background, respectively) (Fig. 1, *d* and *e*). Production of IgM and IgG2b were additionally decreased in BALB/c RP105$^{-/-}$ mice. These findings suggest that RP105 is important in regulating serum levels of innate antibodies, and especially those of the IgG3 class.

B cells lacking RP105 are impaired in proliferative responses to TLR2 but not TLR9 ligand

We previously demonstrated that RP105$^{-/-}$ B cells are hyporesponsive to LPS (15). Given that RP105$^{-/-}$ mice were more impaired in serum IgG3 production than TLR4$^{-/-}$ mice (Fig. 1), we asked a possibility that RP105/MD-1 has a role in responses to TLR ligands other than LPS. We stimulated RP105$^{-/-}$ B cells with Pam_3CSK_4 (TLR2/TLR1 ligand), MALP-2 (TLR2/TLR6 ligand), or the TLR9 ligand CpG. B cell proliferation in response to lipid A stimulation was impaired in RP105$^{-/-}$ B cells by at least an order of magnitude (Fig. 2*A*). We now show that RP105$^{-/-}$ B cells have defective proliferative responses to TLR2 ligands, either Pam_3CSK_4 or MALP-2 (Fig. 2*B*). Proliferation induced by these ligands could not be due to LPS contamination, because TLR2 ligands did not stimulate TLR2$^{-/-}$ B cells (Fig. 2*B*), which can respond to LPS (21). Hyporesponsiveness was more apparent in response to Pam_3CSK_4 than to MALP-2. As is the case with lipid A, the impairment of B cell proliferation in RP105$^{-/-}$ B cells seemed to be dose-dependent. The impairment was more apparent at lower concentrations of TLR2 ligands but not significant at higher concentration of Pam_3CSK_4 (Fig. 2, *B* and *C*). Interestingly, RP105$^{-/-}$ B cells responded normally to the TLR9 ligand CpG (Fig. 2*D*). CpG used in the present study did not stimulate B cells lacking TLR9, excluding contamination of LPS or other TLR ligands (data not shown). TLR9 is distinct from TLR2 or TLR4/MD-2 and similar to TLR7 in its ligand recognition in endosomal/lysosomal compartments (17, 22). Therefore, we stimulated RP105$^{-/-}$ B cells with the TLR7 ligand loxoribine (17). RP105$^{-/-}$ B cells proliferated normally (data not shown). RP105/MD-1 regulates B cell responses to LPS or TLR2 ligands but not those involving TLR7 or TLR9 ligands.

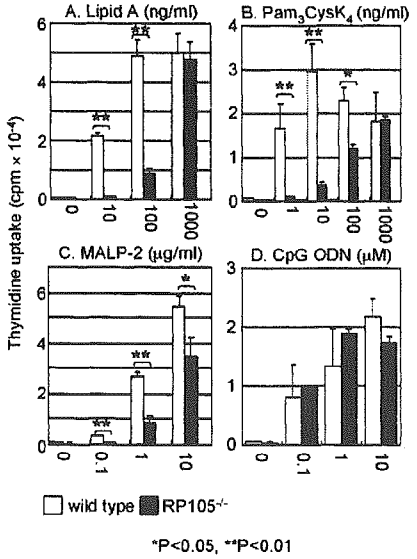

FIGURE 2. Responses to TLR2 ligands but not to the TLR9 ligand CpG are impaired in RP105$^{-/-}$ B cells. Enriched splenic B cells from wild-type, RP105$^{-/-}$, TLR4$^{-/-}$, and TLR2$^{-/-}$ mice were stimulated with lipid A (*A*), Pam_3CSK_4 (*B*), MALP-2 (*C*), and CpG (*D*) at concentrations indicated in the figure. After 3 days culture, B cell proliferation was determined by thymidine uptake. Results represent mean values with SEs from triplicate cultures. Experiments were conducted five times with similar results.

RP105$^{-/-}$ mice are impaired with respect to LPS-induced polyclonal Ab production

Serum Ig production is thought to be driven by TLR ligands from microbial flora. To study a role for RP105 in Ab production in vivo, we next investigated whether RP105 is important for polyclonal Ab production in vivo induced by i.p. injection of LPS or TLR2 ligands. Mice at 4–5 wk of age were injected weekly for 4 weeks with LPS (50 μg/mouse) or Pam_3CSK_4 (50 μg/mouse), and serum Ig levels were determined by ELISA. Significant difference in serum titers between wild-type and RP105$^{-/-}$ mice injected with Pam_3CSK_4 or MALP-2 was seen only in IgG3 (Fig. 3, *right panel*, and data not shown). Because such a difference in IgG3 was also seen without any stimulation at 11–12 wk of age (Fig. 1), it was difficult to exclude a possibility that the difference in IgG3 was due to age-dependent increase in IgG3. In contrast, LPS induced significant differences between wild-type and RP105$^{-/-}$ mice not only in IgG3 but also in IgM and IgG2b despite C57BL/6 background (Fig. 3, *left panel*), which appeared to be distinct from serum Ig titers at 11–12 wk of age (Fig. 1) or from the results with Pam_3CSK_4 stimulation (Fig. 3, *right panel*). RP105 is important for mediating polyclonal Ab production in vivo induced by LPS.

RP105$^{-/-}$ mice are impaired in production of Abs specific to LPS, TLR2 ligands, and bacteria

We then studied specific TI responses to the hapten, FITC conjugated to defined TLR2 ligands. These were injected into mice i.p.

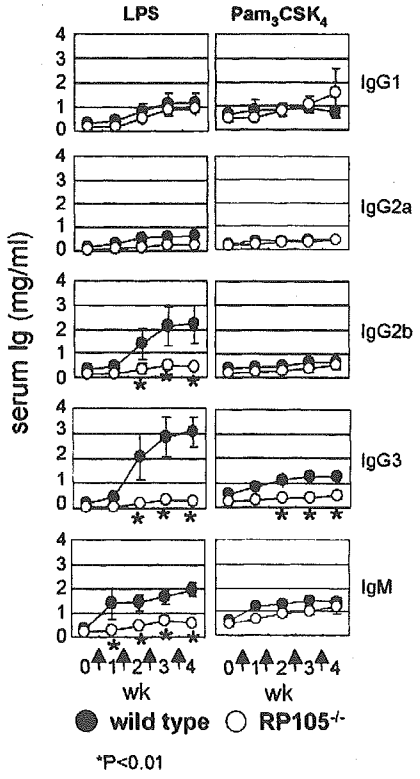

FIGURE 3. RP105$^{-/-}$ mice were impaired in polyclonal Ab production stimulated with LPS or TLR2 ligands. RP105$^{-/-}$ mice and age-matched C57BL/6 mice (4–5 wk of age) were i.p. injected weekly with LPS or Pam_3CSK_4 (50 μg/mouse) four times. The arrows show the times when TLR ligands were injected. Blood was collected 3 days after each injection, and serum Ig concentrations were determined by ELISA. Each group contained 4–5 mice. The results were represented by mean values with SD. Significant difference between wild-type and RP105$^{-/-}$ mice were shown by asterisks (*, $p < 0.01$ by Student's *t* test). These experiments were repeated twice.

and FITC-specific Ab production was determined by ELISA (Fig. 4). Mice were also immunized with TNP-LPS, which induces TNP-specific Ab production in wild-type but not RP105$^{-/-}$ mice (15). The TLR2 ligands, FITC-Pam_3CSK_4 and FITC-MALP-2, induced FITC-specific IgM, IgG3, and IgG2b in normal animals, but these responses were all severely impaired in RP105$^{-/-}$ mice (Fig. 4). Similar results were obtained with TNP-Pam_3CSK_4 (data not shown). Because the immunostimulatory activity of microbial membranes is mostly explained by LPS and TLR2 ligands, we also assessed the importance of RP105 for primary Ab production against bacteria. Mice were immunized i.p. with heat-killed *Escherichia coli* and titers of Ab to *E. coli* were determined by ELISA. IgM, IgG3, and IgG2b Abs to *E. coli* were produced in wild-type but not in RP105$^{-/-}$ mice (Fig. 4). Thus, RP105/MD-1 is very important for Ab production against microbial membranes.

RP105 is expressed on B1, MZ B, and follicular B, but not germinal center (GC) B cells

Although the above results indicate that RP105 regulates TI type 1 responses, we previously showed that RP105$^{-/-}$ mice were not impaired with respect to thymus-dependent (TD) responses (15). Therefore, we wondered whether this molecule might be differentially expressed by lymphocyte subsets. All B cell subsets expressed similar amounts of TLR2. However, MZ B cells had 3-fold higher mean fluorescence intensities of RP105 than B1 or follicular B cells (Fig. 5, *a* and *b*). TLR4/MD-2 was barely detectable. We previously found that GC B cells in human tonsils had less cell surface RP105 than follicular B cells (23). We now show that the same is true for mice. RP105 was almost absent in PNA-positive GC B cells in the spleens of mice immunized with SRBC (Fig. 5*c*). Whereas GC B cells are committed to T-dependent Ab responses, MZ B cells contribute to TI Ab production against microbial membranes (24), in which IgG3 is a principal Ig isotype. This is consistent with a unique role for RP105 in TI responses.

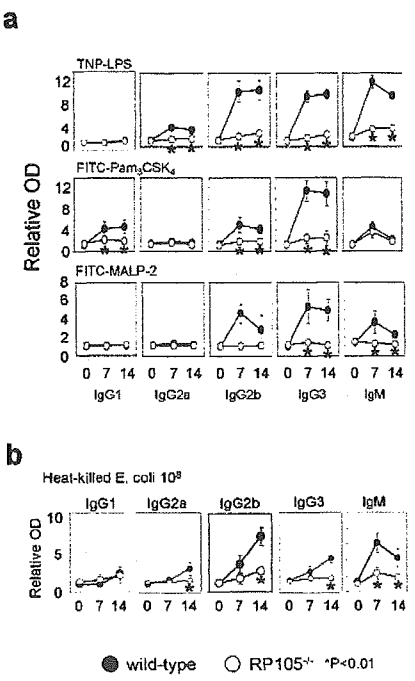

FIGURE 4. RP105$^{-/-}$ mice are impaired in Ag-specific Ab production to LPS, TLR2 ligands, and bacteria. Wild-type mice (●) or RP105$^{-/-}$ mice (○) were injected i.p. with TNP-LPS (50 μg/mouse), FITC-Pam_3CSK_4 (50 μg/mouse), FITC-MALP-2 (50 μg/mouse) in *a*, or heat-killed *E. coli* (10^8/mouse) in *b*. Sera were collected at indicated times (0, 7, and 14 days), and hapten-specific or *E. coli*-specific Ab production was determined by Ag-specific ELISA (see *Materials and Methods*). Relative ODs were calculated by dividing OD values by the OD from a well with PBS. Each group contained four to five mice. The results were represented by mean values with SD. Significant difference between wild-type and RP105$^{-/-}$ mice was shown by asterisks (*, $p < 0.01$ by Student's t test). These experiments were repeated twice.

