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Research paper

Enhancement of antibody responses to native G protein-coupled receptors using *E. coli* GroEL as a molecular adjuvant in DNA immunization

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ABSTRACT

Antibody-based drug research involves the preparation of polyclonal and monoclonal antibodies, especially those that are reactive with native G protein-coupled receptors (GPCRs) on the cell membrane. Here, we report that DNA immunization of mice with a plasmid that encodes endothelin A receptor (ETAR) fused to *Escherichia coli* (*E. coli*) GroEL at its C-terminus (ETAR–GroEL) induced very strong and specific antibody responses to native ETAR. Co-injection of plasmids that expressed ETAR and GroEL (ETAR + GroEL) induced significantly lower antibody responses compared with the ETAR–GroEL plasmid. Monoclonal antibodies that are prepared by using GroEL as a molecular adjuvant could be used in immunoassays, such as flow cytometry, western blotting, and immunoprecipitation, to detect both exogenous and endogenous ETAR. The adjuvant activity of GroEL might involve inflammatory cytokine mediators via Toll-like receptor 4 in addition to the anticipated carrier effect. DNA immunization using GroEL might become a standard method for producing antibodies that are useful for the functional analysis of GPCRs.

1. Introduction

G protein-coupled receptors (GPCRs), which are one of the largest protein families, are involved in many types of stimulus–response pathways in diverse processes, ranging from intercellular communication to physiological sensations. As a result, GPCRs are common drug targets. Furthermore, since antibodies (Abs) can be used to predict the function, localization, and ligands of GPCRs, extensive antibody-based drug research has attempted to develop Abs that are reactive with native GPCR. However, until recently and still, the production of anti-GPCR Abs those have biological activity has been difficult. The reactivity of most Abs that is obtained by immunization with a synthetic peptide is usually limited to the immunizing peptide rather than native proteins on cell surfaces. On the other hand, DNA immunization via electroporation is a useful method to induce native-formed antigens in vivo, which may induce Abs that are reactive with native proteins. Also that DNA immunization is uniquely suited for this effort since Ag is encoded as a native protein. In addition, this method is simple, useful, inexpensive, and safe for introducing DNA into cells (Aihara and Miyazaki, 1998).

DNA immunization usually induces a very low anti-GPCR response, increasing the antigenicity of GPCR is important to produce Abs that are reactive with native GPCR. Although several complementary strategies have been developed to enhance the potency of DNA immunizations (Berzofsky et al., 2001; Bins et al., 2007), the development of immunization protocols and a molecular adjuvant is still needed for the production of Abs that are reactive with low antigenic proteins, such as GPCR. In this specific field, many biotechnology companies were founded and one such company in German is claiming that they are supplying functional anti-GPCR Abs for ten years. However, judging from their home page data, only several Abs are available for distribution. To

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overcome this problem, we developed more efficient DNA immunization method to produce anti-GPCR Abs.

Here, we designed a efficient DNA immunization protocol that uses an endothelin A receptor (ETAR) antigen fused with *Escherichia coli* GroEL at its C-terminus. Endothelin receptors are GPCRs that increase intracellular calcium levels upon activation. GroEL, which is a heat shock protein (HSP), is a molecular chaperone that is responsible for the transportation and refolding of proteins. GroEL fusion proteins are highly expressed in the soluble fraction of *E. coli* and are assembled into double-ring complexes, which suggest that they encapsulate fusion proteins (Furutani et al., 2005). HSPs are usually intracellular proteins; however, when they act as danger signals during infections, they need to be present in the extracellular environment. Many other bacterial HSPs have been found in the extracellular milieu and implicated in immune reactions to infections (Tsuzuki et al., 2002; Hennequin et al., 2001; Frisk et al., 1998). We hypothesized that *E. coli* GroEL could be an immunostimulatory molecule and a carrier protein. In addition, GPCR encased in GroEL might form a native structure that has good in vivo antigenicity. Here, we report the efficient generation of mouse Abs that are reactive with native GPCRs on cell surfaces and show that GroEL may be one of a potentially useful molecular adjuvant for DNA immunization.

2. Materials and methods

2.1. Construction of expression vectors and purification of plasmid DNA

Expression plasmids were constructed by using routine molecular biology techniques. The expression vector pCADEST1 (Fujimoto et al., 2009), which was constructed on the basis of pCADEST2.2 (Ainai et al., 2006), included a CAG promoter and LR-cloning sites. The ETAR, ETBR, ETAR–OVA, ETAR–HSP60, ETAR–GroEL, ETBR–GroEL, ETAR–FLAG, and the GroEL genes were subcloned into the expression vector pCADEST1 (Fig. S1) by using the LR recombination reaction with the Gateway LR Clonase II enzyme (Invitrogen). Plasmid DNAs were isolated and purified from *E. coli* DH5α cells by using the endotoxin-free Gigaprep kit (QIAGEN).

2.2. Cell culture conditions and transient and stable expression of G protein-coupled receptors

Human embryonic kidney 293T and B300-19 cells were maintained in DMEM supplemented with 10% FCS or RPMI1640 medium supplemented with 10% FCS and 50 μM 2-mercaptoethanol, respectively. For transient transfections, FuGENE6 transfection reagent (Roche) was used to transfect B300-19 cells with plasmids that encoded ETAR, ETAR–GroEL, ETBR, and ETBR–GroEL. For stable expression, the ETAR, ETBR, ETAR–GroEL, ETBR–GroEL, ETAR–HSP60, ETAR–EGFP, or ETAR–OVA genes were subcloned into the pMRX-IRES-Puro vector (Saitoh et al., 2003) and B300-19 cells that stably expressed these genes were established by retroviral infection, as described previously (Morita et al., 2000). The infected cells were selected for 3 d with 1 μg/ml of puromycin (SIGMA). All cells were grown at 37 °C in a humidified incubator containing 5% CO2.

