Inhibition of antibody production in vivo by pre-stimulation of Toll-like receptor 4 before antigen priming is caused by defective B-cell priming and not impairment in antigen presentation

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Abstract

Stimulation of Toll-like receptor 4 (TLR4) induces not only innate but also adaptive immune responses, and has been suggested to exert adjuvant effects. Additional to such positive effects, pre-stimulation of TLR4 induces endotoxin tolerance where animals are unresponsive to subsequent lethal challenges with lipopolysaccharide (LPS). We examined the effects of pre-stimulation of TLR4 using an agonistic anti-TLR4 mAb (UT12) on antibody production in vivo. Pre-injection of UT12 prior to both primary and secondary immunization completely inhibited antigen-specific antibody responses. Cellular analysis revealed that the inhibition was not due to impairment of T-cell activation. Accordingly, T-helper activities in UT12 pre-injected mice were not impaired. In contrast, B-cell priming was defective in UT12 pre-injected mice. The observation that the expression of activation markers such as CD69 and CD86 on B cells was blocked by UT12 pre-injection supports this. Interestingly, UT12 pre-injection only showed inhibitory effects at the primary and not the secondary immunization. These results provide important information concerning the regulatory mechanisms of antibody production, especially in endotoxin-tolerant states.

Keywords: agonistic monoclonal antibody, endotoxin tolerance, innate immunity, LPS

Introduction

Recognition of pathogen-associated molecular patterns by Toll-like receptors (TLRs) induces both innate and adaptive immune responses (1, 2). Immediately after an invasion by pathogens, innate signals through TLRs on macrophages and dendritic cells induce the production of pro-inflammatory cytokines. The stimuli through TLRs also enhance the expression of MHC class II (MHCII) and co-stimulatory molecules on antigen-presenting cells (APCs) for efficient antigen presentation to activate T cells. These activated T cells induce specific B cells to produce antibodies that combat foreign pathogens. Such effects of TLR stimulation on the adaptive immune response suggest the application of TLR agonists as adjuvants in experimental and clinical situations (3).

Lipopolysaccharide (LPS), a Gram-negative bacterial cell wall component, is known to be recognized by TLR4 (4), one of the best-studied TLR family members, with an associated MD-2 molecule (5). LPS is known to be a potent inducer of inflammation (6). Injection of large amounts of LPS will induce animal death, probably due to excessive secretion of cytokines. However, animals pre-treated with small amounts
of LPS become tolerant to subsequent lethal LPS challenges. This phenomenon is called LPS- or endotoxin-tolerance and is defined as the reduced capacity of the host or cultured macrophages/monocytes to respond to LPS following initial stimulation (7, 8).

Previous analysis has suggested a general suppression of pro-inflammatory cytokine production as a possible mechanism for endotoxin tolerance (7). However, recent molecular analysis suggests the involvement of negative regulators for TLR signaling, anti-inflammatory cytokines, microRNAs and chromatin modifications in endotoxin tolerance (8–12). These findings prompted the term `LPS-induced gene reprogramming` (9) instead of `endotoxin tolerance`.

Induction of adaptive immune responses was also reported to be impaired during endotoxin tolerance. Thus, the treatment of cells or animals with LPS induces the reduction of MHCI and co-stimulatory molecule expression on macrophages and dendritic cells (13–15). These findings imply that T- and/or B-cell immunity is also suppressed in an endotoxin-tolerant state (13–16), although precise studies have not been reported to date.

Previously, we established an agonistic anti-TLR4 mAb (UT12) that induces secretion of pro-inflammatory cytokines in vivo and in vitro (17, 18). Pre-injection of UT12 in vivo induced long-term endotoxin tolerance (18, 19) and attenuated airway allergic inflammation (14). In this study, we describe the adjuvant function of UT12 in antigen-specific antibody production in vivo. Additionally, pre-stimulation of TLR4 by UT12 prior to immunization inhibits antibody production. This inhibitory effect was not due to impairment of the helper activities of T cells, but was likely due to a defect in B-cell antigen priming. Interestingly, the recall responses of immune B cells were not affected by UT12 pre-injection. These results provide important information concerning the novel regulatory mechanisms of antibody production, especially during the endotoxin-tolerant state.

