*Immunology* 2004 **111** 19–26 doi: 10.1046/j.1365-2567.2003.01787.x

# Co-administration of CD40 agonistic antibody and antigen fails to overcome the induction of oral tolerance

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## SUMMARY

**T-cell stimulation in the absence of a second, costimulatory signal can lead to anergy or deletion. There is growing evidence that peripheral tolerance to an exogenous antigen might be caused by the lack of costimulatory molecules on antigen-presenting cells (APCs). In the present study, we examined whether tolerance against orally administered antigen could be reversed by maturation of APCs via CD40 signalling. Monoclonal antibody (mAb) to CD40 efficiently induced costimulatory molecules on APCs. Treatment with anti-CD40 mAb potentiated the division of ovalbumin-specific T cells in response to oral ovalbumin in secondary lymphoid organs. However, such treatment did not prolong the presentation of oral ovalbumin on APCs. Surprisingly, treatment of anti-CD40 mAb at the time of oral administration of ovalbumin did not reverse the induction of tolerance to ovalbumin in either the high- or low-dose regimens. Furthermore, the induction of oral tolerance in our model is not the result of negative signalling by cytotoxic T-lymphocyte antigen-4. These results indicate that tolerance for oral antigen could be established regardless of APC maturation by a CD40-specific mAb, suggesting that there could be a unique mechanism to regulate immunity versus tolerance to encountered antigen in the gut-associated lymphoid tissue.**

## INTRODUCTION

Onset of T-cell immunity against an antigen requires the delivery of two signals. The first signal involves the specific engagement of the T-cell receptor by peptides presented by major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs). The second signal provides costimulation and involves ligation of another receptor on the T-cell surface in an antigen non-specific manner. Delivery of signal one without signal two does not fully activate the T cell but instead directs it to a non-responsive state known as anergy.[1,2]

Peripheral tolerance to sequestered self-antigen has been explained in this context. Non-professional APCs do not bear costimulatory molecules, such as B-7s, under normal conditions and thus cannot deliver signal two.[1] Furthermore, it is widely accepted that peripheral tolerance to an exogenous antigen might be caused by the lack of costimulatory molecules on APCs.[3–5]

Providing costimulatory molecules on APCs would reverse the T-cell anergy. In addition, it has been reported that activation of APCs by CD40 ligation delayed the clonal deletion of antigen-specific T-cell and enhanced T-cell clonal expansion in response to super-antigen.[6] Thus it is a reasonable assumption that providing signal two would ablate the induction of peripheral tolerance to an exogenous antigen and lead to immunity against the antigen.[3–5,7–12] Signalling via CD40 has been used as an efficient tool to activate APCs *in vivo*. Indeed, accumulating evidence has shown that activation of APC via CD40 reverses tolerance induction and leads to immunity to tumour antigen or injected soluble antigen.[9–12] Garza *et al*. reported that glycoprotein (gp) peptide treatment with CD40 ligation led to autoimmunity instead of tolerance in the RIP-gp/P14 mouse model.[5] Furthermore, CD40 signalling can replace the assistance of CD4 in inducing cytotoxic T-lymphocyte responses in mouse models.[10,12] Extensive approaches are underway to utilize this property in tumour immunotherapy.

A large population of immune cells resides in mucosa-associated lymphoid tissue (MALT) such as gut, nasal tissue and trachea. Since these areas are the site of pathogen entry and have unique physiological and anatomical properties, MALT has been thought to be of importance for both vaccine developments against pathogens and for tolerance induction to those

Received 22 July 2003; revised 23 September 2003; accepted 24 October 2003.

Abbreviations: GALT, gut-associated lymphoid tissue; ILN, inguinal lymph nodes; MALT, mucosa-associated lymphoid tissue; MLN, mesenteric lymph nodes; PP, Peyer's patch; Tr, regulatory T cells.

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proteins that cause autoimmune or allergic disorders.[13,14] This is especially the case for oral administration of antigen, which is one of the oldest approaches for inducing immune tolerance. Many studies have shown that oral administration of antigen blocked the development of autoimmune or allergic disorders.[15–18] Despite its importance, the mechanism of immunity and tolerance through the MALT is poorly understood. In particular, the role of APCs in establishing tolerance to encountered antigen is not defined although APCs are the platform of such immune regulation.

In this report, it was examined whether the maturation status of APCs would affect the induction of oral tolerance. To this aim, studies were designed to stimulate APCs by CD40 signals and then test whether tolerance to oral antigen would be ablated *in vivo*. The involvement of CTLA-4 was also examined in this model of oral tolerance. The results indicate that tolerance to oral antigen could be established regardless of APC maturation by CD40 ligation.

## MATERIALS AND METHODS

*Mice*

Female BALB/c mice were purchased from Charles River (Biogenomics, Seoul, South Korea) and used at the age of 6–10 weeks. Four to six mice per group were used in all experiments. Breeding pairs of DO11.10 mice were purchased from JAX (the Jackson Laboratory Bar Harbour, ME) and were bred in our animal centre. Offspring were identified as transgenic by polymerase chain reaction of genomic DNA or by clonotypic monoclonal antibody (mAb; KJ) staining of peripheral blood lymphocytes by flow cytometer. Mice were housed at Seoul National University until use and were kept in specific-pathogen-free conditions during the entire period.

