(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).

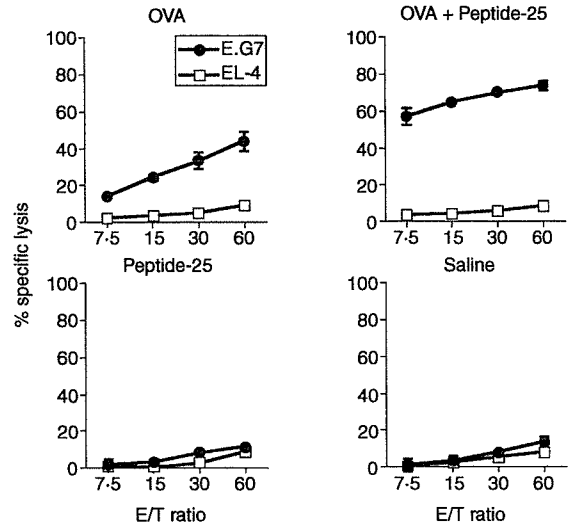
ELISA for anti-OVA antibody titration

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

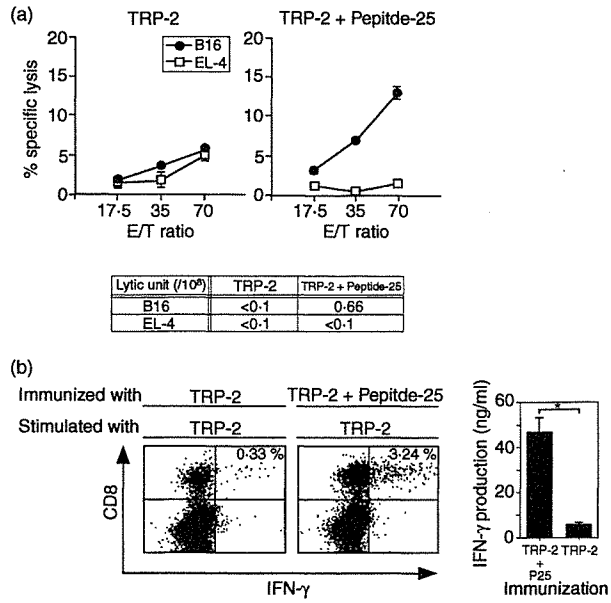
Figure 1. Enhanced induction of OVA-specific $CD8^+$ CTL response in spleen by coimmunization with OVA and Peptide-25. Three groups of mice were immunized with OVA (10 μg) in IFA, OVA (10 μg) and Peptide-25 (10 μg) in IFA or Peptide-25 (10 μg) in IFA subcutaneously. Spleen cells from each group of mice 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

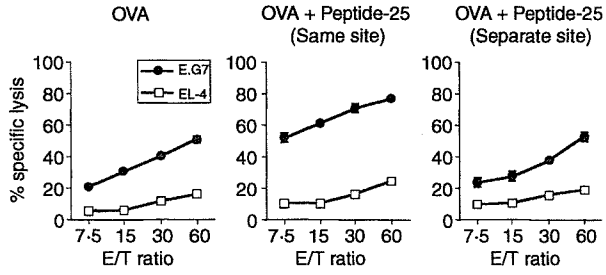
Figure 2. Enhancing effect of Peptide-25 on TRP-2-specific $CD8^+$ CTL response by coimmunization with TRP-2. (a) Two groups of mice were immunized subcutaneously with TRP-2 (10 μg) in IFA or TRP-2 (10 μg) and Peptide-25 (P25) (10 μg) in IFA. Spleen cells from each group of mice were subjected to TRP-2-specific 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 (/10⁶)	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.

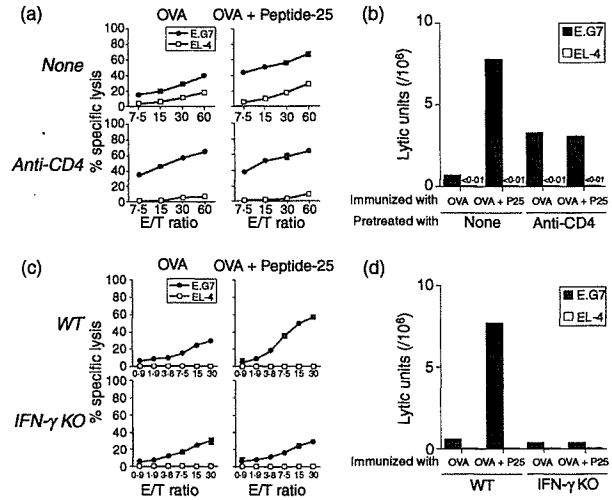


Figure 4. Role of $CD4^+$ T cells and IFN-γ in the enhancing effect of Peptide-25 on CTL response. (a,b) Two groups of wild-type mice were injected with anti-CD4 mAb (GK1.5) on days −13, −12, −11, −6, −5, −4, +1, +2 and +3 relative to immunization. Each group of mice was immunized with OVA (10 μg) in 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).