**Methods**

**Animals**

C57BL/6 (B6) and BALB/c mice were purchased from SLC Japan (Hamamatsu, Japan). The mice were 6–10-week-old females. A breeding pair of TLR4−/− mice (20) was a kind gift from Dr S. Akira (Osaka University, Osaka, Japan). They were backcrossed for more than eight generations in our laboratory onto the BALB/c genetic background. The mice were maintained in the Center for Laboratory Animals at Saga Medical School. All animal experimental procedures were conducted in accordance with the Regulations on Animal Experimentation at Saga University.

**Reagents and antibodies**

Agonistic UT12 (IgG3) and non-agonistic UT15 (IgG1) mAbs against mouse TLR4 were prepared and purified as described previously (17, 18). Ovalbumin (OVA) (grade V), BSA, CFA, aluminum hydroxide gel (alum) and an isotype control IgG3 antibody (Y5606) were purchased from Sigma–Aldrich (St Louis, MO, USA). *Escherichia coli* LPS was purchased from Wako Pure Chemicals (Osaka, Japan). KLH and 7-amino-actinomycin D (7-AAD) were purchased from Calbiochem (San Diego, CA, USA). Fluorescein isothiocyanate (FITC)-anti-mouse CD11c, PE-anti-mouse CD11c, FITC-anti-mouse MHCII, PE-anti-mouse CD40, PE-anti-mouse CD69, PE-anti-mouse CD86 and anti-mouse CD16/32 mAbs were purchased from eBioscience (San Diego, CA, USA). PE-rat anti-mouse CD138 was purchased from BD Biosciences (San Jose, CA, USA). Alkaline phosphatase (ALP)-conjugated goat anti-mouse IgG (H&L) was purchased from American Qualex Scientific Products (San Clemente, CA, USA). ALP-conjugated goat anti-mouse IgG2a and IgG2b were purchased from Bethy Laboratories (Montgomery, TX, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 and IgG3 were purchased from SouthernBiotech (Birmingham, AL, USA).

**Immunization and serum collection**

Mice (five to six mice per group except where otherwise indicated) were immunized with 10 μg OVA plus 3 μg of UT12 in 0.2 ml PBS subcutaneously (s.c.) at the inter-scapular area and boosted 4 weeks later, unless otherwise stated. Blood samples were collected from the tail vein at the indicated times and the serum anti-OVA IgG antibody titer determined by ELISA.

**Antibody production in vitro**

Culture medium consisted of RPMI1640 with 10% FCS, 30 μM β-mercaptoethanol, 100 U ml−1 penicillin and 100 μg ml−1 streptomycin. L-Glutamine was added to a final concentration of 2 mM prior to use. RBCs from the spleen were lysed by osmotic shock using RBC-lysis buffer (0.15 M NH4Cl, 1.0 mM KHCO3 and 0.1 mM Na2EDTA). For antibody production in vitro, 2 × 106 spleen cells were stimulated with antigen in 0.5 ml of culture medium/well in a 48-well culture plate (Corning Inc., NY, USA). After 2 days, cells were washed three times with HBSS to remove antigen. Care was taken to remove the antigen completely by tapping the culture plates on a sterile gauze after each wash. Cells were re-cultured with fresh medium for a further 5 days, and the amount of antibody in the culture medium was measured by ELISA. For the antibody production assay, antigen-specific long-term cultured T cell lines were used (see below); 7 × 105 spleen cells were mixed with 6 × 105 T cell lines and cultured as above.

**ELISA**

The amounts of serum antigen-specific IgG antibodies were measured by ELISA using Immuno 96 MicroWell Solid Plates (Nunc, Roskilde, Denmark). The plates were coated with antigen followed by blocking with BSA. To each well, 50 μl of appropriately diluted serum sample was added, incubated for 2h at room temperature and washed three times with 0.05% Tween 20 in PBS. The plates were incubated with 50 μl of appropriately diluted ALP- or HRP-conjugated anti-mouse IgG antibody, washed three times and developed by 4-nitrophenyl phosphate disodium salt hexahydrate substrate or by o-phenylenediamine dihydrochloride substrate (Sigma–Aldrich). Plates were read at OD405 or at OD490 using a SpectraMax 190
mixed lymphocyte culture reactions, 4 × 10⁵

In primary in vitro use.

the T-cell lines were checked for antigen specificity before sometimes deviated; at these times cells became autoreactive and proliferated in the absence of antigen. Therefore, the amounts of antibody in the non-diluted culture supernatants were measured by the same procedure and expressed as OD₄⁵⁰. The amounts of TNF-α and IL-6 in serum were measured using the Mouse ELISA Ready-SET-Go! kit (eBiosciences), according to the manufacturer’s instructions.

