T<sub>h</sub>1 cell adjuvant therapy combined with tumor vaccination: a novel strategy for promoting CTL responses while avoiding the accumulation of Tregs

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Keywords: Th1 cell, Treg, tumor-specific CTL, tumor vaccination therapy

Abstract

We have previously described a method for adoptive immunotherapy of cancer based on antigen-specific Th1 cells. However, efficient induction of anti-tumor 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, ovalbumin (OVA)-expressing EG-7 cells. Tumor-bearing mice were intradermally 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 ~50–60% of tumor-bearing mice were completely cured. Tumor eradication was crucially dependent on the generation of OVA/H-2K<sup>b</sup>-specific CTLs in the tumor DLNs and tumor site. The injected Th1 cells were mainly distributed in tumor DLNs, where they vigorously proliferated and enhanced the activation of dendritic cells. Strikingly, we also found that the accumulation of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs) was significantly inhibited in tumor DLNs by Th1 cell adjuvant therapy and this abrogation was associated with IFN<sub>γ</sub> 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 dendritic cells (DCs), 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 CTLs in these patients (7, 8). Thus, tumor vaccine therapy focused only on CTL activation appeared to be insufficient to induce potent anti-tumor immunity in tumor-bearing hosts (8–10). A major problem in such a strategy is that effective CD8<sup>+</sup> T cell responses require concomitant activation of CD4<sup>+</sup> Th<sub>1</sub> cells. CD4<sup>+</sup> Th<sub>1</sub> cells play a critical role in the induction, migration and maintenance of CD8<sup>+</sup> CTLs (11–13) and in the generation of memory CTL responses (14–16).

In a series of studies (17–22), we have demonstrated a critical role of Th<sub>1</sub> immunity for the induction of tumor-specific CTLs in tumor-bearing mice. In a previous report (17), we demonstrated that adoptive transfer of antigen-specific Th<sub>1</sub> cells into mice bearing established, MHC class II-expressing A20-ovalbumin (OVA) tumors resulted in the induction of tumor-specific CTLs, which were able to completely eradicate the tumor mass. In contrast to MHC class II<sup>+</sup> tumors, it has been demonstrated that MHC class II<sup>−</sup> tumors are refractory to immunotherapy. We have also demonstrated that in contrast to MHC class II<sup>−</sup> A20-OVA tumors, it was hard to eradicate established, MHC class II<sup>−</sup> EG-7 tumors from mice by Th<sub>1</sub> cell transfer alone (18). The resistance of MHC class II<sup>−</sup> EG-7 tumors against Th<sub>1</sub> cell therapy may be due to the lack of direct interaction with tumor cells at the local tumor site. However,
Th1 cell adjuvant therapy for tumor vaccination

Injection of the model tumor antigen, OVA, into the tumor tissue was able to overcome this problem and augmented the interaction between antigen-presenting cells (APCs) and Th1 cells and promoted Th1 cell proliferation and CTL generation in the tumor-draining lymph nodes (DLNs), which is essential for complete tumor eradication (18). 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 DLNs play an important role in initiating anti-tumor immune responses. In addition, it has been reported that the tumor DLNs contain tumor-specific T cell precursors (23–26). These findings suggest that tumor DLNs 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 DLNs together with a tumor model antigen, OVA. Tumor regression was demonstrated in all mice treated with this vaccination and ~50–60% of all mice showed complete tumor rejection. Tumor eradication was dependent on host-derived CD8+ CTLs. Moreover, we found that Th1 cells injected i.d. with OVA vigorously proliferated and accelerated the activation of DCs and tumor-specific CTLs predominantly in the tumor DLNs, 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+ regulatory T cells (Tregs) in DLNs, and this down-regulation is controlled by IFNγ 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.

