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Th1 Cell Adjuvant Therapy Combined with Tumor Vaccination: A Novel Strategy for Promoting CTL Responses while Avoiding the Accumulation of Tregs

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Abstract

We have previously described a method for adoptive immunotherapy of cancer based on antigen-specific Th1 cells. However, efficient induction of antitumor responses using Th1 cells remains a formidable challenge, especially for MHC class II-negative tumors. In the present study, we sought to develop a novel strategy to eradicate established tumors of the MHC class II-negative, OVA-expressing EG-7. Tumor-bearing mice were intradermally (i.d.) treated with OVA-specific Th1 cells, combined with the model tumor antigen (OVA), near the tumor-draining lymph node (DLN). We found that tumor growth was significantly inhibited by this strategy and about 50~60% of tumor-bearing mice were completely cured. Tumor eradication was crucially dependent on the generation of OVA/H-2K^b-specific CTL in the tumor DLN and tumor site. The injected Th1 cells were mainly distributed in tumor DLN, where they vigorously proliferated and enhanced the activation of DC. Strikingly, we also found that the accumulation of $CD4^+$ $CD25^+$ Treg cells was significantly inhibited in tumor DLN by Th1 cell adjuvant therapy and this abrogation was associated with IFN-γ secreted by Th1 cells. These results identify Th1 cell adjuvant therapy combined with tumor vaccination as a novel approach to the treatment of human cancer.

Introduction

There is considerable interest in developing immunotherapeutic approaches to elicit tumor-specific CTL responses in tumor-bearing hosts suffering from strong immunosuppression (1-3). Many investigators have attempted to overcome this problem by active immunization with MHC class I-binding peptides or peptide-pulsed DC, based on the observation that most tumors are MHC class I positive but lack MHC class II molecules (4-6). However, recently, it has been demonstrated that tumor-vaccine therapy using MHC class I-binding peptides showed very low clinical response rates, although this approach was able to induce peptide-specific CTL in these patients (7,8). Thus, tumor-vaccine therapy focused only on CTL activation appeared to be insufficient to induce potent antitumor immunity in tumor-bearing hosts (8-10). A major problem in such a strategy is that effective $CD8^+$ T cell responses require concomitant activation of $CD4^+$ T helper (Th) cells. $CD4^+$ Th cells play a critical role in the induction, migration, and maintenance of $CD8^+$ CTL (12-14) and in the generation of memory CTL responses (15-17).

In a series of studies (18-23), we have demonstrated a critical role of Th1 immunity for induction of tumor-specific CTL in tumor-bearing mice. In a previous report (18), we demonstrated that adoptive transfer of antigen-specific Th1 cells into mice bearing established, MHC class II-expressing A20-OVA tumors resulted in the induction of tumor-specific CTL, which were able to completely eradicate the tumor mass. In contrast to MHC class II^+ tumors, it has been demonstrated that MHC class II^- tumors are refractory to immunotherapy. We have also

demonstrated that, in contrast with MHC class II^+ A20-0VA tumors, it was hard to eradicate established, MHC class II^- EG-7 tumors from mice by Th1 cell transfer alone (19). The resistance of MHC class II^- EG-7 tumors against Th1 cell therapy may be due to the lack of direct interaction with tumor cells at the local tumor site. However, injection of the model tumor antigen, OVA, into the tumor tissue was able to overcome this problem and augmented the interaction between APC and Th1 cells and promoted Th1 cell proliferation and CTL generation in the tumor draining LN, which is essential for complete tumor eradication (19). These findings suggested a promising new strategy for tumor immunotherapy, with the caveat that this approach requires knowledge regarding the relevant tumor-associated antigens.

It has been well recognized that tumor-draining lymph nodes (DLN) play an important role in initiating antitumor immune responses. In addition, it has been reported that the tumor DLN contain tumor-specific T cell precursors (24-27). These findings suggest that tumor DLN represent potent cell sources for specific immunotherapy against cancer. Based on these considerations, we developed a novel tumor immunotherapy technique, termed Th1 cell adjuvant therapy. In this model, tumor-specific Th1 cells are intradermally (i.d.) injected near the tumor DLN together with a tumor model antigen, OVA. Tumor regression was demonstrated in all mice treated with this vaccination and about 50~60% of all mice showed complete tumor rejection. Tumor eradication was dependent on host-derived $CD8^+$ CTL. Moreover, we found that Th1 cells injected i.d. with OVA vigorously proliferated and accelerated the activation of dendritic cells (DC) and

tumor-specific CTL predominantly in the tumor DLN, but not in distal lymphoid organs. We also provide the first evidence that injection of Th1 cells together with tumor antigen can suppress accumulation of $CD4^+$ $CD25^+$ Treg cells in DLN, and this downregulation is controlled by IFN-γ secreted by Th1 cells.

Thus, we have established a novel protocol for tumor immunotherapy, which permits the generation of potent tumor-specific CTL responses and avoids the generation of tumor-specific Tregs.

Materials and methods

Mice. All mice were age- and sex- matched female C57BL/6. C57BL/6 mice were obtained from Charles River Japan (Yokohama, Japan). OT-II TCR transgenic mice, expressing a TCR specific for the $I\text{-}A^b$-restricted 323-339 peptide from OVA, were kindly provided by Dr. F.R. Carbone (University of Melbourne, VIC, Australia) (28). IFN-$\gamma R^{-/-}$ mice on a C57BL/6 background were kindly provided by Dr. Y. Iwakura (University of Tokyo, Tokyo). Mice at 5 to 6 weeks of age were used in experiments. All animals were maintained in specific pathogen-free conditions.

Reagents. IL-12 was kindly donated by Wyeth Research (Cambridge, MA). IL-2 was supplied by Dr. T. Sawada (Shionogi Pharmaceutical Institute Co. Ltd., Osaka, Japan). IFN-γ was purchased from Pepro Tech EC ltd. (London, England). Anti-IL-4 mAb (11B11) was purchased from American Type Culture Collection (Rockville, MD). PE-anti-CD4 mAb, PE-anti-CD11b, PE-anti-B220, PE-anti-NK1.1, PE-Cy7-anti-CD8, FITC-anti-CD80, FITC-anti-CD86, FITC-anti-CD45RB mAb,

FITC-anti-CD8 mAb, FITC-anti-CD69 mAb, and PE-anti-CD11c mAb were purchased from PharMingen (San Diego, CA). Anti-CD8 mAb-conjugated microbeads for the MACS system were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-CD8 mAb for $CD8^+$ T cell depletion was purchased from MEIJINYUGYO (Kanagawa, Japan). PE-labeled tetrameric H-$2K^b$ molecules loaded with the $OVA_{257-264}$ peptide SIINFEKL (OVA Tetramer) were purchased from MBL (Nagoya, Japan). OVA protein was purchased from Sigma-Aldrich Japan (Tokyo, Japan).

Generation of OVA-specific Th1 cells from OT-II TCR-transgenic mice. $CD4^+$ $CD45RB^+$ naïve T cells were isolated from nylon-passed spleen cells of OT-II TCR-transgenic mice using FACS Aria (Becton Dickinson, San Jose, CA). Purified $CD4^+$ $CD45RB^+$ cells were stimulated with 5μg/mL $OVA_{323-339}$ peptide in the presence of mitomycin C (MMC)-treated spleen cells, 100U/mL IL-2, 20U/mL IL-12, 1ng/mL IFN-γ, and 50μg/mL anti-IL-4 mAb for Th1 development. At 48hr, cells were restimulated with $OVA_{323-339}$ under the same conditions and used at 9-12 days of culture.

Detection of CFSE-labeled Th1 cells in vivo. CFSE was purchased from Molecular Probes (Eugene, Oregon) and used for monitoring daughter cell generation in vivo as indicated in the manufacturer's instructions. In brief, 2 μL of a CFSE stock solution (5 mmol/L in DMSO) was incubated with 10 mL of Th1 cells (1 x 10^7/mL) in PBS for 5 minutes at room temperature. Cells were washed thrice with 10% FCS-containing medium. Three days after the transfer of CFSE-labeled Th1 cells (2 x 10^7 per mouse), the generation of daughter cells by the labeled Th1 cells in DLN, distant LN, spleen, or tumor tissue was analyzed using FACSCalibur. Fluorescence

data were collected with logarithmic amplification.