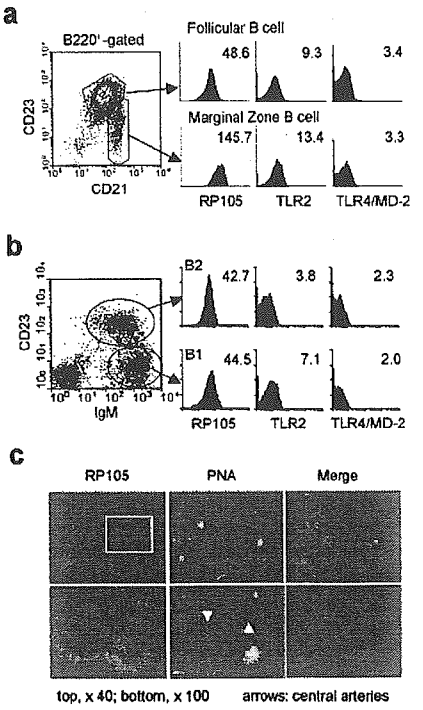

FIGURE 5. RP105/MD-1 expression on MZ B, follicular, B1, and GC B cells. *a*, Spleen cells were stained with Abs to B220, CD23, and CD21 together with RP105, TLR2, or TLR4/MD-2. Cell surface expression of RP105, TLR2, or TLR4/MD-2 on follicular ($B220^+$ $CD23^{high}$ $CD21^{low}$) B cells and MZ ($B220^+$ $CD23^{low}$ $CD21^{high}$) B cells is shown in the histograms with mean fluorescence intensities. Experiments were conducted three times with similar results. *b*, Peritoneal cells were collected and stained with Abs to CD23 and IgM together with RP105, TLR2, or TLR4/MD-2. Expression of RP105, TLR2, or TLR4/MD-2 was shown on B-1 ($CD23^-sIgM^{high}$) or B-2 ($CD23^+sIgM^{med}$) cells. Experiments were conducted three times with similar results. *c*, Mice were immunized with SRBC, and spleens were stained with anti-RP105 (left) or PNA (middle). Merged images are also shown (right). *Top panels* are lower magnification views (×40). *Bottom panels* are higher magnification (×100) views of the area indicated in the *top left panel*. Arrows indicate central arteries.

RP105$^{-/-}$ mice are not impaired in expression of TLR4, MD-2, and TLR2, or in macrophages responses to lipid A or TLR2 ligands

To address the mechanism by which B cell response to TLR4 and TLR2 ligands were impaired in RP105$^{-/-}$ mice, we first studied expression of TLR2, TLR4, and MD-2 in RP105$^{-/-}$ B cells. Although an Ab to the TLR4/MD-2 complex did not give significant staining on normal splenic B cells (Fig. 5*a*), expression of mRNAs for TLR4 and MD-2 were confirmed in RP105$^{-/-}$ B cells by RT-PCR (Fig. 6*a*). TLR2 densities were similar on B cells from wild-type and RP105$^{-/-}$ mice (Fig. 6*b*). RP105 is expressed not only on B cells but also on bone marrow-derived macrophages and dendritic cells (Fig. 6*c* and data not shown). We could not see any difference between wild-type and RP105$^{-/-}$ macrophages in cell surface TLR4/MD-2 and TLR2 as judged by flow cytometry (Fig. 6*c*).

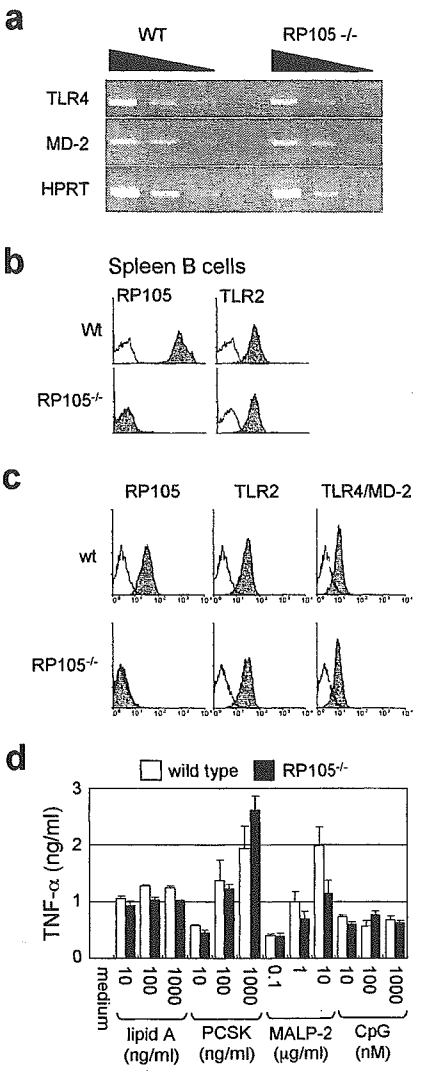

FIGURE 6. Expression of TLR2, TLR4, and MD-2 in RP105$^{-/-}$ B cells or macrophages and TNF-α production in RP105$^{-/-}$ macrophages. *a*, Total RNA was extracted from enriched splenic B cells from wild-type or RP105$^{-/-}$ mice. RT-PCR was conducted to detect mRNA encoding TLR4, MD-2, and HPRT. *b*, Enriched splenic B cells from wild-type or RP105$^{-/-}$ mice were stained with biotinylated RP105 or TLR2 mAb, followed by streptavidin-PE. Open histograms depict those stained with the second reagent alone. *c*, Bone marrow-derived macrophages were prepared as in *Materials and Methods*, and stained with biotinylated mAbs to RP105, TLR2, or TLR4/MD-2, followed by streptavidin-PE. Open histograms depict staining with the second reagent alone. *d*, Bone marrow macrophages were plated in 96-well plates (1×10^5/well) and stimulated with indicated TLR ligands for 24 h. Concentration of TNF-α in culture supernatants was determined by ELISA. The results were represented by the mean values with SD from triplicate wells. All the experiments were conducted three times with similar results.

RP105$^{-/-}$ bone marrow-derived macrophages showed sharp contrast to B cells in that they were not impaired in TNF-α production induced with lipid A or TLR2 ligands (Fig. 6*d*). RP105$^{-/-}$ bone marrow-derived dendritic cells were similar to macrophages in that they were not impaired in IL-12 production or up-regulation of costimulatory molecules in response to lipid A or TLR2 ligands (data not shown). Coexpression of RP105/MD-1 did not necessarily influence the expression of TLR4/MD-2 and TLR2.

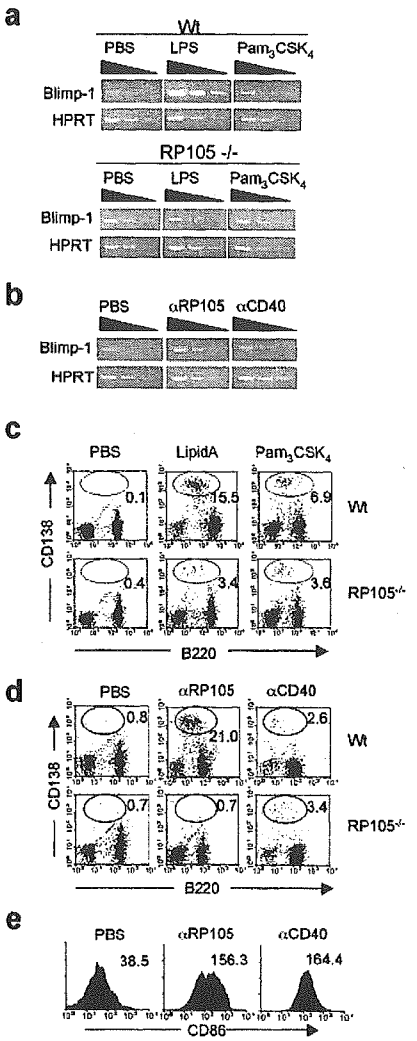

FIGURE 7. RP105/MD-1 mediated signals drive B cells to differentiate into Ab-secreting cells. *a* and *b*, Wild-type or RP105$^{-/-}$ mice were injected i.p. with PBS, lipid A (100 μg), Pam_3CSK_4 (100 μg), anti-RP105 mAb (500 μg), or anti-CD40 mAb (500 μg). Mice were sacrificed 4 days later, and RNA was extracted from spleen. RT-PCR was conducted to detect mRNA encoding Blimp-1 or HPRT. *c* and *d*, Wild-type or RP105$^{-/-}$ mice were injected i.p. with PBS, lipid A (100 μg), Pam_3CSK_4 (100 μg), anti-RP105 (500 μg), or anti-CD40 (500 μg) mAb. Mice were sacrificed 4 days later, and cell surface expression of CD138 and B220 on spleen cells was analyzed by flow cytometry. The percentages of B220$^+$ CD138$^+$ plasmacytic cells are also shown. *e*, Cell surface expression of CD86 on spleen cells obtained in *b* is shown. The mean fluorescence intensities are also indicated. All the experiments were conducted three times with similar results.

Signals transmitted via RP105/MD-1 induce plasma cell differentiation

CD19 plays an important role in regulating signal transduction through RP105 (25). In keeping with this, mAb-mediated RP105 ligation induced potent activation in B cells (12) but not in macrophages and dendritic cells, as judged by TNF-α production in macrophages, IL-12 production in dendritic cells, and up-regulation of costimulatory molecules on dendritic cells (Fig. 6*d* and data not shown). B cell-specific defect in TLR responses of RP105$^{-/-}$ mice seemed to correlate with cell activation induced by RP105 ligation. RP105-dependent B cell activation is likely to have a role in Ab production in response to LPS or TLR2 ligands. To address

this possibility, we studied plasma cell differentiation in vivo. B lymphocyte-induced maturation protein (Blimp-1) is a transcriptional repressor that drives the terminal differentiation of B cells into Ig-secreting plasma cells (26). Furthermore, Blimp-1 is up-regulated early during the transition of mature B cells to IgM-secreting plasma cells (27). Transcripts for Blimp-1 increased dramatically in wild-type but not in RP105$^{-/-}$ spleens following immunization with LPS (Fig. 7*a*). Much lower but significant up-regulation of transcripts for Blimp-1 was observed in wild-type mice stimulated with Pam_3CSK_4 but not in RP105$^{-/-}$ mice (Fig. 7*a*). Soro et al. (27) recently demonstrated that Blimp-1 expression in B cells were up-regulated by LPS but not by anti-CD40 mAb and IL-4. In keeping with this, Blimp-1 expression was not up-regulated by anti-CD40 mAb injection but clearly up-regulated by anti-RP105 mAb injection (Fig. 7*b*).