Methods

Mice

All mice were age- and sex-matched female C57BL/6, C57BL/6J mice were obtained from Charles River Japan (Yokohama, Japan). OT-II TCR transgenic mice (Yokohama, Japan). OT-II TCR transgenic mice, expressing a TCR specific for the I-A^b-restricted 323–339 peptide from OVA, were kindly provided by T. Sawada (Shionogi Pharmaceutical Institute Co. Ltd, Osaka, Japan). Mice were obtained from Charles River Japan (Yokohama, Japan). OT-II TCR transgenic mice, expressing a TCR specific for the I-A^b-restricted 323–339 peptide from OVA, were kindly provided by T. Sawada (Shionogi Pharmaceutical Institute Co. Ltd, Osaka, Japan). All mice were age- and sex-matched female C57BL/6. C57BL/6 mice were age- and sex-matched female C57BL/6. All mice were age- and sex-matched female C57BL/6. Mice at 5–6 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, USA). IL-2 was supplied by T. Sawada (Shionogi Pharmaceutical Institute Co. Ltd, Osaka, Japan). IFNγ was purchased from Pepro Tech EC Ltd (London, UK). Anti-IL-4 mAb (11B11) was purchased from American Type Culture Collection (Rockville, MD, USA). 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, USA). Anti-CD8 mAb-conjugated microbeads for the magnetic cell sorting 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^d molecules loaded with the OVA^{325–339} 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+ naive T cells were isolated from nylon-passed spleen cells of OT-II TCR transgenic mice using FACSAria (Becton Dickinson, San Jose, CA, USA). Purified CD4+CD45RB+ cells were stimulated with 5 μg ml^-1 OVA^{323–339} peptide in the presence of mitomycin C-treated spleen cells, 100 U ml^-1 IL-2, 20 U ml^-1 IL-12, 1 ng ml^-1 IFNγ and 50 μg ml^-1 anti-IL-4 mAb for Th1 development. At 48 h, cells were re-stimulated with OVA^{323–339} under the same conditions and used at 9–12 days of culture.

Detection of carboxyfluorescein diacetate succinimidyl ester-labeled Th1 cells in vivo

Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Molecular Probes (Eugene, OR, USA) and used for monitoring daughter cell generation in vivo as indicated in the manufacturer’s instructions. As described previously (20), tetramer-blocking assays were carried out by the co-culture of 51Cr-labeled tumor cells with effector cells, which were pre-labeled with CFSE-labeled Th1 cells (2 × 10^6 per mouse). The generation of daughter cells by the labeled Th1 cells in DLNs, distant lymph nodes (LNs), spleen, or tumor tissue was analyzed using FACS Calibur. 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 DLNs or the tumor site were washed twice with PBS and stained with 3.3 μl ml^-1 of OVA–MHC tetramers per 1 × 10^6 cells at 4°C for 15 min, followed by staining with FITC-labeled anti-CD8 mAb. The cells were washed again with PBS and analyzed by flow cytometry. As described previously (20), tetramer-blocking assays for determining the fine MHC-restricted peptide antigen specificity were carried out by the co-culture of ^31^-Cr-labeled target cells with effector cells, which were pre-labeled with a tetramer reagent.

Flow cytometric analysis

Detailed procedures for staining and sorting have been described previously (17). Fluorescence data were collected on a FACS Calibur (Becton Dickinson), and analyzed using CellQuest software (BD Biosciences, Mountain View, CA, USA). The mAbs used for experiments are listed in the agent section.
**Th1 cell adjuvant therapy**

A total of 2 x 10^6 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 DLNs with saline, OVA protein (200 µg per mouse), OVA-specific Th1 cells (2 x 10^7 per mouse) or Th1 cells plus OVA. Anti-tumor 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 (17). Tumor-bearing mice that survived for >60 days after therapy were considered completely cured. The mean of five mice per group is indicated in the figures.

**Cytotoxicity assay**

The cytotoxicity mediated by tumor-specific CTLs was measured by a 6-H 51Cr-release assay as described previously (28). 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-2Kb-restricted CTLs, 51Cr-labeled target cells were incubated with CD8+ CTLs pre-treated with OVA tetramer, which blocks the recognition of antigen by CTLs. CD8+ T cells were enriched by MACS system according to the manufacturer’s protocol. The percent cytotoxicity was calculated as described previously (28).