Enrichment of T and B cells from spleen cells

To obtain a T or B cell enriched population, T or B cells were depleted from the spleen using Dynabeads Mouse pan T (Thy1.2) or Mouse pan B (B220) (Invitrogen Dynal AS, Oslo, Norway), respectively, according to the manufacturer’s instructions. After depletion, the purity of the T or B cells was >90%, as assessed by flow cytometry.

Long-term cultured allo-reactive and antigen-specific T-cell lines

Allo-reactive and antigen-specific long-term cultured T-cell lines were established as described previously (21, 22). T-cell lines were maintained by re-stimulation every 2 weeks. For the T-cell proliferation assay, 1 × 10⁴ T cells were stimulated using varying numbers of RBC-lysed, irradiated (3.3 Gy) spleen cells as stimulator cells or APCs in the presence or absence of antigen for 3 days. The proliferative responses were measured by the uptake of tritiated thymidine ([³H]TdR) during the final 16 h. During long-term culture of the antigen-specific T-cell lines, the specificity sometimes deviated; at these times cells became autoreactive and proliferated in the absence of antigen. Therefore, the T-cell lines were checked for antigen specificity before use.

Mixed lymphocyte culture reaction

In primary in vitro mixed lymphocyte culture reactions, 4 × 10⁶ lymph node (LN) cells were stimulated with varying numbers of RBC-lysed, irradiated (3.3 Gy) spleen cells as stimulator cells for 5 days. The proliferative responses were measured as above.

Flow cytometry

All staining procedures were performed using staining buffer (HBSS with 4% FCS and 0.1% NaN₃) at 4°C. RBC were lysed using RBC-lysis buffer. After blocking the Fc receptor (FoR) by anti-CD16/32 mAb for 20 min, the cells were stained with FITC- and/or PE-conjugated antibodies for 30 min, washed twice with FACS buffer and analyzed on a FACSScan (BD Biosciences). Dead cells were gated out by staining with 7-AAD. The data were analyzed using the WinMDI software (J. Trotter, The Scripps Research Inst. La Jolla, CA, USA).

Statistics

Experimental results were analyzed for their significance using Student's t-test. Statistical significance was established at the 95% confidence level (P < 0.05).

Results

Adjuvant effects of agonistic anti-TLR4 mAb

Stimulation of TLRs using natural or synthetic agonists has been shown to have an adjuvant effect on a variety of immune responses (23, 24). To examine how the agonistic anti-TLR4 mAb UT12 functions as an adjuvant for antibody production, we immunized BALB/c mice with 10 µg OVA plus 3 µg of UT12 s.c. and administered a booster 4 weeks later. The amounts of IgG anti-OVA antibodies in serum samples collected at various times after immunization were measured by ELISA.

As shown in Fig. 1A, immunization of OVA with UT12 increased anti-OVA antibody production compared with OVA in PBS. The amounts of anti-OVA antibody generated were similar to that of the samples immunized with 50 µg of LPS adjuvant. Dose-response experiments showed that 1.0 µg of UT12 exhibited a small but significant adjuvant effect (Fig. 1B). Increasing the amounts of UT12 to >5 µg sometimes induced mouse death, therefore 3 µg of UT12 was employed as the adjuvant dose in the following experiments. Neither an isotype control IgG3 mAb (Y5606) nor a non-agonistic anti-TLR4 mAb (UT15) exhibited adjuvant effects (Fig. 1C). As a specificity control, UT12 mAb did not show adjuvant effects in TLR4⁻/⁻ mice (data not shown). Interestingly, immunization of OVA and UT12 at different sites, i.e. OVA s.c. and UT12 intra-peritoneally (i.p.) or vice versa, showed a level of antibody production similar to that which resulted from injection at the same site, i.e. OVA and UT12 s.c. or OVA and UT12 i.p. (Fig. 1D). Because UT12 injection was shown to suppress the expansion of T,2 and T,17 cells (14), it was interesting to examine the IgG subclass profile of serum obtained by immunization with various adjuvants. Figure 1E shows the relative amounts of each subclass antibody in the serum obtained with various adjuvants compared with those of UT12 adjuvant.