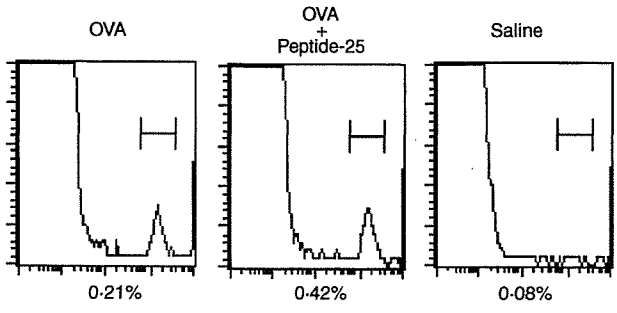


Figure 5. Frequency analysis of OVA-specific CTL. Two groups of mice were immunized with either OVA in IFA or OVA and Peptide-25 in IFA. Spleen cells from each group of mice were prepared 10 days after the immunization and stained with 4 μg of OVA peptide (SIINFEKL)-loaded H-2K^b:Ig protein and incubated 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*.

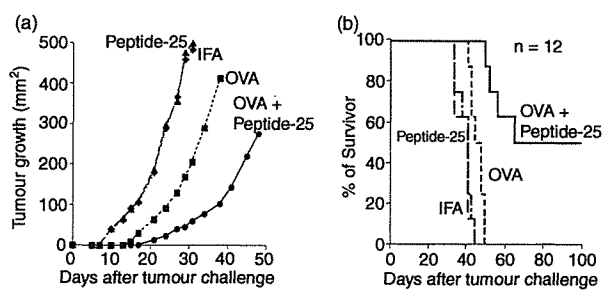


Figure 6. Enhancement of antitumour immunity by Peptide-25. (a) Suppression of E.G7 growth by augmented induction of E.G7-specific immunity. Three groups of mice were immunized with OVA (10 μg) in IFA, OVA (10 μg) and Peptide-25 (10 μg) in IFA or Peptide-25 (10 μg) in IFA subcutaneously. As a control, 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

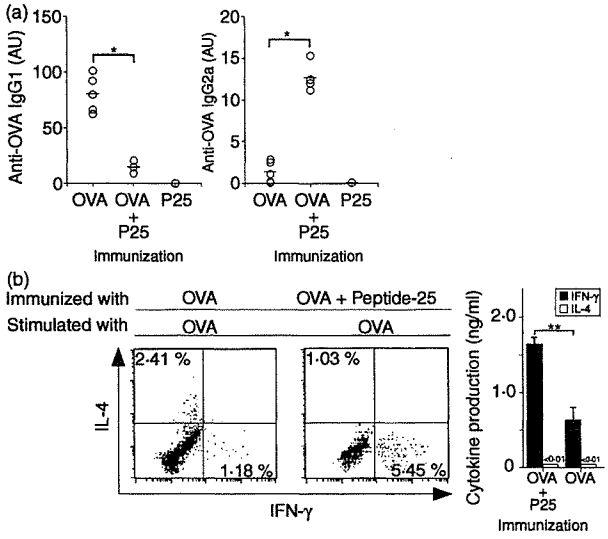


Figure 7. Enhancement of OVA-specific Th1 response by coimmunization of C57BL/6 mice with OVA and Peptide-25. Two different groups of mice were immunized with OVA (10 μg) in IFA, OVA (10 μg) and Peptide-25 (P25) (10 μg) in IFA, or Peptide-25 (10 μg) in IFA subcutaneously. (a) Serum anti-OVA IgG1 and 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

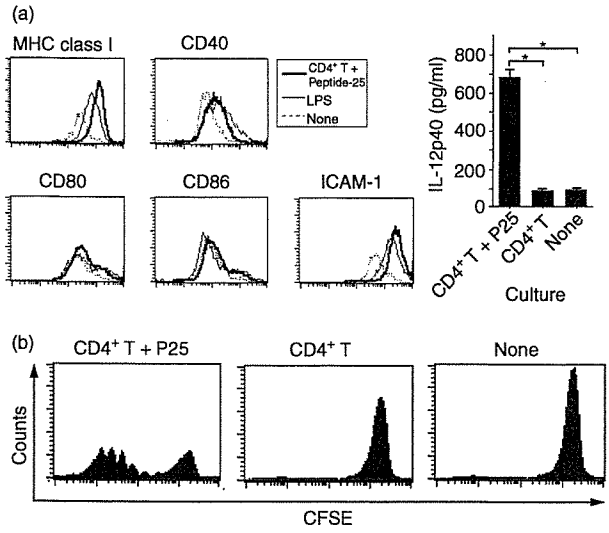


Figure 8. Activation of DCs by culturing with Peptide-25 in the presence of $CD4^+$ T cells from P25 TCR-Tg mice. (a) Immature DCs were propagated by culturing bone marrow cells with GM-CSF (20 ng/ml) and IL-3 (20 ng/ml) for 6 days. The cells recovered (5×10^5) were cultured with lipopolysaccharide (LPS; 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 $I\text{-}A^b$ 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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Macrophage scavenger receptor down-regulates mycobacterial cord factor-induced proinflammatory cytokine production by alveolar and hepatic macrophages

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Abstract

We aimed to reveal the regulatory function of macrophage scavenger receptor-A (MSR-A) in proinflammatory cytokine production by macrophages stimulated with mycobacterial cord factor (CF). By the culture with CF, MSR-A (+/+) alveolar macrophages and Kupffer cells produced TNF-α/MIP-1α in a time- and dose-dependent manner. However, the amounts of cytokines produced by them were much less compared to those produced by MSR-A (−/−) macrophages. Consistent with this, treatment of MSR-A (+/+) macrophages with anti-MSR-A antibody increased TNF-α production. Binding of CF to MSR-A was demonstrated by measuring the binding affinity. These results indicate that CF binds MSR-A, and MSR-A down-regulates TNF-α/MIP-1α production by activated macrophages, suggesting the role of this receptor in suppression of excessive inflammatory responses during mycobacterial infection.