**Immunoistochemical analysis**

Snap-frozen, Tissue-Tek-embedded lymph nodes (five for each group) were cut at 5–7 mm, fixed for 10 min in cold acetone and used for immunoistochemical analysis. We used an already established double-immunofluorescence staining protocol for Foxp3 and CD4 (29). Briefly, the primary antibodies were diluted in 1% BSA (polyclonal rabbit antibody against murine Foxp3 at a concentration of 5 µg ml⁻¹ 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 and anti-rabbit IgG-Alexa 488; Alexa, Leiden, The Netherlands). Finally, the samples were analyzed with an 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 2 x 10^6 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 (200 µg per mouse), OVA-specific Th1 cells (2 x 10^7) or Th1 cells plus OVA, near the tumor DLNs. OVA-specific Th1 cells were derived from T cell receptor transgenic (OT-II) mouse spleen cells. In prior studies (17), we have demonstrated that Th1 cells cannot induce the regression of MHC class II-negative tumors, even when Th1 cells were intravenously (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 (18). This finding indicated that activation of APCs 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 DLNs. After three rounds of therapy, tumor-bearing mice were treated with Th1 cell adjuvant combined with OVA showed significant regression of their tumor mass and ~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 2 x 10^7 Th1 cells but not with 2 x 10^6, 5 x 10^6 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 anti-tumor 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 DCs in DLNs 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 (DLNs, distal LNs 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 LNs, spleen and tumor tissue (data not shown). In contrast, high numbers of CFSE-positive Th1 cells were present in tumor DLNs. 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 DLNs (Fig. 3A, b). Such extensive proliferation of CFSE-labeled Th1 cells was not observed in distal LNs, spleen and tumor tissues at the same time (3 days after first therapy), though Th1 cell proliferation was observed at a 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 DLNs are critical lymphoid organs for the migration and activation of injected Th1 cells in our immunotherapy model.
It is now well established that tumor DLNs play an important role in initiating early immune responses against tumor cells (25, 26). APCs, especially DCs, 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 DCs in tumor DLNs. 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 DCs. In contrast, i.d. injection of Th1 cells with OVA had a strong impact on the activation of DCs, as evidenced by profound up-regulation of the expression of co-stimulatory 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 DCs were increased (Fig. 3C). In addition to DCs, it was demonstrated that various immunoregulatory cells including CD8^+ T cells, NK cells, macrophages and B cells were activated at DLNs (Fig. 4). These results indicate that Th1 cell adjuvant therapy can activate both innate and acquired immunity cells in tumor DLNs.

Generation of tumor antigen-specific CTLs in tumor-bearing mice by treatment with Th1 cell adjuvant and tumor antigen

The interaction of host DCs with Ag-specific Th1 cells in tumor DLNs might facilitate the induction of tumor-specific CTLs derived from naive, endogenous CD8^+ T cells in tumor-bearing mice. To address this possibility, lymphocytes were prepared from DLNs or tumor tissue 3 days after the second round of therapy and examined for the generation of OVA-specific CTLs by staining with OVA257–264/H-2K^b tetramers. The frequency of tetramer^+ CD8^+ CTLs in mice treated with Th1 cell adjuvant plus tumor antigen was markedly elevated in tumor DLNs (2.7 ± 0.42%) and within the tumor.
tissue (35.5 ± 5.15%) compared with that in the mice treated with Th1 cells alone (b) or Th1 + OVA (a). Th1 cells were pre-labeled with CFSE as described in Methods. Three days after the primary therapy, the tumor DLNs 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; open square), OVA (hatched square) or Th1 + OVA (filled square). Results represent the mean and standard error 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+ DCs from DLNs of tumor-bearing mice 24 h 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 are shown in the figures.

Taken together, these data demonstrate that vaccination of tumor-bearing mice with Th1 cell adjuvant plus model tumor antigen (OVA) effectively induces OVA/H-2Kb-specific CD8+ CTLs 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+ CTLs 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 CTLs are critical for complete tumor eradication.
Th1 cell adjuvant therapy inhibits the accumulation of Foxp3+ CD4+ Tregs in DLNs of tumor-bearing mice

Immunotherapy against large tumor masses has generally been ineffective because tumors themselves often elicit strong immunosuppressive responses (20, 30–32). The success of our strategy indicated that the 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 Treg subsets in the maintenance of immune tolerance against self- and tumor antigens (33–35). To investigate the possible impact of Th1 cell adjuvant therapy on these regulatory cells, we examined the frequency of Foxp3+ CD4+ Treg in tumor DLNs. DLNs were separated from tumor-bearing mice 3 days after the second round of therapy and the frequency of Tregs was examined by immunohistochemical analysis. The number of Tregs in DLNs 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 Tregs was significantly reduced (Fig. 7c). A slight decrease of Treg accumulation was noted in tumor DLNs 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 DLNs via a small number of tumor antigen-pulsed DCs migrated from the tumor tissues. To determine which factors are involved in this blockade in Treg accumulation, we utilized IFNγR−/− mice. As shown in Fig. 7f and g, the number of Tregs increased in IFNγR−/− tumor-bearing mice compared with untreated IFNγR−/− mice. In contrast to wild-type mice, a strong Treg accumulation was induced in the IFNγR−/− tumor-bearing hosts even when the mice were treated with the Th1 cell adjuvant therapy (Fig. 7h). Taken together, these results suggest that Th1 cell adjuvant therapy has the capacity to suppress Treg accumulation at the local tumor site, which might be due to IFNγ produced by Th1 cells.

