specific antibody, which consists with that expected for extracellular domains of H-2K^d heavy chain plus the Fc region of IgG2a. The sandwich ELISA assay with antibodies specific for Fc portion and for H-2K^d indicated the fusion protein consists of both Fc portion and H-2K^d. Peritoneal Mϕ of C57BL/6 (H-2K^b) can be stained with H-2K^d specific monoclonal antibody (mAb) after incubated with the H-2K^d/IgG2aFc fusion protein. These results demonstrate the fusion protein can be used to attach the H-2K^d molecule to the surface of murine Mϕ, and provides a novel means to manipulate the T cell recognized epitope on the surface of murine Mϕ, which can be applied to activate antigen-specific cytotoxic T lymphocyte (CTL). *Cellular & Molecular Immunology*. 2007;4(2):147-151.

Key Words: H-2K^d/IgG2aFc, divalent MHC, fusion protein, Fc receptor

Introduction

CD8$^+$ T lymphocyte plays a critical role in the cellular immunity. The T cell receptor (TCR) on CD8$^+$ T cell recognizes the antigen-derived peptide-MHC class I complex (pMHC) on the surface of antigen-presenting cells (APCs) or target cells; the recognition takes place through receptor-ligand (pMHC/TCR) and adjuvant molecule interactions, which include that of coreceptor CD8, costimulatory molecules and adhesion molecules. The MHC class I molecule contains a light chain (β_2 microgloblin, β_2m) and a heavy chain including an antigen-binding groove with antigenic peptide fragment resident in it. It plays the central role in the antigen-specific T cell activation (1-3).

Recently, soluble MHC complexes have gained a lot of attention as a sensitive tool to identify antigen-specific T cells. Multimeric MHC complexes are also used to enhance or block specific immune responses *in vitro* (4-10). However, soluble MHC molecules do not induce effective T cell response unless cross-linked. In order to solve this problem, Joseph's group first developed a divalent MHC I/IgG fusion molecule. In this molecule, the IgG heavy chain was used as the scaffold to form fusion protein between MHC class I heavy chain extracellular part and IgG Fc fragment. But these fusion molecules can not cause effective T cell activation because of the absence of costimulatory signal from APCs. The activating properties of anti-TCR mAbs administered *in vivo* result from TCR cross-linking mediated by FcR$^+$ cells of delivering a costimulatory signal (4, 7). On the other hand, the application of dimeric or multimeric pMHC may be limited by shortages of a spectrum of allelic MHC molecules, for MHC shows extreme polymorphism (10). For example, the H-2K^d dimer is not commercially available yet.

In the present study, we developed a divalent H-2K^d/IgG2aFc fusion molecule, consisting of extracellular domains

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Received January 31, 2007. Accepted April 3, 2007.

of a murine MHC class I molecule, H-2K^d, and the Fc region of IgG2a. The IgG2a Fc region binding to FcR$^+$cells (such as macrophage, Mϕ) is expected to attach the H-2K^d to the surface of these cells.

Materials and Methods

Mice

BALB/c mice (H-2K^d) and C57BL/6 mice (H-2K^b) at 8 to 10 weeks of age, which were housed in cages under specific pathogen-free condition, were purchased from Hubei Provincial Center for Disease Control and Prevention.

Cell lines

The J558L cell line (BALB/c mouse myeloma cells, which express λ immunoglobulin light chain but can not express any endogenous immunoglobulin heavy chain) and the HB-95 cell line (BALB/c mouse myeloma cells) were obtained from American Type Culture Collection (ATCC). All the cell lines were cultured at 37^{o}C in 5% CO_2 atmosphere in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate.

Primers

The sequence of PCR sense primer for mouse H-2K^d was P1 (5'-GGG TGA CAA GCT TAT GGC ACC CTG CAC GCT G-3') with a restrictive endonuclease *Hind* III site (underlined), and that of anti-sense primer was P2 (5'-TAG CTG GGC TCC CAT CTC AGG GTG AGA GGC T-3'). The sequence of PCR sense for IgG2a Fc gene was P3 (5'-CCT GAG ATG GAG CCC AGA GTG CCC ATA ACA CAG AA-3') and the anti-sense primer was P4 (5'-AAG CAT TCT AGA TCA TTT ACC CAG AGA CCG GGA G-3') containing a restrictive endonuclease *Xba* I site (underlined).

