$CD4^+$ Th1 cells promote $CD8^+$ Tc1 cell survival, memory response, tumor localization and therapy by targeted delivery of interleukin 2 via acquired pMHC I complexes

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Summary

The cooperative role of $CD4^+$ helper T (Th) cells has been reported for $CD8^+$ cytotoxic T (Tc) cells in tumor eradication. However, its molecular mechanisms have not been well elucidated. We have recently demonstrated that $CD4^+$ Th cells can acquire major histocompatibility complex/peptide I (pMHC I) complexes and costimulatory molecules by dendritic cell (DC) activation, and further stimulate naïve $CD8^+$ T cell proliferation and activation. In this study, we used $CD4^+$ Th1 and $CD8^+$ Tc1 cells derived from ovalbumin (OVA)-specific T cell receptor (TCR) transgenic OT II and OT I mice to study $CD4^+$ Th1 cell's help effects on active $CD8^+$ Tc1 cells and the molecular mechanisms involved in $CD8^+$ Tc1-cell immunotherapy of OVA-expressing EG7 tumors. Our data showed that $CD4^+$ Th1 cells with acquired pMHC I by OVA-pulsed DC (DC_{OVA}) stimulation are capable of prolonging survival and reducing apoptosis formation of active $CD8^+$ Tc1 cells *in vitro*, and promoting $CD8^+$ Tc1 cell tumor localization and memory responses *in vivo* by 3-folds. A combined adoptive T-cell therapy of $CD8^+$ Tc1 with $CD4^+$ Th1 cells resulted in regression of well-established EG7 tumors (5 mm in diameter) in all 10/10 mice. The $CD4^+$ Th1's help effect is mediated via the helper cytokine IL-2 specifically targeted to $CD8^+$ Tc1 cells *in vivo* by acquired pMHC I complexes. Taken together, these results will have important implications for designing adoptive T-cell immunotherapy protocols in treatment of solid tumors.

Keywords: Th1; pMHC I; IL-2; apoptosis; Tc1 cell therapy

Introduction

$CD8^+$ cytotoxic T (Tc) lymphocytes which are a major immunological effector cell population mediating resistance to cancer can eradicate the growth and metastasis of malignant tumor cells[1]. Effective cancer immunotherapy with adoptively transferred tumor-sensitized Tc cells has been well documented in animal models[2,3]. $CD8^+$ Tc cells can be polarized to effector subsets with distinct cytokine production profiles (Tc1 cells producing IFN-γ and Tc2 cells secreting IL-4 and IL-5)[4]. Dobrzanski *et al.* previously reported that tumor-specific Tc1 cells were relatively more effective reduction of lung metastasis of OVA-transfected B16 melanoma than Tc2 cells[5]. Recently, they have further shown that these Tc1 and Tc2 can also cure intradermally transplanted OVA-transfected B16 melanomas, but only in their palpable sizes[6]. In clinical trials, only a limited number of patients have responded to T-cell therapy[7,8] partly due to lacking of T cell helper arm and/or only very small fractions of transferred T cells accumulating in tumors[9,10].

$CD4^+$ T helper (Th) cells can also be subdivided into Th1 cells producing IL-2 and IFN-γ and Th2 cells secreting IL-4, IL-5 and IL-10. It has been reported that Th2 cells responsible for humoral immunity[11] neither enhanced nor suppressed antitumor $CD8^+$ cytotoxic T lymphocyte (CTL) responses[12] whereas Th1 cells essential for cellular immunity play an important role in

Abbreviations: APC, antigen-presenting cells; CTL, cytotoxic T lymphocyte; DC, dendritic cell; PCR, polymerase chain reaction; pMHC, major histocompatibility complex/peptide; TCR, T cell receptor; Tc, $CD8^+$ cytotoxic T; Th, $CD4^+$ helper T.

priming CTL-mediated antitumor responses[9]. The traditional explanation is that $CD4^+$ Th1 cells provide IL-2 or helper to $CD8^+$ Tc cells[13]. $CD4^+$ Th cells have also been shown to have another role in induction of $CD8^+$ Tc cell responses through DC activation by CD40/CD40L interactions[14]. Recently, it has also been reported that $CD4^+$ Th cells are required in determining the magnitude and persistence of CTL responses[15] and for $CD8^+$ T cell infiltration of tumors[16]. However, the underlying immune mechanisms of these $CD4^+$ Th's effects in adoptive $CD8^+$ Tc-cell therapy are still largely unknown.