Tetramer staining. We used a slightly modified version of the original manufacturer's instructions to stain cells with OVA-MHC tetramers. Lymphocytes isolated from tumor DLN or the tumor site were washed twice with phosphate-buffered saline and stained with 3.3 μL/mL of OVA-MHC tetramers per $1x10^6$ cells at 4°C for 15min, followed by staining with FITC-labeled anti-CD8 mAb. The cells were washed again with phosphate-buffered saline and analyzed by FCM. As described previously (21), tetramer-blocking assays for determining the fine MHC-restricted peptide antigen specificity was carried out by co-culture of ^{51}Cr-labeled target cells with effector cells, which were prelabeled with tetramer reagent.

Flow cytometric analysis. Detailed procedures for staining and sorting have been described previously (18). Fluorescence data were collected on a FACSCalibur (Becton Dickinson, San Jose, CA, USA), and analyzed using CellQuest software (BD Biosciences, Mountain View, CA, USA). mAbs used for experiments are listed in the reagent section.

Th1 cell adjuvant therapy. A total of $2x10^6$ MHC class II-negative EG-7 cells were i.d. inoculated into C57BL/6 mice to generate OVA^+ tumors. When the tumor mass became large (7~8 mm), the tumor-bearing mice were i.d. injected nearby the tumor DLN with saline, OVA protein (200μg/mouse), OVA-specific Th1 cells ($2x10^7$/mouse) or Th1 cells plus OVA. Antitumor activity mediated by the transferred cells was determined by measuring tumor size in perpendicular

diameters. Tumor volume was calculated by the following formula: tumor volume= 0.4 x length (mm) x [width (mm)] ^{2} (18). Tumor-bearing mice that survived for more than 60 days after therapy were considered completely cured. The mean of 5 mice per group is indicated in the figures.

Cytotoxicity assay. The cytotoxicity mediated by tumor-specific CTL was measured by 6 hr-^{51}Cr-release assay as described previously (29). Tumor-specific cytotoxicity was determined using EG-7 cells (OVA gene transfected EL-4 cells) as target cells. Parental EL-4 cells were used as control target cells. To confirm the antigen-specificity of H-2K^{b}-restricted CTL, ^{51}Cr-labeled target cells were incubated with CD8^{+} CTL pretreated with OVA-tetramer, which blocks the recognition of antigen by CTL. CD8^{+} T cells were enriched by MACS system according to the manufacturer's protocol. The percent cytotoxicity was calculated as described previously (29).

Immunohistochemical analysis. Snap-frozen, Tissue-Tek-embedded lymph nodes (five for each group) were cut at 5-7mm, fixed for 10min in cold acetone and used for immunohistochemical analysis. We used an already established double-immunofluorescence staining protocol for Foxp3 and CD4 (30). Briefly, the primary antibodies were diluted in 1% BSA (polyclonal rabbit antibody against murine Foxp3 at a concentration of 5 μg/mL and rat anti-mouse CD4 antibody from BD Pharmingen (diluted 1：500). The secondary antibody was applied at a 1：500 dilution (anti-rat IgG-Alexa 546, anti-rabbit IgG-Alexa 488; Alexa, Leiden, The Netherlands). Finally, the samples were analyzed with Olympus FV500 confocal laser scanning microscope system (Olympus Optical, Tokyo, Japan).

Results

Eradication of established, MHC class II-negative tumors by Th1 cell adjuvant therapy combined with tumor antigen vaccination

C57BL/6 mice were inoculated with $2x10^6$ MHC class II-negative EG-7 cells expressing OVA as a model tumor antigen. When the tumor mass became large (7~8 mm), the tumor-bearing mice were treated with i.d. injection of saline, OVA protein (200μg/mouse), OVA-specific Th1 cells ($2x10^7$) or Th1 cells plus OVA, near the tumor DLN. OVA-specific Th1 cells were derived from T-cell receptor-transgenic (OT II) mouse spleen cells. In prior studies (18), we have demonstrated that Th1 cells cannot induce the regression of MHC class II-negative tumors, even when Th1 cells were i.v. transferred into tumor-bearing mice. However, adoptively transferred Th1 cells were able to eradicate the tumor mass when combined with intratumor (i.t.) injection of a model tumor antigen (19). This finding indicated that activation of APC that had processed tumor antigens by Th1 cells at the local tumor site might be essential for inducing potent tumor-specific immunity in vivo that can eradicate an established tumor mass. To test our working hypothesis, we developed a novel tumor-vaccine protocol using the model tumor antigen (OVA), together with tumor-specific Th1 cells as a potent cell adjuvant. Th1 cells, together with tumor antigen, were injected i.d. near the tumor DLN. After 3 rounds of therapy, tumor-bearing mice treated with Th1 cell adjuvant combined with OVA showed significant regression of their tumor mass and about 50~60% of mice were completely tumor-free. However, immunotherapy with Th1 cells or protein alone had little effect on tumor growth and the survival rate of the tumor-challenged mice (Fig. 1). Such complete

cure of tumor-bearing mice was induced only when mice were treated with i.d. injection of OVA with $2x10^7$ Th1 cells but not with $2x10^6$, $5x10^6$ or 10^7 Th1 cells (Fig. 2). In this protocol, we used OVA protein antigen for Th1 cell adjuvant cell therapy. However, we have already confirmed that MHC class II-binding OVA peptide, but not MHC class I OVA peptide, can promote antitumor immunity to block the growth of tumor combined with Th1 cell adjuvant though it caused no complete cure of tumor-bearing mice. A significant enhancement of Th1 cell adjuvant therapy was observed when synthetic MHC class II and class I peptides were used for antigenic vaccination (data not shown). These results indicated that Th1 cells act as a potent cell adjuvant for tumor antigen to induce complete regression of a well-established, MHC class II-negative tumor.

Th1 cell adjuvant therapy in the presence of antigen-specific vaccination promotes Th1 cell proliferation and potently activates DC in DLN of tumor-bearing mice

Th1 cells labeled with CFSE were injected i.d. together with OVA into tumor-bearing C57BL/6 mice to monitor the number of cell divisions of the injected Th1 cells in vivo. Lymphoid tissues (DLN, distal LN and spleen) and the tumor mass were collected from the mice 3 days after treatment and analyzed for CFSE dilution by flow cytometry. We found that injected Th1 cells were present at low, yet detectable levels in the distal LN, spleen and tumor tissue (data not shown). In contrast, high numbers of CFSE-positive Th1 cells were present in tumor DLN. When Th1 cells were co-injected with OVA antigen, the CFSE dilution profiles indicated that Th1 cells had undergone multiple rounds of cell division in tumor DLN (Fig. 3A-b). Such extensive proliferation of

CFSE-labeled Th1 cells was not observed in distal LN, spleen, and tumor tissues at the same time (3 days after 1st therapy) though Th1 cell proliferation was observed at later time (data not shown). Treatment of tumor-bearing mice with Th1 cells alone caused no significant cell proliferation of CFSE-labeled Th1 cells (Fig. 3A-a). These data indicate that tumor DLN are critical lymphoid organs for the migration and activation of injected Th1 cells in our immunotherapy model.

