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Immunological basis of M13 phage vaccine: Regulation under MyD88 and TLR9 signaling

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ABSTRACT

Peptide-displaying bacteriophages induce mimotope-specific antibody responses, suggesting a novel application of phage-display library as bacteriophage vaccine. We examined the antibody response against M13 phage in mice induced by an i.p. administration of M13 phage in phosphate-buffered saline. We showed here that firstly, mice showed strong IgG antibody responses, particularly, in IgG2b, IgG2c, and IgG3 subclasses even in primary responses. Secondly, IgG production in primary response is totally dependent on MyD88 signaling. These responses were almost comparable, but slightly weaker, in TLR2-, TLR4- and TLR7-deficient mice relative to wild-type mice, suggesting that this enhancing effect is not due to plausible LPS contamination. Thirdly, although primary IgG1 response was not detected in wild-type mice, remarkable IgG1 response was induced in TLR9-deficient mice, suggesting that TLR9 pathway functions as regulatory, but not a simple augmenting signaling cascade, and furthermore, the enhanced IgG1 response was not due to adjuvant effect of single-stranded DNA derived from M13 phage. Thus, innate immunity including TLR regulation is crucial for M13 phage vaccine design.

1. Introduction

Phage-displaying technology was reported by Smith [1]. Lerner and Winter attempted to display an antibody on bacteriophage at 1989 [2] and 1990 [3], respectively. Since then, a peptide- or an antibody-displaying phage library has become a powerful tool to develop various recombinant peptides or human antibodies applicable for revolutionary therapeutics.

Filamentous phages are highly immunogenic and are known to induce humoral and cellular immune responses directed to their naive coat proteins [4]. It is well known that B cells can be efficiently activated by antigens with a highly ordered, repetitive structure that can very effectively cross-link B cell surface antibodies (BCR), resulting in a strong activation response [5,6], employing virus-like particles (VLPs) derived from the coat protein of the RNA bacteriophage Q beta. Using VPL, fel d 1-conjugated VLP has been reported to be useful for a desensitization vaccine against cat allergies [7].

Recent dramatic progress in immunology demonstrated the importance of innate immunity involved with various pattern-recognition molecules [8]. Accordingly with these studies, a recent report showed that tumor-specific filamentous phages could induce tumor destruction through activation of tumor-associated macrophages in an MyD88-dependent manner [9]. Mori et al. have reported that administration of M13 phage DNA induced IFNs and protected mice against vaccinia virus infection [10]. Furthermore, it was shown that Qbeta-VLPs contain naturally packaged bacterial RNA, a TLR7/8 ligand, which induced strong IgG2a/c-dominated antibody responses [11]. These data indicate that the antigen-displaying phage clone itself derived from a phage-display library is a powerful immunogen for bacteriophage vaccine immunotherapy.

M13 phage proteins pIII and pVIII have been frequently used in phage displays. Foreign DNA can be inserted into gene 3 or gene 8 of filamentous phages to create a fusion protein that is exported to the surface and displayed on the coat of the bacteriophage [1,12]. In the case of the M13 phage, the antibody response against the phage is restricted to the 12 N-terminal residues of pVIII and the outer domains of pIII [13]. It was also reported that peptide-displaying bacteriophages induce mimotope-specific antibody responses [6,14].

In this study, we investigated the antibody response against M13 phage *in vivo* in order to characterize them for bacteriophage vaccine vehicles.

2. Materials and methods

2.1. Mice

All animal experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of Kagoshima

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University. C57BL/6 mice (male, 6-week old) were purchased from Charles River Japan, Inc. Mice deficient in TLR2, TLR4, TLR7, TLR9, or MyD88 (male, 7- to 10-week old, C57BL/6 background) [15] were purchased from Oriental Bio-Service (Kyoto, Japan). All mice were housed under specific pathogen-free conditions.