Syndecan-1 (CD138) is normally expressed on bone marrow pre-B cells and IgM-secreting plasma cells but not on mature B cells (27). Lipid A injection induced B220$^+$ CD138$^+$ plasma cells in wild-type but much less so in RP105$^{-/-}$ spleen cells (Fig. 7*c*). Much lower but significant increase in plasma cells were observed with stimulation by TLR2 ligand Pam_3CSK_4 in wild-type mice but not in RP105$^{-/-}$ mice (Fig. 7*c*). Furthermore, RP105 mAb injection induced CD138$^+$ plasma cells only in wild-type spleens (Fig. 7*d*). Although CD40 mAb caused CD86 up-regulation in vivo (Fig. 7*e*) and drove B cells to proliferate in vitro (28), anti-CD40 alone induced much less B220$^+$ CD138$^+$ plasma cells than anti-RP105 (Fig. 7*d*), which is consistent with a previous report (27). These results strongly suggested that a signal via RP105/MD-1 drove B cells to differentiate into Ab-secreting cells expressing Blimp-1 and CD138.

Discussion

Low-affinity polyreactive Abs of the IgM and IgG3 classes provide first line protection against microbial pathogens, and the experiments conducted in this study addressed their dependence on particular TLRs. Animals lacking the RP105/MD-1 containing receptors, previously known to be essential to LPS responses, were IgG3 deficient. Normal polyclonal Ig production in response to LPS required the same complex as did synthesis of hapten-specific, thymus-independent Abs. MZ B cells displayed high densities of RP105/MD-1, and ligation of this receptor complex on spleen B cells promoted terminal differentiation. We will now discuss the importance of RP105/MD-1 relative to other TLR family-containing receptors and speculate how they might be functionally coupled.

The lack of RP105 did not seem to influence expression of TLR4, MD-2, and TLR2, as revealed by RT-PCR and flow cytometry (Fig. 6, *a–c*). RP105/MD-1 is expressed not only on B cells but also on macrophages and dendritic cells, but the phenotypes of RP105$^{-/-}$ mice are B cell specific. That is, macrophages and dendritic cells from RP105$^{-/-}$ mice were not impaired in response to LPS or TLR2 ligands (Fig. 6*d* and data not shown). It is of note that the agonistic activity of the RP105 mAb is also B cell specific in that it did not activate macrophages and dendritic cells (Fig. 6*d* and data not shown). We prefer a possibility that a signal via RP105/MD-1 is required for TI responses to LPS or TLR2 ligands. This is supported by the following results. Blimp-1^{high} CD138$^+$Ab-secreting cells appeared in response to lipid A stimulation in wild-type but much less so in RP105$^{-/-}$ mice (Fig. 7). Injection of RP105 mAb induced Blimp-1^{high} CD138$^+$ plasmacytic cells in the spleen (Fig. 7).

RP105$^{-/-}$ B cells were able to proliferate in response to higher concentrations of lipid A or TLR2 ligands (Fig. 2). In contrast, we could not detect polyclonal Ab production even after repeated injection of LPS (Fig. 3). Moreover, RP105$^{-/-}$ mice were comparable with MyD88$^{-/-}$ mice in serum IgG3 deficiency. TLR signals without RP105 are able to induce B cell proliferation particularly at high concentration of LPS or TLR2 ligands but not differentiation into plasma cells. We would like to conclude that RP105/MD-1-mediated signal is indispensable for B cell differentiation into plasma cells during TI responses.

The present findings beg the important question of what molecular mechanisms couple RP105/MD-1 with TLR4/MD-2 or with TLR2. Given that not only LPS but also TLR2 ligands stimulate the RP105/MD-1 complex, it is unlikely that RP105/MD-1 directly interacts with these TLR ligands. Tsuneyoshi et al. (29) recently demonstrated that LPS interacts with MD-2 but not with MD-1. In keeping with this, LPS was coprecipitated with TLR4/MD-2 but not with coexpressed RP105/MD-1 (data not shown). LPS or TLR ligands stimulate TLR4/MD-2 and TLR2, and these activated TLRs might then activate RP105/MD-1 by an as yet unknown mechanism. TLR9 and TLR7 have no obvious functional link with RP105/MD-1. TLR9 and TLR7 reside in the endoplasmic reticulum (17, 22), and TLR9 is recruited to early endosomes, where interaction with ligand and subsequent signaling occurs. Cell surface expression seems to be important for use of RP105/MD-1. As one possibility, RP105/MD-1 could be physically associated with TLR4/MD-2 and/or TLR2 on the cell surface. Physical association may lead to coclustering of RP105/MD-1 with TLR4/MD-2 or with TLR2 upon stimulation with TLR ligands.

There were marked differences between Pam_3CSK_4 and MALP-2 in inducing B cell responses. MALP-2 required ~100–1000 times higher concentration than lipid A or Pam_3CSK_4 (Fig. 2). Ab titers induced by FITC-MALP-2 were also lower than those induced by FITC-Pam_3CSK_4 (Fig. 4). Given that TLR6 is required for response to MALP-2, cell surface expression of TLR6 may be low on the splenic B cell surface. Although FITC-specific Ab production was significantly induced by Pam_3CSK_4 in a manner dependent on RP105 (Fig. 4*a*), Pam_3CSK_4 was much lower than lipid A in inducing plasmablasts (Fig. 7) and we were able to detect polyclonal Ab production with LPS but not with Pam_3CSK_4 (Fig. 3). In contrast, Pam_3CSK_4 was as potent as lipid A in inducing B cell proliferation (Fig. 2). Pam_3CSK_4-induced B cell proliferation might be differentially regulated from B cell differentiation. It is of note that CD19 is shown to regulate RP105-signaling (25). CD19$^{-/-}$ B cells were reported to be lower than wild-type B cells in Ab production to TNP-LPS (30), suggesting that CD19 activation in LPS response are directly linked with Ab production. It is possible that CD19 activation during Pam_3CSK_4 response is much lower than that in LPS response. CD19 was shown to be phosphorylated when a B cell lymphoma A20 was stimulated with LPS (25). We tried to examine CD19 phosphorylation in normal B cells activated by LPS or Pam_3CSK_4, but we could not detect it (data not shown). CD19 phosphorylation may be too low in normal B cells for biochemical detection. Further studies are under way.

For primary defense, Abs in the serum or produced during TI responses must bind to microbial membranes. The immunostimulatory activity of these membranes is explained by the presence of ligands for TLR4/MD-2 and TLR2. LPS and TLR2 ligands were both able to induce Ag-specific Ab production in vivo (Figs. 3 and 4): RP105$^{-/-}$ mice were impaired in mounting Ab production not only against LPS and TLR2 ligands but also against heat-killed bacteria (Figs. 3 and 4). Taken together, primary Ab production to microbial membranes is regulated by TLR4/MD-2, TLR2, and RP105/MD-1. TLR4$^{-/-}$ and TLR2$^{-/-}$ mice were only modestly impaired in serum IgG3 production when compared with RP105$^{-/-}$ and MyD88$^{-/-}$ mice (Fig. 1). TLR2 and TLR4 would compensate for a lack of TLR4 or TLR2, respectively. In contrast,

TI responses to TLR4 and TLR2 ligands were all impaired in RP105$^{-/-}$, leading to low serum IgG3 titer. It is important to study serum Ig titers of mice lacking both TLR4 and TLR2.

RP105$^{-/-}$ mice have normal levels of serum IgG1 and IgG2a (Fig. 1), and were not impaired with respect to TD Ab production (15). In keeping with this, RP105/MD-1 was nearly absent from GC B cells that were committed to TD Ab production (Fig. 5*c*). In sharp contrast, the density of RP105/MD-1 was, among B cell subsets, brightest on MZ B cells, which greatly contribute to TI responses (Fig. 5*a*) (24). RP105$^{-/-}$ mice are quite different from CD40$^{-/-}$ mice that are impaired in TD but not in TI responses (31). An additional difference between these two types of receptors was found by injection of mAb. Injection of RP105 but not CD40 mAb induced plasma cells in spleen (Fig. 7). These differences between RP105/MD-1 and CD40 could represent important distinctions between TI and TD responses.

In conclusion, TLR2- and TLR4/MD-2-containing receptors are essential for recognition of lipoproteins and LPS. Whereas RP105/MD-1 may not directly recognize these microbial products, it is displayed at high levels on MZ B cells and is essential for both polyclonal and specific IgG3 Ab responses to TI type 1 vaccines. These observations add to our understanding of innate immune mechanisms and should be important for developing new immunization strategies.

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Disclosures

The authors have no financial conflict of interest.

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Augmented induction of CD8$^+$ cytotoxic T-cell response and antitumour resistance by T helper type 1-inducing peptide

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Summary

The effector CD8$^+$ T cells recognize major histocompatibility complex (MHC) class I binding altered self-peptides expressed in tumour cells. Although the requirement for CD4$^+$ T helper type 1 (Th1) cells in regulating CD8$^+$ T cells has been documented, their target epitopes and functional impact in antitumour responses remain unclear. We examined whether a potent immunogenic peptide of *Mycobacterium tuberculosis* eliciting Th1 immunity contributes to the generation of CD8$^+$ T cells and to protective antitumour immune responses to unrelated tumour-specific antigens. Peptide-25, a major Th epitope of Ag85B from *M. tuberculosis* preferentially induced CD4$^+$ Th1 cells in C57BL/6 mice and had an augmenting effect on Th1 generation for coimmunized unrelated antigenic peptides. Coimmunization of mice with Peptide-25 and ovalbumin (OVA) or Peptide-25 and B16 melanoma peptide [tyrosinase-related protein-2 (TRP-2)] for MHC class I led to a profound increase in CD8$^+$ T cells specific for OVA and TRP-2 peptides, respectively. This heightened response depended on Peptide-25-specific CD4$^+$ T cells and interferon-γ-producing T cells. In tumour protection assays, immunization with Peptide-25 and OVA resulted in the enhancement of CD8$^+$ cytotoxic cell generation specific for OVA and the growth inhibition of EL-4 thymoma expressing OVA peptide leading to the tumour rejection. These phenomena were not achieved by immunization with OVA alone. Peptide-25-reactive Th1 cells counteractivated dendritic cells in the presence of Peptide-25 leading them to activate and present OVA peptide to CD8$^+$ cytotoxic T cells.

Keywords: antigen presentation; cytotoxic T cells; peptide; T helper 1 cells; tumour immunity

Introduction

The identification of tumour antigens has renewed interest in immunotherapy for cancer. There is a body of evidence that tumour-specific T cells recognize tumour-associated antigens on the cancer cells and play an essential role in inhibiting tumour growth and eradicating cancer cells.[1–3] CD8$^+$ cytotoxic T lymphocytes (CTL) from specifically immunized mice are capable of destroying tumour target cells *in vitro*[4] and adoptive transfer of CD8$^+$ T cells from immunized donors confers resistance to tumour transplants on naive mice.[5–7] As CD8$^+$ CTL can lyse tumour cells directly and destroy large tumour masses *in vivo*, much attention has focused on the role of CD8$^+$ T cells in the immunotherapy of cancer. Over the past two decades, a wide range of peptides derived from tumour cells of mice and humans that bind major histocompatibility complex (MHC) class I and are recognized by CD8$^+$ T cells has been defined.[1,8,9] However, in both clinical and animal studies, therapeutic strategies focused on the use of CD8$^+$ T cells and MHC class I-restricted tumour antigens have not been effective in eliminating cancer cells.