Construction of H-2K^d/IgG2aFc hybrid gene

The H-2K^d/IgG2aFc hybrid gene was constructed by recombinant PCR. Firstly, two separate PCR reactions were used to amplify the appropriate domains of H-2K^d (extra) and IgG2aFc. The cDNA encoding H-2K^d extracellular sequence was cloned from total mRNA of BALB/c mouse spleen by RT-PCR with primers P1 and P2. This amplified H-2K^d (extra) sequence contains a *Hind* III site at the 5' end, and a 7-bp sequence at the 3' end homologous to the 5' end of the IgG2aFc hinge region. The cDNA of IgG2a Fc was amplified by RT-PCR with P3 and P4 from total mRNA of the HB-95 cell line. The IgG2a Fc sequence has an *Xba* I site at the 3' end, and a 10-bp sequence on the 5' end homologous to the 3' end of the H-2K^d (extra). Then, the PCR products of both H-2K^d (extra) and IgG2a Fc were annealed in the next PCR reaction with primers P1 and P4 to generate the H-2K^d/IgG2aFc fusion gene. The recombinant PCR products were purified and digested by restriction endonucleases *Hind* III and *Xba* I, and ligated into eukaryotic expression vector pcDNA 3.1(+) which was digested by the same restriction endonucleases. At last, the recombinant plasmid was transformed into JM110, followed by identification of the transformants.

Transfection and purification of the H-2K^d (extra)/IgG2aFc fusion protein

To generate stable transfectants, the H-2K^d/IgG2aFc recombinant plasmids were transfected into J558L cell line by electroporation using a gene pulser with a capacitance extender (Bio-Rad) at 960 μF, 260 V. Cells were rested for 24 h after electroporation, followed by selection in 800 μg/ml G418 (Gibco). High expressing clones were selected and grown under 800 μg/ml G418 in RPMI 1640 containing 10% FBS and adapted to multiply in serum-free media for collection. The cultural supernatant was passed over a Staphylococcal Protein (SPA) column to purify the dimeric H-2K^d/IgG2aFc fusion protein, eluted with 0.1 M sodium acetate, pH 5.0, and dialyzed against PBS.

SDS-PAGE and Western blotting assay

The supernatant of J558L transfected with the H-2K^d/IgG2aFc recombinant plasmids was fractionated in 12% SDS-PAGE, and the fractionated proteins were electrophoretically transferred to a nitrocellulose membrane. Then, the supernatant of J558L without transfection was used as negative control and the mAb W6/32 (mouse anti-human HLA class I mAb, whose isotype was IgG2a) was used as positive control. Immunoblotting was performed by using horseradish peroxidase-conjugated goat anti-mouse IgG Fc (Boster). Bands were visualized by using diaminobenzidine (DAB).

ELISA with antibodies specific for Fc portion and for H-2K^d

Sandwich-ELISA was performed in 96-well plate, and the wells were coated with goat anti-mouse IgG Fc at a concentration of 5 μg/ml in PBS and incubated overnight at 4^{o}C. After washing with PBS-Tween, the well was blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at 37^{o}C, then washed again and incubated with samples in triplicate wells at 37^{o}C for 1 h. Samples for experimental group were serially diluted fusion protein, the blank control and positive control were PBS, while the negative control was the supernatant of J558L. Wells were washed again, 100 μl murine serum was added and incubated at 37^{o}C for 2 h to block the well-coated, unoccupied goat anti-mouse IgG, except the wells of the positive control into which 100 μl PBS was added. After washing, biotin-labeled anti-mouse H-2K^d (34-1-2s, eBioscience) was added at a concentration of 1 μg/ml, and incubated at 4^{o}C for 30 min. After washing, avidin-labeled HRP (Biodisign) was added. After washing again, the wells were developed with ortho-phenylene-diamine (OPD) and read at 492 nm with a microplate reader (TECAN), and triplicates were averaged.