2.3. Animals and immunization and hybridoma selection protocols

Six-week-old female BALB/c, C3H/HeN, or C3H/HeJ mice (Sankyo Lab Service, Tokyo, Japan) were immunized with a plasmid that encoded ETAR, ETAR–GroEL, ETAR + GroEL, ETBR, or ETBR–GroEL. The plasmids were injected intramuscularly and electroporated in vivo, as we described previously (Fujimoto et al., 2009; Takatsuka et al., 2010). One, 3, and 4 weeks after the primary immunization, the DNA immunization was repeated in the same manner. Blood samples were collected from the mice every week. Following separation by centrifugation, the sera were stored at 4 °C for subsequent assays.

Splenocytes from ETAR–GroEL or ETBR–GroEL immunized mice and SP2/0 mouse myeloma cells were fused as described previously (Nagata et al., 2003). Hybridomas were selected by identifying B300-19 cells that stably expressed ETAR or ETBR with FCM analysis. All experiments that involved the confirmation of the expression of ETAR and ETBR used anti-ETAR mAb clone 3G8 or anti-ETBR mAb clone 14F11, respectively.

2.4. Flow cytometry analysis and antibody titration

The Ab response was assessed by using FCM analysis. The sera were reacted with ETAR/B300 or ETBR/B300 cells at 4 °C for 30 min, and then the cells were washed and incubated with phycoerythrin-conjugated goat $F(ab')_2$ fragment anti-mouse IgG (H + L) (Beckman Coulter) at 4 °C for 30 min. Afterwards, the cells were washed and incubated with 2 μg/ml of propidium iodide (PI) (SIGMA) at room temperature for 10 min and analyzed with the FACSCalibur flow cytometry system (BD). Data were analyzed from PI negative cells. All of the staining and washing steps were carried out in PBS supplemented with 0.5% FCS, 5 mM EDTA, and 0.1% NaN_3.

Ab titration was performed by using a FCM assay. Briefly, 1×10^5 of ETAR/B300 cells were incubated with a 1/200–1/256,000 dilution of mouse antiserum at 4 °C for 1 h. The cut-off value was defined to be 1.5 times the MFI in the immunized serum controls (1/200 dilution of preimmune serum).

2.5. Intracellular calcium analysis

To measure $[Ca^{2+}]_i$ levels in ETAR/B300, ETBR/B300, ETAR–GroEL/B300, ETBR–GroEL/B300, ETAR–HSP60/B300, ETAR–EGFP/B300, and ETAR–OVA/B300 cells, the cells were loaded with Fluo-4, a fluorescent dye, (Dojindo) and incubated in Hank's balanced salt solution at 37 °C for 1 h. Subsequently, they were seeded at a density of 1×10^5 cells/ml on a 96-well plate in recording buffer (20 mM HEPES, 115 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 13.8 mM glucose, pH 7.4). The changes in $[Ca^{2+}]_i$ were monitored by measuring Fluo-4 fluorescence, and $[Ca^{2+}]_i$ imaging and data acquisition were performed by using an Envision multilabel reader (PerkinElmer), as described previously (Tomic et al., 2002).

2.6. Complement-dependent cytotoxicity assay

The mAb potency was assessed by using a complement-dependent cytotoxicity assay, as described previously (Lamon

et al., 1975). Briefly, ETAR/B300, ETBR/B300, or mock cells were washed twice with PBS and then suspended at a final concentration of 1×10^6 cells/ml with cytotoxicity medium (RPMI with 1.6 g/l of sodium bicarbonate, 25 mM HEPES, and 0.3% BSA) in 96-well microplates. Then, the cells were incubated with Ab at 4 °C for 30 min. After washing the cells, 50 μL of rabbit complement (Pel-freez) was added, diluted 1/10 with cytotoxicity medium, and then incubated at 37 °C for 30 min. The CDC reaction was stopped by lowering the temperature to 4 °C. Cell viability was monitored by trypan blue exclusion. The CDC was calculated by using the following formula:

$$\text{CDC}\,(\%) = \text{Number of dead cells}/(\text{Total number of cells}) \times 100.$$

All experiments were performed in triplicate.

2.7. Immunoprecipitation

Human embryonic kidney 293 T cells were transfected with 6 μg of pCADEST1–ETAR–FLAG. Subsequently, 1×10^6 transfected cells were lysed with 1 mL of HBST buffer (20 mM HEPES, 150 mM NaCl, 1% Triton X-100, 200 μM PMSF), 10 μg/ml leupeptin, 0.15 U/ml aprotinin, pH 7.4). Equal amounts of ETAR–FLAG were used for immunoprecipitation, as described previously (Hosomi et al., 1994), with minor modifications. Anti-FLAG M2 mAb (2 μg, SIGMA), anti-GroEL mAb (1 μl, Stressgen), or anti-ETAR mAb (1 μg) were added to 1 ml of lysed protein, and then incubated at 4 °C for 1 h on a rotator. Subsequently, 20 μL of a 50% slurry of protein G-sepharose beads (GE) was added to each sample and incubated for another 1 h at 4 °C. The sample were centrifuged briefly and washed 3 times in the lysis buffer. Then, the samples were resuspended in 20 μL SDS sample buffer for analysis.

2.8. Western blotting

Protein samples in SDS sample buffer were electrophoresed with 10% SDS polyacrylamide gel electrophoresis, and then transferred to an Immobilon-P polyvinylidene fluoride membrane (Millipore). The membrane was blocked for 1 h with 100% Block Ace (Dainippon Sumitomo Pharma), and then incubated with anti-ETAR mAb (1 μg/ml) for 1 h at room temperature. Subsequently, the blot was washed and incubated with alkaline phosphatase (ALP)-conjugated goat anti-mouse IgG + IgM Ab (Biosource) at room temperature for 1 h. After washing, the blots were visualized by using 0.03% Nitro blue tetrazolium chloride and 0.016% 5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) substrate (Wako) to detect the ALP activity, and then scanned.