2.2. Bacteriophage injection

M13 phage was partially purified from the culture supernatant by two polyethylen glycol (PEG) precipitations as described [16]. PEG-precipitated virions were further purified by CsCl equilibrium density gradient centrifugation and phase separation using Triton X-114 [17,18]. Wild-type or knockout mice were injected i.p. with 10^{11} plaque-forming unit (pfu) of M13 phage. Blood was taken from the lateral tail vein, and sera were stored at −20 °C until measured by ELISA. M13 phage were not detected in feces of M13 phage-injected mice when it was examined until day 9. There are no influences on the estimation of the body weights of M13 phage-injected mice.

2.3. Elisa

ELISA plates (Nunc-Immuno MaxiSorp; Nunc, Denmark) coated with 10^{10} cfu/well of M13 phage were blocked with 0.5% gelatin in PBS for 2 h at room temperature (RT). A serum sample diluted 1/2000 was added to the plates and incubated for 2 h at RT. Bound antibodies were detected using anti-mouse IgG, IgM or isotype-specific secondary antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) conjugated to alkaline phosphatase at a dilution of 1:2000, followed by incubation with 50 μl of a *p*-nitrophenylphosphate/10% diethanol amine solution [19]. Absorbance was measured at 405 nm by the use of a microplate reader (NJ-2300; Nunc, Tokyo, Japan).

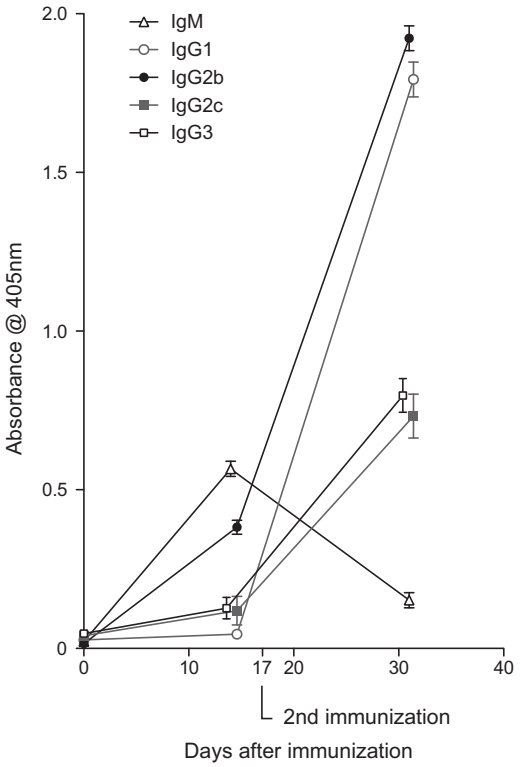

Fig. 1. Primary and secondary antibody responses against the M13 phage. C57BL/6 mice ($n = 6$) were immunized i.p. with 1011 pfu of the M13 phage in PBS on days 0 and 17. The sera (diluted 1:2000) was tested for M13 phage-specific IgM and IgG subclass responses by ELISA as described in Materials and Methods. Results are expressed as the mean ± SD.

3. Results and discussion

3.1. Primary anti-M13 phage IgG response was induced by a single administration of M13 phage without an adjuvant in mice

C57BL/6j mice were administered 10^{11} cfu of the M13 phage i.p. on day 0 and boosted with the same amount on day 17 (Fig. 1). Antibody response to M13 phage was measured on days 14 and 31. It was noteworthy that IgG2b was markedly induced despite it was injected in PBS solution and was a primary response. A weak IgG3 response, but not the IgG1, was observed. The IgM response was comparable to the IgG2b response. These responses are very characteristic of M13 phage-induced antibody response. In the case of secondary responses induced with the M13 phage, both IgG2b and IgG1 were highly produced as in similar way to the antibody response induced with usual immunogen such as SRBC. Thus, it is possible to generate a high IgG antibody response by injecting the M13 phage in mice, even in the absence of an adjuvant, and this may become a promising feature for a phage vaccine.