It is now well established that tumor DLN play an important role in initiating early immune responses against tumor cells (26,27). APC, especially DC, have an essential role in the regulation of primary immunity. To investigate the role of Th1 cells in DC activation, we examined the expression of co-stimulatory molecules and CD69, an early activation marker, on DC in tumor DLN. Mice injected i.d. with OVA protein or Th1 cells alone did not induce a significant change in the surface expression levels of CD86, CD80, or CD69 on DC. In contrast, i.d. injection of Th1 cells with OVA had a strong impact on the activation of DC, as evidenced by profound up-regulation of the expression of costimulatory molecules such as CD86 and CD80 in addition to early activation marker, CD69 (Fig. 3B). We further showed that, when the mice were treated with Th1 cell adjuvant and tumor antigen, the absolute numbers of DC were increased (Fig. 3C). In addition to DC, it was demonstrated that various immunoregulatory cells including CD8+ T cells, NK cells, MΦ, and B cells were activated at DLN (Fig. 4). These results indicate that Th1 cell adjuvant therapy can activate both innate and acquired immunity cells in tumor DLN.

Generation of tumor antigen-specific CTL in tumor-bearing mice by treatment with Th1 cell

adjuvant and tumor antigen

The interaction of host DC with Ag-specific Th1 cells in tumor DLN might facilitate the induction of tumor-specific CTL derived from naive, endogenous $CD8^+$ T cells in tumor-bearing mice. To address this possibility, lymphocytes were prepared from DLN or tumor tissue 3 days after the second round of therapy and examined for the generation of OVA-specific CTL by staining with $OVA_{257-264}$/H-$2K^b$-tetramers. The frequency of $tetramer^+$ $CD8^+$ CTL in mice treated with Th1 cell adjuvant plus tumor antigen was markedly elevated in tumor DLN ($2.7 \pm 0.42\%$) and within the tumor tissue ($35.5 \pm 5.15\%$) compared with that in the mice treated with Th1 cells or tumor antigen alone (Fig. 5A).

The cells recovered from DLN were also assayed for cytotoxic activity against OVA-positive EG-7 and OVA–negative EL-4 tumor cells. Vaccination with Th1 cells alone or OVA antigen alone induced weak or negative cytotoxic responses against EG-7. In contrast, lymphocytes isolated from mice treated with Th1 cell adjuvant combined with OVA showed potent cytotoxicity against EG-7 cells (Fig. 5B-a). In addition, the $CD8^+$ CTL isolated from DLN of tumor-bearing mice treated with Th1 cell adjuvant plus OVA exhibited strong specific cytotoxicity against EG-7 tumor cells. Moreover, this cytotoxicity was strongly blocked by OVA/H-$2K^b$-tetramer (Fig 5B-c). No significant cytotoxicity against OVA-negative EL-4 parental tumor cells was detected in the cytotoxicity assay of any experimental groups (Fig 5B-b and d).

Taken together, these data demonstrate that vaccination of tumor-bearing mice with Th1 cell

adjuvant plus model tumor antigen (OVA) effectively induces OVA/H-2K^b-specific CD8$^+$ CTL that can exhibit strong tumor-specific cytotoxicity in tumor-bearing mice.

Requirement of host-derived CD8$^+$ T cells for tumor eradication by treatment with Th1 cell adjuvant plus OVA

To directly assess the possible requirement of CD8$^+$ CTL in the protective effect induced by Th1 cell adjuvant plus OVA, mice were treated with anti-CD8 mAb injection to deplete CD8$^+$ CTL precursors prior to treatment. As shown in Fig. 6, depletion of CD8$^+$ T lymphocytes from tumor-bearing mice caused a complete ablation of the protective effect of Th1 cell adjuvant therapy combined with tumor antigen vaccination. Thus, we concluded that CD8$^+$ tumor-specific CTL are critical for complete tumor eradication.

Th1 cell adjuvant therapy inhibits the accumulation of Foxp3$^+$ CD4$^+$ Treg cells in DLN of tumor-bearing mice

Immunotherapy against large tumor masses has generally been ineffective because tumors themselves often elicit strong immunosuppressive responses (21,31-33). The success of our strategy indicated that Th1 cell adjuvant could effectively induce anti-tumor responses by overcoming the strong immunosuppressive environment in the tumor-bearing host. Although mechanisms underlying tumor-induced immunosuppression are complex and diverse, recent studies have implicated a number of regulatory T cell subsets in the maintenance of immune tolerance against self and tumor antigens (35-37). To investigate the possible impact of Th1 cell

adjuvant therapy on these regulatory cells, we examined the frequency of $Foxp3^+$ $CD4^+$ Treg in tumor DLN. DLN were separated from tumor-bearing mice 3 days after the second round of therapy and the frequency of Treg cells was examined by immunohistochemical analysis. The number of Treg cells in DLN of tumor-bearing mice was markedly increased (Fig. 7b) as compared with normal mice (Fig. 7a). When tumor-bearing mice were treated with Th1 cell adjuvant plus OVA, the number of Treg cells was significantly reduced (Fig. 7c). A slight decrease of Treg accumulation in tumor DLN of the tumor-bearing mice treated with Th1 cells alone but not OVA alone (Fig. 7d and e). This might be because that slight activation of Th1 cells was induced in tumor DLN via small number of tumor-antigen-pulsed DC migrated from the tumor tissues. To determine which factors are involved in this blockade in Treg cell accumulation, we utilized IFN-$\gamma R^{-/-}$ mice. As shown in Fig. 7f and g, the number of Treg increased in IFN-$\gamma R^{-/-}$ tumor-bearing mice compared with untreated IFN-$\gamma R^{-/-}$ mice. In contrast to wild-type mice, a strong Treg cell accumulation was induced in the IFN-$\gamma R^{-/-}$ tumor-bearing hosts even when the mice were treated with Th1 cell adjuvant therapy (Fig. 7h). Taken together, these results suggest that Th1 cell adjuvant therapy has the capacity to suppress Treg cell accumulation at the local tumor site, which might be due to IFN-γ produced by Th1 cells.

Discussion

It has been well-established that tumor DLN play an important role in cancer immunotherapy (19,26-27). The immune defense to tumor cells is initiated in these nodes, where antigen-presenting DC migrate and activate $CD4^+$ and $CD8^+$ T cells specifically reactive to tumor antigen (19,24-27). Based on this evidence, in the present paper, we developed a novel tumor-vaccine therapy model using Th1 cell adjuvant. When tumor-bearing mice were treated with i.d. injection of tumor-specific Th1 cells and tumor antigen near the tumor DLN, complete tumor-regression was induced concomitantly with efficient induction of tumor-specific CTL (Fig. 1 and 5), in the absence of Treg accumulation in the tumor DLN (Fig. 7). Adoptively transferred Th1 cells dominantly distributed into DLN but not in spleen and distal LN (data not shown). This preferential distribution of Th1 cells in DLN permitting these cells to facilitate the initiation of antitumor responses within the local tumor site. Th1 cell adjuvant therapy facilitated the migration and activation of DC in tumor DLN (Fig. 3B and C). Tumor-specific CTL were induced in vaccinated mice, and these cells were also enriched in DLN and the tumor site, as compared with other lymphoid organs (data not shown). More than 50% of tumor-bearing mice treated with Th1 cell adjuvant therapy were completely cured from the tumor (Fig. 1). The improved antitumor response observed in these mice was strongly dependent on activation of $CD8^+$ Ag-specific CTL, because OVA/H-$2K^b$ specific CTL activity was enriched in $CD8^+$ T cells (Fig. 5), and depletion of $CD8^+$ T cells in vivo by anti-CD8 mAb injection completely abrogated the therapeutic effect of Th1 cell adjuvant therapy (Fig. 6).

The exact mechanism(s) underlying Th1 cell adjuvant therapy combined with tumor antigen remains unclear. However, it is possible that transferred Th1 cells first migrate into tumor DLN and specifically interact with DC that had taken up tumor antigens. The enhanced interactions between DC and Th1 cells via costimulatory molecules and cytokines further promote the differentiation of host-derived naïve $CD8^+$ T cells into tumor-specific CTL, which are the relevant effector cells for complete regression of the tumor mass (19,21).