*Flow cytometry for costimulatory molecules on dendritic cells (DC) and B cells*

Anti-CD40 mAb (FGK 45.5) was purified from hybridoma culture supernatant (Ultradoma, Biowhittaker, MD) using a protein G column. Mice were treated with 200 μg of anti-CD40 mAb, or with rat immunoglobulin G (IgG) as a control, and secondary lymphoid organs were isolated at various times thereafter. DCs were isolated from the mesenteric lymph nodes (MLN) or spleen using anti-CD11c microbeads and a magnetic antibody cell-sorting (MACS) column (Miltenyi Biotec, Bergisch Gladbach, Germany). To stain B cells, single-cell suspensions of spleen, MLNs, Peyer's patches (PP), or inguinal lymph nodes (ILN) were stained in phosphate-buffered saline (PBS) containing 1% fetal bovine serum, with phycoerythrin-conjugated rat anti-mouse B220 (PharMingen, San Diego, CA). Additional staining with biotin-conjugated rat anti-mouse CD40, CD80, CD86 and I-A/E (PharMingen) and streptavidin-conjugated phycoerythrin (Pierce, Rockford, IL) was performed. Cells were analysed on a PAS III flow cytometer (Partec, Munster, Germany) using FLOW MAX software or a FACSort using CELLQUEST (Becton Dickinson, Mountain View, CA) software.

*Tolerance induction and CD40 mAb treatment*

*Kinetic study of CD40 ligation on oral tolerance.* Groups of mice were fed 20 mg of ovalbumin (OVA; Grade V; Sigma, St Louis, MO) and received intravenous (i.v.) injections of 200 μg anti-CD40 mAb either 24 hr before feeding or at 0, 2, 6, or 24 hr after feeding. Positive control mice were fed PBS and negative control mice were fed 20 mg of OVA and received rat IgG. After 2 weeks, these mice were primed and boosted with 50 μg OVA emulsified in complete or incomplete Freund's adjuvant, respectively, at 2-week intervals. Ten days later, sera and spleens were obtained from mice and tested.

*Dosage study.* Groups of mice were fed 0·2, 2, or 20 mg of OVA or PBS alone and received 200 μg anti-CD40 mAb or rat IgG i.v., simultaneously. These mice were primed, boosted and tested using the same basic protocol described above.

*CTL antigen-4 blocking study.* The anti-CTL antigen-4 (CTLA-4) mAb 4F10 was purified from hybridoma culture supernatant using a protein G column. Groups of mice were fed 20 mg of OVA or PBS and received 200 μg anti-CTLA-4 mAb or hamster IgG at the time of feeding and 1, 2, 3 and 5 days after feeding. One group of mice also received 200 μg anti-CD40 mAb at the time of feeding.

*Antigen uptake study*

For uptake studies, OVA was conjugated to fluorescein isothiocyanate (FITC; Sigma). Mice received 200 μg anti-CD40 mAb or rat IgG at −24 hr or 0 hr and were injected i.v. with 3 mg/mouse of OVA-FITC. Non-fluorescent native OVA was injected into control mice to provide the DC background for FITC labelling.[19] After 1 hr, spleens were removed from the mice and DCs were isolated by using anti-CD11c microbeads and a MACS column. Analysis of DC showing uptake of fluorescent antigens was performed by flow cytometer.

*Enzyme-linked imunosorbent assay (ELISA) for OVA-specific IgG*

OVA-specific IgG in serum was measured as described previously.[20] IgG concentration in tested serum was determined from standard curves constructed using immunoaffinity-purified anti-OVA IgG.

*Proliferation assay*

Single cell suspensions from the spleen were plated at $5 \times 10^5$ spleen cells per well in 96-well, round-bottomed microtitre plates, and cultured for 4 days with 5, 50, or 500 μg/ml OVA or alone in 200 μl of medium. After 96 hr of incubation, including a final 22-hr pulse with [$^3$H]thymidine (1 μCi/well), cells were harvested and label incorporation was measured.

*Cytokine ELISA*

Interleukin-5 (IL-5), IL-4, or interferon-γ (IFN-γ) was quantified from splenocyte culture supernatant. Cells were plated, at $8 \times 10^6$ cells per well, with 1 ml aliquots. Cells were cultured for 72 hr with 40 μg/ml OVA or alone. Culture supernatants were tested for the presence of IL-5, IL-4, or IFN-γ by sandwich ELISA. Purified rat anti-mouse IL-5 mAb (clone TRFK5), biotinylated rat anti mouse IL-5 mAb (clone TRFK4), and recombinant mouse IL-5 (PharMingen) were used for IL-5 sandwich ELISA. For IL-4 sandwich ELISA, purified rat anti-mouse IL-4 mAb (clone 11B11), biotinylated rat anti-mouse IL-4 mAb (clone BVD6-24G2), and recombinant mouse IL-4 (PharMingen) were used and for IFN-γ sandwich ELISA

purified rat anti-mouse IFN-γ mAb (clone R4-6A2), biotinylated rat anti-mouse IFN-γ mAb (clone XMG1.2), and recombinant mouse IFN-γ were used.