3.2. Impaired antibody response against M13 phage in the absence of MyD88

To examine the involvement of innate immune response [8] to M13 phage *in vivo*, we first analyzed the induction of antibody responses to M13 phage in MyD88-knockout B6-background mice. Wild-type and MyD88-deficient mice were administered a single i.p. injection with 10^{11} cfu of the M13 phage. The primary anti-M13 phage IgG responses in these mice were examined on day 13 (Fig. 2A). Surprisingly, MyD88-deficient mice gave no response, in contrast to wild-type mice, which produced strong IgG2b, IgG2c, and IgG3 antibody responses. In the case of IgG1, the response was very weak. These results indicated that the M13 phage induce a strong primary response composed of the following classes, IgG2b, 2c, and 3, with the essential involvement of MyD88.

These mice were boosted 97 days later with i.p. injection of M13 phage. When IgM, IgG1 and IgG2c responses against M13 phage were examined 14 days after the second immunization, MyD88-deficient mice gave no responses while wild-type mice showed significantly higher responses than the primary responses (Fig. 2B).

3.3. IgG1 production in primary response against the M13 phage was elevated in TLR9-deficient mice

We next attempt to investigate the role of MyD88-dependent TLR signaling on the induction of IgG response against M13 phage. Wild-type mice and mice lacking TLR2, TLR4, TLR7, or TLR9 were used in this experiment. The response was examined on day 13 of the primary response. As shown in Fig. 3, TLR2-, TLR4-, and TLR7-deficient mice showed relatively comparable responses to wild-type mice in spite of complete abolishment of the IgG response in MyD88-deficient mice. We expected that the minute amount of LPS in the M13 phage preparation would have an influence as a ligand for TLR4 signaling; however, we found that TLR4-deficient mice had a similar response to wild-type mice. Thus, it was unlikely that endotoxin was responsible for the augmented antibody responses. Another possible factor might be single-strand (ss) DNA of the M13 phage, in which the presence of CpG motifs in their genome might stimulate the TLR9 signaling. When the