Current immunotherapeutic strategies have demonstrated promising results for tumor treatment. Experimental evidence clearly shows that vaccines elicit effective responses against early, microscopic tumors, but vaccines have been far less successful against established, large tumor masses (11). These poor antitumor responses in mice bearing a large tumor mass may be because tumors themselves can produce immunosuppressive factors such as TGF-β, PGs, and IL-10 (38,39). Indeed, TGF-β derived from tumors is a critical factor for the differentiation of $CD4^+$ $CD25^+$ regulatory T (Treg) cells, which have been recognized as a major immunosuppressive component in the tumor-bearing host. Treg cells play a critical role in immunologic self-tolerance as well as in antitumor immune responses and organ rejection during transplantation (41,42). Several studies have reported an increase in the number of Treg cells in tumor-bearing hosts in both animal and human systems (31,44-46). An increase in Treg cell numbers was also seen in our experimental model (Fig. 7b). It has been demonstrated that Treg cells can impair the induction of both antigen-specific and non-specific T cell immunity (34,43) and an increase in

these cells is predictive of reduced survival in cancer patients (31). Moreover, it has been shown that Tregs can inhibit NK and NKT cell-mediated innate immune responses. Consistent with these findings, depletion of Tregs by anti-CD25 mAb resulted in enhancement of effective tumor immune responses via removal of strong immunosuppression (11). In the present study, we found that the frequency of $Foxp3^+$ $CD4^+$ regulatory T (Treg) cells in tumor-bearing mice was significantly decreased by Th1 cell adjuvant therapy combined with tumor antigen vaccination. The observed blockade in Treg cell accumulation appeared to be due to IFN-γ produced in tumor-bearing mice following Th cell adjuvant therapy, because such inhibitory effect was not observed when IFN-$\gamma R^{-/-}$ mice were used as tumor-bearing hosts (Fig. 7c and h). These data suggested that Th1 cells are the major source of IFN-γ for overcoming Treg cell accumulation. We have never examined whether EG-7-bearing IFN-$\gamma R^{-/-}$ mice treated with Th1 cell adjuvant and OVA showed deficiency in tumor eradication in parallel with the increase of Treg populations. However, depletion of $CD4^+$ $CD25^+$ Treg by treatment with anti-CD25 mAb completely inhibited the growth of EG-7 tumor cells (data not shown), indicating that cancellation of Treg-dependent down-modulation in IFN-$\gamma R^{-/-}$ may deteriorate antitumor effects induced by Th1 cell adjuvant therapy. We are now investigating about this issue. It should be noted that the frequency of Treg in DLN of tumor-bearing IFN$\gamma R^{-/-}$ mice was increased rather than suppressed by treatment with Th1 cell adjuvant plus OVA, as compared with untreated control mice. This increase in Tregs may be accounted for by IL-2, another important cytokine secreted by Th1 cells. IL-2, regarded as the main growth factor necessary for the

proliferation and survival of T lymphocytes, has been used as an adjuvant to stimulate the immune system for treatment of multiple tumors (36). However, clinical trials using IL-2 showed limited efficacy for tumor immunotherapy (36,37). Accumulating evidence from knockout mice has suggested that IL-2 is crucial for the homeostasis and function of $CD4^+$ $CD25^+$ regulatory T cells in vivo (42). Clinical research also has shown that the frequency of Treg cells was significantly increased after IL-2 treatment (40). It is possible that IL-2 administration may impede antitumor immune responses through activation of Tregs. Therefore, the balance between the differentiation of tumor-reactive effector T cells and Treg, both of which are controlled by IL-2, may determine the clinical outcome for tumor patients. In our Th1 cell adjuvant therapy model, IFN-γ may antagonize the effect of IL-2 on Treg cells, and provide help for the induction of antigen-specific CTL.

In this paper, we emphasized the importance of tumor DLN for the activation of Th1 cells and the subsequent generation of $tetramer^+$ CTL, which are essential for complete cure of tumor-bearing mice. However, when Th1 cells were i.d. injected with OVA distantly from tumor, detectable number of $tetramer^+$ CTL (1.7%) were also induced at DLN of vaccination site though higher percentage of CTL (2.56%) were demonstrated at tumor DLN even by this systemic Th1 cell adjuvant therapy (data not shown). Therefore, i.d. injection of Th1 cell adjuvant with OVA near tumor DLN may be the best protocol for inducing antitumor immunity to cure tumor-bearing mice. But Th1 cell adjuvant appeared to be effective to induce tumor-specific CTL at tumor local site even when it was injected at distal site from the tumor tissues. This might be because Th1 cells

function as cytokine-producing cell adjuvant, which facilitate their own active migration into tumor-DLN via lymphatic system. In contrast to Th1 cell adjuvant, systemic treatment of tumor-bearing mice with CpG-containing liposomes co-encapsulated with OVA caused a significant inhibition of tumor, but this strategy did not induce complete cure of the mice (data not shown). Thus, Th1 cell adjuvant appeared to be superior to CpG, a powerful, well-known adjuvant, in systemic vaccination therapy. In summary, the data presented here indicate that tumor-vaccine therapy using tumor-specific Th1 cells combined with tumor antigen can efficiently cure established, large MHC class II-negative tumors. In the previous paper, we reported that i.v. injection of Th1 cells combined with i.t. injection of model tumor antigen (OVA) was an efficient method to induce tetramer^{+} CTL at tumor local site to cure tumor-bearing mice (19). However, the application of that method is limitted to visible tumor mass on skin such as melanoma. In contrast, i.d. injection of Th1 cell adjuvant near tumor DLN is appicable to every tumor-bearing hosts. Moreover, systemic antitumor immunity is induced in tumor-bearing mice even when Th1 cell adjuvant is i.d. injected with tumor antigen at distal site of tumor tissue. Both protocols for Th1 cell therapy combined with tumor antigen may induce antitumor immunity by the same mechanisms including (i) acceleration of DC/Th1 cell interaction, (ii) Th1 cell activation, and (iii) acceleration of tumor-specific CTL induction. Here, in addition to promotion of both innate and acquired antitumor immune responses, we initially demonstrate that our novel Th1 cell adjuvant therapy inhibits the accumulation of Treg cells in tumor DLN.

Thus, our data presented here identify Th1 cell adjuvant therapy combined with tumor antigen vaccination as a promising therapeutic strategy for cancer. In our tumor therapy model, we used tumor cell expressing a strong xenogeneic antigen (OVA). We have never demonstrated whether our developed Th1 cell adjuvant therapy combined with endogenous tumor antigen is applicable to tumor expressing weak native tumor antigen. However, it has been reported that activation of DC in vivo with adjuvant plus weak tumor antigen protein (carcinoembryonic antigen) induced a strong antitumor immunity (47). Thus, natural tumor-antigen was shown to have a capability of inducing tumor-specific CTL in vivo, although it is a weak self-antigen. Therefore, we believe that Th1 cell adjuvant therapy, which can activate both DC and CTL function in vivo, may also induce an efficient antitumor immunity against weak tumor antigen and may be applicable to clinical trial although there are still unresolved problems.

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Abbreviation

OVA	ovalbumin
CTL	cytotoxic T lymphocyte
i.d.	intradermally
DC	dendritic cell
DLN	draining lymph node
Treg	$CD4^+CD25^+$ regulatory T cells
APC	antigen-presenting cells

References

1. Clark, W.R., Walsh, C.M., Glass, A.A., Hayashi, F., Matloubian, M., Ahmed,.R. 1995. Molecular pathways of CTL-mediated cytotoxicity. *Immunol Rev*.146:33.

2. Ward, P.L., Koeppen, H.K., Hurteau, T., Rowley, D.A., Schreiber, H. 1990. Major histocompatibility complex class I and unique antigen expression by murine tumors that escaped from $CD8^+$ T-cell-dependent surveillance. *Cancer Res*. 50:3851.

3. Golumbek, P.T., Lazenby, A.J., Levitsky, H.I, Jaffee, L.M., Karasuyama, H., Baker, M., Pardoll, D.M. 1991. Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4. *Science*. 254:713.

4. Melief, C.J., Toes, R.E., Medema, J.P., S.H. van der Burg, S.H., Ossendorp, F., Offringa, R. 2000. Strategies for immunotherapy of cancer. *Adv Immunol*. 75:235.