Keywords: Cord factor; Macrophage scavenger receptor; Tumor necrosis factor alpha; Macrophage inflammatory protein-1 alpha; Alveolar macrophage; Kupffer cell

1. Introduction

In mycobacterial infection, the cell-surface cord factor (CF), or trehalose dimycolate, plays an important role in the development of granulomatous inflammation. This function is mediated by tumor necrosis factor-alpha (TNF-α) and macrophage inflammatory protein 1 alpha (MIP-1α), both potent inflammatory cytokines, secreted by activated macrophages [1,2]. In vitro experiments demonstrated TNF-α/MIP-1α production by direct stimulation of cultured macrophages with CF [3], and in vivo studies revealed high levels of TNF-α/MIP-1α in CF-induced pulmonary granulomas in mice [4]. These data indicate that granulomatous inflammation induced by mycobacterial infection seems to be closely related to TNF-α/MIP-1α production by CF-activated macrophages. There are, however, few reports on the regulatory mechanism of cytokine production induced by CF stimulation.

It is recently demonstrated that mice lacking class-A macrophage scavenger receptor (MSR-A) [5] or cyclophilin C-associated protein, a member of scavenger receptor cysteine-rich domain superfamily [6], are more susceptible than wild-type mice to endotoxin shock due to overproduction of TNF-α.

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This fact indicates a suppressive effect of MSR-A on lipopolysaccharide (LPS)-induced TNF-α production. MSR-A was originally reported as receptors that recognize LPS of gram-negative bacteria [7] and lipoteichoic acid of gram-positive bacteria [8]. MSR-A deficient mice were accordingly more susceptible than wild-type mice to infection with *Listeria monocytogenesis* [9] and *Staphylococcus aureus* [10].

In mycobacterial infection as well, MSR-A seems to be engaged in binding of *Mycobacterium tuberculosis* to macrophages [11]. Consistent with this interpretation, macrophages obtained from pulmonary granulomas in the patients with tuberculosis highly express MSR-A [12] and contain numerous bacilli [13]. There are, however, few reports on the macrophage receptors involved in the recognition of CF. In this study, we aimed to reveal the binding of CF to MSR-A and a regulatory role of MSR-A in TNF-α/MIP-1α production by CF-stimulated macrophages, using alveolar macrophages (AMs) and Kupffer cells (KCs) prepared from MSR-A (+/+) and (−/−) mice.

2. Results

2.1. Time- and dose-dependent production of TNF-α by MSR-A (+/+) AMs and KCs after stimulation with CF

We first determined the optimal culture condition for TNF-α production by cultured macrophages. Both AMs and KCs from MSR-A (+/+) mice produced large amounts of TNF-α by treatment with CF, while they produced negligible amounts without stimulation (Fig. 1). Cytokine levels in the culture medium reached a plateau at 24 h after stimulation. When compared to KCs, AMs secreted approximately 7-fold higher amounts of TNF-α. Such TNF-α production was dose-dependent, ranging from 0.1 to 1 μg CF/well, in both AMs and KCs. It, however, considerably decreased at 5 μg/well possibly due to CF cytotoxicity [14]. From these results, we cultured AMs and KCs with a dose of 1 μg/well of CF, and measured TNF-α/MIP-1α at 24 h in the following studies.

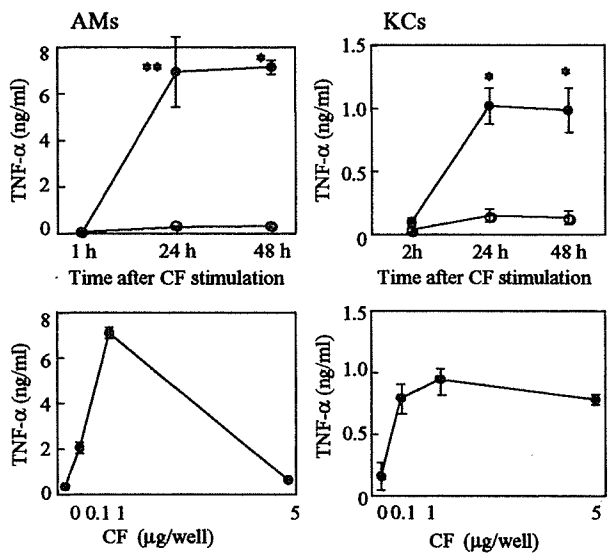

Fig. 1. (Upper columns) Timecourse of TNF-α production by AMs and KCs from MSR-A (+/+) mice after the culture on non-coated (○) and 1 μg CF-coated (●) wells. (Lower columns) Dose-response of TNF-α production by AMs and KCs from MSR-A (+/+) mice at 24 h after the culture. After plating 1.2×10^5 AMs or KCs on each well, the medium was subjected to ELISA analysis. Data represent the mean ± SD for three experiments. *$p<0.01$, **$p<0.05$ vs. non-coated wells.