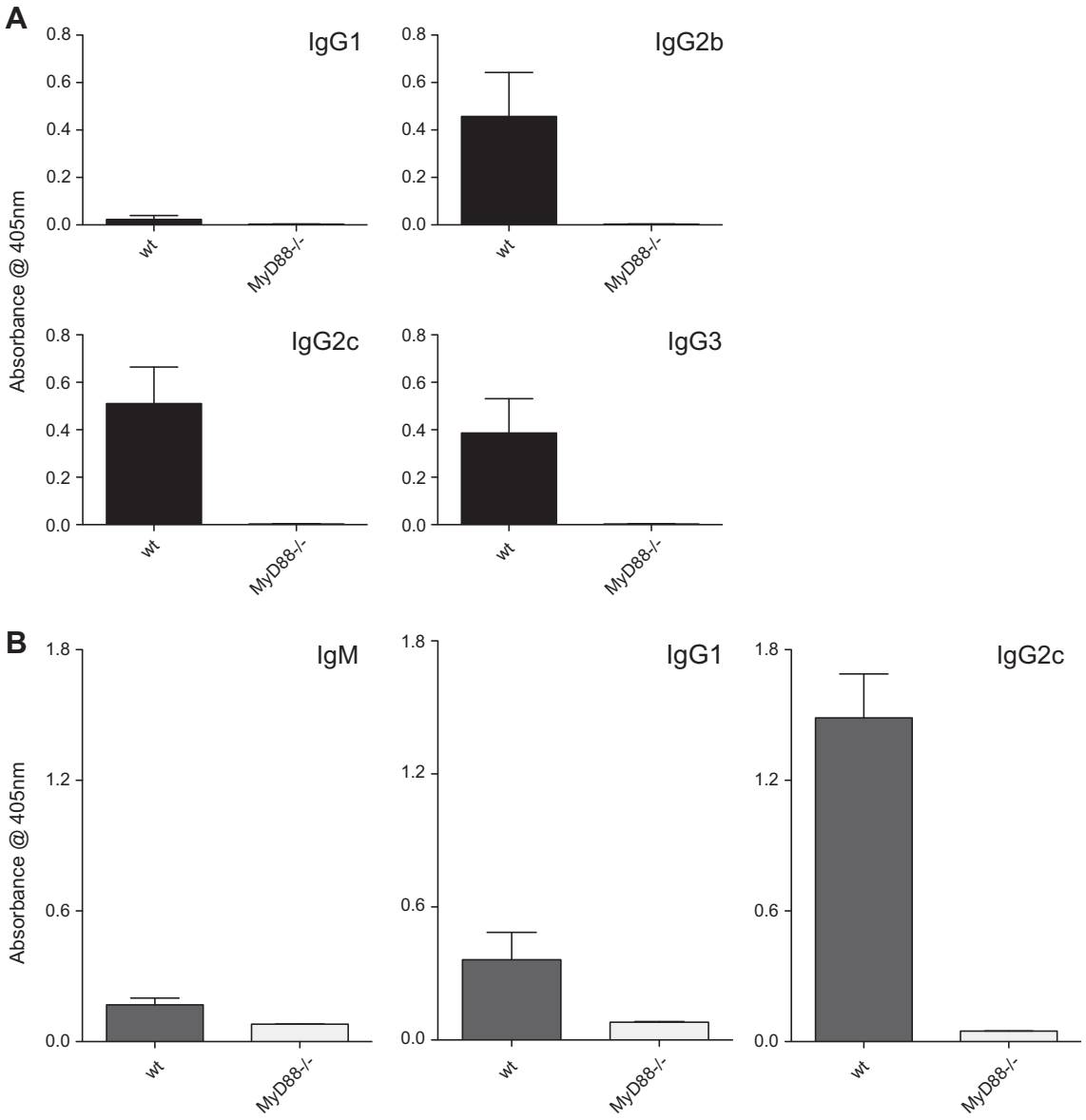

Fig. 2. Antibody response to M13 phage is dependent on MyD88 signals. Mice (4–5/group) were immunized i.p. with 10^{11} pfu of the M13 phage in PBS. M13 phage-specific IgG responses were measured by ELISA on day 13 for the primary response (A). Secondary immunization was performed at day 97. Sera were harvested two weeks later (B). All sera were tested at a dilution of 1:2000. The absorbance was measured at 405 nm 4 h (A) and 0.5 h (B) after the development. Results are expressed as the mean ± SD, with each group of mice.

responses of TLR9-deficient mice were investigated, contrary to our expectation, IgG1 response was dramatically induced in contrast to an almost undetectable response in wild-type mice even in the primary response. IgG2b and IgG3 responses were also significantly higher than they were in wild mice. In the case of IgG2c, no significant enhancement was observed relative to that in wild-type mice. These results demonstrated that TLR9 signaling does not always augment the antibody response and plays, at times, a regulatory role, which is particularly strong in the IgG1 and significantly so in the IgG2b and IgG3 responses to the M13 phage. A similar down-regulatory effect of TLR9 has been reported in murine lupus models *in vivo* [20]. These results suggested that TLR9 signaling might be involved in immunoglobulin class switching.

Thus, the following is shown in this study. First, mice had strong IgG antibody responses to M13 phage, particularly, in IgG2b, 2c, and 3 even in the primary response. Secondly, IgG production in the primary response is completely dependent on MyD88 signaling. Thirdly, although the primary IgG1 response was not detected in wild mice, a remarkable IgG1 response was induced in TLR9-deficient mice, suggesting that TLR9 pathway functions as a regulatory cascade, and not a simple augmenting signaling one, and, furthermore, the enhanced IgG1 response was not due to an adjuvant effect of single-strand DNA derived from the M13 phage. Finally, MyD88-deficient mice did not respond to M13 phage immunization in both primary and secondary responses, indicating that evoking innate immunity is essential to generate the following acquired immunity against M13 phage. The molecular mechanism of M13 phage-induced innate immunity remains to be elucidated. Our findings demonstrate that MyD88 signaling is dispensable in M13 phage-induced IgG responses and present new information on the role of TLR9 signaling for the development of an M13 phage-based vaccine.