2.9. Histology

A biopsy sample was obtained from the constricted lumen of the coronary artery of an arteriosclerotic patient by endoscopic operation. A paraffin section was treated with pepsin for antigen retrieval, and then standard immunohistochemistry was performed by using 1 μg/ml anti-ETAR mAb. Informed consent was obtained from this patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the human research committee of Showa University, Japan.

2.10. Determination of carrier effect

BALB/c mice were immunized with a plasmid that encoded ETAR–EGFP, ETAR–OVA, or ETAR–HSP60. Since OVA is often used as a carrier protein (David, 1988) to generate Abs against epitopes, we used OVA as a positive control. We used HSP60, which is a mouse homolog of *E. coli* GroEL, as a negative control.

2.11. In vitro stimulation of dendritic cells and quantification of cytokine secretion

DCs were generated from murine bone marrow cells as described by (Lutz et al., 1999), with minor modifications. On day 10, the cells were pretreated with 200 μg/ml of polymyxin B (SIGMA), and then 1×10^6 DCs were stimulated with 10 μg/ml LPS (SIGMA) or 5 μg/ml GroEL (Stressgen) at 37 °C for 48 h. Then, the cytokines in the cell-free culture supernatant of the DCs were quantified with an ELISA and matched Ab pairs that were specific for IL-12p70, IL-23, TNF-α, and IFN-γ (BD).

3. Results

3.1. Expression of endothelin receptor GroEL fusion proteins

First, we determined the feasibility of developing an immunization method with GPCRs by expressing endothelin receptor GroEL fusion proteins. Specifically, we constructed 7 plasmids that encoded ETAR or endothelin B receptor (ETBR) with or without a carrier protein (Fig. S1). As shown by western blotting (WB), the molecular weights of ETAR and ETAR–GroEL were 50 and 110 kDa, respectively, as expected (Fig. 1A). In addition, the expression of ETAR and ETBR on the cell surface was confirmed by flow cytometry (FCM) (Fig. 1B and D). We did not western blot ETBR and ETBR–GroEL because an anti-ETBR Ab was not available. In some cases, such as C–C chemokine receptor 2B (CCR2B), the fusion of GroEL hindered protein expression on the cell surface (Fig. S2).

We also investigated whether ETAR–GroEL and ETBR–GroEL were expressed functionally on the surface of murine pre-B B300-19 cells (B300) by analyzing calcium influx to determine whether they could bind ligands and transduce signals. There were no significant differences in ligand reactivity or signaling in either fusion protein (Fig. 1C and E). These results indicated that ETAR and ETBR proteins that are fused to GroEL are functional.

3.2. E. coli GroEL robustly enhances antibody response in DNA immunization

We investigated whether GroEL might act as an adjuvant in vivo to enhance the production of Abs against native GPCRs on the cell surface. As shown by FCM, no immunospecific Abs were produced in mice that were immunized with ETAR (Fig. 2A). In contrast, mice that were immunized with ETAR–GroEL or ETAR + GroEL exhibited the highest Ab response 6 weeks after immunization (Fig. 2B, C, H, and I).

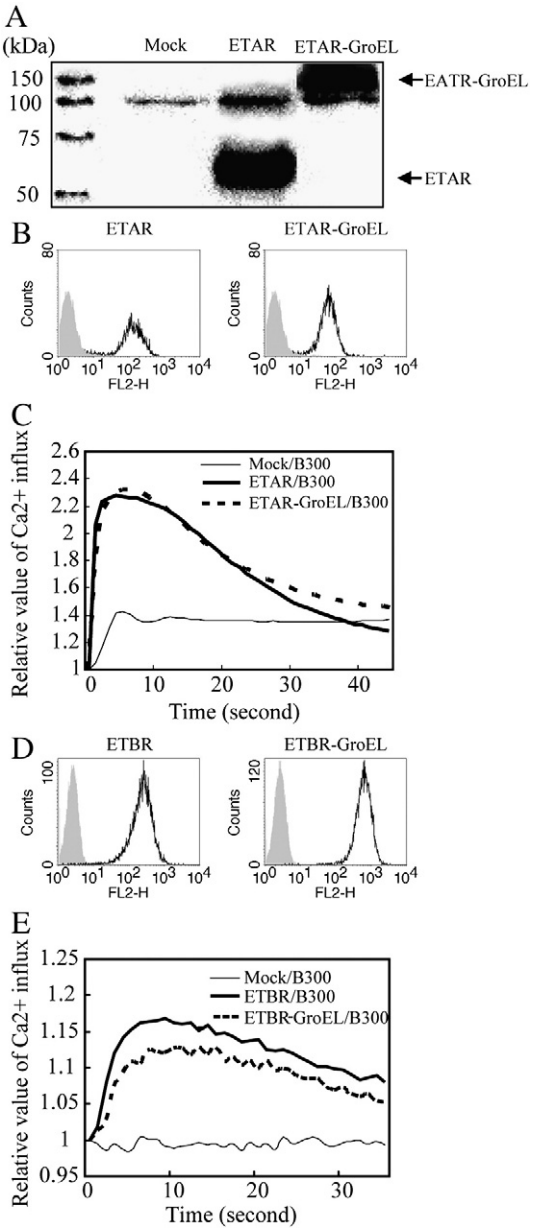

Fig. 1. Endothelin receptor GroEL fusion proteins are functional. (A) The expression of ETAR and ETAR–GroEL fusion protein was analyzed by western blotting by using an anti-ETAR mAb. (B and D) The expression of ETAR, ETAR–GroEL, ETBR, and ETBR–GroEL fusion protein on the cell surface was analyzed by FCM. Open histogram, ETAR, ETBR, ETAR–GroEL, or ETBR–GroEL transfected B300-19 murine pre-B cells; filled histogram, mock-transfected B300-19 cells. (C and E) The calcium influx of Fluo-4AM-stained B300-19 cells was measured in the presence of 100 nM endothelin 1 (ET-1). Bold line, ETAR/B300 or ETBR/B300; dotted line, ETAR–GroEL/B300 or ETBR–GroEL/B300; thin line, mock.