Fig. 4. Th1 cell adjuvant therapy activates both innate and acquired immunity cells in DLNs. DLNs were prepared from tumor-bearing mice 48 h after treatment with saline (control), OVA, Th1 cells or Th1 cells + OVA. Then, surface expression levels of CD69 on CD8+ T cells, NK1.1+ TCRγδ NK cells, CD11c+ DCs, CD11b+ macrophages (MΦ) and B220+ B cells in the DLNs were examined by FACS analysis. The number attached in each profile denotes the percentage of each CD69+ cell population indicated as CD8 cells, NK cells, DCs, MΦ and B cells. Similar results were obtained in three separate experiments and representative data are shown in the figures.
It has been well established that tumor DLNs play an important role in cancer immunotherapy (18, 25, 26). The immune defense to tumor cells is initiated in these nodes, where antigen-presenting DCs migrate and activate CD4+ and CD8+ T cells specifically reactive to tumor antigen (18, 23–26). 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 DLNs, complete tumor regression was induced concomitantly with efficient induction of tumor-specific CTLs (Figs 1 and 5), in the absence of Treg accumulation in the tumor DLNs (Fig. 7). Adoptively transferred Th1 cells dominantly distributed into DLNs but not in spleen and distal LN (data not shown). This preferential distribution of Th1 cells in DLNs effectively promoted the initiation of anti-tumor responses within the local tumor site. Th1 cell adjuvant therapy also facilitated the migration and activation of DCs in tumor DLNs (Fig. 3B and C). Tumor-specific CTLs were induced in vaccinated mice, and these cells were also enriched in DLNs 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 anti-tumor response observed in these mice was strongly dependent on the...
activation of CD8+ Ag-specific CTLs, because OVA/H-2Kb-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 mechanisms underlying Th1 cell adjuvant therapy combined with tumor antigen remains unclear. However, it is possible that transferred Th1 cells first migrate into tumor DLNs and specifically interact with DCs that had taken up tumor antigens. The enhanced interactions between DCs and Th1 cells via co-stimulatory molecules and cytokines further promote the differentiation of host-derived naive CD8+ T cells into tumor-specific CTLs, which are the relevant effector cells for complete regression of the tumor mass (18, 20).
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 (36). These poor anti-tumor responses in mice bearing a large tumor mass may be because tumors themselves can produce immunosuppressive factors such as transforming growth factor (TGF) β, prostaglandins and IL-10 (37, 38). Indeed, TGFβ derived from tumors is a critical factor for the differentiation of CD4+CD25+ Tregs, which have been recognized as a major immunosuppressive component in the tumor-bearing host. Tregs play a critical role in immunologic self-tolerance as well as in anti-tumor immune responses and organ rejection during transplantation (39, 40). Several studies have reported an increase in the number of Tregs in tumor-bearing hosts in both animal and human systems (30, 41–43). An increase in Treg numbers was also seen in our experimental model (Fig. 7b). It has been demonstrated that Tregs can impair the induction of both antigen-specific and non-specific T cell immunity (44, 45) and an increase in these cells is predictive of reduced survival in cancer patients (30). 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 an anti-CD25 mAb resulted in enhancement of effective tumor immune responses via removal of strong immunosuppression (36). In the present study, we found that the frequency of Foxp3+ CD4+ Tregs in tumor-bearing mice was significantly decreased by T(h)1 cell adjuvant therapy combined with tumor antigen vaccination. The observed blockade in Treg accumulation appeared to be due to IFNγ produced in tumor-bearing mice following T(h)1 cell adjuvant therapy, because such inhibitory effect was not observed when IFNγR−/− mice were used as tumor-bearing hosts (Fig. 7c and h). These data suggested that T(h)1 cells are the major source of IFNγ for overcoming Treg accumulation. We have never examined whether EG-7-bearing IFNγR−/− mice treated with T(h)1 cell adjuvant and OVA showed a deficiency in tumor eradication in parallel with the increase of Treg populations. However, depletion of CD4+CD25+ Tregs 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 IFNγR−/− may deteriorate anti-tumor effects induced by T(h)1 cell adjuvant therapy. We are now investigating this issue. It should be noted that the frequency of Tregs in DLNs of tumor-bearing IFNγR−/− mice was increased rather than suppressed by treatment with T(h)1 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 T(h)1 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 the treatment of multiple tumors (34). However, clinical trials using IL-2 showed limited efficacy for tumor immunotherapy (34, 35). Accumulating evidence from knockout mice has suggested that IL-2 is crucial for the homeostasis and function of CD4+CD25+ Tregs in vivo (40). Clinical research has also shown that the frequency of Tregs was significantly increased after IL-2 treatment (46). It is possible that IL-2 administration may impede anti-tumor immune responses through activation of Tregs. Therefore, the balance between the differentiation of tumor-reactive effector T cells and Tregs, both of which are controlled by IL-2, may determine the clinical outcome for tumor patients. In our T(h)1 cell adjuvant therapy model, IFNγ may antagonize the effect of IL-2 on Tregs and provide help for the induction of antigen-specific CTLs.