*CFSE labelling and adoptive transfer study*

OVA-specific $CD4^+$ T cells were isolated from DO11 mice using a negative selection CD4 T-cell comlumn (R&D System, Minneapolis, MN) following the manufacturer's instructions. Briefly, red-blood-cell-depleted single cell splenocyte was prepared and stained with the antibody mixture and loaded onto the column. Eluted cells were harvested (85% pure) and 5- (and 6-) carboxyfluorescein diacetate, succinimidyl ester (CFSE) labelling was performed following the manufacturer's instructions. Briefly, purified T cells were resuspended in PBS containing 0·1% bovine serum albumin (Sigma) at $10^7$ cells/ml. For fluorescence labelling, 2 μl of a CFSE (Molecular Probes, Eugene, OR) stock solution (5 mM in dimethyl sulphoxide) was incubated with $10^7$ cells for 10 min at 37°.

CFSE-labelled DO11 T cells were transferred i.v. into naive BALB/c mice ($1 \times 10^7$/mouse). The next day, recipient mice were fed 20 mg OVA or PBS with anti-CD40 mAb or rat IgG. Three days later, the secondary lymphoid organs were removed and analysed by flow cytometer for their division.[21]

*Statistics*

Results are expressed as the means ± SE. Statistical analyses were performed upon comparisons made between the treated groups and the positive controls using Student's *t*-test. Each experiment was repeated at least twice.

## RESULTS

### Signal through CD40 agonistic mAb induces up-regulation of costimulatory molecules on APCs

Initial studies sought to examine the effects of CD40 ligation on APC populations. Triggering with anti-CD40 mAb caused the up-regulation of CD40, B7-1, B7-2 and MHC class II on DCs in the MLN, (Fig. 1a) as well as the spleen within 24 hr. MHC class II was up-regulated as early as 12 hr in anti-CD40-treated mice. B cells also up-regulated such molecules in secondary lymphoid organs including ILN and PP (data not shown). Up-regulation of such molecules on DCs and B cells persisted for at least 72 hr after mAb treatment. The ability of anti-CD40 mAb to induce the activation of APCs described above was comparable over a range of 100–500 μg mAb per mouse and 200 μg was used during the following experiments.

Overall, these data indicated that agonistic CD40 mAb could efficiently induce costimulatory molecules on APCs.

### CD40 ligation enhanced the proliferation of OVA-specific $CD4^+$ T cells in response to oral OVA *in vivo*

It has been reported that antigen-specific T cells proliferate primarily in PPs and MLNs in response to oral antigen.[22,23] It seems reasonable to assume that providing costimulatory molecules on APCs would enhance such activation and/or proliferation of antigen-specific T cells. Thus, it was next asked whether the ligation of CD40 could affect the proliferation of T cells *in vivo*. CFSE-labelled DO11 T cells were transferred into naive mice. The next day, recipient mice were fed OVA and received an i.v. injection of anti-CD40 mAb simultaneously. Three days later, secondary lymphoid organs were removed and analysed by flow cytometer.

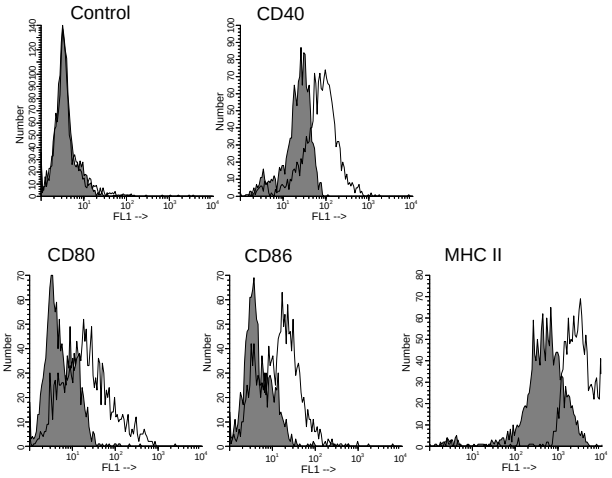


**Figure 1.** Anti-CD40 mAb induced costimulatory molecules. Mice received 200 μg of anti-CD40 mAb (open areas) or rat IgG (filled areas). Twenty-four hours later, mesenteric lymph nodes were removed and $CD11^+$ DCs were isolated and analysed for the expression of CD40, CD80, CD86 and MHCII(I-A/E) by flow cytometer.

As previously reported by others, OVA-specific $CD4^+$ T cells proliferated primarily in the PPs and MLNs after oral administration of OVA. In mice that received OVA plus anti-CD40 mAb, the proliferation of DO11 T cells was enhanced compared with that of OVA plus rat IgG-treated mice in most lymphoid tissues (Fig. 2). In PPs, a higher proportion of DO11 T cells proliferated in anti-CD40 mAb-treated mice. In MLNs, the proportion of DO11 T cells dividing was similar for both groups, though there seemed to be a slight enhancement in the rate of division in anti-CD40 mAb-treated mice. In summary, CD40 ligation weakly enhanced the division of DO11 T cells in the secondary lymphoid organs in response to oral OVA.