2.2. Production of TNF-α/MIP-1α by MSR-A (+/+) and (−/−) AMs and KCs after stimulation with CF

Both AMs and KCs from MSR-A (+/+) and those from MSR (−/−) mice produced large amounts of TNF-α/MIP-1α by treatment with CF, whereas they did not produce them without stimulation (Fig. 2). Interestingly, CF-stimulated macrophages from MSR-A (−/−) mice produced significantly higher levels of TNF-α/MIP-1α than those from MSR-A (+/+) mice except for MIP-1α production by KCs. There was no significant difference in cell viability between MSR-A (+/+) and MSR-A (−/−) macrophages or between AMs and KCs (data not shown). Differences in TNF-α/MIP-1α production seen here were 'therefore' not due to differences in susceptibility of macrophages to CF toxicity.

2.3. Binding of CF to MSR-A

To determine whether CF binds MSR-A on cultured macrophages in the present experiment, we conducted a binding assay using a macrophage cell line J774. By stimulating with 1 μg/ml of LPS, J774 cells showed enhanced expression of MSR-A as demonstrated by western blot analysis (Fig. 3a). We cultured these LPS-stimulated J774 cells in either

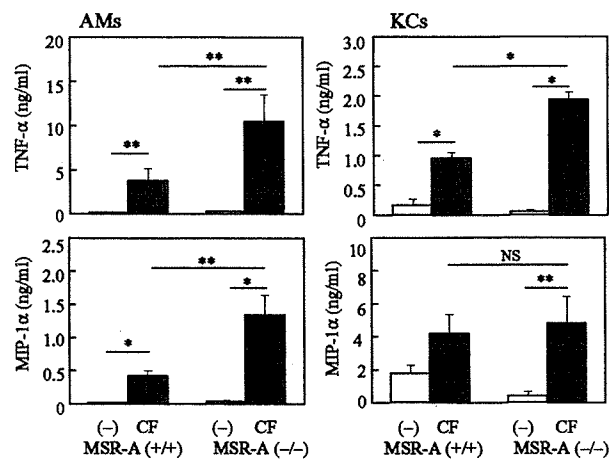

Fig. 2. TNF-α and MIP-1α production by AMs and KCs from MSR-A (+/+) and MSR-A (−/−) mice after incubation for 24 h in the presence (CF) or absence (−) of 1 μg CF. Data represent the mean ± SD for three experiments. *$p<0.01$, **$p<0.05$. NS, not significant.

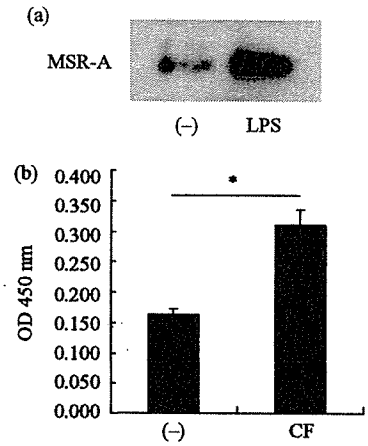

Fig. 3. Binding of CF to MSR-A. a. MSR-A expression is enhanced in LPS-stimulated J774. Western blot analysis. b. Binding affinity of CF to MSR-A. LPS-stimulated J774 was incubated for 24 h in the presence (CF) or absence (−) of 1 μg CF. Binding affinity was determined by ELISA using anti-MSR-A mAb 2F8. Data represent the mean ± SD for three experiments. $*p<0.01$.

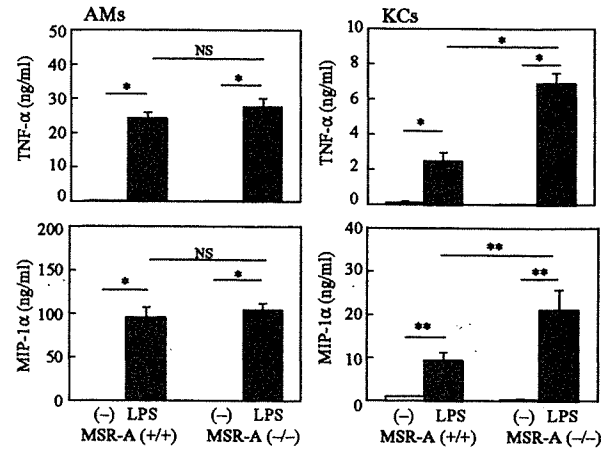

Fig. 5. TNF-α and MIP-1α production in AMs and KCs from MSR-A (+/+) and MSR-A (−/−) mice after incubation for 24 h in the presence (LPS) or absence (−) of 1 μg LPS. Data represent the mean ± SD for three experiments. $*p<0.01$, $**p<0.05$. NS, not significant.

CF-coated or uncoated wells, and measured MSR-A that was bound to the wells. The amount of MSR-A bound to CF-coated wells was significantly larger than that of uncoated wells (Fig. 3b).