5. Scupoli, M.T., Sartoris, S., Tosi, G., Ennas, M.G., Nicolis, M., Cestari, T., Zamboni, G., Martignoni, G., Lemoine, N.R., Scarpa, A., Accolla, R.S. 1996. Expression of MHC class I and class II antigens in pancreatic adenocarcinomas. *Tissue Antigens.* 48:301.

6. Bander, N.H., Yao, D., Liu, H., Chen, Y., Steiner, M., Zuccaro, W., Moy, P. 1997. MHC class I and II expression in prostate carcinoma and modulation by interferon-alpha and -gamma. *Prostate.* 33:233.

7. Marchand, M., van Baren, N., Weynants, P., Brichard, V., Dreno, B., Tessier, M.H., Rankin, E., Parmiani, G., Arienti, F., Humblet,Y., Bourlond, A., Vanwijck, R., Lienard, D., Beauduin, M., Dietrich,

P.Y., Russo, V., Kerger, J., Masucci, G., Jager, J. De Greve, E., Atzpodien, J., Brasseur, F., Coulie, P.G., van der Bruggen, P., Boon, T. 1999. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. *Int J Cancer.* 80:219.

8. Rosenberg, S.A., Yang, J.C., Restifo, N.P. 2004. Cancer immunotherapy: moving beyond current vaccines. *Nat Med.* 10:909.

9. Ribas, A., Butterfield, L.H., Glaspy, J.A., Economou, J.S. 2003. Current developments in cancer vaccines and cellular immunotherapy. *J Clin Oncol.* 21:2415

10. Coulie, P.G., van der Bruggen, P. 2003. T-cell responses of vaccinated cancer patients. *Curr Opin Immunol.* 15:131.

11. Nicholl, M., Lodge, A., Brown, I., Sugg, S.L., Shilyansky, J. 2004. Restored immune response to an MHC-II-Restricted antigen in tumor-bearing hosts after elimination of regulatory T cells. *J Pediatr Surg.* 39:941.

12. Hunziker, L., Klenerman, P., Zinkernagel, R.M., Ehl, S. 2002. Exhaustion of cytotoxic T cells during adoptive immunotherapy of virus carrier mice can be prevented by B cells or $CD4^+$ T cells. *Eur J Immunol.* 32:374.

13. Baxevanis, C.N., Voutsas, I.F., Tsitsilonis, O.E., Gritzapis, A.D., Sotiriadou, R., Papamichail, M. 2000. Tumor-specific $CD4^+$ T lymphocytes from cancer patients are required for optimal induction of cytotoxic T cells against the autologous tumor. *J Immunol.* 164:3902.

14. Marzo, A.L., Kinnear, B.F., Lake, R.A., Frelinger, J.J., Collins, E.J., Robinson, B.W., Scott, B. 2000. Tumor-specific $CD4^+$ T cells have a major "post-licensing" role in CTL mediated anti-tumor immunity. *J Immunol.* 165:6047.

15. Janssen, E.M., Droin, N.M., Lemmens, E.E., Pinkoski, M.J., Bensinger, S.J., Ehst, B.D., Griffith, T.S., Green, D.R., Schoenberger, S.P. 2005. $CD4^+$ T-cell help controls $CD8^+$ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature.* 434:88.

16. Sun, J.C., Bevan, M.J. 2003. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science.* 300:339.

17. Gao, F.G., Khammanivong, V., Liu, W.J., Leggatt, G.R., Frazer, I.H., Fernando, G.J. 2002. Antigen-specific $CD4^+$ T-cell help is required to activate a memory $CD8^+$ T cell to a fully functional tumor killer cell. *Cancer Res*. 62:6438.

18. Nishimura, T., Iwakabe, K., Sekimoto, M, et al. 1999. Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. *J Exp Med.*190:617.

19. Chamoto, K., Wakita, D., Narita, Y., Zhang, Y., Noguchi, D., Ohnishi, H., Iguchi, T., Sakai, T., Ikeda, H., Nishimura, T. 2006. An essential role of antigen-presenting cell/T-helper type 1 cell-cell interactions in draining lymph node during complete eradication of class II-negative tumor tissue by T-helper type 1 cell therapy. *Cancer Res.* 66:1809.

20. Ikeda, H., Chamoto, K., Tsuji,T., Suzuki, Y., Wakita, D., Takeshima, T., Nishimura, T. 2004. The critical role of type-1 innate and acquired immunity in tumor immunotherapy. *Cancer Sci*. 95:697.

21. Wakita, D., Chamoto, K., Zhang, Y, Narita, Y., Noguchi, D., Ohnishi, H., Iguchi, T., Sakai, T., Ikeda,H., Nishimura,T. 2006. An indispensable role of type-1 IFNs for inducing CTL-mediated complete eradication of established tumor tissue by CpG-liposome co-encapsulated with model tumor antigen. *Int Immunol*. 18:425.

22. Suzuki, Y., Wakita, D., Chamoto, K, Narita, Tsuji, T., Takeshima, T., Gyobu, H., Kawarada,Y., Kondo, S., Akira, S., Katoh, H., Ikeda, H., Nishimura, T. 2004. Liposome-encapsulated CpG oligodeoxynucleotides as a potent adjuvant for inducing type 1 innate immunity. *Cancer Res*. 64:8754.

23. Chamoto, K., Tsuji, T., Funamoto, A., Kosaka, H., Matsuzaki, J., Sato, T., Abe, H., Fujio, K., Yamamoto, K., Kitamura, T., Takeshima, T., Togashi, Y., Nishimura, T. 2004. Potentiation of tumor eradication by adoptive immunotherapy with T-cell receptor gene-transduced T-helper type 1 cells. *Cancer Res*. 64:386.

24. Rennert, P.D., Hochman, P.S., Flavell, R.A, Chaplin, D.D., Jayaraman, S., Browning, J.L., Fu, Y.X. 2001. Essential role of lymph nodes in contact hypersensitivity revealed in lymphotoxin-alpha-deficient mice. *J Exp Med*.193:1227.

25. Miller, M.J., Wei, S.H., Parker, I., Cahalan, M.D. 2002. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science.* 296:1869.

26. Bousso, P., Robey, E. 2003. Dynamics of $CD8^+$ T cell priming by dendritic cells in intact lymph nodes. *Nat Immunol*. 4:579.

27. Mempel, T.R., Henrickson, S.E., Von Andrian, U.H. 2004. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature*. 427:154.

28. Barnden, M.J., Allison, J., Heath, W.R., Carbone, F.R. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based a- and h-chain genes under the control of heterologous regulatory elements. *Immunol Cell Biol.* 6:34.

29. Nishimura, T., Burakoff, S.J., Herrmann, S.H. 1987. Protein kinase C required for cytotoxic T lymphocyte triggering. *J Immunol*.139:2888.

30. Hontsu, S., Yoneyama, H., Ueha, S., et al. 2004. Visualization of naturally occurring $Foxp3^+$ regulatory T cells in normal and tumor-bearing mice. *Int Immunopharmacol*. 4:1785.

31. Curiel, T.J., Coukos, G., Zou, L., et al. 2004. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med*.10:942.

32. Berthier-Vergnes, O., Bermond, F., Flacher, V., Massacrier, C., Schmitt, D., Peguet-Navarro, J. 2005. TNF-alpha enhances phenotypic and functional maturation of human epidermal Langerhans cells and induces IL-12 p40 and IP-10/CXCL-10 production. *FEBS Lett*. 579:3660.

33. Yang, L., DeBusk, L.M., Fukuda, K., Fingleton, B., Green-Jarvis, B., Shyr, Y., Matrisian, L.M., Carbone, D.P., Lin, P.C. 2004. Expansion of myeloid immune suppressor Gr^+CD11b^+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell*. 6:409.