There has been a recent reappraisal of the role and importance of CD4$^+$ T helper (Th) cells in antitumour responses, because CD4$^+$ Th cells are required for generating and maintaining potent antitumour immunity.[5,6,10] The role of CD8$^+$ and CD4$^+$ T cells in tumour systems has been the object of intense interest. A major obstacle for the development of optimal cancer vaccines is the lack of effective methods for identifying MHC class

II-restricted tumour antigens that can stimulate $CD4^+$ T cells.[11,12] Identification of such antigens would provide new opportunities for developing effective $CD8^+$ CTL and would improve our understanding of the mechanisms by which $CD4^+$ T cells regulate the host immune system.

A variety of tumour-derived antigens have been defined by immunoglobulin G (IgG) antibodies in sera taken from tumour bearers with serological identification of antigens by recombinant expression cloning (SEREX).[13–16] The SEREX repertoire can be considered a reflection of the $CD4^+$ T-cell repertoire. Shiku and his colleagues reported that coimmunization of mice with plasmids encoding these SEREX-defined wild-type antigens and mutated mitogen-activated protein kinase 2 (mERK2; containing tumour-specific CTL epitope 9m of CMS5) led to a profound increase in $CD8^+$ T cells specific for mERK2.[13] This heightened response depends on $CD4^+$ T cells and on the copresentation of SEREX-defined wild-type antigens and the CTL epitope. Their results indicate the essential role of $CD4^+$ T cells in mediating the increased $CD8^+$ T-cell response and tumour inhibition induced by coimmunization with SEREX-defined antigens.

We have reported that immunization of *Mycobacterium tuberculosis*-primed mice with purified protein derivative (PPD)-modified attenuated X5563 myeloma cells induces an X5563-specific $CD8^+$ CTL response and antitumour immunity.[17–19] We infer from these results that *M. tuberculosis*-derived proteins or peptides may enhance the $CD8^+$ CTL response and antitumour immunity by coimmunization with tumour antigen or neo-tumour antigen. Ag85B, one of the major proteins secreted by *M. tuberculosis*, elicits a strong Th1 response *in vitro* in T cells from both PPD-positive asymptomatic human subjects and Ag85B-primed cells of C57BL/6 ($I\text{-}A^b$) mice. Peptide-25 (amino acids 240–254) of Ag85B, which is the most potent antigen species yet purified for both humans and mice, is a major Th1 cell epitope of Ag85B. Active immunization of C57BL/6 mice with Peptide-25 induces the differentiation of $CD4^+$ T-cell receptor (TCR) $V\beta11^+$ T cells that produce interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α)[20–23].

We investigated whether Th1-inducible Peptide-25 intensifies the $CD8^+$ CTL response to unrelated tumour-specific antigens through stimulation of a $CD4^+$ Th1 cell response leading to the induction of antitumour immunity that is effective in eliminating cancer cells. We also discuss the possible mechanisms of Peptide-25-induced enhancement of the $CD8^+$ CTL response.

Materials and methods

Mice

C57BL/6 mice were purchased from Charles River Japan (Tokyo, Japan). Peptide-25-reactive TCR transgenic (Tg) (P25 TCR-Tg) mice were generated and maintained as described previously.[24] IFN-γ deficient ($IFN\text{-}\gamma^{-/-}$) mice[25] were kindly provided by Dr Y. Iwakura (Institute of Medical Science, University of Tokyo, Tokyo, Japan). Ovalbumin (OVA)-specific TCR-Tg (OT-1) mice were kindly provided by Dr T. Hirano (Osaka University, Suita, Japan). These mice were housed in the animal facility at the Institute of Medical Science, University of Tokyo, under specific pathogen-free conditions, and were used at 8–12 weeks of age.

Antigens and reagents

Peptide-25 (FQDAYNAAGGHNAVF), Peptide-9 (DWY SPACGKAGCQTY), and Peptide-18 (AGGYKAADM WGPSSD) of Ag85B were synthesized by Funakoshi Co., Ltd (Tokyo, Japan). Purified chicken OVA was purchased from Sigma-Aldrich, Co. (St Louis, MO). MHC class I-binding OVA Peptide (SIINFEKL) and B16 melanoma peptide tyrosinase-related protein-2 (TRP-2) (VYDF FVWL)[26] were also synthesized by Funakoshi Co., Ltd.

Culture medium

RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Sigma-Aldrich, Co.), 50 μM 2-mercaptoethanol, 100 IU/ml penicillin G and 50 μg/ml streptomycin was used as the complete medium for cultures throughout the present experiment.

Cell lines

The murine thymoma line, EL-4 ($H\text{-}2K^b$) was purchased from the American Type Culture Collection (Rockville, MD). EL-4 transfectant of the OVA gene (E.G7 cells) was kindly provided by Dr H. Udono (Nagasaki University School of Medicine, Nagasaki, Japan) and the B16 melanoma cell line was kindly provided by Dr H. Tahara (Institute of Medical Science, University of Tokyo, Tokyo, Japan).

Immunization

Mice were immunized by subcutaneous injection on the abdomen with OVA (10 μg/mouse) emulsified in incomplete Freund's adjuvant (IFA), Peptide-25, or its related peptide (10 μg/mouse) in IFA or a mixture of OVA (10 μg/mouse) and Peptide-25 (10 μg/mouse) in IFA as described previously.[21] In some experiments, mice were immunized with OVA (10 μg/mouse) in IFA on the left-hand side of the abdomen and with Peptide-25 (10 μg/mouse) in IFA on the right-hand side of the abdomen. We also immunized mice with MHC class I-binding TRP-2 peptide (10 μg/mouse) in place of OVA.

In vivo *and* in vitro *T-cell depletion*

$CD4^+$ T cells were depleted *in vivo* by the administration of 0·3 mg monoclonal antibodies (mAbs) against CD4 (GK1.5) on days −13, −12, −11, −6, −5, −4, +1, +2 and +3 relative to immunization. Fluorescence-activated cell sorter (FACS) analysis of blood mononuclear cells from GK1.5-treated mice at the time of immunization confirmed the effectiveness of the $CD4^+$ T-cell depletion. *In vitro* T-cell depletion was achieved by the incubation of spleen cells with either the IgM subclass of mAb against CD4 or CD8 and guinea-pig complement. FACS analysis of the treated spleen cells confirmed the effectiveness of the depletion.

In vitro *CTL induction and* $CD8^+$ *cytotoxic T-cell assay*

In vitro CTL induction and $CD8^+$ CTL assay were carried out according to previously described methods[17,18] with slight modification. Ten days after immunization with OVA in IFA or OVA and Peptide-25 in IFA, spleen cells (1×10^7) were cultured *in vitro* with γ-irradiated (20 000 rad) E.G7 cells (8×10^5). Spleen cells from TRP-2-immunized mice were stimulated *in vitro* with TRP-2 (10 μg/ml). After 5 days in culture, the CTL activity of the resulting effector cells was assayed. Target cells (E.G7, EL-4, and B16 melanoma cells) were labelled with ^{51}Cr (Perkin Elmer Life Science, Boston, MA) at 37° for 40 min. After washing, ^{51}Cr-labelled target cells (1×10^4) were incubated with effector cells at various effector cell to target cell ratios. Release of ^{51}Cr was measured in the supernatants that were harvested after 4 hr incubation. Maximum release was measured by resuspending the target cells in lysis buffer containing 0·1% Triton-X-100. Spontaneous release was obtained from target cells incubated with medium alone and was less than 10% of maximum ^{51}Cr release. The percentage specific lysis was calculated according to the following formula, where c.p.m. represents counts per minute: percentage specific lysis = $[(\text{c.p.m.}_{\text{experimental release}} - \text{c.p.m.}_{\text{spontaneous release}})/(\text{c.p.m.}_{\text{maximum release}} - \text{c.p.m.}_{\text{spontaneous release}})] \times 100$.

A dose–response curve of effector cells was established in all experiments and the number of lytic units (LU) was calculated as previously described.[19] In these calculations 1 LU was arbitrarily defined as the number of spleen cells required to achieve 50% lysis of 1×10^4 ^{51}Cr-labelled target cells during a 4-hr incubation.

Tumour challenge experiments

Three groups of 12 mice were immunized by subcutaneous injection of the abdomen with OVA (10 μg/mouse) in IFA, Peptide-25 (10 μg/mouse) in IFA, or a mixture of OVA (10 μg/mouse) and Peptide-25 (10 μg/mouse) in IFA. Twelve mice were injected with IFA without any protein or peptide to act as a control group. Ten days after the immunization, all mice were challenged by subcutaneous injection with E.G7 (5×10^5 cells/mouse) on their backs. In some experiments, B16 melanoma cells (5×10^5 cells/mouse) were transplanted in TRP-2-immunized mice. Tumour size was assessed using a microcaliper a 2-day to 3-day intervals and was expressed as the square of the smallest diameter of the tumour multiplied by its largest diameter. The survival of the mice was also monitored periodically.

Frequency analysis of OVA-specific CTL

The frequency of OVA-specific CTL in spleen cells after immunization was measured using OVA peptide-loaded H-$2K^b$:Ig protein (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer's instructions. Spleen cells prepared from mice 10 days after immunization, were stained with 4 μg OVA peptide-loaded H-$2K^b$:Ig protein and incubated for 60 min at 4°. After washing, cells were stained with anti-mouse IgG1 (A85-1)–phycoerythrin (PE; BD Biosciences Pharmingen) and anti-CD8 (53-6.7)–fluorescein isothiocyanate (FITC; BD Biosciences Pharmingen) and incubated for 30 min at 4°. After washing, cells were analysed using FACSCalibur (Becton Dickinson, Mountain View, CA).