Next we examined whether CD40 ligation affected the persistence of antigen presentation. Mice were fed OVA and received antibody simultaneously. Then CFSE-labelled DO11 T cells were transferred at 1, 2, or 3 days thereafter, respectively. Three days after transfer, MLNs were removed. Figure 3 reveals strong proliferation of DO11 T cells 1 day after antigen administration, a weak response after 2 days and nothing after 3 days in both rat IgG-treated and anti-CD40 mAb-treated groups. As expected, CD40 ligation enhanced the proliferation of DO11 T cells (Fig. 3, day 1 and day 2), but it did not prolong the presentation of oral OVA in MLNs. Thus, oral OVA persisted for approximately 2 days in MLN and anti-CD40 mAb treatment did not affect this.

### Administration of CD40 mAb before, but not after, OVA feeding blocked the induction of oral tolerance

Since mAb against CD40 efficiently induced costimulatory molecules on APCs, it was investigated next whether such

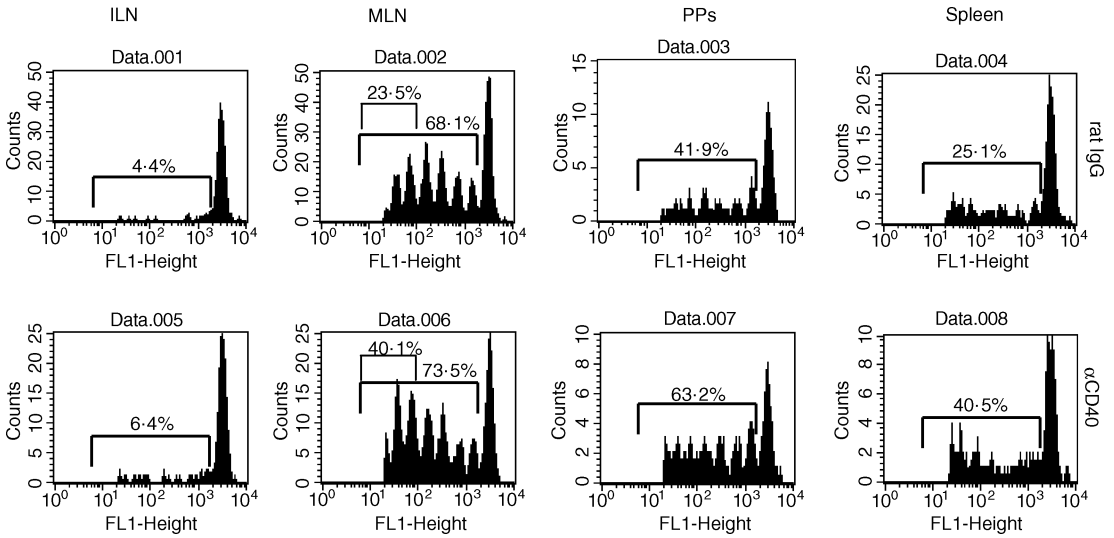


**Figure 2.** Effect of anti-CD40 mAb on OVA-specific CD4 T-cell division in response to oral OVA. CFSE-labelled DO11 T cells ($1 \times 10^7$) were transferred into syngenic BALB/c mice. Next day, the recipient mice were given 20 mg of oral OVA and received i.v. injections of anti-CD40 mAb or rat IgG simultaneously. Three days later, the indicated lymphoid organs were removed and analysed by flow cytometer.

maturation of APCs can ablate the induction of peripheral tolerance by oral antigen. Although proliferation of OVA-specific T cells was not greatly enhanced, it was still possible that APCs stimulated by CD40 ligation could reverse tolerance induction and lead to immunity against oral OVA.

Groups of mice were fed 20 mg of OVA and received mAb at 24 hr before or at 0, 2, 6 or 24 hr after feeding and then were primed and boosted at 2-week intervals with OVA in complete and incomplete Freund's adjuvant, respectively. As expected,

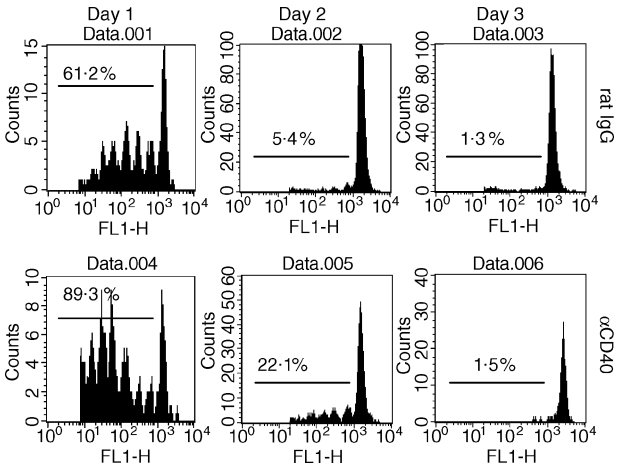


**Figure 3.** Effect of anti-CD40 mAb on the persistence of oral OVA in APCs in mesenteric lymph nodes. Mice were given 20 mg of oral OVA and received the indicated antibody simultaneously. After 1, 2, or 3 days, respectively, $1 \times 10^7$ CFSE-labelled DO11 T cells were transferred into the OVA-fed mice. Three days later, mesenteric lymph nodes were removed and analysed by flow cytometer.