Use of LPS adjuvant shows almost similar subclass profile except low IgG3 as that of UT12 adjuvant. Alum, a TLR-independent adjuvant, seems to preferentially help IgG1 production compared with UT12. CFA, which may contain agonistic ligands against various TLRs, seems to help increased IgG1 and IgG2b production compared with UT12.

Inhibition of antibody production by pre-stimulation of TLR4

Our previous report demonstrated that UT12 induced long-term endotoxin tolerance (18). Thus, pre-injection with sub-lethal amounts of UT12 resulted in reduced secretion of TNF-α and IL-6 in serum and prevented death on subsequent lethal LPS challenge. To examine the effects of pre-injection of
UT12 on antibody production in vivo, UT12 was pre-injected into BALB/c mice prior to OVA plus UT12 immunization, and the serum IgG anti-OVA antibody titer was assessed.

As shown in Fig. 2A, pre-injection of 3 µg UT12 i.p. 3 days prior to both the primary and secondary immunizations showed almost complete inhibition of antibody production. A potent inhibitory effect was observed with 1 µg of UT12, although 0.3 µg of UT12 was ineffective. Similar results were obtained using either a B6 strain of mice or KLH as the antigen (data not shown). Time course analysis of UT12 pre-injection demonstrated that pre-injection 1 week before immunization showed weak but significant inhibitory activity (Fig. 2B). This effect was not seen at 2 weeks before immunization. Pre-injection between 3 days and 8 h before immunization showed almost complete inhibition. Injection at 24 h after immunization did not show any inhibitory
activity, indicating that the inhibition of antibody production was not due to the poor general health condition of hypercytokinemia (18) caused by the UT12 injection. As a control, pre-injection of 50 µg of LPS inhibited antibody production almost completely, but pre-injection of IgG3 mAb (Y5606) or non-agonistic anti-TLR4 mAb (UT15) did not show any inhibition (data not shown). UT12 pre-injection showed mild, but significant, inhibition of the antibody response in mice immunized with OVA plus LPS (Fig. 2C). However, antibody production by immunization with OVA in CFA was not affected by the UT12 pre-injection (Fig. 2C). The reason for these results is the presence of contaminated agonistic materials in the LPS and CFA preparations used in our experiments that stimulate innate receptors other than TLR4 to which UT12 pre-injection could not exert tolerant effects.

Fig. 2. Pre-injection of UT12 inhibition of antibody production in vivo. (A) BALB/c mice were pre-injected i.p. with varying amounts of UT12 3 days before primary and secondary immunization with 10 µg of OVA plus 3 µg of UT12. (B) BALB/c mice were injected i.p. with or without 3 µg of UT12 at the indicated time before or after both primary and booster immunizations. (C) BALB/c mice were pre-injected i.p. with or without 3 µg of UT12 3 days before primary and secondary immunization with 10 µg of OVA plus 50 µg of LPS (left panel) or 50 µg of OVA in CFA (right panel). (D) WT or TLR4−/− BALB/c mice were pre-injected with or without 3 µg of UT12 3 days before primary and booster immunization with 50 µg of OVA in alum. Serum anti-OVA IgG titer 1 week after booster immunization was measured as in Fig. 1. ****P< 0.001, ***P< 0.005, **P< 0.01, *P< 0.05, n.s., not significant.
To confirm that the inhibitory effect of UT12 pre-injection was mediated through TLR4 signaling, we pre-injected UT12 and immunized wild-type (WT) or TLR4–/– mice with OVA in alum, the adjuvant effect of which is not mediated by the TLR family of receptors (25). TLR4–/– mice with a BALB/c background were used in this experiment to exclude the effects of strain differences in the responsiveness to LPS through TLR4 (26). As expected, anti-OVA antibody production was not inhibited by immunization with alum in both UT12 pre-injected WT and TLR4–/– mice (Fig. 2D).