In addition, the Ab titer of ETAR–GroEL immunized mice was significantly higher than that of ETAR immunized mice (64,000-fold difference, $P<0.001$) (Fig. 2D and E). Anti-ETAR Abs also were detected in the serum of mice that were immunized with ETAR + GroEL (3800-fold difference vs. ETAR immunized mice, $P<0.03$), although their Ab titers were 8–10 times lower than those that were immunized with ETAR–GroEL (Fig. 2E and F). Finally, no specific Abs were detected in the preimmune serum from mice that were immunized with ETAR, ETAR–GroEL, or ETAR + GroEL after 6 weeks (Fig. 2G–I). These results indicated that the ETAR–GroEL fusion protein robustly induces an Ab response in mice.

Next, we determined whether GroEL can be used as an adjuvant for other antigens. As shown by FCM, a strong Ab response was observed after 5–6 weeks in mice that were immunized with ETBR–GroEL (Fig. S2A), which is similar to that from mice that were immunized with ETAR–GroEL. Six weeks after immunization, the anti-ETBR Ab titer in mice that were immunized with ETBR–GroEL was significantly higher than that in mice that were immunized with ETBR (Fig. S2B). No significant Ab response was induced by immunization with ETBR. The FCM histograms of sera that were collected 6 weeks after mice were immunized with ETBR or ETBR–GroEL are shown in Fig. S2C. Similar results were observed in mice that were immunized with other GPCR antigens, namely C–X–C chemokine receptor 4 or retinoic acid-inducible gene 1 (Fig. S3).

3.3. Immunospecific recognition of native G protein-coupled receptors on the cell surface

To determine whether the mAbs produced by DNA immunization recognized the native antigen, we incubated B300-19 cells that stably expressed ETAR or ETBR (ETAR/B300 or ETBR/B300 cells, respectively) with serial dilutions (1–1000 ng/ml) of anti-ETAR or anti-ETBR Ab. As shown by FCM, the mean fluorescence intensity (MFI) of both mAbs decreased in an Ab concentration-dependent manner (Fig. 3A and D). We also performed an Ab absorption test to determine whether the mAbs were specific for the immunized antigen. In this test, anti-ETAR or anti-ETBR mAbs were incubated with ETAR/B300, ETBR/B300, or mock-transfected cells, and then the remaining Abs were analyzed by FCM. The Abs were absorbed in an antigen volume-specific manner, which indicated that anti-ETAR or anti-ETBR mAb reacted specifically with cells that expressed the immunized antigen and did not cross-react with mock-transfected cells or cells that expressed other antigens (Fig. 3B and E). Finally, we used a complement-dependent cytotoxicity (CDC) assay to confirm that the mAbs recognized native GPCRs that were expressed on the cell surface. No cytotoxicity was observed in cells that did not express the immunized antigen; however, significant cytotoxicity was observed in cells that expressed the immunized antigen (Fig. 3C and F). Furthermore, cytotoxicity increased in an Ab concentration-dependent manner. Collectively, these results showed that both mAbs and polyclonal Abs that were produced by immunizing mice with endothelin receptor GroEL fusion proteins recognized native GPCRs on the cell surface.

3.4. Immunization with endothelin A receptor fused with enhanced green fluorescent protein, ovalbumin, or heat shock protein 60 does not induce a strong antibody response

We analyzed the expression of several ETAR fusion proteins with enhanced green fluorescent protein (ETAR–EGFP), ovalbumin (ETAR–OVA), and heat shock protein 60 (ETAR–HSP60) in B300-19 cells by using WB, FCM, and a