OVA-specific tolerance was induced in both the humoral response and splenocyte proliferation in OVA-fed rat IgG-treated mice (indicates–control in Fig. 4a,b). Treatment with anti-CD40 mAb 24 hr before oral OVA caused remarkable blocking of OVA-specific tolerance induction. However, the level of OVA-specific proliferation was still less than that in the PBS-fed group when the concentration of OVA was high ($P < 0{\cdot}05$) and the level of OVA-specific antibody in the primary response revealed that the immune response against OVA was not primed by this regimen (data not shown). Surprisingly, anti-CD40 mAb at the time of OVA feeding could not abrogate tolerance induction by oral OVA. As shown in Fig. 4, the levels of OVA-specific IgG and OVA-specific proliferation of splenocytes were similar to those of OVA-fed rat IgG-treated mice. CD40 ligation after oral administration of OVA also failed to reverse the induction of oral tolerance. The observed suppression in OVA-fed mice was OVA-specific because immune response to an irrelevant antigen was not affected in OVA-fed mice (data not shown).

Ligation of CD40 before antigen administration blocked the induction of tolerance by oral antigen. One possible explanation for this could be that the stimulation of APCs via CD40 signals hampers the uptake of antigen. To test this possibility, mice were injected with anti-CD40 mAb or rat IgG as a control. Twenty-four hours later, these mice were injected with OVA-FITC or OVA alone. DCs were isolated from the spleen and the uptake of OVA-FITC was determined by flow cytometer. Indeed, uptake of OVA-FITC was greatly reduced in DCs isolated from anti-CD40 mAb-pretreated mice compared with rat IgG-treated mice (Fig. 5a). Consistent with this result, proliferation of DO11 T cells in response to oral OVA was reduced when cells from mesenteric lymph nodes of anti-CD40 mAb pretreated mice were used as stimulator (Fig. 5b).

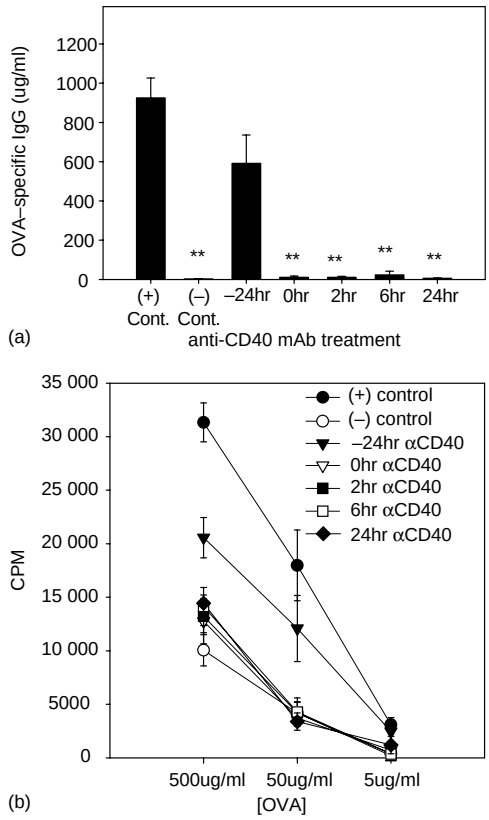


**Figure 4.** Anti-CD40 mAb treatment at the inductive phase of oral tolerance. Groups of BALB/c mice were fed 20 mg of OVA and received mAb 24 hr before or 0, 2, 6, or 24 hr after feeding. After 2 weeks, these mice were primed and boosted at 2-week intervals. Ten days later, sera and splenocytes were obtained. (a) Concentration of OVA-specific IgG in serum was assessed by ELISA. (b) Proliferation of splenocytes were measured using the standard [$^3$H]thymidine incorporation method. (+) control indicates the PBS-fed and rat IgG-injected group, (−) control indicates the OVA-fed and rat IgG-injected group. $^{**}P < 0{\cdot}01$ in comparison with positive control.

Collectively, stimulation of APCs by CD40 ligation at the time of oral administration of antigen did not reverse the induction of tolerance to that antigen.

### CD40 triggering failed to prime immune response to oral OVA

Since CD40 ligation enhanced the response of DO11 T cells to oral OVA, we next examined whether ligation of CD40 primes the immune response to oral OVA. Mice received anti-CD40 mAb at the time of oral administration. The proliferation of splenocytes in the presence of OVA was examined without further immunization. As shown in Table 1, CD40 ligation did not prime the immune response to oral OVA. Since OVA-specific CD4 T cells initially proliferated (Fig. 2) and they did not prime immunity to oral OVA (Table 1), it was concluded that OVA-specific T cells became anergic after early activation by oral OVA and that CD40 triggering did not reverse this anergy.