Decrease of MHCII and co-stimulatory molecule expression on APCs by pre-injection of agonistic anti-TLR4 mAb

To analyze the mechanism underlying the inhibition of antibody production by UT12 pre-injection, we examined the expression of MHCII, CD40 and CD86 on CD11c+ cells in the spleen of mice pre-injected 3 days before. As shown in Fig. 3, UT12 pre-injected CD11c-high spleen cells showed decreased MHCII, CD40 and CD86 expression compared with the non-injected controls. These results suggest that expression of antigen presentation-associated molecules on CD11c+ APCs, such as dendritic cells and macrophages, decreased due to pre-stimulation of TLR4.

No impairment of antigen-presenting function by pre-injection of agonistic UT12 mAbs

To examine whether such a decreased expression of antigen presentation-associated molecules on APCs affects the stimulatory activities of T cells, UT12 pre-injected spleen cells were used as stimulator cells in primary mixed lymphocyte culture reactions (MLR). In spite of the decreased expression of MHCII and co-stimulatory molecules, the stimulatory activities of UT12 injected cells were similar to those of non-injected cells in both B6 anti-BALB/c and BALB/c anti-B6 primary MLR responses (Fig. 4A). Additionally, allo-stimulatory activities of these UT12 pre-injected and non-injected spleen cells were similar when assayed on B6 anti-BALB/c (Fig. 4B) and BALB/c anti-B6 (data not shown) long-term cultured allo-reactive T cell lines, although the responses declined sharply when a high number of UT12 pre-injected stimulator cells were administered, probably due to the accelerated consumption of nutrients.

Antigen-presenting activities of spleen cells from UT12 pre-injected or non-injected mice were assayed using long-term cultured OVA-specific T cell lines. In our experiments it was difficult to make antigen-specific T cell lines from BALB/c mice for unknown reasons; therefore, we used T cell lines from B6 mice. We found that although the optimal APC numbers for maximal proliferation varied between UT12 pre-injected (0.3–3 × 10^5 cells per well) and non-injected (1–10 × 10^5 cells per well) spleen cells (see Discussion), the degree of maximal response was similar between these APC populations (Fig. 4C).

No impairment of T-helper function but defective B-cell antibody production of spleen cells from UT12 pre-injected mice

To examine the functional activities of the T and B cells of UT12 pre-injected spleen cells in vitro. To analyze the helper function, we used the T cell lines from B6 mice. We found that although the optimal APC numbers for maximal proliferation varied between UT12 pre-injected (0.3–3 × 10^5 cells per well) and non-injected (1–10 × 10^5 cells per well) spleen cells (see Discussion), the degree of maximal response was similar between these APC populations (Fig. 4C).
function of the T cells, BALB/c mice were pre-injected with or without UT12. After 3 days they were immunized with OVA plus UT12. One week after immunization, T-enriched cells from the UT12 pre-injected spleen cells were cultured with B-enriched cells from BALB/c mice hyper-immunized with OVA. As shown in Fig. 5A, T cells obtained from non-injected and UT12 pre-injected mice showed similar helper activities on OVA-stimulated antibody production by hyper-immune B cells, indicating that UT12 injection does not impair helper-T-cell activities. This is in accordance with our observation that there is no functional impairment in the antigen-presenting function of UT12 pre-injected spleen cells (Fig. 4).

To analyze B-cell function, spleen cells from B6 mice pre-injected with or without UT12 and immunized with OVA plus UT12 were cultured with B6-derived OVA-specific T-cell lines. We used long-term cultured OVA-specific T-cell lines to provide potent helper activity (27). As shown in Fig. 5B, non-injected spleen cells produced a significant increase in anti-OVA antibody level in the presence of helper cells as APCs from non- or UT12-injected B6 mice for 3 days. Proliferative responses were measured by the uptake of [3H]ThdR for the final 16 h. Proliferative responses without antigen were usually below 1000 c.p.m. (data not shown). Results are expressed as the mean of triplicate cultures. Each standard deviation was <10%.

Representative results of more than three independent experiments with similar results are presented.