Assay for dendritic cell activation

Immature dendritic cells (DCs) were propagated *in vitro* by culturing $CD11c^+$ bone marrow cells with granulocyte–macrophage colony-stimulating factor (GM-CSF) (20 ng/ml) and interleukin-3 (IL-3) (20 ng/ml) for 6 days. To assess the expression of surface molecules and IL-12 production of DCs after Peptide-25 treatment, the cells obtained (5×10^5) were cocultured with Peptide-25 (10 μg/ml) in the presence of $CD4^+$ T cells (5×10^5) from P25 TCR-Tg mice for 48 hr. The expression of surface molecules on DCs was analysed by FACS. The IL-12 production was assessed by enzyme-linked immunosorbent assay (ELISA). To assess the antigen-presenting activity of DCs after Peptide-25 treatment, the cells obtained (5×10^5) were cocultured with $CD4^+$ T cells (5×10^5) from P25 TCR-Tg mice and 5-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled $CD8^+$ T cells (5×10^5) from OT-1 mice for 96 hr in the presence of Peptide-25 (10 μg/ml) and OVA (10 μg/ml). After the culture, cell division cycles were determined by FACS analysis.[27]

Assay for cytokine production by intracellular cytokine staining and ELISA

For assessment of cytokine production of spleen cells from OVA- or TRP-2-immunized mice, spleen cells

(1×10^6/ml) prepared from mice 10 days after immunization, were stimulated with 10 μg/ml OVA or 10 μg/ml TRP-2. After the stimulation, IFN-γ- and IL-4-producing cells were examined by intracellular staining according to previously described methods.[22,24] In brief, 2 μM of monensin (BD Biosciences Pharmingen) was added for the last 4 hr of the culture. The cells were harvested and stained with 7-amino-actinomycin D and anti-CD4 (GK1·5)- or anti-CD8- allophycocyanin (BD Biosciences Pharmingen). Then, the cells were washed in 0·05% azide–1%FCS–phosphate-buffered saline, fixed with 1·6% formaldehyde, made permeable with 0·1% saponin and stained with anti-IFN-γ-FITC (XMG1.2) (BD Biosciences Pharmingen) and anti-IL-4-PE (11B11) (BD Biosciences Pharmingen) or isotype control antibodies. Stained cells were gated on live $CD4^+$ or $CD8^+$ cells and analysed by FACSCalibur. The amounts of IFN-γ and IL-4 in the culture supernatant after OVA or TRP-2 stimulation *in vitro* were quantified by ELISA following the manufacturer's instructions. The mAbs specific for mouse IFN-γ and IL-4 that were used for capture and detection of cytokines were purchased from BD Biosciences Pharmingen. ELISA of IL-12p40 was conducted using a murine IL-12 p40 OptEIA™ ELISA kit (BD Biosciences Pharmingen).

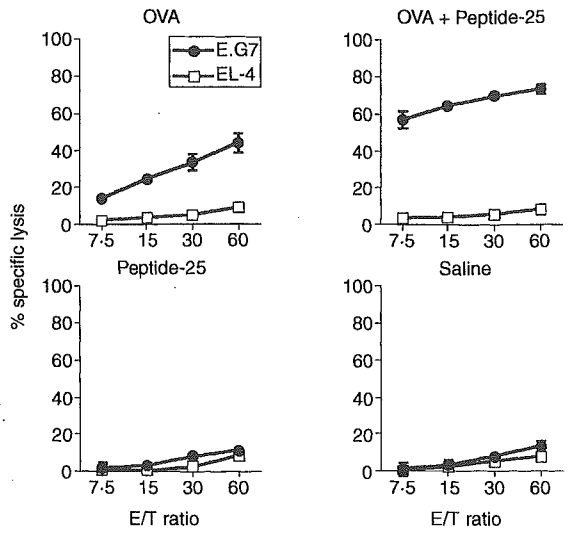
ELISA for anti-OVA antibody titration

For assessment of anti-OVA IgG1 and IgG2a levels, serum was collected from the immunized mice at 10 days after immunization and added to the OVA-coated plate. Biotinylated goat anti-mouse IgG1 antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) or biotinylated goat anti-mouse IgG2a (5.7.2) was applied and detection was performed using streptavidin-peroxidase (Zymed Laboratories Inc., San Francisco, CA).

Results

Peptide-25 enhances the generation of OVA-specific $CD8^+$ CTL response

Peptide-25, a 15-mer peptide of Ag85B is a major T-cell epitope recognized by $CD4^+$ I-A^b-restricted Th1 cells specific for Ag85B of *M. tuberculosis*.[20,21] Immunization of C57BL/6 mice with Peptide-25 induced the generation of IFN-γ- and TNF-α-producing Th1 cells that preferentially express TCRVβ11.[21] As $CD4^+$ Th1 cells can augment the $CD8^+$ CTL response[28,29] we examined whether immunization with a mixture of OVA and Peptide-25 can enhance the generation of an OVA-specific CTL response compared to OVA immunization. Three groups of mice were immunized with OVA in IFA, Peptide-25 in IFA, or a mixture of OVA and Peptide-25 in IFA. As a control, a group of mice was treated with IFA. Ten days after the immunization, spleen cells from each group of mice were



Lytic unit (/10^6)	OVA	OVA + Peptide-25	Peptide-25	Saline
E.G7	1·0	29·1	<0·01	<0·01
EL-4	<0·01	<0·01	<0·01	<0·01

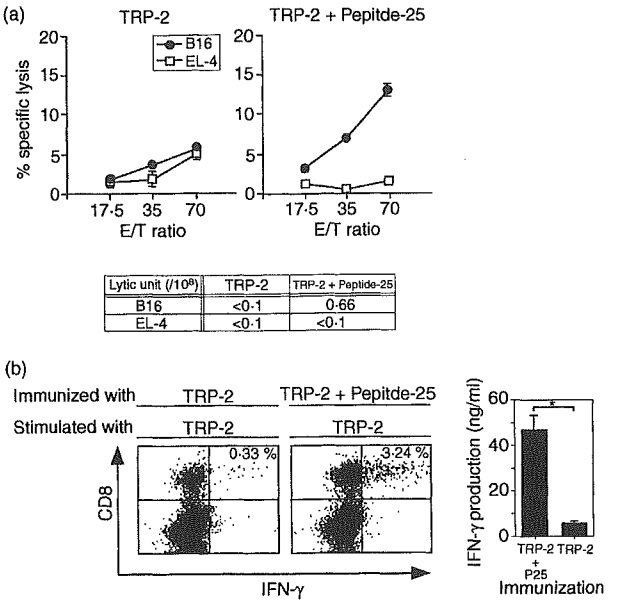
Figure 1. Enhanced induction of OVA-specific $CD8^+$ CTL response in spleen by coimmunization with OVA and Peptide-25. Three groups of mice were immunized with OVA (10 μg) in IFA, OVA (10 μg) and Peptide-25 (10 μg) in IFA or Peptide-25 (10 μg) in IFA subcutaneously. Spleen cells from each group of mice 10 days after the immunization were subjected to OVA-specific CTL assay. CTL assay was conducted by incubating sensitized cells with ^{51}Cr-labelled E.G7 or ^{51}Cr-labelled EL-4 cells of various effector to target ratios at 37° for 4 hr. The values represent the geometric means and standard deviation of percentage specific lysis in triplicate cultures. Background lysis of the target cells was less than 10% of maximum ^{51}Cr-release. The number of LU was calculated from the dose–response curve obtained for each group. A representative result of a series of five experiments is shown.

stimulated *in vitro* for 5 days with heavily irradiated E.G7 as stimulator cells that express OVA linked to the MHC class I molecule. The responding cells recovered after the culture were subjected to OVA-specific CTL assay as effector cells. The CTL activity was assessed on a 4-hr ^{51}Cr-release assay using ^{51}Cr-labelled E.G7 or EL-4. Results clearly revealed that the spleen cells from OVA-immunized mice mounted a significant CTL response to E.G7, but not to EL-4 upon *in vitro* stimulation with irradiated E.G7 (Fig. 1). Interestingly, a robust OVA-specific CTL response was induced in the culture of the spleen cells from mice immunized with a mixture of OVA and Peptide-25 in IFA upon E.G7 stimulation. The enhancement of the OVA-specific CTL response by coimmunization of OVA with Peptide-25 was quantitatively confirmed by calculating the lytic unit. Spleen cells from Peptide-25-immunized mice or from IFA-treated mice did not mount a significant CTL response to E.G7 upon E.G7 re-stimulation *in vitro*, indicating that Peptide-25 immunization does not induce a polyclonal CTL response.

We confirmed that an enhanced OVA-specific CTL response was observed after coimmunization with Peptide-25 and MHC class I-binding OVA peptide (data not shown). The OVA-specific CTL activity in effector cells was abrogated completely by the depletion of $CD8^+$ T cells using anti-CD8 mAb plus complement treatment before CTL assay, while the CTL activity remained the same in the treatment of spleen cells with anti-CD4 mAb plus complement (data not shown).

To examine the enhancing effect of I-A^b-binding peptides other than Peptide-25 on OVA-specific CTL generation, three groups of mice were immunized with OVA in IFA, a mixture of OVA and Peptide-25 in IFA, or a mixture of OVA and Peptide-9 of Ag85B in IFA. As a control, a group of mice was immunized with OVA and Peptide-18 (non-I-A^b-binding peptide of Ag85B) in IFA. The generation of an OVA-specific CTL response in spleen cells was assessed 10 days after the immunization. While immunization with OVA and Peptide-25 induced a potent OVA-specific CTL response, the CTL response observed in spleens from mice immunized with OVA and Peptide-9 was much less, if present at all (data not shown). Immunization with a mixture of OVA and Peptide-18 did not show enhancement of the OVA-specific CTL response.

In separate experiments, we analysed the augmenting effect of Peptide-25 on the CTL response specific for TRP-2 peptide, which is an MHC class I-binding peptide of murine melanoma. We immunized C57BL/6 mice with TRP-2 in IFA or a mixture of TRP-2 and Peptide-25 in IFA twice with a 10-day interval. Spleen cells from each group of mice were stimulated *in vitro* with TRP-2 for 5 days and TRP-2-specific CTL assay was conducted. Co-immunization with TRP-2 and Peptide-25 induced a significant $CD8^+$ CTL response in T cells to TRP-2 and IFN-γ production, while TRP-2 immunization was ineffective (Fig. 2a,b).



Lytic unit (/10^6)	TRP-2	TRP-2 + Peptide-25
B16	<0·1	0·66
EL-4	<0·1	<0·1

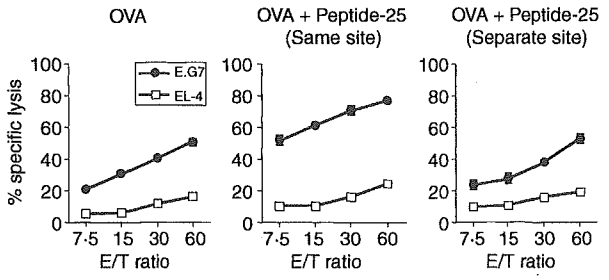
Figure 2. Enhancing effect of Peptide-25 on TRP-2-specific $CD8^+$ CTL response by coimmunization with TRP-2. (a) Two groups of mice were immunized subcutaneously with TRP-2 (10 μg) in IFA or TRP-2 (10 μg) and Peptide-25 (P25) (10 μg) in IFA. Spleen cells from each group of mice were subjected to TRP-2-specific CTL assay 10 days after the immunization. CTL assay was conducted by incubating sensitized cells with ^{51}Cr-labelled B16 melanoma or ^{51}Cr-labelled EL-4 cells of various effector to target ratios at 37° for 4 hr. The values represent the geometric means and standard deviation of percentage specific lysis in triplicate cultures. Background lysis of the target cells was less than 9% of maximum ^{51}Cr-release. The number of LU was calculated from the dose–response curve obtained with each group. A representative result of a series of three experiments is shown. (b) Ten days after immunization, spleen cells were stimulated with TRP-2 (10 μg/ml) for 2 days. Intracellular staining of IFN-γ was carried out on the recovered cells and they were examined by FACSCalibur. The percentages of IFN-γ-producing $CD8^+$ cells are presented in the upper right region. IFN-γ production of spleen cells in the culture supernatants was quantified by ELISA. The values represent the mean and standard deviation of the triplicate cultures. *$P < 0.01$ by Student's *t*-test.