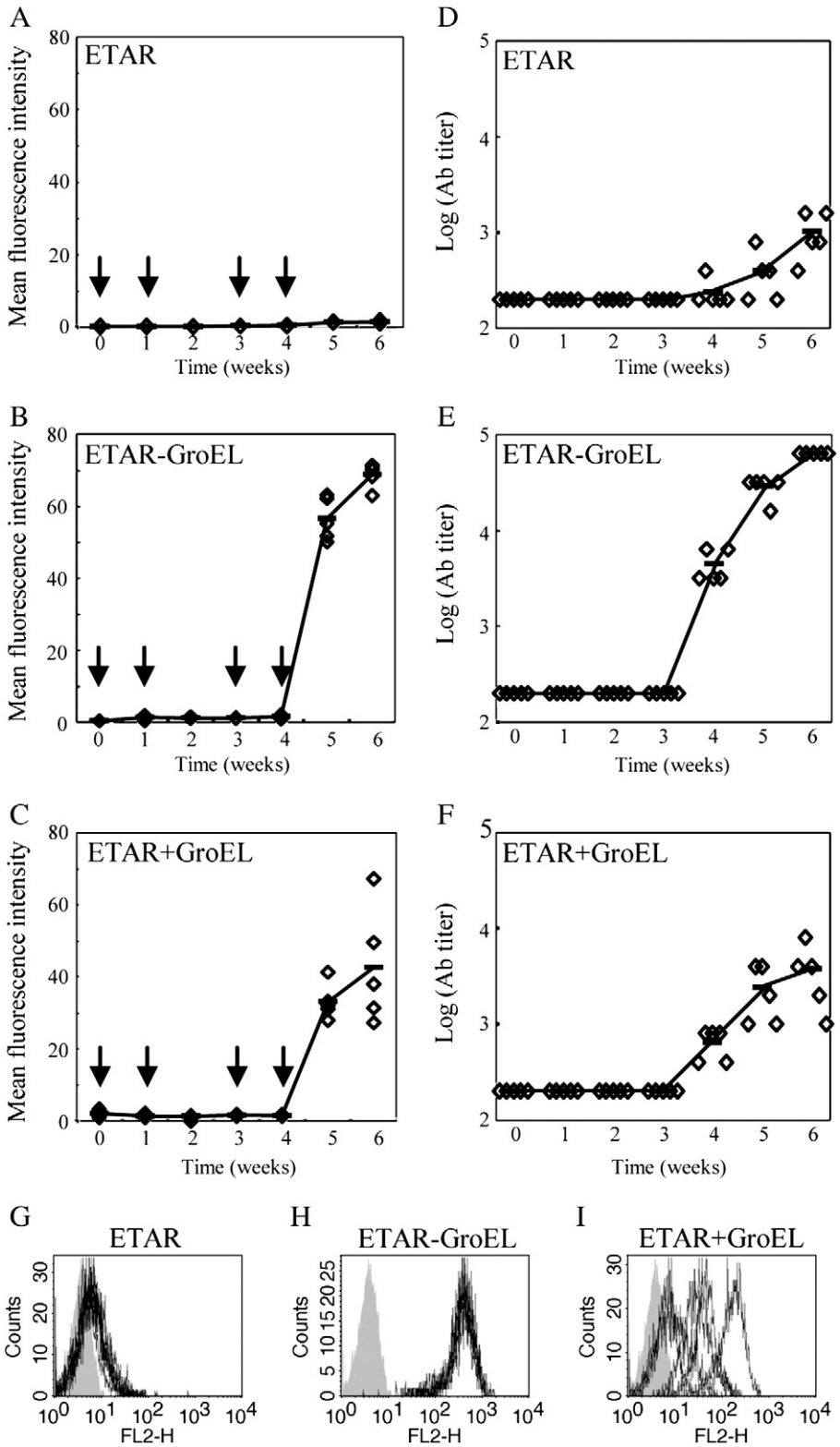

Fig. 2. Antibody response to endothelin A receptor in immunized mice sera. The anti-ETAR Ab response was analyzed by FCM in mice that were immunized with a plasmid that expresses ETAR (A, D, and G), ETAR–GroEL (B, E, and H), or coexpresses ETAR + GroEL (C, F, and I). (A, B, and C) Time course of the MFI, which is a measure of the anti-ETAR Ab response in a 1/1000 dilution of mice sera. (D, E, and F) Time course of ETAR-specific Ab titer, as measured by FCM. Each diamond-shaped data point represents a mouse. The black arrows indicate the first, second, third, and fourth immunizations. (G, H, and I) FCM histograms of mice sera (1/100 dilution) 6 weeks after immunization. Results are representative of 3 experiments. Filled histogram, preimmune serum; open histogram, immunized sera.