Fig. 4. Allo-stimulatory and antigen-presenting activities of UT12-injected spleen cells. (A) Primary MLR. 4 × 10⁵ LN cells from B6 (left panel) or BALB/c (right panel) mice were stimulated with varying numbers of RBC-lysed irradiated spleen cells from non-injected B6 (○), non-injected BALB/c (△), UT12-injected B6 (●) or UT12-injected BALB/c (▲) mice as stimulator cells for 5 days. (B) Long-term cultured B6 anti-BALB/c allo-reactive T cells (1 × 10⁴) were stimulated with varying numbers of RBC-lysed irradiated spleen cells from non-injected B6 (○), non-injected BALB/c (△) or UT12-injected BALB/c (▲) mice for 3 days. (C) Long-term cultured anti-OVA T cells (1 × 10⁴) from B6 mice were stimulated with 50 µg ml⁻¹ of OVA in the presence of varying numbers of RBC-lysed irradiated spleen cells as APCs from non- (○) or UT12- (●) injected B6 mice for 3 days. Proliferative responses were measured by the uptake of [3H]ThdR for the final 16 h. Proliferative responses without antigen were usually below 1000 c.p.m. (data not shown). Results are expressed as the mean of triplicate cultures. Each standard deviation was <10%.

Representative results of more than three independent experiments with similar results are presented.
markers by this treatment. As shown in Fig. 6A, CD138-positive B220-low cells appeared to be induced 3 days after UT12 injection, probably reflecting the maturation of some B cells to plasmablasts. The frequency of such CD138-positive B220-low cells was 13.4% in the total spleen cells 3 days after UT12 pre-injection. The expression of activation markers CD69 and CD86 by immunization of OVA plus UT12 was completely suppressed on B220-high B cells by pre-injection of UT12 (Fig. 6B). These results also support the idea that pre-stimulation of TLR4 by UT12 inhibits B-cell activation.

Inhibition of antibody production by pre-injection of TLR4 at primary but not at secondary immunization

We further examined whether pre-injection of UT12 exerts inhibitory activity at the primary and/or secondary immunizations. As shown in Fig. 7B, pre-injection of UT12...
during primary immunization significantly inhibited antibody production, although the degree of inhibition was weak compared with that observed after pre-injection at both the primary and secondary immunizations (Fig. 2). In sharp contrast, UT12 pre-injection at the secondary immunization did not inhibit antibody production significantly (Fig. 7C). This was not due to the ineffectiveness of UT12 to induce endotoxin tolerance when injected during the secondary immunization, because TNF-α and IL-6 production were completely inhibited in UT12 pre-injected mice (Fig. 7D). This suggests that the mechanisms of inhibition of antibody production by pre-stimulation of TLR4 function during the

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**Fig. 6.** Change of surface markers on B cells by UT12 pre-injection. (A) Spleen cells from BALB/c mice pre-injected with (UT12 pre-inj.) or without (non-inj.) 3 µg of UT12 i.p. 3 days before were subjected to FACS analysis. After RBC lysis, FcRs were blocked with anti-mouse CD16/32 mAb, stained with FITC-anti-B220 plus PE-anti-CD138. The frequency of CD138 positive B220 low cells in the region R1 was indicated in each panel. (B) BALB/c mice were pre-injected with (UT12 pre-inj.) or without (non-inj.) 3 µg of UT12 i.p. 3 days before and were immunized (shaded histograms) or not (open histograms) with 50 µg OVA plus 3 µg UT12. After 24h, FCRs of RBC-lysed spleen cells were blocked and stained with FITC-anti-B220 plus PE-anti-CD69 or PE-anti-CD86. Histogram profiles of CD69 and CD86 expression on B220-high cells are shown. Dead cells were gated out by staining with 7-AAD.
priming phase of the immune response, but not during recall memory responses.