2.4. Augmentation of TNF-α production by blocking MSR-A with mAb

In order to demonstrate that enhanced TNF-α production by MSR-A (−/−) macrophages is due to absence of MSR-A, we measured TNF-α in MSR-A (+/+) AMs after treatment with anti-MSR-A mAb 2F8. Twenty four hours after the culture in CF-coated wells, mAb 2F8-treated AMs produced larger amounts of TNF-α than AMs treated with control IgG-treated ones (Fig. 4).

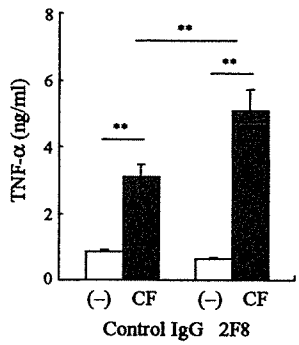

Fig. 4. Increased TNF-α production by blocking MSR-A with mAb. AMs from MSR-A (+/+) stimulated in the presence (CF) or absence (−) of 1 μg CF were treated with 20 μg/ml anti-MSR-A mAb 2F8 or control IgG2b. Data represent the mean ± SD for three experiments. ** $p<0.05$.

2.5. Production of TNF-α and MIP-1α by MSR-A (+/+) and (−/−) AMs and KCs after stimulation with LPS

We next compared CF-induced TNF-α/MIP-1α production with LPS-induced one in MSR-A (+/+) and (−/−) AMs and KCs. By stimulation with 1 μg/well of LPS, AMs and KCs from either MSR-A (+/+) or (−/−) mice produced large amounts of TNF-α and MIP-1α (Fig. 5). The amount of TNF-α and MIP-1α generated by LPS stimulation was larger than that produced by CF stimulation. There was no significant difference in TNF-α and MIP-1α production from AMs between MSR-A (+/+) and (−/−) mice, whereas MSR-A

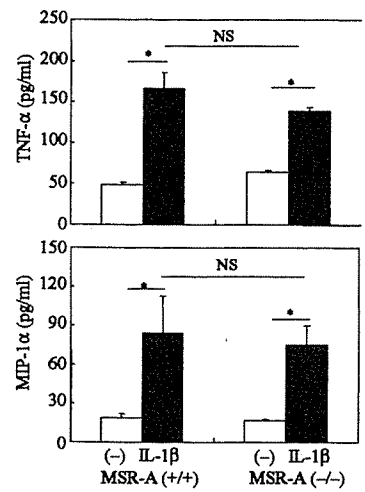

Fig. 6. TNF-α and MIP-1α production in AMs and KCs from MSR-A (+/+) and MSR-A (−/−) mice after incubation for 24 h in the presence (IL-1β) or absence (−) of 1 μg/ml IL-1β. Data represent the mean ± SD for two experiments. $*p<0.01$. NS, not significant.

(−/−) KCs showed enhanced production of TNF-α/MIP-1α compared to MSR (+/+) ones. Toll-like receptor (TLR)-4 is the receptor for lipopolysaccharide (LPS) and mediates signals via myeloid differentiation protein (MyD88) [15], although MSR-A cannot bind to IL-1. In addition, IL-1 utilizes MyD88 as signal transduction [16,17]. To clarify the role of MSR-A, we have examined the effect of IL-1β on macrophages derived from MSR-A (+/+) and (−/−) mice. The results showed that the levels of TNF-α and MIP-1α production from MSR-A (+/+) mice were comparable to those from MSR-A (−/−) mice (Fig. 6). Thus, there were no differences in the production between wild-type and MSR-A deficient mice.

3. Discussion and conclusion

It has been demonstrated that mycobacteria bind multiple distinct macrophage receptors, i.e. complement receptors [18], CD44 [19], CD14 [20], surfactant protein A receptors [21], mannose receptors [20,22], TLR-2 [23] and MSR-A[11]. Among mycobacterial components, CF is essential for the development of granulomatous inflammation because it directly stimulates macrophages to produce TNF-α and MIP-1α [3]. However, macrophage receptors that recognize CF have not been identified. In this study, we have demonstrated that CF binds MSR-A, and induces TNF-α/MIP-1α production in macrophages. Furthermore, experiments using MSR-A (−/−) and (+/+) macrophages revealed that MSR-A down-regulates CF-induced TNF-α/MIP-1α production. Such down regulation of CF-induced TNF-α production was blocked by treatment with anti-MSR-A mAb and the level of TNF-α/MIP-1α production induced by IL-1 β stimulation was not affected by MSR-A that could not bind to IL-1. These findings suggest that absence of MSR-A, but neither the defect of associated molecules like MyD88 in the downstream of a signaling pathway nor compensation by other receptors, is responsible for enhanced TNF-α/MIP-1α production in MSR-A (−/−) mice.