Co-immunization of a mixture of OVA and Peptide-25 at the same site is required for the enhanced $CD8^+$ CTL response

We examined whether enhanced OVA-specific CTL generation by coimmunization with Peptide-25 can be induced when OVA and Peptide-25 are immunized separately. A group of mice was immunized with a mixture of OVA and Peptide-25 in IFA subcutaneously at the same site on the right-hand side of the abdomen. A group of mice was immunized with OVA in IFA and Peptide-25 in IFA separately (left and right sides of the abdomen, respectively). The CTL assay was conducted using spleen cells from each group of mice 10 days after the immunization. As shown in Fig. 3, the enhancement of the OVA-specific CTL response by Peptide-25 was observed only when a mixture of OVA and Peptide-25 in IFA was immunized at the same site. These results suggest that OVA and Peptide-25 need to be presented by the same antigen-presenting cells (APCs) for antigen processing to occur.

The enhancement of the OVA-specific cytolytic T-cell response by Peptide-25 depends on $CD4^+$ T cells and IFN-γ

To understand the efficacy of Peptide-25 to enhance OVA-specific cytotoxic activity, we examined whether IFN-γ-producing $CD4^+$ T cells contribute to the above enhancing effect of Peptide-25 on OVA-specific CTL generation. First, we depleted $CD4^+$ T cells *in vivo* by administering anti-CD4 mAb (GK1.5) to two groups of mice as



Lytic unit (/10^6)	OVA	OVA + Peptide-25 (Same site)	OVA + Peptide-25 (Separate site)
E.G7	1·8	15·1	1·7
EL-4	<0·01	<0·01	<0·01

Figure 3. Enhanced OVA-specific $CD8^+$ T-cell response induced by coimmunization with OVA and Peptide-25 at the same site. A group of mice was immunized with OVA (10 μg) and Peptide-25 (10 μg) in IFA at the same site subcutaneously. Another group of mice was immunized with OVA (10 μg) in IFA and Peptide-25 (10 μg) in IFA at two distant sites (separate sites). As a control, we also immunized a group of mice with OVA (10 μg) in IFA. Spleen cells from each group of mice 10 days after the immunization were subjected to OVA-specific CTL assay. CTL assay was conducted by incubating sensitized cells with ^{51}Cr-labelled E.G7 or ^{51}Cr-labelled EL-4 cells at various effector to target ratios at 37° for 4 hr. The values represent the geometric means and standard deviation of percentage specific lysis in triplicate cultures. Background lysis of the target cells was less than 10% of maximum ^{51}Cr-release. The number of LU was calculated from the dose–response curve obtained with each group. A representative result of a series of three experiments is shown.

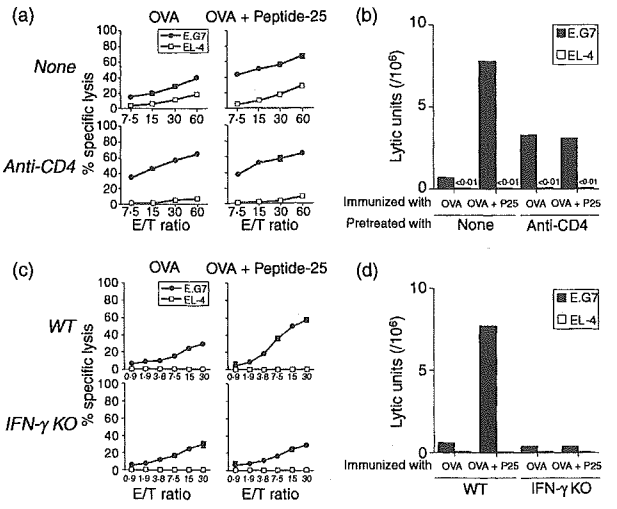


Figure 4. Role of $CD4^+$ T cells and IFN-γ in the enhancing effect of Peptide-25 on CTL response. (a,b) Two groups of wild-type mice were injected with anti-CD4 mAb (GK1.5) on days −13, −12, −11, −6, −5, −4, +1, +2 and +3 relative to immunization. Each group of mice was immunized with OVA (10 μg) in IFA or a mixture of OVA (10 μg) and Peptide-25 (10 μg) in IFA on Day 0. As controls, two other groups of mice had been treated with normal rat IgG in place of GK1.5 and immunized with OVA in IFA or a mixture of OVA and Peptide-25 in IFA. Spleen cells from each group of mice were subjected to *in vitro* OVA-specific CTL assay 10 days after the immunization. (c,d). Either wild-type or IFN-$\gamma^{-/-}$ mice with C57BL/6 background were immunized with OVA (10 μg) in IFA or a mixture of OVA (10 μg) and Peptide-25 (P25) (10 μg) in IFA. Spleen cells from each group of mice were subjected to OVA-specific CTL assay 10 days after the immunization. CTL assay (a,c) and LU calculations (b,d) were conducted as described in Figure 1.

described in the Materials and methods. As a control, rat IgG was administered in place of anti-CD4 to another two groups of mice. All groups of mice were immunized with OVA in IFA or with a mixture of OVA and Peptide-25 in IFA. As shown in Fig. 4(a,b), an enhanced CTL response specific for OVA, mounted in a culture of spleen cells taken from mice immunized with a mixture of OVA and Peptide-25 in IFA, was abrogated by the anti-CD4 mAb treatment before immunization, and the level of CTL response, measured as LU, was similar to that mounted in spleen cells from OVA-immunized mice. Unexpectedly, an enhanced OVA-specific CTL response, mounted in a culture of spleen cells from OVA-immunized mice, was observed when anti-CD4 mAb mice had been treated before OVA immunization.

To examine the roles of IFN-γ-producing cells in the CTL response, we immunized both wild-type and IFN-$\gamma^{-/-}$ mice with a mixture of OVA and Peptide-25 in IFA. Spleen cells from each group of mice were then subjected to the *in vitro* CTL assay specific for OVA. The results revealed that an enhanced CTL response specific for OVA was mounted in spleen cells from wild-type mice immunized with a mixture of OVA and Peptide-25 in IFA, while the enhancement was not observed in spleen cells from IFN-$\gamma^{-/-}$ mice (Fig. 4c,d). These results indicate that Peptide-25-reactive IFN-γ-producing T cells contribute to the enhancing effect of Peptide-25 on OVA-specific CTL generation.

Co-immunization with a mixture of OVA and Peptide-25 increases proportions of OVA-specific CTL precursors

To examine the cellular mechanisms of the enhancing effect of coimmunization of mice with a mixture of OVA and Peptide-25 on CTL generation following *in vitro* OVA stimulation, we examined the frequencies of OVA-specific CTL precursors. Spleen cells from mice were immunized with either OVA in IFA or a mixture of OVA and Peptide-25 in IFA 10 days before the experiments. The cells were then stained with OVA peptide-loaded H-$2K^b$:Ig protein and anti-mouse IgG1–PE and analysed using FACSCalibur. The results revealed that frequencies for OVA-specific CTL precursors in spleen cells (0·42%) from mice immunized with a mixture of OVA and Peptide-25 were about two-fold higher than those in spleen cells (0·21%) from OVA-immunized mice (Fig. 5).

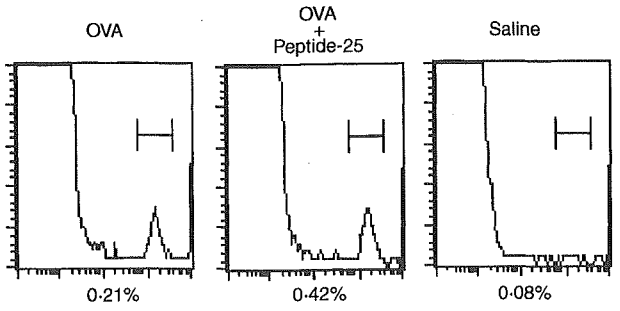


Figure 5. Frequency analysis of OVA-specific CTL. Two groups of mice were immunized with either OVA in IFA or OVA and Peptide-25 in IFA. Spleen cells from each group of mice were prepared 10 days after the immunization and stained with 4 μg of OVA peptide (SIINFEKL)-loaded H-2K^b:Ig protein and incubated for 60 min at 4°. After washing with staining buffer, cells were resuspended in 100 μl staining buffer containing appropriately diluted anti-mouse IgG1-PE and anti-CD8-FITC. After washing twice with staining buffer, cells were analysed using FACSCalibur.

Co-immunization of mice with a mixture of OVA and Peptide-25 can suppress E.G7 growth, leading to tumour rejection *in vivo*

We examined whether the immunization of C57BL/6 mice with a mixture of OVA and Peptide-25 was effective as a prophylactic intervention into the growth of E.G7. Three groups of 12 mice each were immunized with OVA in IFA, Peptide-25 in IFA, or a mixture of OVA and Peptide-25 in IFA. As a control, a fourth group of 12 mice was injected with IFA. Ten days after the immunization, we transplanted viable E.G7 tumour cells onto the backs of the mice and monitored tumour growth (Fig. 6a) and survival (Fig. 6b) after tumour challenge. As shown in the figures, the transplanted E.G7 established tumour masses within days of the tumour challenge in the abdomen of both the control group and the Peptide-25-immunized group of mice, leading to the death of all animals by day 30. Tumour growth in mice immunized with OVA in IFA was slightly delayed compared with that in the control mice however, all mice died by day 40 after the tumour challenge. Interestingly, mice immunized with a mixture of OVA and Peptide-25 in IFA showed significantly delayed tumour progression. Among these 12 mice, the E.G7 tumours of six of them were eradicated by Day 60 after the tumour challenge and the lifetimes of the mice were continued, tumour-free, for up to 100 days. By contrast, all mice in the other groups died within 40 days after the tumour challenge. These results indicate that coimmunization of Peptide-25 with OVA enhances not only an OVA-specific CTL response *in vitro* but also induces a potent antitumour immunity against OVA-expressing tumour cells *in vivo*.