34. Mukherji, B., Guha, A., Chakraborty, N.G., Sivanandham, M., Nashed, A.L., Sporn, J.R., Ergin, M.T.1989. Clonal analysis of cytotoxic and regulatory T cell responses against human

melanoma. *J Exp Med*. 169:1961.

35. Viguier, M., Lemaitre, F., Verola, O., Cho, M.S., Gorochov, G., Dubertret, L., Bachelez, H., Kourilsky, P., Ferradini, L. 2004. Foxp3 expressing $CD4^{+}CD25$ (high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J Immunol*. 173:1444.

36. Grande, C., Firvida, J.L., Navas, V., Casal, J. 2006. Interleukin-2 for the treatment of solid tumors other than melanoma and renal cell carcinoma. *Anticancer Drugs*. 17:1.

37. Rosenberg, S.A., Yang, J.C., Topalian, S.L., Schwartzentruber, D.J., Weber, J.S., Parkinson, D.R., Seipp, C.A., Einhorn, J.H., White. D.E. 1994. Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2. *JAMA*. 271:907.

38. Gajewski, T.F., Meng, Y., Harlin, H. 2006. Immune suppression in the tumor microenvironment. *J Immunother*. 29:233.

39. Wang, D., Dubois, R.N. 2006. Prostaglandins and cancer. *Gut*. 55:115.

40. Ahmadzadeh, M., Rosenberg, S.A. 2006. IL-2 administration I ncreases $CD4^{+}CD25$(hi) Foxp3+ regulatory T cells in cancer patients. *Blood.*107:2409.

41. Sakaguchi, S. 2000. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell*. 101:455.

42. Antony, P.A., Restifo, N.P. 2005. $CD4^{+}CD25^{+}$ T regulatory cells, immunotherapy of cancer, and interleukin-2. *J Immunother*. 28:120.

43. Chakraborty, N.G., Twardzik, D.R., Sivanandham, M., Ergin, M.T., Hellstrom, K.E., Mukherji, B. 1990. Autologous melanoma-induced activation of regulatory T cells that suppress cytotoxic response. *J Immunol*. 145:2359.

44. Woo, E.Y., Chu, C.S., Goletz, T.J., Schlienger, K., Yeh, H., Coukos, G., Rubin, S.C., Kaiser, L.R., June, C.H. 2001. Regulatory $CD4^+CD25^+$ T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res*. 61:4766.

45. Ichihara, F., Kono, K., Takahashi, A., Kawaida, H., Sugai, H., Fujii, H. 2003. Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. *Clin Cancer Res*. 9:4404.

46. Liyanage, U.K., Moore, T.T., Joo, H.G., Tanaka, Y., Herrmann, V., Doherty, G., Drebin, J.A., Strasberg, S.M., Eberlein, T.J., Goedegebuure, P.S., Linehan, D.C. 2002. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol*. 169:2756.

47. Okano, F., Merad, M., Furumoto, K., Engleman, E.G. 2005. In Vivo Manipulation of Dendritic Cells Overcomes Tolerance to Unmodified Tumor-Associated Self Antigens and Induces Potent Antitumor Immunity. *J Immunol.* 174:2645.

Figure legends

Figure 1. Eradication of established MHC class II-negative tumors by Th1 cell adjuvant therapy combined with tumor antigen vaccination.

EG-7 cells were intradermally (i.d.) inoculated into C57BL/6 mice. When the tumor mass became palpable (7~8mm), the mice were i.d injected with saline (control, open cycle in A and B; photo C), OVA protein (200μg/mouse; filled triangle in A and B; photo D), OVA-specific Th1 cells ($2x10^7$; open triangle in A and B; photo E), or Th1 cells plus OVA protein (filled cycle in A and B; photo F) near the DLN. The anti-tumor effect was determined by measuring the tumor size (A) and by assessing the survival ratio (B). Results are shown as the mean and SD of two independent experiments, with five mice per a group at each time point.

Figure 2. Th1 cell adjuvant therapy remarkably inhibits growth of the tumors, dose dependently.

MHC class II-negative EG-7 cells were i.d. inoculated into C57BL/6 mice. When the tumor mass became palpable (7~8mm), the tumor-bearing mice were i.d. injected with saline (control, filled cycle) or various numbers of Th1 cells ($2x10^6$/mouse; filled triangle, $5x10^6$/mouse; open triangle, $1x10^7$/mouse; filled square, $2x10^7$/mouse; open cycle) combined with OVA protein (200μg/mouse) near the DLN. Growth of the tumors was monitored every other day for 4 weeks. Two independent experiments were carried out with five mice per a group. Data are shown as the mean and SE of

five mice in each experimental group.

Figure 3. Th1 cell adjuvant therapy promotes Th1 cell proliferation and activates DC in DLN of tumor-bearing mice.

(A) Tumor-bearing mice were treated with Th1 cells alone (b) or Th1+OVA (a). Th1 cells were prelabeled with CFSE as described in Materials and Methods. Three days after the primary therapy, the tumor DLN were examined for the rate of proliferation of CFSE-labeled Th1 cells. (B) Absolute cell numbers in DLN of the tumor-bearing mice were determined 3 days after treatment with saline (control; opened square), OVA (striped square), or Th1+OVA (filled square). Results represent the mean and SE of five mice in each experimental group. Similar results were obtained in three separate experiments. (C) Surface expression levels of CD86, CD80, and CD69 on CD11c+ DC from DLN of tumor-bearing mice 24 hours after treatment with saline (control; fine line), OVA, Th1, or Th1+OVA (bold lines) were examined by FACS analysis. Similar results were obtained in three separate experiments and representative data were shown in the figures.

Figure 4. Th1 cell adjuvant therapy activates both innate and acquired immunity cells in DLN.

DLN were prepared from tumor-bearing mice 48 hours after treatment with saline (control), OVA, Th1 cells, or Th1 cells + OVA. Then, surface expression levels of CD69 on CD8+ T cells, NK1.1+

TCR- NK cells, CD11c+ dendritic cells (DC), CD11b+ macrophages (MΦ) and B220+ B cells in the DLN were examined by FACS analysis. The number attached in each profile means the percentage of each CD69+ cell population indicated as CD8, NK, DC, MΦ, and B cells. Similar results were obtained in three separate experiments and representative data were shown in the figures.

Figure 5. Generation of tumor-specific CTL in tumor-bearing mice by treatment with Th1 cell adjuvant combined with tumor antigen vaccination

(A) Induction of tumor-specific CTL in tumor-bearing mice was carried out by the treatment with Th1+OVA. The population of H-2K^{b}/OVA$_{257-264}$-tetramer^{+} CD8^{+} T cells in DLN (a-d) and TIL (e-h) of tumor-bearing mice was examined 3 days after the 2nd round of therapy with saline (control; a and e), OVA (b and f), Th1 (c and g), or Th1+OVA (d and h). The percentage of OVA-tetramer positive cells among the total CD8^{+} T cell population is plotted in the figure. (B) Mice bearing EG-7 tumors were treated twice with saline (control, open cycle), OVA antigen (filled triangle), Th1 cells (open triangle), or Th1+OVA (filled cycle) as described in the legend of Figure 1. Unfractionated DLN cells in each group were prepared 3 days after the 2nd round of therapy and the cytotoxicity against EG-7 (a) or EL-4 (b) cells was measured by 6-hour ^{51}Cr-release assay. CD8- and CD8^{+} T cells were isolated from DLN of EG-7-bearing mice, which were treated with Th1+OVA. To determine specificity against H-2K^{b}-restricted peptides, CD8^{+} T cells were further treated with

H-2K^{b}/OVA-tetramer. Then, their cytotoxicity of CD8- T cells (filled triangle), CD8+ T cells (filled circle), and the CD8+ T cells plus OVA-tetramer against EG-7 (c) or EL-4 (d) cells were examined by ^{51}Cr-release assay. Results indicate the mean and SE of triplicate samples.