Several studies have demonstrated that the amount of TNF-α produced by LPS binding to MSR-A is much less compared to that produced by binding to CD14 or TLR [5,24,25], and most of LPS binds to MSR-A and remaining free LPS binds CD14 [5,6]. It is thus likely that LPS preferentially binds to MSR-A in MSR-A (+/+) mice, resulting in low production of TNF-α. By contrast, LPS binds to CD14 in MSR-A (−/−) mice, leading to more abundant TNF-α production. This is consistent with our present results. [Different from TNF-α/MIP-1α production by LPS-stimulated KCs, that by AMs was not affected by the presence of MSR-A, probably because AMs produce larger amounts of TNF-α and MIP-1α than KCs [26], and MSR-A might not sufficiently inhibit CD14-mediated TNF-α/MIP-1α production [25]. Similar mechanism might be present in the down-regulation of TNF-α/MIP-1α production by MSR-A from CF-stimulated macrophages. Thus, CF might bind to other receptors, for example TLR of MSR-A (−/−) macrophages, and produce more abundant TNF-α. However, there is still controversy about the involvement of TLR, because mycobacterial lipids, including CF, induce TNF-α production in a TLR-2-dependent manner [27], and similar cytokine production is observed in wild-type mice and in mice lacking either TLR-2 or TLR-4 [28].

In conclusion, the present study implies that MSR-A down-regulates TNF-α/MIP-1α production by macrophages stimulated with CF and LPS, and plays a preventive role in excessive inflammatory responses during microbial infection.

4. Materials and methods

4.1. Mice

MSR-A (+/+) and (−/−) mice, 15–20 weeks old, were gifted from Chugai Pharmaceut. Co. Ltd (Tokyo, Japan). They were housed in a specific pathogen-free condition and fed chow pellet and water ad libitum. Experiments were conducted according to the guideline for animal experiments of Osaka City University Graduate School of Medicine.

4.2. Preparation of CF and LPS

CF was prepared as previously described [4]. Briefly, *M. tuberculosis* AOYAMA-B was cultivated in Sauton medium for 5–6 weeks at 37 °C. Bacterial cells were autoclaved at 121 °C for 30 min, and then harvested. The lipids were serially extracted with chloroform–methanol (4:1, vol/vol), chloroform-methanol (3:1, vol/vol) and chloroform–methanol (2:1, vol/vol). CF (C78-88, C26-α-unit) was purified by developing the lipids on a thin-layer plate of silica gel with chloroform–methanol–acetone–acetic acid (90:10:6:1, vol/vol/vol/vol) and then with chloroform–methanol–water (90:10:1, vol/vol/vol). Purified CF did not contain detectable levels of LPS. LPS of *Salmonella enteritidis* by a phenol–water method was purchased from Sigma-Aldrich (St Louis, MO).

4.3. Isolation of AMs and KCs

AMs were obtained by a repeated lavage of the lung with phosphate-buffered saline (PBS) of anesthetized mice with an intraperitoneal injection of pentobarbital. Lavaged cells were centrifuged at 200×*g* for 10 min and suspended in Tris–NH4Cl solution to remove erythrocytes. After washing with PBS twice, they were suspended in RPMI 1640 containing 10% fetal calf serum and 100 μg streptomycin and 100 U of penicillin per milliliter. KCs were isolated by the pronase–collagenase method [29]. The liver was perfused via the portal vein with SC-1 solution (NaCl 8 g/l, KCl 400 mg/l, NaH_2PO_4–$2H_2O$ 88 mg/l, Na_2HPO_4 120 mg/l, HEPES 2,380 mg/l, $NaHCO_3$ 350 mg/l, EGTA190 mg/l, glucose 900 mg/l, pH 7.4) for 3 min, with SC-2 solution (NaCl 8 g/l, KCl 400 mg/l, NaH_2PO_4–$2H_2O$ 88 mg/l, Na_2HPO_4 120 mg/l, HEPES 2,380 mg/l, $NaHCO_3$ 350 mg/l, CaCl2–$2H_2O$ 560 mg/l, pH 7.4) containing 0.05% pronase E for 5 min, and then with SC-2 solution containing 0.05% collagenase for 10 min. The liver was excised, cut into small pieces, and incubated in the SC-2 solution containing 0.05% pronase E, 0.05% collagenase, and 20 μg/ml of DNase for 45 min at 37 °C. The cell suspension

was filtered through a steel mesh with a pore size of 150 μm, and centrifuged on 17% Nycodenz cushion. A non-parenchymal cell-enriched fraction containing KCs was obtained from the upper layer. This fraction was subjected to the elutriation with a CR20B2 elutriator (Hitachi, Tokyo, Japan) at a pump speed of 40–68 ml/min in 200 ml. Cells were cultured on a plastic dish in RPMI 1640 containing 10% fetal calf serum for 45 h. More than 95% of AMs and KCs obtained by these procedures phagocytozed 0.8 μm latex beads, and the purity was always more than 95%. Cells obtained from 3 to 5 mice were pooled, and used for each experiment.

4.4. Stimulation of macrophages with either CF or LPS

Ninety six-well plastic plates were coated with 0.1, 1, 5 μg/well of CF dissolved in isopropyl alcohol. Control plates were prepared by placing isopropyl alcohol alone. These plates were dried overnight in a biosafety cabinet. AMs or KCs in the number of 1.2×10^5 were cultured. The similar number of AMs or KCs was stimulated with 1 μg/well of LPS or 1 μg/ml of IL-1β (Peprotech EC, UK). The culture supernatant was stored at −80 °C until cytokine assays. After removing the culture supernatant, the cell viability was determined by measuring the quantity of formazan product using a Cell Proliferation Assay Kit (Promega, Madison, WI).

4.5. Cytokine assay

The concentrations of TNF-α and MIP-1α were determined using the ELISA kit (Genzyme Techne, Minneapolis, MN). Sensitivity limits of ELISAs were as 5 pg/ml for TNF-α and 1.5 pg/ml for MIP-1α.