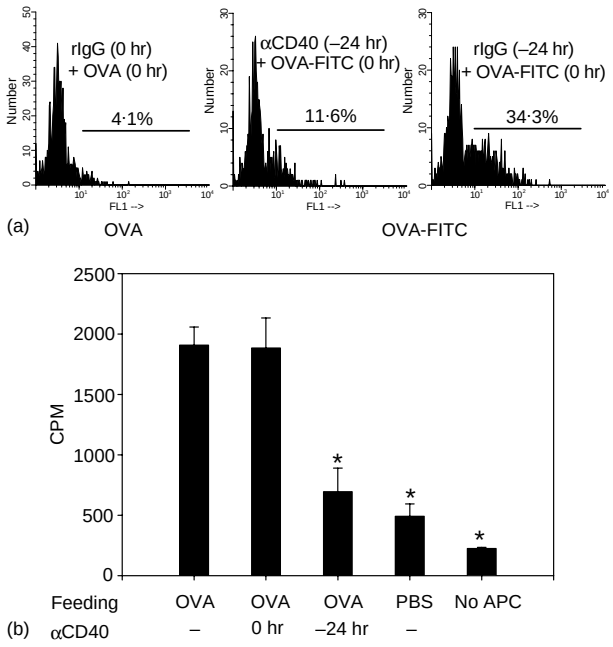


**Figure 5.** Preactivation of APCs with anti-CD40 mAb reduces the uptake of antigen by DCs. (a) Mice received 200 μg anti-CD40 mAb or rat IgG at −24 hr or 0 hr and were injected i.v. with 3 mg/mouse of OVA-FITC. Non-fluorescent native OVA was injected into control mice to provide the DC background for FITC labelling (left). After 1 hr spleens were removed, DCs were isolated and uptake of fluorescent OVA was performed by flow cytometer. (b) Mice received 200 μg anti-CD40 mAb rat IgG at −24 hr or 0 hr and were fed 20 mg OVA at 0 hr. Eight hours later, total lymph node cells were harvested and treated with mitomycin C. Then these cells ($1 \times 10^6$ cells/well) were cultured in the presence of purified DO11 T cells ($5 \times 10^4$ cells/well). After 96 hr, including 18-hr [$^3$H]thymidine incorporation, cells were harvested and tested. $^{*}P < 0{\cdot}01$ versus OVA-fed anti-CD40 mAb non-treated group.

**Table 1.** Anti-CD40 mAb does not prime the immune response to oral OVA*

| | Proliferation (counts/minute) | |
|---|---|---|
| Antibody | PBS | OVA |
| Rat IgG | 5871 ± 1081 | 5122 ± 1078 |
| Anti-CD40 | 3919 ± 916 | 3787 ± 667 |

*Mice were fed 20 mg OVA or PBS and received the indicated antibody simultaneously. Two weeks later, splenocytes were prepared and cultured in the presence of 50 μg/ml of OVA for 96 hr, including 18-hr [$^3$H]thymidine incorporation.

### Agonistic CD40 mAb did not reverse the induction of oral tolerance regardless of antigen dose

Antigen dose has been reported to influence the mechanism of oral tolerance, with high doses causing clonal deletion and anergy and low doses inducing active suppression.[15] Thus our next study was designed to examine the effect of CD40 ligation on oral tolerance induced by different doses of OVA. Again,