Figure 6. Requirement of host-derived CD8^{+} T cells for tumor eradication by treatment with Th1 cell adjuvant plus OVA

Mice bearing EG-7 tumors were treated twice with saline (filled triangle in A and B) or Th1+ OVA (open cycle, filled cycle) at 6, 9, and 12 days after tumor inoculation. Mice were also treated with i.v. injection of saline (filled triangle in A and B; photo C), anti-CD8 mAb (open cycle in A and B; photo D) or rat immunoglobulin M (IgM; filled cycle; photo E) at 5, 6, 9,12, 15, 18, and 21 days. Tumor size was measured as described in the legend to Figure 1. The anti-tumor effect was determined by measuring the tumor size (A) and by assessing the survival ratio (B). Data are shown as the mean and SD of representative of two independent experiments, with three or four mice per a group at each time point.

Figure 7. Th1 cell adjuvant therapy suppresses the accumulation of Foxp3^{+} CD4^{+} Treg cells in DLN of tumor-bearing mice

Tissue sections of DLN from untreated (a and f), tumor-bearing (b and g), Th1+OVA-treated (c and h), OVA-treated (d), or Th1-treated (e) C57BL/6 and IFN-gR-/- mice were stained with anti-Foxp3

mAb, followed by goat anti-rabbit IgG Alexa 488. The Foxp3+ cells were visualized as green signals. Anti-CD4 mAb was used to determine the T cell area of LN. The sections treated with anti-CD4 mAb were sequentially stained with goat anti-rat IgG Alexa 546, indicated as red signals. Representative data (original magnification, x 200) are shown in the figures.

Fig.1

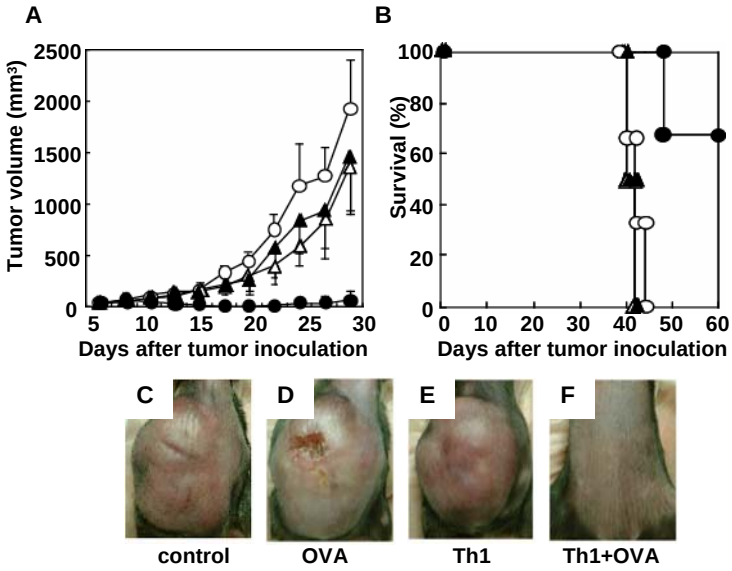
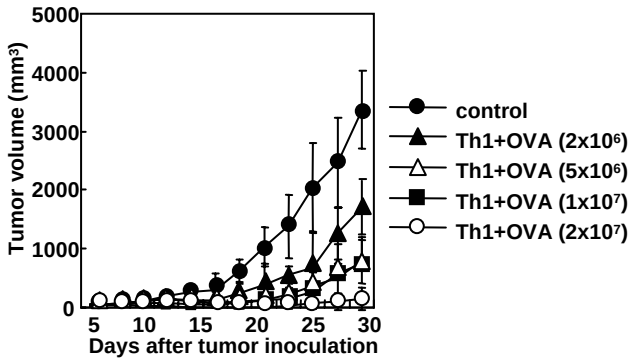

A
B
Tumor volume (mm³)
2500
2000
1500
1000
500
0
5
10
15
20
25
30
Days after tumor inoculation
Survival (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Days after tumor inoculation
C
D
E
F
control
OVA
Th1
Th1+OVA

Fig.2


5000
4000
3000
2000
1000
0
Tumor volume (mm³)
5
10
15
20
25
30
Days after tumor inoculation
control
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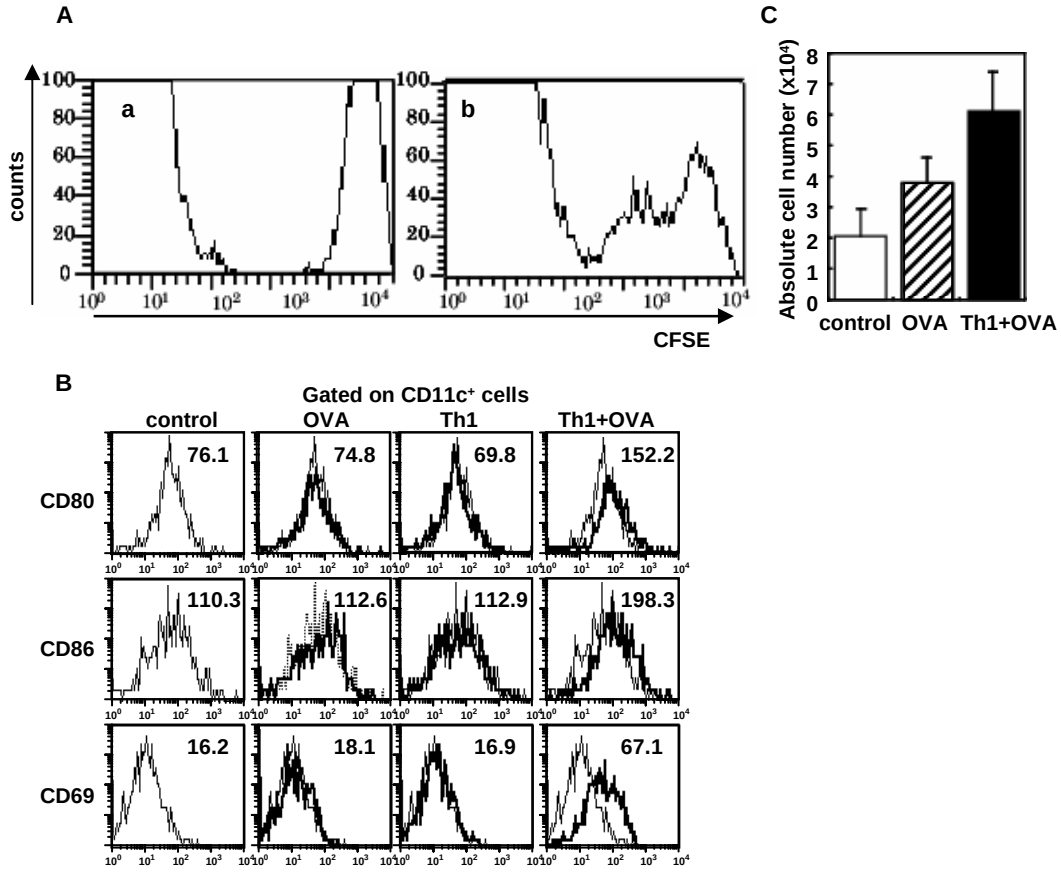
Th1+OVA (5x10⁶)
Th1+OVA (1x10⁷)
Th1+OVA (2x10⁷)