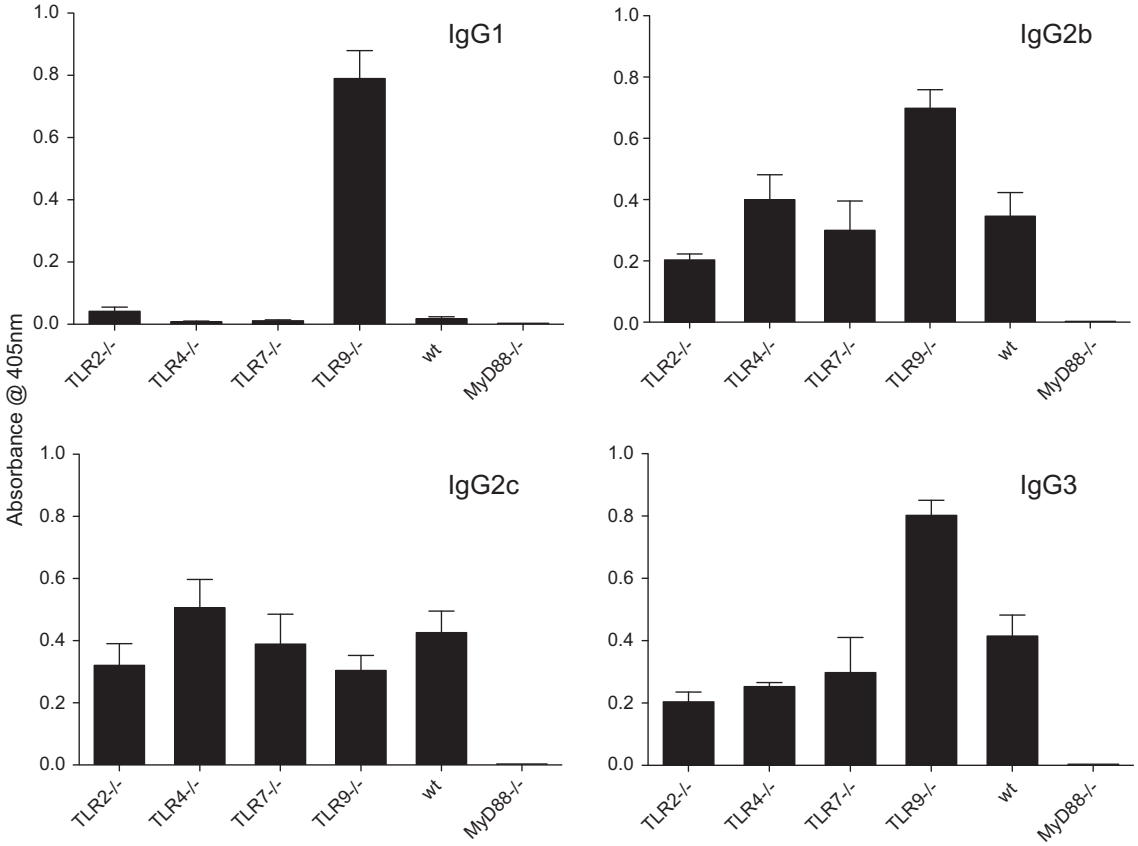

Fig. 3. Antibody response to M13 phage in TLR2-, TLR4-, TLR7-, TLR9-, or MyD88-deficient mice. Mice (4–5 mice/group) were immunized i.p. with 1011 pfu of the M13 phage in PBS. Sera were harvested on day 13 and tested at a dilution of 1:2000 by ELISA. The absorbance was measured at 405 nm 4 h after the development. Results are expressed as the mean ± SD, with each group of mice.

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