Discussion

We examined the effects of TLR4 signaling on antibody production in vivo using an agonistic anti-TLR4 mAb (UT12). Immunization of the antigen together with UT12 induced an increase in specific antibody production in vivo, demonstrating that UT12 exerts an adjuvant effect. Pre-stimulation of TLR4 alone by UT12, however, inhibited the antibody production induced by a subsequent immunization with antigen. Cellular analysis revealed that although expression of antigen presentation-associated molecules, such as MHCII, CD40 and CD86, on APCs decreased, pre-injection of UT12 did not impair antigen presentation. In accordance with this, antigen-specific T cells from mice pre-injected with UT12 showed similar helper activities on antibody production compared with those from non-injected mice. In contrast, antigen-primed spleen cells from UT12 pre-injected mice showed defects in antibody production upon stimulation with specific T cells and antigens, suggesting that the in vivo decrease in antibody production caused by TLR4 pre-stimulation might be due to impaired B cell priming. The lack of expression of activation markers such as CD69 and CD86 on B cells by UT12 pre-injection supports this.

In this study, we used agonistic mAbs instead of LPS to stimulate TLR4. The advantage of using agonistic mAb over LPS was because there could be no contamination of materials. Additionally, the agonistic mAb shows longer effectiveness in vivo (18, 19), and does not require LPS-binding protein (LBP) or CD14 for its TLR4 signal-transducing activity. Also, the effects of RP105, a TLR4 homolog (28), on

![Diagram](https://academic.oup.com/intimm/article-abstract/25/2/117/2950761)

**Fig. 7.** Inhibition of antibody production by UT12 pre-injection at primary but not secondary immunization. (A) Protocol of UT12 pre-injection and immunization. BALB/c mice received primary (1º) and booster immunization (2º) with 10 µg of OVA plus 3 µg of UT12 s.c. (Gr. I), 3 µg of UT12 pre-injected i.p. 3 days before primary immunization (Gr. II) or 3 days before secondary immunization (Gr. III). (B and C) Mice in each group were bled 1 week after booster immunization and anti-OVA IgG titers were measured by ELISA and expressed as mean percentages of the standard anti-OVA antiserum (% std) ± SD. Representative results of more than three independent experiments with similar results are shown. (D) Mice in each group were bled 2 h after secondary immunization. The amounts of serum TNF-α (▲, left scale) and IL-6 (●, right scale) were measured by ELISA and expressed as means ± SD. ***P < 0.005, n.s., not significant.
Inhibited antibody production by pre-stimulation of TLR4

B-cell function can be excluded by using agonistic anti-TLR4 mAbs. The most important findings in this study is that in spite of the reduced expression of antigen presentation-associated molecules on APCs by pre-stimulation of TLR4, which is consistent with reports by other researchers, antigen-presenting and allo-stimulatory abilities of TLR4-pre-stimulated spleen cells were not impaired. Thus, the proliferative responses of anti-OVA T-cell lines and long-term cultured B6 anti-BALB/c allo-reactive T-cell lines to spleen cells from UT12 pre-injected mice were almost identical to those from spleen cells from non-injected mice. The optimal cell number for maximal antigen-specific T-cell proliferative responses differed between UT12 pre-injected and non-injected APCs. The reason for this is not clear, but may be due to the fact that injection of UT12 increases the total spleen cell number and the frequency of CD11b- and CD11c-positive cells so that the absolute number of CD11b and CD11c cells increases by >3-fold (data not shown), which might compensate for the reduced expression of antigen presentation-associated molecules. Although the number of APCs that induced maximal responses was different, the maximal T-cell proliferation was comparable between APCs from both UT12 pre-injected and non-injected mice. Therefore, we believe that pre-injection of UT12 did not impair antigen presentation or allo-stimulatory activities.

Because these antigen-specific or allo-reactive T-cell lines were already primed, the threshold for activation would be low compared with that of naive T cells due to the relatively low dependency on the amount of antigen presentation-associated molecules. It could be argued that this might be because there was no difference in the antigen-presenting abilities of UT12 pre-injected and non-injected APCs. However, this possibility can be dismissed because the primary MLR of B6 anti-BALB/c and BALB/c anti-B6 LN responses were virtually identical in the stimulator cells from UT12-injected and non-injected cells.