Fig.3


A
a
b
counts
CFSE
C
Absolute cell number (x10⁴)
control
OVA
Th1+OVA
B
Gated on CD11c⁺ cells
control
OVA
Th1
Th1+OVA
CD80
CD86
CD69
76.1
74.8
69.8
152.2
110.3
112.6
112.9
198.3
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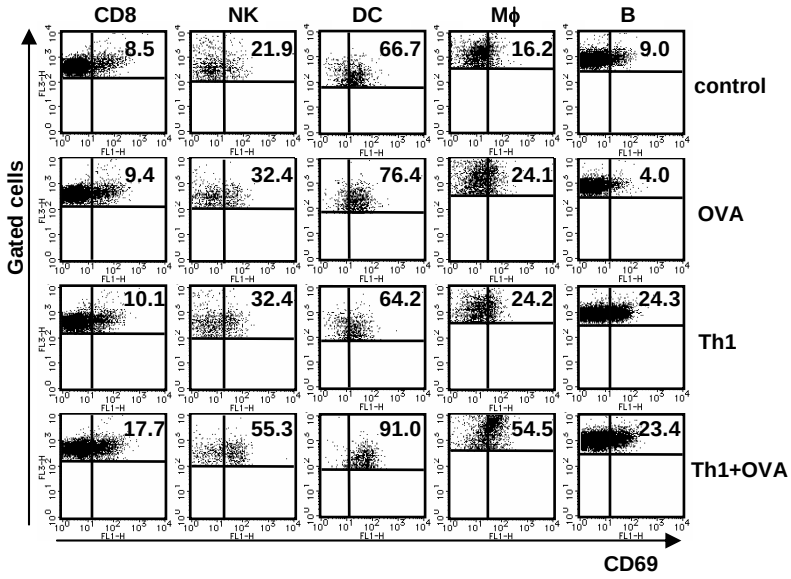
16.9
67.1

Fig.4


CD8
NK
DC
Mϕ
B
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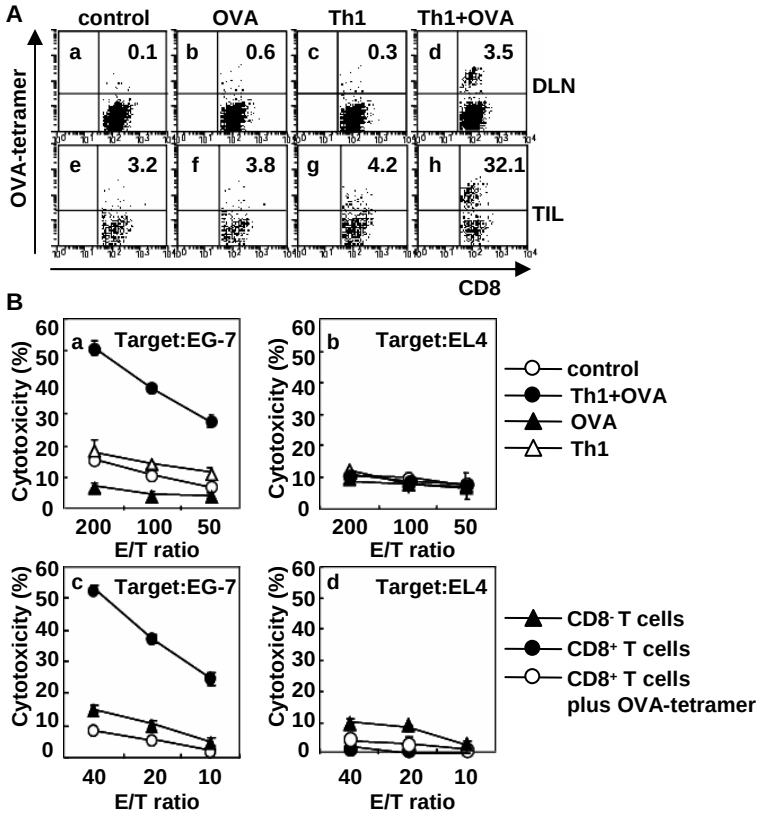
control
9.4
32.4
76.4
24.1
4.0
OVA
10.1
32.4
64.2
24.2
24.3
Th1
17.7
55.3
91.0
54.5
23.4
Th1+OVA
Gated cells
CD69
FL1-H
FL3-H

Fig.5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control
OVA
Th1
Th1+OVA
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DLN
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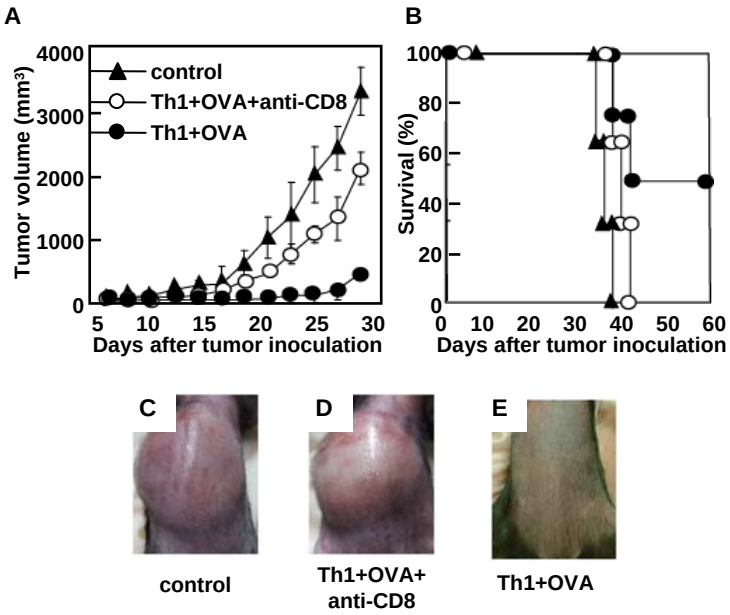
g 4.2
h 32.1
TIL
OVA-tetramer
CD8
B
a Target:EG-7
b Target:EL4
c Target:EG-7
d Target:EL4
Cytotoxicity (%)
E/T ratio
control
Th1+OVA
OVA
Th1
CD8- T cells
CD8+ T cells
CD8+ T cells plus OVA-tetramer

Fig.6


A
B
Tumor volume (mm³)
4000
3000
2000
1000
0
control
Th1+OVA+anti-CD8
Th1+OVA
5 10 15 20 25 30
Days after tumor inoculation
Survival (%)
100
80
60
40
20
0
0 10 20 30 40 50 60
Days after tumor inoculation
C
D
E
control
Th1+OVA+
anti-CD8
Th1+OVA

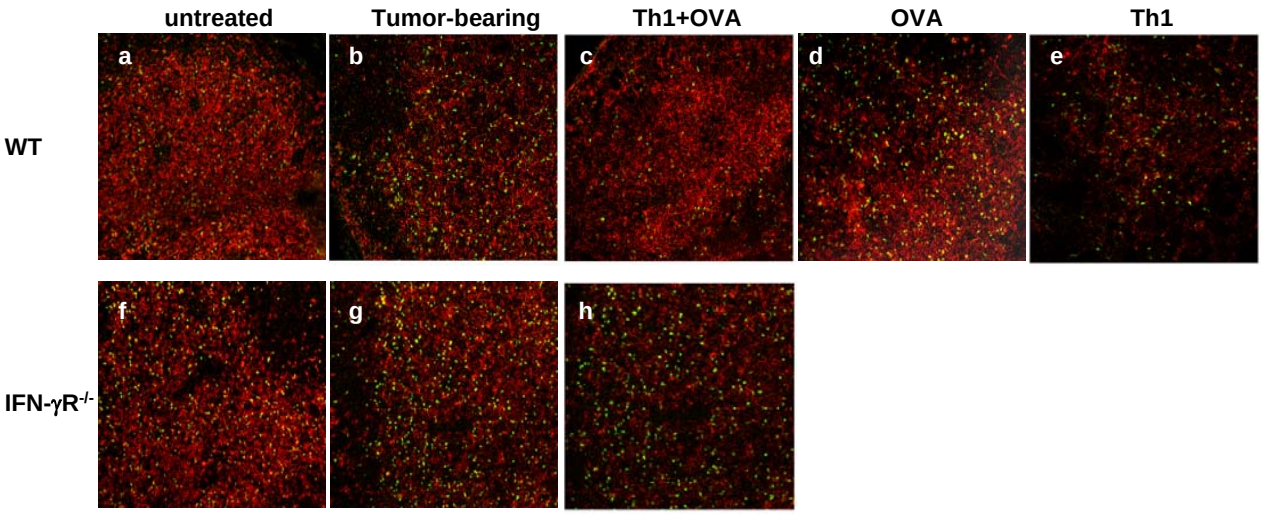



Fig.7