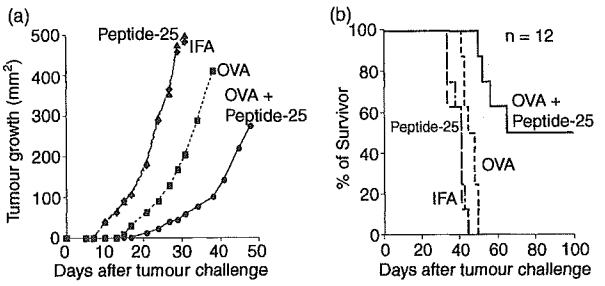


Figure 6. Enhancement of antitumour immunity by Peptide-25. (a) Suppression of E.G7 growth by augmented induction of E.G7-specific immunity. Three groups of mice were immunized with OVA (10 μg) in IFA, OVA (10 μg) and Peptide-25 (10 μg) in IFA or Peptide-25 (10 μg) in IFA subcutaneously. As a control, a group of mice was injected with IFA. All groups of mice were challenged with 5×10^5 viable E.G7 cells subcutaneously 10 days after the immunization. Growth of E.G7 tumour was monitored by measuring its size periodically (2- to 3-day intervals) and expressed as mm^2. (b) Survival of E.G7-bearing mice. The percentages of survivors in the respective groups shown in (a) are displayed.

The enhancement of the Th1 response to OVA by coimmunization with Peptide-25

To understand the mechanisms of the enhancing effect of Peptide-25 on the OVA-specific CTL response, we examined the Th1 generation specific for OVA when C57BL/6 mice were immunized with OVA in IFA, Peptide-25 in IFA, or a mixture of OVA and Peptide-25 in IFA. First, we measured anti-OVA antibody in the serum of each group of mice by ELISA 10 days after immunization. As shown in Fig. 7(a), OVA-immunized mice produced predominantly anti-OVA IgG1 antibody and produced lower levels of the anti-OVA IgG2a antibody. In contrast, mice immunized with a mixture of OVA and Peptide-25 produced predominantly anti-OVA IgG2a antibody. We could not detect any anti-OVA antibody in sera from Peptide-25-immunized mice (Fig. 7a).

To evaluate the cytokine-producing profiles in CD4$^+$ T cells in the spleen, spleen cells from each group of immunized mice were stimulated *in vitro* with OVA for 48 hr, and the proportions of IFN-γ- and IL-4-producing cells were examined by intracellular cytokine staining. Cells stained were gated on live CD4$^+$ cells and analysed by FACS. The results revealed that we could detect IFN-γ-producing CD4$^+$ T cells but not IL-4-producing cells when spleen cells from mice immunized with a mixture of OVA and Peptide-25 were stimulated with OVA (Fig. 7b). In contrast, OVA stimulation of spleen cells from OVA-immunized mice could become both IFN-γ- and IL-4-producing cells. We confirmed the enhancement of OVA-induced IFN-γ production by ELISA (Fig. 7b). Co-immunization of OVA with Peptide-9 slightly enhanced the proportions

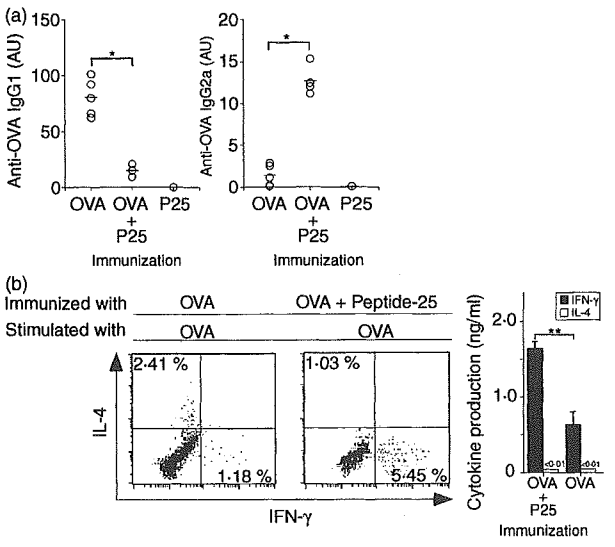


Figure 7. Enhancement of OVA-specific Th1 response by coimmunization of C57BL/6 mice with OVA and Peptide-25. Two different groups of mice were immunized with OVA (10 μg) in IFA, OVA (10 μg) and Peptide-25 (P25) (10 μg) in IFA, or Peptide-25 (10 μg) in IFA subcutaneously. (a) Serum anti-OVA IgG1 and IgG2a were titrated by ELISA 10 days after the immunization. Each open circle represents the results of an individual mouse. The horizontal bar represents the mean value of six mice. $*P < 0{\cdot}01$ by Student's *t*-test. (b) Ten days after immunization, spleen cells were stimulated *in vitro* with OVA (10 μg/ml) for 4 days. Intracellular staining of IL-4 and IFN-γ was carried out to the recovered cells. Cells stained were gated on live $CD4^+$ cells and examined by FACSCalibur. The percentages of IL-4- and IFN-γ-producing $CD4^+$ T cells are presented in the upper left and lower right regions, respectively. IL-4 and IFN-γ produced in the culture supernatants were titrated by ELISA. The values represent the mean and standard deviation of the triplicate cultures. $**P < 0{\cdot}05$ by Student's *t*-test.

of IFN-γ-producing cells, while coimmunization with Peptide-18 did not (data not shown). We did not observe differences in proportion of $CD4^+$ $CD25^+$ T cells between Peptide-25-immunized and Peptide-9-immunized T cells. These results suggest that the weak Th1-inducing ability of Peptide-9 correlates with a weak ability to enhance OVA-specific CTL generation when coimmunized with OVA.

The involvement of Peptide-25-reactive T cells in the induction of an OVA-reactive Th1 response was examined by using the rat anti-mouse clonotypic TCR mAb KN7 that can recognize TCR expressed on the Peptide-25-reactive Th1 clone of C57BL/6 mice. Enhanced induction of an OVA-specific IFN-γ-producing T-cell response in mice immunized with a mixture of OVA and Peptide-25 was impaired when we injected KN7 intraperitoneally 1 day before the immunization. In contrast, the control group that received rat IgG injection in place of KN7 did not show this suppressive effect (data not shown).

Peptide-25-reactive T cells can activate DCs through Peptide-25 leading to enhanced induction of OVA-presenting activity

The enhancement of an OVA-specific CTL response by coimmunization of Peptide-25 with OVA may be because the Peptide-25 directly or indirectly affects the OVA-presenting activity of APCs. To investigate this issue, we first evaluated the antigen-presenting activity of DCs. We propagated immature DCs *in vitro* by culturing bone marrow cells with GM-CSF and IL-3 for 6 days. They were stimulated for 48 hr with Peptide-25 in the presence of splenic $CD4^+$ T cells from P25 TCR-Tg mice. As a control, DCs were also stimulated with lipopolysaccharide. The expression of surface markers such as CD40, CD80, CD86, MHC class I antigen and intracellular adhesion molecule-1 (ICAM-1) was analysed using FACSCalibur. The results revealed that Peptide-25 stimulation alone did not alter the expression of the above surface markers on DCs (data not shown). When we cocultured DCs with $CD4^+$ T cells from P25 TCR-Tg mice together with Peptide-25, the expressions of MHC class I and ICAM-1 were enhanced to a similar extent to that found under lipopolysaccharide stimulation and this led to the induction of IL-12p40 production (Fig. 8a). Such DCs showed more effective OVA presentation to $CD8^+$ T cells from OT-1 mice and enhanced OT-1 cell divisions (Fig. 8b). These results suggest that Peptide-25-reactive $CD4^+$ T cells directly activate DCs in the presence of Peptide-25, leading to effective OVA cross-presentation for the activation of $CD8^+$ T cells.

Discussion

Antitumour immune responses involve complex interactions among various immunocompetent cells. $CD8^+$ CTLs are major effector cells capable of direct tumour destruction both *in vivo* and *in vitro*, and they recognize MHC class I binding peptides derived from molecules with altered expression in tumour cells.[28–30] Although the need for $CD4^+$ Th cells in regulating $CD8^+$ T cells has been documented, their target epitopes and functional impact in antitumour responses remain unclear.

There has been a recent reappraisal of the role and importance of $CD4^+$ Th cells in antitumour responses. $CD4^+$ Th cells are considered to contribute to the activation of $CD8^+$ CTLs through the expression of the CD40 ligand and production of cytokines, such as IL-2 and IFN-γ that are essential for the proliferation and maturation of $CD8^+$ CTL precursors. $CD4^+$ Th cells recognize MHC class II binding peptides on APCs and their interaction may result not only in the activation and priming of $CD4^+$ Th cells, but also in the activation of APCs themselves.[31–33] Consequent to these mutual activations, APCs prime and activate $CD8^+$ CTLs specific for the

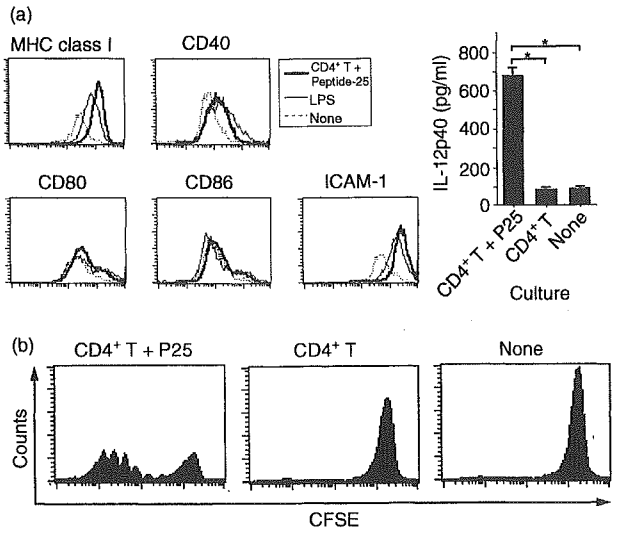


Figure 8. Activation of DCs by culturing with Peptide-25 in the presence of $CD4^+$ T cells from P25 TCR-Tg mice. (a) Immature DCs were propagated by culturing bone marrow cells with GM-CSF (20 ng/ml) and IL-3 (20 ng/ml) for 6 days. The cells recovered (5×10^5) were cultured with lipopolysaccharide (LPS; 5 μg/ml), Peptide-25 together with $CD4^+$ T cells (5×10^5) from P25 TCR-Tg mice or left untreated for 48 hr. The expression of surface markers was assessed by FACS analysis. IL-12p40 in the cultured supernatant was assessed by ELISA. (b) CFSE-labelled $CD8^+$ T cells (5×10^5) from OT-1 mice were cultured with immature DCs (5×10^5) and OVA (10 μg/ml) for 4 days. The cells were cocultured with $CD4^+$ T cells (5×10^5) from P25 TCR-Tg mice and Peptide-25 (P25) (10 μg/ml) (left panel) or $CD4^+$ T cells from P25 TCR-Tg mice (middle panel). Subsequently, cell division of the $CD8^+$ T cells was monitored by FACSCalibur.

tumour antigen or peptides. This scenario of cellular interaction assumes that $CD4^+$ Th cells and $CD8^+$ CTLs may not necessarily be in direct or close association. $CD4^+$ T cells are also implicated in the activation of tumoricidal macrophages that are involved in tumour clearance. Cytokines produced by $CD4^+$ T cells can recruit and activate macrophages and eosinophils, linking the T-cell response with the innate immune response.