Binding assay of the H-2K^d/IgG2aFc fusion protein to Fc receptor on murine peritoneal Mϕ

C57BL/6 peritoneal Mϕ was harvested 3 days after the mice were intraperitoneally injected with 1 ml 10% sterile

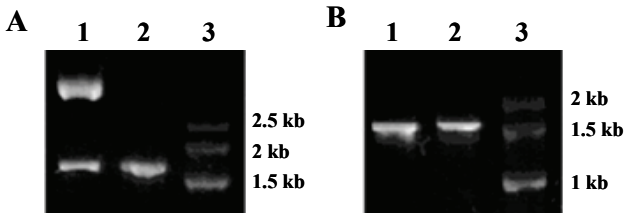

Figure 1. The recombinant plasmid pcDNA3.1(+)-H-2K^d/IgG2aFc was verified by double restriction endonuclease digestion and RT-PCR with primers P1 and P4. (A) Lane 1, *Xba* I and *Hind* III double digested products of the recombinant plasmid, the size of the small fragment was the same as that of PCR product. Lane 2, PCR product (1,602 bp) of recombinant plasmid using primers P1 and P4. Lane 3, DNA ladder (Marker VII, MD105-01, TIANGEN). (B) RT-PCR products of J558L cells transfected with the recombinant plasmids, using primers P1 and P4. The result suggests the expression of H-2K^d/IgG2aFc hybrid gene. Lane 1, RT-PCR product of J558L cell line transfected with the recombinant plasmids. Lane 2, PCR product of the recombinant plasmids. Lane 3, DNA ladder (Marker V, MD107-01, TIANGEN).

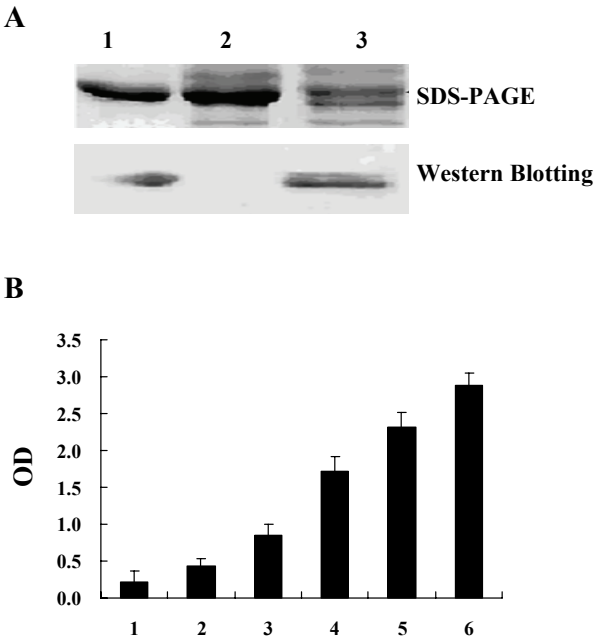

Figure 2. The culture supernatant of J558L cell line transfected with the recombinant plasmids contains the H-2K^d/IgG2aFc fusion protein. (A) SDS-PAGE of the culture supernatant, a newly ocurring protein band of around 58.4 kDa could be observed in Lane 3, the H-2K^d/IgG2aFc fusion protein had an expected molecular weight of approximately 58.4 kDa. Lane 1, mAb W6/32 was used as positive control. Lane 2, the supernatant of J558L cell line without transfection was used as negative control. (B) Western blotting using HRP-conjugated goat anti-mouse IgG Fc, the 58.4 kDa band can be stained with anti-mouse IgG Fc in Lane 3. The positive control (W6/32, Lane 1) shows the band, while negative control (supernatant of the J558L without transfection, Lane 2) doesn't. (C) Sandwich ELISA with an H-2K^d-specific and an immunoglobulin-specific antibodies. (1) PBS was used as a blank control. (2) The supernatant of J558L cell line without transfection was used as a negative control. (3-5) Serially diluted samples of purified H-2K^d/IgG2aFc fusion protein, (3) 1:2500, (4) 1:500 and (5) 1:100. (6) The sample without murine serum blocking was used as a positive control, for the well-coated goat anti-mouse IgG is able to bind to the H-2K^d-specific mAb, and the latter is of murine IgG2a isotype.