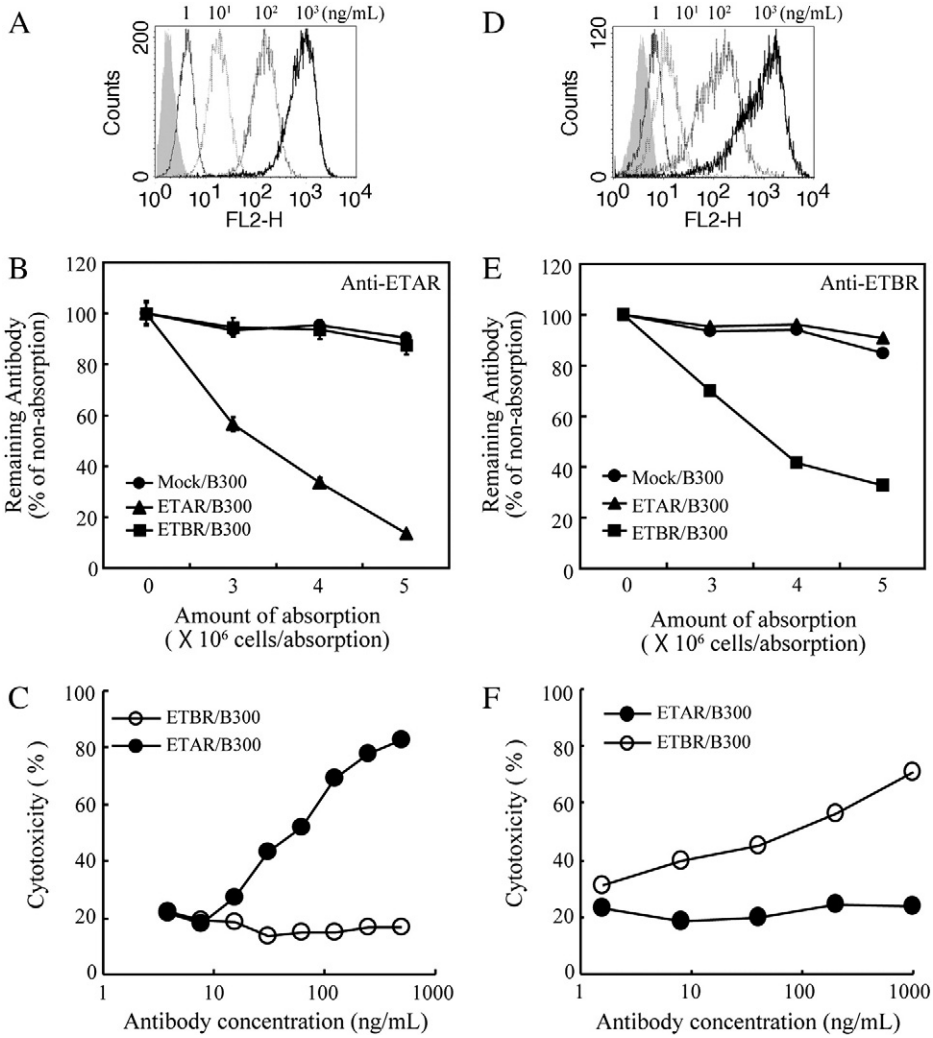

Fig. 3. Recognition of native GPCRs on the cell surface by monoclonal antibodies. The specificity of mAbs was analyzed with FCM and a complement-dependent cytotoxicity assay. (A and D) ETAR/B300 (A) or ETBR/B300 (D) cells were reacted with serial dilutions (1–1000 ng/ml) of anti-ETAR (A) or anti-ETBR (D) mAb, respectively, and then analyzed with FCM. (B and E) In each Ab absorption test, 100 ng of anti-ETAR (B) or anti-ETBR (E) mAb was incubated with 1×10^6 ETAR/B300 (▲), ETBR/B300 (■), or mock-transfected (●) cells for 1 h at 4 °C. Subsequently, the residual Abs in the supernatant were analyzed with FCM. Data are expressed as the mean percentage of non-absorption (SD). (C and F) ETAR/B300 or ETBR/B300 cells (1×10^6) were reacted with various concentrations of anti-ETAR (C) or anti-ETBR (F) mAb in a CDC assay. Data are expressed as the mean percentage of cytotoxicity (SD) of 2 independent experiments.

calcium influx assay. As shown by WB, the molecular weights of ETAR–EGFP, ETAR–OVA, and ETAR–HSP60 were 75, 110, and 120 kDa, respectively (Fig. 4A). In addition, FCM confirmed their expression on the cell surface (Fig. 4B). Furthermore, there were no significant differences in the amount of calcium influx among these fusion proteins (Fig. 4C). These results indicated that the expression of ETAR was not affected by the fusion of a 25–60 kDa carrier protein to its C-terminus.

We also investigated whether the Ab response that was enhanced by *E. coli* GroEL might have been due to a potent carrier effect. The FCM histograms and titers of anti-ETAR Ab responses in mice sera 6 weeks after immunization are shown in Fig. 4D and E, respectively. No Ab responses were observed in mice that were immunized with ETAR–OVA or ETAR–HSP60; however, a weak Ab response was detected in mice that were immunized with ETAR–EGFP. In addition, no specific Ab response was detected in the preimmune serum. These results indicated that *E. coli* GroEL enhanced the Ab response by not only the carrier effect but also other mechanisms.

3.5. GroEL stimulates dendritic cells to produce inflammatory cytokines in vitro

Finally, we determined whether *E. coli* GroEL could stimulate innate immunity. After exposing bone marrow-derived dendritic cells to LPS, substantial amounts of interleukin (IL)-12p70, interferon (IFN)-γ, IL-23, and tumor necrosis factor (TNF)-α were detected in culture supernatants (Fig. 5A). In comparison, the cells that were stimulated with GroEL produced significantly more IL12p70 and IL-23 than those stimulated by LPS; however, the secretion of TNFα was similar in both cases. In addition, polymyxin B, which is a potent inhibitor of LPS (Ohashi et al., 2000), markedly