In this study using OVA as neo-tumour antigen in a mouse model, we investigated the contribution of the Th1 epitope within the 15 amino acid residues of the Ag85B protein. We then examined the significance of the defined Th1 epitope regarding $CD8^+$ CTL generation and tumour eradication. We demonstrated that the coimmunization of Peptide-25 and the OVA CTL epitope resulted in a marked increase in the OVA-specific $CD8^+$ CTL response (Fig. 1). The selection of immunization site for Peptide-25 and OVA is important and both should be immunized at the same site (Fig. 3). The augmenting effect of Peptide-25 on $CD8^+$ CTL generation was cancelled by *in vivo* administration of the anti-CD4 mAb and was diminished in IFN-$\gamma^{-/-}$ mice (Fig. 4), indicating that the $CD4^+$ T cells and IFN-γ-producing T cells are required. It remains elusive why the enhancement of OVA-specific CTL activity was abrogated when IFN-$\gamma^{-/-}$ mice were immunized with the mixture of Peptide-25 and OVA. As we reported, the generation of Peptide-25-reactive $CD4^+$ T cells significantly decreases in IFN-$\gamma^{-/-}$ mice as compared with wild-type mice. Furthermore, Peptide-25 stimulation of Peptide-25-immunized cells induces IL-2 production that is also impaired in IFN-$\gamma^{-/-}$ cells.[22] IFN-γ-dependent IL-2 production may be indispensable to enhance the OVA-specific $CD8^+$ CTL generation and expansion in this particular system. Whilst we favour the possibility that impaired production of IFN-γ by $CD4^+$ T cells contributes to the reduction of CTL activity seen in mice primed with Peptide-25 and OVA we cannot exclude the possibility that ablation of IFN-γ production by $CD8^+$ T cells also plays a role.

Another explanation is that IFN-γ may play an important role in a cell-to-cell contact between Peptide-25-specific $CD4^+$ Th cells and APCs to enhance the induction of the OVA-specific $CD8^+$ CTL response. APCs are capable of processing and presenting exogenous antigens along with MHC class I molecules to $CD8^+$ T cells, which is termed antigen cross-presentation. The antigenic peptide generation for cross-presentation appears to be dependent on both the ubiquitin–proteasome system and the transporter associated with antigen processing.[34–37] Imai and his colleagues recently demonstrated that exogenously added OVA is accumulated in microsomal fractions, including the endoplasmic reticulum and late endosomes followed by retrograde transport to the cytoplasm through the Sec61 transporter complexes.[38] IFN-γ may facilitate these processes of OVA cross-presentation by APCs leading to enhancement of the induction of the OVA-specific $CD8^+$ CTL response.

Unexpectedly, our result showed that anti-CD4 mAb treatment before OVA immunization in mice enhanced the OVA-specific CTL response. Although we do not have concrete evidence, anti-CD4 mAb treatment may eliminate OVA-specific $CD4^+$ T cells that negatively regulate OVA-specific $CD8^+$ CTL generation.

Among I-A^b-binding peptides, Peptide-25 showed the most potent effect that correlated with potency for the *in vivo* induction of Th1 generation to each peptide. These results indicate that a Th1 epitope, regardless of the difference in its molecular origin, exhibits a helper activity for *in vivo* $CD8^+$ CTL generation. It is unclear why I-A^b binding Peptide-9 does not augment the induction of the OVA-specific CTL response. Peptide-9 showed lower immunogenicity for Th1 induction specific for its own compared with Peptide-25.[22] We analysed the Foxp3 expression in Peptide-9-reactive T cells by FACS and found no significant increase in the $Foxp3^+$ T regulatory cell population compared with Peptide-25-reactive T cells (data not shown). These results imply that the weak

potency of Peptide-9 to augment the OVA-specific CTL response is well correlated with its weak Th1-inducing potency instead of a T regulatory cell induction.

Both quantitative and qualitative changes may be involved in the Th effect of Peptide-25 in the CTL response. We think that a two-fold increase in the frequency of OVA-specific $CD8^+$ T cells and an increase of the lytic unit are qualitatively correlated with the Th effect of Peptide-25 (Figs 1–4). In addition, Peptide-25 stimulation of spleen cells from Peptide-25-immunized wild-type mice induces secretion of IL-2 that is impaired in IFN-$\gamma^{-/-}$ mice.[22] IL-2 produced by the Th1 cells may also enhance and expand the CTL maturation quantitatively. To evaluate qualitative changes more directly, we examined Granzyme B expression in OVA-reactive $CD8^+$ T cells. Results revealed that we did not observe significant increases in Granzyme B-expressing cells in the OVA-stimulated $CD8^+$ T cells in the presence of Peptide-25-primed Th cells compared with the precursors induced in the absence of the primed Th cells (data not shown). These results suggest that OVA-specific $CD8^+$ T-cell precursors induced in the presence of primed Th cells may differ quantitatively from these induced in the absence of primed Th cells.

The basis for the strong 'helper' function of Peptide-25 in the $CD8^+$ CTL response to E.G7 rejection is unknown. There are several possibilities to account for the enhancing effect of Peptide-25 on Th1 and CTL generation specific for OVA when Peptide-25 and OVA were coimmunized at the same site. Peptide-25 may directly activate APCs through unknown molecules resulting in effective cross-presentation of OVA molecules to both Th1 and CTL precursors. To address this issue, we propagated bone-marrow-derived DCs *in vitro*, stimulated them with Peptide-25 and analysed the surface expression of activation markers on DCs. The results revealed that expression of MHC class I, ICAM-1, CD40, CD80 and CD86 was not enhanced upon Peptide-25 stimulation (data not shown). We found that myeloid differentiating factor (MyD)88-deficient mice also showed enhanced CTL generation specific for OVA when coimmunized with Peptide-25 and OVA (data not shown). The MyD88-dependent signalling pathway may not be required for exerting helper activity by Peptide-25. On the whole, we do not support the hypothesis that Peptide-25 directly activates APCs.

As Peptide-25-reactive $CD4^+$ Th cells recognize MHC class II binding cognate Peptide-25 on APCs and their interaction may result in the activation of APCs, consequent to these mutual activations, APCs prime and activate $CD8^+$ CTLs specific for OVA peptides. The requirement for coimmunization of Peptide-25 and OVA in the same site suggests that the physical proximity of OVA and Peptide-25 is crucial for intracellular events in APCs. Cell-to-cell contact between Peptide-25-specific $CD4^+$ Th1 cells and APCs may be required or the two antigens need to be processed and presented by the same APC to augment the OVA-specific $CD8^+$ CTL response. To simplify the experimental system for further evaluation, we established an *in vitro* culture system using T cells from P25 TCR-Tg mice, CFSE-labelled T cells from OT-1 mice and bone-marrow-derived immature DCs propagated *in vitro*. As we reported, $CD4^+$ T cells from P25 TCR-Tg mice preferentially develop IFN-γ-producing T cells upon Peptide-25 stimulation in the presence of I-A^b splenic APCs under neutral conditions.[24] Surface expression of the MHC class I molecule, ICAM-1, CD40, CD80 and CD86 on DCs as well as IL-12p40 production was enhanced when DCs were cultured with $CD4^+$ T cells from P25 TCR-Tg mice and Peptide-25 (Fig. 8a). Furthermore, enhanced cell divisions of CFSE-labelled OT-1 T cells were observed when the cells were stimulated with OVA in the presence of DCs, Peptide-25 and $CD4^+$ T cells from P25 TCR-Tg mice (Fig. 8b). These results support the notion that $CD4^+$ Th1 cell-derived factor may augment antigen processing by APCs leading to the enhancement of the cross-priming of the antigenic peptide for $CD8^+$ T cells.

A number of approaches to augment $CD4^+$ T-cell help have been investigated.[2,10,13,39] One involves modifying the immunizing antigen itself by, for instance, haptenizing the antigen[40] or linking heterologous immunogenic peptides directly to the antigen.[17,41] The second involves coimmunization with tumour antigens and molecules with strong helper determinants.[42,43] The third, the discovery of a range of molecular signals, such as the CD40 ligand and other costimulatory signals[31–33] involved in the helper function of $CD4^+$ T cells, provides other ways to augment the $CD8^+$ T-cell response. Finally, broadly expressed wild-type molecules in murine tumour cells eliciting humoral immunity contribute to the generation of $CD8^+$ T cells and protective antitumour immune responses to unrelated tumour-specific antigen.[13]

It is important to ask whether the enhancing effect of Peptide-25 on $CD8^+$ CTL responses is beneficial, detrimental, or insignificant to the tumour-bearing host. It is likely that the phenomena that we have described in the mouse of a heightened $CD8^+$ CTL response to tumour antigens by corecognition of Peptide-25 has its counterpart in humans, occurring as a consequence of the simultaneous uptake of complex antigenic mixtures from disintegrating tumour cells by APCs. As coimmunization with Peptide-25 and tumour antigens also results in heightened resistance to tumour challenge in the mouse, this approach may be an attractive strategy for human cancer immunotherapy. In fact, there are ongoing clinical trials utilizing either heterologous helper antigens, such as keyhole limpet haemocyanin or tumour-derived helper antigens to augment antitumour immune responses by $CD8^+$ CTLs directed against tumour-derived CTL

epitopes.[44,45] As Ag85B and Peptide-25 are stimulatory to human $CD4^+$ T cells from PPD^+ healthy donors in a certain proportion of the Japanese population, Ag85B or peptide-25 may be applicable to augment antitumour immune responses by $CD8^+$ CTLs against tumour cells. Along with the scenario, we re-examined the effect of preimmunization with *M. tuberculosis* or Peptide-25 on the antitumour CTL response and found that preimmunization of mice with heat-killed *M. tuberculosis* or Peptide-25 followed by immunization with a mixture of OVA and Peptide-25 produced a significant increase in the number of $CD8^+$ CTL s (data not shown).

While it is obvious that $CD8^+$ T cells recognize MHC class I binding peptides derived from tumour target cells based on the subsequent tumour-specific destruction, peptide derivation from tumour cells may not be an absolute requirement for $CD4^+$ T cells because they do not directly interact with tumour cells that mostly lack MHC class II expression. Co-immunization of mice with TRP-2 of B16 melanoma and Peptide-25 in IFA enhances CTL generation specific for TRP-2 (Fig. 2). However, mice that had been immunized with a mixture of TRP-2 and Peptide-25 in IFA failed to eradicate B16 melanoma, a less immunogenic tumour, although B16 melanoma growth was reduced to a certain extent (data not shown), suggesting that enhancing the effect of Peptide-25 in the TRP-2-specific CTL may not be potent enough to eradicate a less immunogenic TRP-2-expressing tumour. We need another regimen together with Peptide-25 immunization that activates effector cells leading to the eradication of tumour cells with weak immunogenicity. This notion has become extremely important in the design of future vaccines aimed at the efficient activation of both T-cell populations involved in antitumour immune responses.

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