thioglycolate medium. The peritoneal cavity was injected with 5 ml ice-cold PBS, and the peritoneal cells were harvested (11). The cells were washed twice with PBS, and incubated with PBS (pH 6.0) at 37^{o}C for 15 min to wash off the pre-existing IgG on the Mϕ. The peritoneal Mϕ was incubated with samples at 4^{o}C for 30 min, including 100 μl purified H-2K^d/IgG2aFc fusion protein for experimental group. Before the fusion protein was added, 100 μl mouse serum was added for block group, 100 μl PBS for the blank control. And the J558L cell (H-2K^d) was used as positive control. Washed twice, the cells were incubated with biotin-labeled anti-mouse H-2K^d at 4^{o}C for 30 min, except for the blank control. Washed twice and the avidin-labeled FITC was added into each well, except for the blank control, then incubated at 4^{o}C for 30 min. The binding of the dimeric H-2K^d/IgG2aFc fusion protein to FcR on murine peritoneal Mϕ was assessed with a flow cytometer (FACSCalibur, BD), fluorescence data were collected using logarithmic amplification on 10,000 visible cells as determined by forward light scatter intensity.

Results

The H-2K^d/IgG2aFc hybrid gene was recombined into pcDNA3.1(+) and able to be expressed in J558L

After the appropriate domains of H-2K^d (extra) and IgG2aFc were cloned by two separate RT-PCR reactions, a recombinant PCR was used to generate a 1602 bp product encoding the H-2K^d (extra)/IgG2aFc fusion protein. Then this hybrid gene was inserted into the plasimid pcDNA3.1(+), to form the recombinant pcDNA3.1(+)-H-2K^d/IgG2aFc. As shown in the Figure 1A, the plasmid digested by *Hind* III and *Xba* I gave rise to a 1602 bp band, which was the same in size as that of PCR products of the plasmid with the primers P1 and P4 (Lane 2). The DNA sequencing data of the H-2K^d (extra)/IgG2aFc hybird gene was consistent with DNA sequences of H-2K^d (extra) and IgG2aFc genes listed in the Genbank (data not shown).

In order to determine if the H-2K^d/IgG2aFc hybrid gene was able to be expressed in a host cell, the pcDNA3.1(+)-H-2K^d/IgG2aFc was transfected into the cell line J558L. The hybrid gene expression was checked by RT-PCR using primers P1 and P4, with total RNA extracted from the plasmid-transfected J558L. As shown in Figure 1B, the result of RT-PCR indicated there was an H-2K^d/IgG2aFc fusion band in Lane 1, with the same size as PCR product (Lane 2) of recombinant plasmid. This suggested the H-2K^d/IgG2aFc hybrid gene could transcript in the cell line J558L.

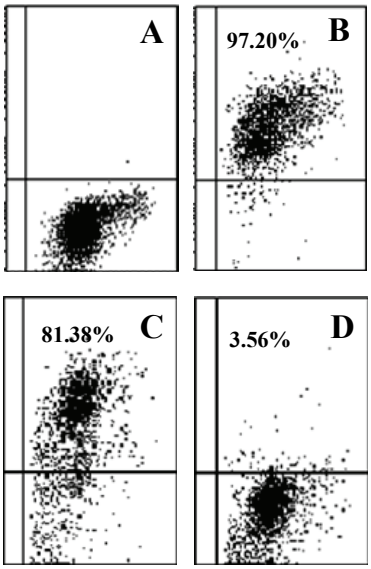

Figure 3. The dimeric H-2K^d/IgG2aFc fusion protein was able to attach the H-2K^d to the surface of H-2K^b Mϕ. Peritoneal H-2K^b Mϕ of C57BL/6 was incubated with the fusion protein, then stained with H-2K^d-specific mAb and visualized by FCM. (A) Blank control (H-2K^b Mϕ only). (B) Positive control (J558L cells, H-2K^d positive) shows 97.20% cells stained. (C) The H-2K^b Mϕ incubated with the fusion protein shows 81.38% stained. (D) The H-2K^b Mϕ blocked by murine serum before incubated with the fusion protein is only 3.56% stained.