In this paper, we emphasized the importance of tumor DLNs for the activation of T(h)1 cells and the subsequent generation of tetramer+ CTLs, which are essential for the complete cure of tumor-bearing mice. However, when T(h)1 cells were i.d. injected with OVA distantly from tumor, detectable number of tetramer+ CTLs (1.7%) were also induced at DLNs of the vaccination site though higher percentage of CTLs (2.56%) were demonstrated at tumor DLNs even by this systemic T(h)1 cell adjuvant therapy (data not shown). Therefore, i.d. injection of T(h)1 cell adjuvant with OVA near tumor DLNs may be the best protocol for inducing anti-tumor immunity to cure tumor-bearing mice. But T(h)1 cell adjuvant appeared to be effective in inducing tumor-specific CTLs at the local tumor site even when it was injected at distal site from the tumor tissues. This might be because T(h)1 cells function as cytokine-producing cell adjuvant, which facilitate their own active migration into tumor DLNs via lymphatic system. In contrast to the T(h)1 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, T(h)1 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 T(h)1 cells combined with tumor antigen can efficiently cure established, large MHC class II-negative tumors. In a previous paper, we reported that i.v. injection of T(h)1 cells combined with i.t. injection of model tumor antigen (OVA) was an efficient method to induce tetramer+ CTLs at the local tumor site to cure tumor-bearing mice (18). However, the application of that method is limited to visible tumor mass on skin such as melanoma. In contrast, i.d. injection of the T(h)1 cell adjuvant near tumor DLNs is applicable to every tumor-bearing hosts. Moreover, systemic anti-tumor immunity is induced in tumor-bearing mice even when T(h)1 cell adjuvant is i.d. injected with tumor antigen at distal site of tumor tissue. Both protocols for T(h)1 cell therapy combined with tumor antigen may induce anti-tumor immunity by the same mechanisms including (i) acceleration of DC-T(h)1 cell interaction, (ii) T(h)1 cell activation and (iii) acceleration of tumor-specific CTL induction. Here, in addition to the promotion of both innate and acquired anti-tumor immune responses, we initially demonstrate that our novel T(h)1 cell adjuvant therapy inhibits the accumulation of Tregs in tumor DLNs.

Thus, our data presented here identify T(h)1 cell adjuvant therapy combined with tumor antigen vaccination as a promising therapeutic strategy for cancer. In our tumor therapy model, we used tumor cells expressing a strong xenogeneic antigen (OVA). We have never demonstrated whether our...
developed T\textsubscript{n1} cell adjuvant therapy combined with endogenous tumor antigen is applicable to tumor cells expressing weak native tumor antigen. However, it has been reported that the activation of DCs \textit{in vivo} with an adjuvant plus weak tumor antigen protein (carcinoembryonic antigen) induced a strong anti-tumor immunity (47). Thus, natural tumor antigen was shown to have a capability of inducing tumor-specific CTLs \textit{in vivo}, although it is a weak self-antigen. Therefore, we believe that T\textsubscript{n1} cell adjuvant therapy, which can activate both DC and CTL function \textit{in vivo}, may also induce an efficient anti-tumor immunity against a weak tumor antigen and may be applicable to clinical trials, although there are still unresolved problems.

Acknowledgements

We would like to thank Luc Van Kaer (Vanderbilt University School of Medicine, Nashville, TN, USA) for reviewing the paper. We also thank Steve Herrmann (Wyeth Research, Cambridge, MA, USA), Takuko Sawada (Shionogi Pharmaceutical Institute Co., Osaka, Japan) and Kouji Matsushima (Department of Molecular Preventive Medicine, School of Medicine, University of Tokyo, Japan) for their kind donations of recombinant IL-12, recombinant IL-2 and anti-Foxp3 mAb, respectively. This work was funded by a Grant-in-Aid from the Ministry of Education Culture, Sports, Science, and Technology, a Grant-in-Aid for Scientific Research on priority Areas, a Grant-in-Aid for Immunological Surveillance and its Regulation and a Grant-in-Aid from the ministry of Education, Culture Sports, Science and Technology Cancer Translational Research Project.

Abbreviations

- APC: antigen-presenting cell
- DC: dendritic cell
- DLN: draining lymph node
- i.d.: intradermally
- i.t.: intratumor
- i.v.: intravenously
- LN: lymph node
- OVA: ovalbumin
- TGF: transforming growth factor
- Treg: regulatory T cell

References