4.6. Western blot analysis

MSR-A expression in macrophages was analysed by western blot analysis using a mAb against MSR-A (2F8; Serotec, Oxford, UK) and ECL Western Blotting Analysis System (Amersham, Aylesbury, UK).

4.7. Assay for the binding affinity of CF to MSR-A

We used LPS-stimulated J774 cells, a mouse macrophage cell line, for the assay [30]. Twenty-four hours after the culture on CF-coated or non-coated wells, they were lysed with 1% Triton X for 1 min, and washed out with 0.05% Tween 20. MSR-A that bound coated or uncoated CF and remained on the plate after washing was colored by subsequent reactions, i.e., incubation with 2F8 overnight at 4 °C, incubation with horseradish peroxidase-conjugated anti-goat antibody for 1 h at room temperature, and addition of the substrate solution. Optical density (OD) was measured at the wavelength of 450 nm.

4.8. Blockade of MSR-A with MAb

MSR-A (+/+) AMs were incubated with 20 μg/ml anti-MSR-A mAb 2F8 or isotype-matched control antibody (Rat IgG2b, Serotec, Oxford, UK) for 10 min at room temperature and subsequent 10 min at 37 °C before the culture on CF-coated or non-coated wells [31]. After 24-h incubation, concentrations of TNF-α in the culture medium were determined by ELISA.

4.9. Statistics

The data were expressed as the mean ± standard deviation (SD). The results were analyzed by the one-way ANOVA and Scheffe test for further analysis and considered significant if P value were less than 0.05.

Acknowledgements

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感染症の現状と制圧戦略

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はじめに

世界の年間総死亡（2004年）は5,700万人，内訳として，循環器疾患：1,670万人，感染症：1,490万人，悪性新生物：710万人であり，感染症は総死亡の１／４強を占め，人類に大きな健康被害を招来している[1)2)]（表－１）。世界保健機関（World Health Organization，WHO）はヒト免疫不全ウイルス（HIV）感染症／後天性免疫不全症候群（AIDS），結核およびマラリアに対し，緊急事態を宣言している。加えて，非感染性疾患として

表－１　世界における死因別死亡数（2004）

死因	死亡数：万人	死亡総数に対する割合：%
全死亡	5、700（108.4）	100（100）
循環器疾患	1、670（ 30.6）	29（ 28.0）
感染症	1、490（ 13.0）	26（ 12.0）
悪性新生物	710（ 32.6）	12（ 30.1）
不慮の事故死	520（ 4.0）	9（ 3.7）

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（ ）内は厚生労働省．平成17年人口動態統計月報年計（概数）の概況．

1) World Health Organization. The world health report 2005, http : //www.who.int/whr/2005/en/.

2) 厚生労働省．平成17年人口動態統計月報年計（概数）の概況．http : //www.mhlw.go.jp/toukei/saikin/hw/jinkou/geppo/nengai 05/toukei 6.html

認識されていた悪性新生物（胃癌：ヘリコバクターピロリ菌，肝細胞癌：ＢおよびＣ肝炎ウイルス，子宮頚癌：ヒト乳頭腫ウイルスなど）にも病原体感染が関与し，すなわち，感染症は広範で甚大な健康被害を惹起している。また，感染症による新たな脅威として，2001年10月，炭疽菌による生物テロリズムが現実となり，米国で肺炭疽患者の５名が死亡した[3]。

最近まで，日本を含めた先進諸国では感染症を解決された過去の疾患と錯覚し，その対策を怠ってきた。しかし，都市化による過密，人口の集中，貧困，交通機関の発達による人民の高速移動，国際化，環境破壊や温暖化など，現代社会の直面している状況が感染症の増加に関与している。感染症は病原体と宿主の生存戦争である。再興病原体は抗微生物薬に耐性を獲得し，また，新興病原体は人類に新たな脅威を提供している[4]。また，宿主側の要因として，感染抵抗力の減弱（人口の高齢化，免疫抑制薬／臓器移植や免疫疾患）が易感染性を惹起している。

これら感染症の脅威に対し，世界保健機関やGroup of Eight（Ｇ８）サミットは３大感染症，すなわち，ヒト免疫不全ウイルス感染症／後天性免疫不全症候群，結核およびマラリアを指定し，積極的に対応することを宣言している[5][6]。本稿では，感染症の脅威や制圧対策について，概説する。

１　感染症による健康被害

世界の年間総死亡（2004年）は5,700万人，内訳として，循環器疾患：

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3) Centers for Disease Control and Prevention. Public health emergency preparedness and response, http : //www.bt.cdc.gov/.

4) Morens, D. M., G. K. Folkers, and A.S. Fauci. 2004. The challenge of emerging and re-emerging infectious diseases. Nature 430 : 242-249.

5) World Health Organization. http : //www.who.int/.

6) The Okinawa summit 2000 archives, http : //www.summit-okinawa.gr.jp/e/index.htm.