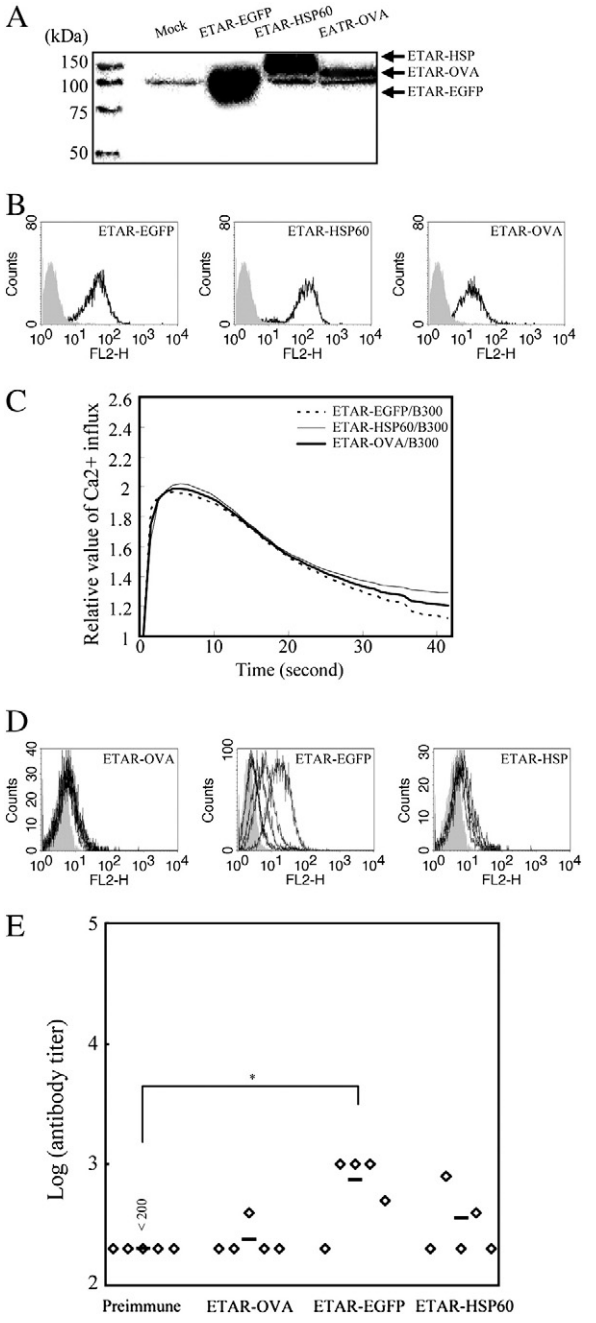

Fig. 4. Antibody response of mice immunized with ETAR fused with enhanced green fluorescent protein, ovalbumin, or heat shock protein 60. (A) WB analysis of the expression of ETAR–EGFP, ETAR–OVA, and ETAR–HSP60 using an anti-ETAR mAb. (B) FCM analysis of the expression of ETAR–EGFP, ETAR–OVA, and ETAR–HSP60 on the cell surface. Open histogram, ETAR transfectants; filled histogram, mock transfectant. (C) Calcium influx of Fluo-4AM-stained B300-19 cells in the presence of 100 nM ET-1. Dotted line, ETAR–EGFP; bold line, ETAR–OVA, thin line, ETAR–HSP. (D) Mice were immunized with a plasmid that expressed ETAR–EGFP, ETAR–OVA, or ETAR–HSP. The resulting sera (1/100 dilution) were collected 6 weeks after immunization, incubated with ETAR/B300 cells, and then the Ab responses were analyzed with FCM. (E) The titer of the antiserum against ETAR was determined by FCM. Each diamond-shaped data point represents a mouse. $^{*}P<0.05$.

inhibited the LPS-stimulated production of cytokines but not GroEL-stimulated production of cytokines.

To test the possibility that the stimulatory effect of *E. coli* GroEL on the induction of the Ab response in vivo may be associated with Toll-like receptor 4 (TLR4), we immunized C3H/HeN (wild-type TLR4) and C3H/HeJ (TLR4 mutant) mice with a plasmid that expressed ETAR–GroEL, and then compared their anti-ETAR Ab responses. FCM analysis showed that the anti-ETAR Ab response of C3H/HeJ mice was less than that of the C3H/HeN mice (Fig. 5B); however, all of the mice exhibited similar anti-GroEL Ab titers (Fig. 5C).

4. Discussion

This study demonstrated that GroEL acts as a potent molecular adjuvant and enhances the Ab response against GPCRs in DNA immunizations. Furthermore, GroEL enables the production of anti-GPCR Abs that are reactive against native GPCRs. In addition to ETAR and ETBR, we succeed to produce polyclonal and monoclonal Abs against other GPCRs by DNA immunization with GroEL (Table 1). These data suggest that GroEL will be capable of universal use for producing Abs against most of GPCR and will contribute to acquisition of functional Ab as we described previously (Takatsuka et al., 2010). In the case of unstable GPCRs, co-immunization of GroEL may be preferable to fusion with GroEL to induce an Ab response because fusion of some carrier proteins decreased their expression. Previous articles showed that the DNA vaccine expressing secretion types of antigen has the higher immunogenicity upon antibody production (Drew et al., 2000), however; ETAR–GroEL expressed in the form of unsecreted type, same with ETAR (data not shown). Our results (Fig. 2) also indicated that the fusion of GroEL with ETAR was critical for an efficient Ab response; however, the carrier effect alone, which targeted more efficiently as expected, did not induce a strong Ab response. Although we predicted 1 helper T cell epitope in human ETAR, 20 in GroEL, 10 in EGFP, and 20 in OVA from their amino acid sequences (data not shown), only GroEL was capable of inducing a robust Ab response in mice. In addition, the observation that strong anti-ETAR Ab responses also were induced in the sera of mice that were immunized with ETAR + GroEL but not ETAR–EGFP and ETAR–OVA (Fig. 4) suggested that GroEL is an effective adjuvant and may be involved in another mechanism that enhances the Ab response in addition to the carrier effect.

The results of Fig. 5 suggested 2 interesting possibilities. First, inflammatory cytokines may be involved in the adjuvant activity of GroEL (Fig. 5A). It is not yet clear whether GroEL treated DCs polarized the Th1 or Th2 type immune response; however, it is clear that intramuscular injection of GroEL DNA induces a polarized Th1 type immune response (Leclerq et al., 2002). Thus, it is possible that the IgG2a subclass of MAbs is predominantly obtained from ETAR–GroEL immunized mice (data not shown). Second, TLR4 signaling may be a stimulatory mechanism in GroEL-mediated DNA immunization (Fig. 5B). Since autologous HSP60 has an important role in infections and autoimmune diseases (Ohashi et al., 2000; Argueta et al., 2006), it is not surprising that *E. coli* GroEL is a potent stimulator of inflammatory cytokines via TLR4. Although there were no differences in the production of anti-GroEL antibodies (Fig. 5C), the difference in