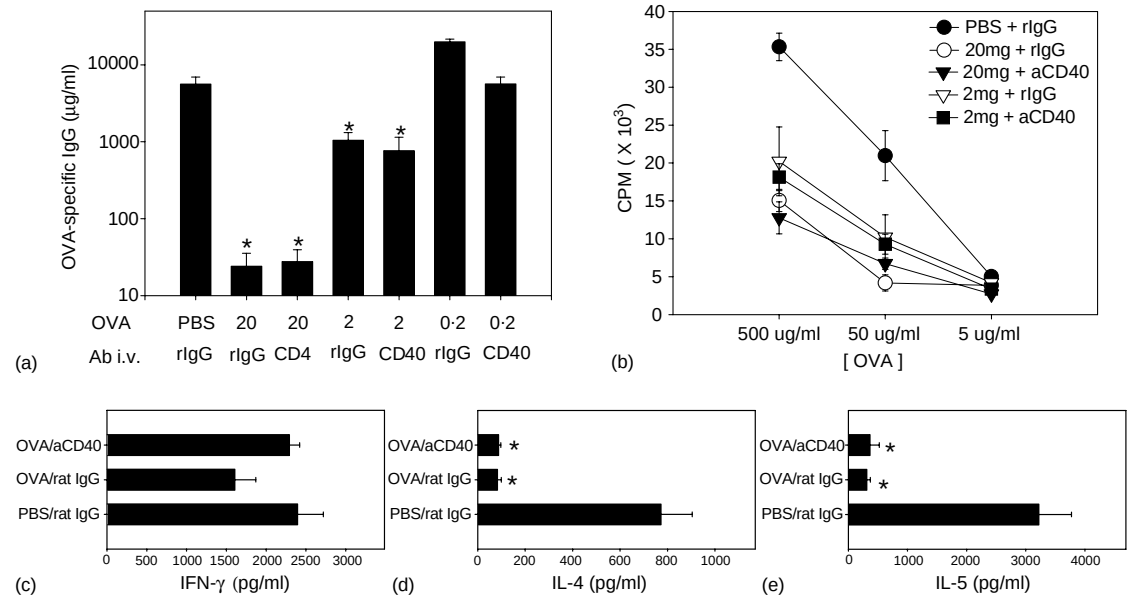


**Figure 6.** Effect of anti-CD40 mAb treatment on induction of oral tolerance with various doses of OVA. Groups of mice were fed 20, 2, or 0·2 mg OVA and received antibody simultaneously. After 2 weeks, these mice were primed and boosted at 2-week intervals. Ten days later, sera and splenocytes were obtained. (a) Concentration of OVA-specific IgG in serum was assessed by ELISA. (b) Proliferation of splenocytes was measured using standard [$^3$H]thymidine incorporation method. (c)–(e) Spleen cells were cultured for 72 hr with 40 μg/ml OVA or alone. Culture supernatants were tested for the presence of IFN-γ (c), IL-4 (d), or IL-5 (e) by sandwich ELISA. $*P < 0{\cdot}01$ versus PBS-fed rat IgG-treated group.

20 mg OVA induced hyporesponsiveness both in humoral and cellular immune responses (Fig. 6) while 2 mg oral OVA led to moderate suppression (Fig. 6a,b). However, activation of APCs by CD40 ligation did not block the induction of tolerance by 2 mg of oral OVA. Data from the mice given both oral PBS and anti-CD40 mAb were similar to those from PBS-fed rat IgG-treated mice, indicating that there was no lingering effect of the anti-CD40 mAb on the following immunization (data not shown). The culture supernatant of splenocytes was tested for cytokines such as IFN-γ, IL-4 and IL-5 by ELISA. Production of IL-4 and IL-5 was also suppressed in mice that received 20 mg (Fig. 6d,e) or 2 mg (data not shown) of oral OVA regardless of treatment with anti-CD40 mAb. Suppression of T helper type 2 cytokines coincided with the suppression of humoral and cellular proliferation. Production of IFN-γ showed a marginal suppression in OVA-fed mice and this was reversed to positive control levels by anti-CD40 mAb (Fig. 6c).

Taken together, these data indicate that CD40 ligation does not block the induction of hyporesponsiveness by either high- or low-dose feeding regimens.

### Induction of oral tolerance in anti-CD40 mAb treated mice is not the result of negative signalling by CTLA-4

As CD28 and CTLA-4 deliver opposing signals, B-7s up-regulated by anti-CD40 mAb may deliver both positive and negative signals to the T cells. It was therefore investigated whether blockade of CTLA-4 signal would leave the up-regulated B-7s on APCs free to interact with CD28 and result in productive immunity in response to oral OVA. Mice were injected with anti-CTLA-4 mAb at the time of oral OVA administration and 1, 2, 3, 5 days after oral OVA administration. As shown in Fig. 7, coadministration of anti-CTLA-4 mAb with oral OVA resulted in a profound suppression of the OVA-specific humoral response, although there was a weak reversal of tolerance suggesting that CTLA-4 did not play a major role in our model of oral tolerance. In mice that received both anti-CD40 mAb and anti-CTLA-4 mAb at the time of oral OVA administration, production of OVA-specific IgG was also significantly suppressed (Fig. 7). These results demonstrated that oral tolerance in anti-CD40 mAb-treated mice was not the result of the ligation of CTLA-4 and B-7s being up-regulated by anti-CD40 mAb.

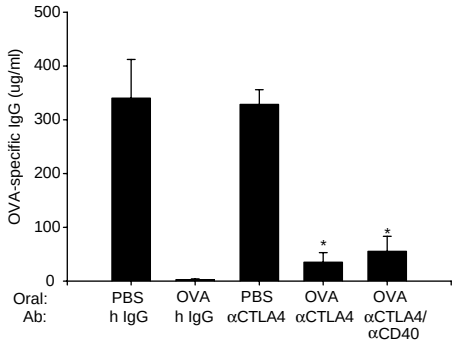


**Figure 7.** CD40 ligation plus CTLA-4 blocking did not break oral tolerance. Mice were fed 20 mg OVA or PBS and received 200 μg anti-CTLA-4 mAb or hamster IgG at 0, 1, 2, 3 and 5 days after feeding. One group of mice additionally received 200 μg anti-CD40 mAb at the time of feeding. $*P < 0{\cdot}01$ versus the PBS-fed hamster IgG-treated group.

## DISCUSSION

APCs are the platform of immune response. Especially, DCs are sentinels of the immune system. They sense 'danger signals' such as invasive pathogens and tissue damage and initiate immunity to remove such danger.[24,25] It seems evident that DCs also regulate tolerance as well as immunity, although it is controversial whether there is a specialized DC for tolerance.[26–28] It is well documented that antigen-specific T cells are anergized or deleted by oral antigen.[15] However, although uptake, processing and presentation of antigen by APCs and their interaction with specific T helper cells are the initial event of tolerance induction, they are none of them well defined. Dissecting the role of APCs in the induction and maintenance of tolerance will provide insights into the mechanism and provide considerations for clinical trial.