The dimeric H-2K^d/IgG2aFc fusion protein was expressed

To make sure the H-2K^d/IgG2aFc fusion protein can be expressed in the J558L transfected with recombinant plasmids, the J558L culture supernatant was analyzed by SDS-PAGE (Figure 2A) and Western blotting (Figure 2B) using goat anti-mouse IgG to detect the dimeric fusion protein. In Lane 3 of Figure 2A, a newly ocurring protein band of around 58.4 kDa could be observed in the SDS-PAGE, which could be bound to by the goat anti-mouse IgG. As the J558L cell line was defective in endogenous immunoglobulin heavy chain, and the molecular weight of the band consisted with that was expected for extracellular domains of H-2K^d heavy chain plus the Fc region of IgG2a, the band should be the H-2K^d/IgG2aFc fusion protein expressed in J558L cell line transfected with the H-2K^d/IgG2aFc hybrid gene.

The dimeric H-2K^d/IgG2aFc fusion protein was further verified by a sandwich ELISA with an H-2K^d-specific and an immunoglobulin-specific antibody. The results were shown in Figure 2C, the OD of the purified dimeric fusion protein was much higher than that of negative control, indicating the fusion protein consists of both H-2K^d part and IgG2a Fc part.

The dimeric H-2K^d/IgG2aFc fusion protein can attach H-2K^d to the surface of peritoneal Mϕ

The H-2K^d-specific mAb was used to detect the binding ability of the dimeric H-2K^d/IgG2aFc fusion protein to the FcR expressed on H-2K^b Mϕ (C57BL/6). When the H-2K^b Mϕ was incubated with the fusion protein, there was 81.38% H-2K^b Mϕ that can be stained with the H-2K^d-specific mAb (Figure 3C). This result shows that the dimeric H-2K^d/IgG2aFc fusion protein is able to attach the H-2K^d to the surface of H-2K^b Mϕ. The binding ability of the fusion protein to FcR is further comfirmed by incubation with mouse serum before the fusion protein was added, in this case, only 3.56% H-2K^b Mϕ can be stained with the H-2K^d-specific mAb (Figure 3D). Because there exists high concentration of IgG in the mouse serum, the binding of the fusion protein to the FcR is blocked by serum IgG. This observation indicates the binding of the fusion protein to the surface of Mϕ is mediated by the Fc-FcR interaction.

Discussion

The activated antigen-specific CTLs have the capacity to remove the virally infected cells and tumor cells. There are some reports showing that the activation of antigen-specific T cell clones *in vivo* might be possible by soluble MHC molecules, but not effective because of the absence of costimulatory signal from antigen-presenting cell (APC) (4, 8). In this study, an H-2K^d/IgG2aFc fusion protein was developed. This is an Ig-like, Y-shaped molecule, the Fc part of IgG2a is able to bind to FcR with high affinity (12-14), and the Fab portion of the molecule is replaced by the extracellular domains of H-2K^d. Apparently, the dimeric H-2K^d of the molecule can work as specific ligand for TCR of CD8$^+$ T cell, and the adjacent IgG2a Fc makes the ligand able to be attached to the surface of FcR$^+$ cells, such as Mϕ.

It is convenient to manipulate the specificity of the dimeric H-2K^d molecule. The antigen-binding groove of H-2K^d/IgG2aFc fusion protein can be loaded with a given H-2K^d restricted peptide, forming a pMHC dimer, which can be applied to identify antigen-specific T cell, since higher stability of the dimeric protein can enhance the interaction between the ligand and TCR, compared with monomeric molecules. Moreover, it can also be used to activate and expand antigen-specific T cells; the ability of H-2K^d/IgG2aFc fusion protein to bind to FcR$^+$ APC allows us to make use of the APC presenting a single peptide to induce antigen-specific CTL for adoptive immunotherapy, such as treatment for cancer and virally infected diseases.

Acknowledgement

This work was supported by a grant from the National Natural Science Foundation of China (NO.30271201).

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