1,670万人，感染症：1,490万人（総死亡の約１／４強）であることは前述したが，感染症死亡の内訳では，呼吸器感染症：396万人，後天性免疫不全症候群（結核合併を含む）：277万人，下痢性疾患：180万人，結核：156万人，マラリア：127万人である[1]（表－２）。呼吸器感染症や下痢性疾患の原因病原体は多種多様であることから，世界保健機関やＧ８サミット[5][6]は１）ヒト免疫不全ウイルス感染症／後天性免疫不全症候群，２）結核および３）マラリア（熱帯熱マラリア）など単一病原体感染症による死亡が年間約500万人，患者発生が３億人であるため，これら３大疾患を最も重要な感染症に認定し，世界が協調して対策を構築することを宣言している（表－２）。

２　感染症の将来発生動向予測

感染症の発生動向予測として，社会基盤の整備，抗微生物化学療法やワクチンで治療・予防可能な疾患は将来的に減少することが考えられるが，その他の感染症（ヒト免疫不全ウイルス感染症／後天性免疫不全症候群や結核）は今後も現状維持あるいは増加することが予測されている。人口増

表－２　世界における感染症による死亡数（2004）

感染症	死亡数：万人
全感染症	１、４９０
急性呼吸器感染症	３９６
ＡＩＤＳ（結核合併を含む）	２７７
下痢性疾患	１８０
結核	１５６
マラリア	１２７
ワクチン予防可能疾患	１１２
性感染症	１８
髄膜炎	１７
ウイルス性肝炎（ＢおよびＣ型）	１６
熱帯性原虫・寄生虫疾患	１３
その他の感染症	１７６

The World Health Report 2005，世界保健機関

加と都市化に伴う自然環境破壊，交通機関の発達に伴う国際交流の激増は，世界の疾病構造に変化を惹起し，特定地域に発生した感染症に対し，自国の問題として迅速に対処しなければならない状況にある。このように，感染症の国際化（globalization）が進む新時代に対応するためには，民族や国境を越えた感染症対策の構築，すなわち，新時代に即応した国際協力体制の確立が必要となる[7]。

3 新興・再興感染症

新興感染症（emerging infectious diseases）は「最近約30年間（1970年以降）に，新たに発見された感染病原体，あるいは，かつては不明であった病原体により惹起され，地域的あるいは国際的に公衆衛生上問題となっている新感染症」，他方，再興感染症（re-emerging infectious diseases）は「既知感染症で，発生数が減少し，公衆衛生上ほとんど問題にならなくなっていたが，近年再び出現／増加している感染症」と定義されている[4]。

新興感染症として，30以上の感染症／病原体が報告され，代表的疾患として，クリプトスポリジウム症，エボラ出血熱，ラッサ熱，レジオネラ症，腸管出血性大腸菌感染症，ヒト免疫不全ウイルス（HIV）感染症／後天性免疫不全症候群（AIDS），H. pylori 感染症（胃炎，胃・十二指腸潰瘍，胃癌），C型ウイルス性肝炎，プリオン病(ウシ海綿状脳症，Creutzfeldt-Jakob 病)，鳥型インフルエンザやウエストナイル熱（ウエストナイル脳炎を含む）や重症呼吸器症候群などがある（表－3）。再興感染症として，結核，ペスト，ジフテリア，劇症型A群溶連菌，マラリア，デング熱

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7) Center for Population and Development Studies at the Harvard School of Public Health. Burden of disease unit, http://www.hsph.harvard.edu/organizations/bdu/index.html.

表－3 代表的な新興感染症

年	感染症
1973	ロタウイルス感染症（乳児下痢症）
1976	クリプトスポリジウム症（急性および慢性下痢症）
1977	エボラ出血熱 レジオネラ症（肺炎）
1982	腸管出血性大腸菌感染症（食中毒、溶血性尿毒症性症候群） ボレリア感染症（ライム病）
1983	*H. pylori* 感染症（胃炎、胃・十二指腸潰瘍、胃癌） ヒト免疫不全ウイルス感染症（後天性免疫不全症候群）
1989	C型ウイルス性肝炎（慢性肝炎、肝硬変、肝細胞癌）
1992	新型コレラ
1996	プリオン病（伝達性海綿状脳症、Creutzfeldt-Jakob 病）
1997	高病原性鳥インフルエンザ（H5N1）
1999	ウエストナイルウイルス感染症（脳炎を含む） ニパウイルス感染症（脳炎を含む）
2003	重症急性呼吸器症候群（SARS）

などがある[8]。

日本において，結核は最大の感染症であり，2004年の新規登録患者数：約29,700（罹患率：23.3／対人口10万人），死亡：約2,300人（死亡率：1.8／対人口10万人）であった[9]。世界的に日本は結核対策において中進国である（アメリカ合衆国：5.1，スウェーデン：4.3／対人口10万人）。結核対策上，我が国が対応を迫られている問題点として，1）一般国民，医療関係者，行政の結核に対する認識の低下，2）特異的，迅速かつ簡便な結核菌感染の検査法の開発，3）高齢結核患者の増加（70歳以上患者：約44%），4）蔓延状況の地域格差（大阪市：61.7，東京都特別区：34.7，長

8) World Health Organization. Communicable disease surveillance and response, http : //www.who.int/emc/.

9) 厚生労働省健康局結核感染症課．平成16年結核発生動向調査年報集計結果（概況），http : //www.mhlw.go.jp/bunya/kenkou/kekkaku-kansenshou 03/04.html.