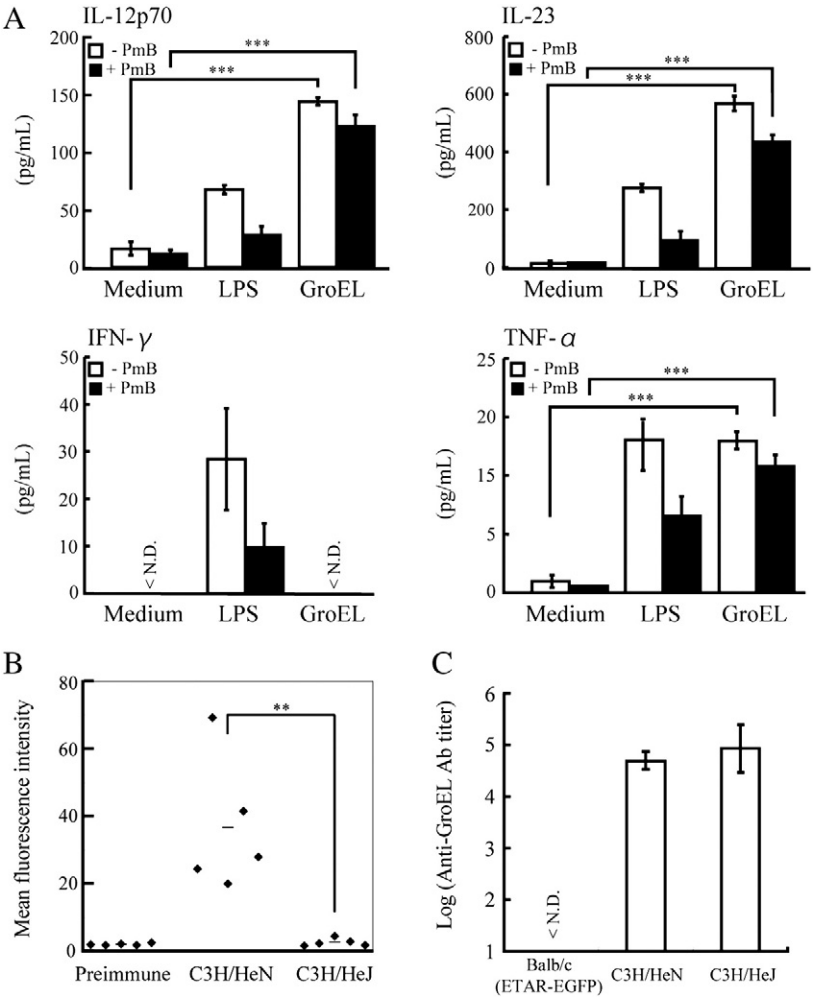

Fig. 5. Cytokine secretion of bone marrow-derived dendritic cells induced by GroEL and Ab responses in Toll-like receptor 4 mutant mice. (A) Bone marrow-derived dendritic cells (1×10^6) were stimulated by LPS (10 μg/ml) or GroEL (5 μg/ml) with or without polymyxin B. An ELISA was used to quantify the amount of cytokines (IL12-p70, IFN-γ, IL-23, and TNF-α) that were secreted into the culture supernatant. The data are expressed as the mean (SD) of 3 independent experiments. (B) C3H/HeN and C3H/HeJ mice were immunized with a plasmid that expressed ETAR–GroEL. The resulting sera (1/100 dilution) was collected 6 weeks after immunization, and then analyzed with FCM. Data are expressed as the MFI (SD) ($n=5$). (C) The anti-GroEL Ab titer in the antiserums from (B) is expressed as the mean (SD). $^{**}P<0.01$.

the production of anti-ETAR antibodies in C3H/HeN and C3H/HeJ mice suggested that TLR4 may be important. In fact, stimulation of the innate immune system through TLR4 was important for the immunoresponsiveness of not only ETAR but also GroEL since activation of the innate immune response with an adjuvant is essential for the enhanced adaptive immune response. Although we performed DNA immunization without any adjuvants except GroEL, we cannot rule out the possibility that our method included an unintentional adjuvant, such as the CpG motif or the plasmid DNA itself (Yasuda et al., 2002; Bauer et al., 2001). Thus, it is logical to assume that, in ETAR–GroEL immunized C3H/HeJ mice, the immune response was elicited only against GroEL but not ETAR because GroEL is strongly antigenic whereas ETAR is weakly antigenic.

Although it is still unclear whether GroEL fusion proteins are capable of stimulating cytokine secretion, or other commonly used cis-adjuvant sequences have a similar effect, our results indicated that GroEL fusion proteins enhance the Ab response in DNA immunization. Since a recent study suggested that IL-23 may be involved in the development of autoimmunity (Iwakura and Ishigame, 2006), IL-23 secreted from macrophages or DCs that are stimulated by GroEL may enhance the antigenicity of ETAR. The lack of an effect of GroEL on IFN-γ secretion suggested that GroEL, rather than LPS, induced the production of inflammatory cytokines, and that the underlying mechanism might be different from that by other members of the HSP family. In this study, we used mouse DC for cytokine production in response to GroEL. Because there are significant differences between mice and humans in immune system development, activation, TLR expression pattern, and response to various pathogens (Mestas and Hughes, 2004), further study using human DC for cytokine production against GroEL would lead to draw solid conclusions. In addition, since our plasmid

Table 1
Antibody responses after immunization with the GroEL fused GPCR[a].

GPCRs	Polyclonal	Monoclonal	Functional
CCR2B	+	+	−
CCR3	+	+	−
CCR5	+	+	−
CCR7	+	+	−
CCR11	+	+	+
CXCR4	+	+	−
CXCR7	+	+	−
RAIG1	+	+	−
FZD5	+	+	−
AT2R	−	N.D.[b]	N.D.
ETAR	+	+	−
ETBR	+	+	−
GPR40	−	N.D.	N.D.
GPR54	−	N.D.	N.D.
GPR85	+	N.D.	N.D.
GPR120	−	N.D.	N.D.

[a] Mice were immunized with plasmid encoding GroEL fused GPCR as described.
[b] Not done.

was prepared in *E. coli*, we also were concerned that the strong Ab response that was produced by DNA immunization might have been due to contamination with LPS. However, we do not think that this possibility is likely because DNA immunization of plasmids that encoded ETAR, which were prepared in the same manner as those that encoded ETAR–GroEL, did not induce any antibody response. In addition, we did not observe any difference between mice that were immunized with 2 plasmid preparations that were purified by using the endotoxin-free Gigaprep kit (QIAGEN).

Although further research is needed to elucidate the mechanisms of our observations, it is clear that GroEL enhances the production of Abs that are reactive against native GPCRs. Furthermore, our results suggested that an ideal molecular adjuvant for DNA immunization will be a multifunctional molecule, such as GroEL. The identification of a minimal functional domain of GroEL may enable the development of a peptide or small molecule adjuvant to induce strong Ab responses to antigens that are highly conserved or difficult to express or purify, such as GPCRs and other cell membrane proteins.

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