It is well documented that T-cell stimulation in the absence of a second signal can lead to tolerance and that peripheral tolerance to an exogenous antigen might be caused by the lack of costimulatory molecules on APCs.[3–5,12] CD40 ligation is generally used to stimulate APCs to prime immunity.[6–9] Indeed, stimulation of APCs with CD40 agonistic mAb enhanced the division of OVA-specific T cells against oral OVA in secondary lymphoid organs in the present study. However, in several oral tolerance models, we found that the stimulation of APCs with CD40 agonistic antibody at the time of oral administration of OVA reversed the induction of tolerance to OVA in neither the high-dose nor the low-dose feeding regimen.

The DO11 T-cell adoptive transfer study showed that orally administered antigen was presented on APCs in mesenteric lymph nodes for 2 days regardless of the activation state of the APCs. To our knowledge, this is the first study to show the duration of antigen presentation after oral administration of antigen. Since up-regulated B7-1 and B7-2 molecules induced by CD40 ligation could bind to CTLA-4 on T cells and thus override the positive signal delivered through CD28, we examined CTLA-4 involvement using anti-CTLA-4 blocking mAb. Our result demonstrated that oral tolerance could be induced in CD40-treated mice in the absence of a CTLA-4 signal. This observation is not consistent with a previous report, which described that CTLA-4 was required for the induction of high dose of oral tolerance.[29] Such discordance is probably the result of the difference in the frequency of antigen feeding.

In most models of peripheral tolerance, immunity to antigen has been shown to occur by CD40 ligation, which coincides with the 'two-signal theory'.[10–12] In this study, however, it was demonstrated that tolerance was efficiently established in mice upon the oral administration of OVA regardless of APCs activation with anti-CD40 mAb (providing costimulatory molecules on APCs). There are several possible reasons for the discordance with the 'two-signal theory' in our oral tolerance model. First, there might be a unique subset of APC that specialized for inducing and maintaining tolerance.[26,27] In this case, this subset of APC (tolerogenic DCs) may overcome other CD40-activating APC (immunogenic DCs) and lead to tolerance to oral antigen. Recently, Wakkach *et al*. reported that a subpopulation of DCs specifically induce tolerance *in vivo* through the differentiation of Tr1 cells.[30] Furthermore, a recent study reported that there are several inhibitory receptors on DCs rendering these cells tolerogenic to CD4 T cells.[31] Characterization of the DC subtype responsible for tolerance in mucosal tissue would be interesting. Second, T cells may use an active mechanism for tolerance induction to harmless antigens rather than the passive mechanism provided by APCs in some unique environments, such as mucosal tissues. Accumulating reports have described cytokines such as transforming growth factor-β and IL-10 from T cells as critical in peripheral tolerance.[32,33] In addition, T cells also express negative regulators such as CTLA-4 and PD-1 in tolerogenic circumstances.[34–36] Such negative regulatory molecules might have a role in inducing oral tolerance. In our study, however, CTLA-4 blockade did not affect the induction of oral tolerance. Third, maturation of APCs by the CD40 signal may not be sufficient to stimulate antigen-specific CD4 T cells to differentiate into effector cells in our model of oral tolerance. Several recent studies demonstrated that mature DCs induce CD4 T-cell tolerance (reviewed in ref. 37). Menges *et al*. described how injection of mature DCs induces antigen-specific protection from autoimmunity in mice.[38] Akbari *et al*. showed that DCs responsible for the induction of intranasal tolerance are phenotypically mature.[39] Furthermore, a recent study demonstrated that maturation of DCs is required for cross-tolerance.[40] Thus it is possible to assume that the DCs of anti-CD40 mAb-treated mice were mature phenotypically but were not activated functionally. Additional signal might be required to overcome tolerance, such as inflammatory cytokines as a third signal.[41,42]

Our results are in agreement with the recent report of Sun and Houten[43] who showed that a single high dose of oral antigen induced T-cell tolerance regardless of CD40 ligation using an adoptive transfer study. In that study, mice were given anti-CD40 mAb twice at the time of adoptive transfer (day −1) and at the time of oral administration of antigen (day 0). Based on our results, treatment of anti-CD40 mAb prior to oral antigen could affect the uptake of the oral antigen by DC. Our study is novel in that we dissected the effects of CD40 ligation in terms of 'antigen-uptake' and 'antigen-presentation' and ruled out the possible involvement of CTLA-4 *in vivo*. Furthermore, our study demonstrates that coadministratioin of CD40 agonistic antibody and antigen fails to reverse the induction of oral tolerance regardless of 'dose'. Our findings suggest that there could be another mechanism in the immune system to regulate immunity versus tolerance to encountered antigen especially in gut-associated lymphoid tissue.

## ACKNOWLEDGMENTS

We thank Dr William R. Heath (WEHI, Australia) for kindly providing the anti-CD40-mAb-secreting hybridoma (FGK45.5) and for discussion and review of this manuscript. This work was supported by grants, HMP-00-CH-06-0006, from the Ministry of Health & Welfare of Korea.

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