In accordance with the above findings, helper activities of T cells were similar between UT12-injected and non-injected mice when examined using an in vitro antibody production assay. In contrast, antibody production by UT12 pre-injected OVA-primed cells was clearly decreased compared with that in non-injected cells when helped by OVA-specific T-cell lines. On the basis of these observations, we suggest that inhibition of antibody production in vivo by pre-stimulation of TLR4 before antigen priming is due not to impaired antigen presentation, but to defective B-cell priming. UT12 pre-injection induced maturation of some B cells to CD138-positive B220-low plasmablasts (Fig. 6A). The remaining B220-high B cells after UT12 pre-injection did not express the activation marker of CD69 and CD86 upon immunization with OVA plus UT12 (Fig. 6B). These observations also support the idea that UT12 pre-injection induced defect in B-cell activation. The reason for the discrepancy between our results and those of others who observed reduced T-cell activities by TLR4 pre-stimulation (13, 15) is not clear, but could be due to the experimental systems employed.

We observed almost complete inhibition of antibody production by pre-injection of UT12 in vivo (Fig. 2A). However, the antibody production by UT12 pre-injected spleen cells was not completely inhibited when stimulated with antigen in the presence of OVA-specific T-helper cells in vitro (Fig. 5B). The reason for this discrepancy is not clear but may suggest that blocking of B-cell priming alone cannot explain the inhibitory effects of UT12 pre-injection. One possible explanation for this would be that UT12 pre-injection induced regulatory T cells for antibody production in vivo, the effects of which were not observed in our in vitro experiments (Fig. 5A). The other but not mutually exclusive possibility would be that UT12 pre-injection induced suppressive macrophages and/or dendritic cells that inhibit antibody production in vivo. Further cellular analysis would be required to clarify the discrepant in vivo and in vitro results.

The functional significance of TLRs to B cells in adaptive immunity has been investigated extensively. However, controversies exist regarding the absolute necessity of B-cell-intrinsic TLR signals for antibody responses. This is probably due to the different experimental systems employed, particularly the different types of antigens. Our data clearly suggest that stimulation of TLR4 induces B cells to be unresponsive or hypo-responsive to subsequent antibody production stimuli. This is probably due to the maturation of B cells into plasmablasts, which do not respond to conventional antigenic stimulation. Also, B220-high B cells become inactive to conventional antigen plus adjuvant stimulation by pre-stimulation of TLR4. These considerations have important implications for clinical vaccine development and practice in that certain types of vaccine materials and/or adjuvants might not be effective when injected within limited intervals.

Another interesting finding is that although UT12 pre-injection only during primary immunization inhibited antibody production, the same injection during secondary immunization did not. This would suggest that inhibition by pre-stimulation of TLR4 functions only during priming of the primary immunization and not at the secondary phase, and implies the existence of different mechanisms in B-cell TLR4 signaling during primary and secondary responses. Further cellular and molecular studies are necessary to analyze the precise mechanisms involved in this process.

The adjuvant activities of stimuli through TLRs were studied extensively in adaptive immune responses and suggested to be useful in clinical vaccine practice. Our results also showed the adjuvant effects of TLR4 stimulation by using agonistic UT12 mAb in antibody production in vivo (Fig. 1). The adjuvant activity for total IgG production of UT12 was almost comparable to that of LPS and about half that of CFA. When we analyze the relative amounts of IgG subclass antibodies produced using various adjuvants, LPS adjuvant seems to show almost similar activities as UT12 except that it stimulates low IgG3. CFA preferentially supported the production of IgG1 and IgG2b compared with UT12. Alum, a TLR-independent adjuvant, showed strong IgG1 production compared with UT12. These subclass preference effects would be due to the different induction of T-helper cell subset(s), such as Th1, Th2 and Th17 by the use of different adjuvants. In fact, a recent report demonstrated the suppression of Th2- and Th17-cell expansion by stimulation of TLR4 with UT12.

It is also worth noting the effects of the antigen and adjuvant injection site. Although co-delivery of antigen and adjuvant was demonstrated to enhance the immune response, our data showed similar antibody responses irrespective of
whether the antigen and adjuvant were administered at the same or different sites. Effective antibody production by separate immunization of antigen and adjuvant was reported (24). It is likely that soluble (UT12 in this report) or nanoparticle (24) adjuvants circulate immediately after injection throughout the body and exert their adjuvant function in combination with antigen.

In summary, we found that pre-stimulation of TLR4 by injection of agonistic anti-TLR4 mAb (UT12) inhibited antibody production, probably because of impairment of B-cell priming. This provides important information concerning B-cell activation in primary and secondary responses, as well as for vaccine development and clinical practice.

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**References**