Stimulation of T cells by antigen-presenting cells (APCs) involves at least two signaling events: one elicited by T cell receptor (TCR) recognition of major histocompatibility complex/peptide (pMHC) complexes and the other one by costimulatory molecule signaling (e.g. T cell CD28/APC CD80)[17]. A consequence of such Ag-specific T cell–APC interactions is the formation an immunological synapse, comprising a central cluster of TCR-pMHC complexes and CD28–CD80 interactions surrounded by rings of engaged accessory molecules (e.g. complexed LFA-1-CD54)[18,19]. One important feature of synapse physiology is that APC-derived surface molecules are transferred to the Th cells during the course of their TCR internalization followed by recycling[20,21]. Recently, we have demonstrated that naive $CD4^+$ T cells acquire DC molecules by DC activation and act as Th-APCs. These Th-APCs with acquired pMHC complexes and costimulatory molecules can stimulate naïve $CD8^+$ T cell proliferation *in vitro* and *in vivo* and induce CTL responses and antitumor immunity[22]. However, the molecular mechanisms responsible for the functional effects of Th-APCs have not been well elucidated, and the critical role the acquired pMHC I complexes play in targeting $CD4^+$ Th's effects to $CD8^+$ T cells *in vivo* has not been clearly defined due to lacking the appropriate control cells such as $CD4^+$ Th(pMHC $I^{-/-}$) cells used in this study.

In this study, we developed a model system with a defined tumor antigen OVA using the OVA-transfected EG7 tumor cells and the OVA-specific TCR transgenic OT I and OT II mice with class I and II specificities, respectively,[23]. Based upon this model system, we investigated the help effects of OT II $CD4^+$ Th1 cells in active $CD8^+$ Tc1-cell immunotherapy of established solid EG7 tumors. We found that $CD4^+$ Th1 cells prolonged active OT I $CD8^+$ Tc1 cell survival and promoted active OT I $CD8^+$ Tc1 cell tumor localization and memory responses. We further elucidated the molecular mechanisms responsible for their help effects in $CD8^+$ Tc1 cell immunotherapy and disclosed the critical role of acquired pMHC I complexes in delivery of $CD4^+$ T help effects to $CD8^+$ Tc1 cells *in vivo* by using the recently established control $CD4^+$ Th(pMHC $I^{-/-}$) cells.

Materials and methods

Antibodies, cytokines, cell lines and animals

Biotin-conjugated antimouse MHC class I (H-$2K^b$) and II (Ia^b), CD4, CD8, CD11c, CD25, CD69 and Vβ5·1,5·2 TCR antibodies (Abs) were obtained from BD Pharmingen Inc. (Mississauga, Ontario, Canada). The FITC-conjugated avidin was obtained from Bio/Can Scientific (Mississauga, Ontario, Canada). PE-labeled H-$2K^b$/$OVA_{257-264}$ (OVA I) tetramer and FITC-labeled anti-CD8 Ab were obtained from Beckman Coulter, Mississauga, Ontario, Canada. The anti-IL-2, -IL-4, -IFN-γ Abs, and the recombinant mouse granulocyte macrophage colony stimulating factor (GM-CSF), IL-2, IL-12 and interferon (IFN)-γ were purchased from R & D Systems (Minneapolis, MN). The anti-H-$2K^b$/OVA I (pMHC I) Ab was obtained from Dr R. Germain, National Institute of Health, Bethesda, MD[24]. The mouse B cell hybridoma cell line LB27 expressing Ia^b, thymoma cell line EL-4 and its derivative OVA-transfected cell line EG7 were obtained from American Type Culture Collection (ATCC), Rockville, MD. OVA I (SIINFEKL) and OVA II (ISQAVHAAHAEINEAGR) peptides were synthesized by Multiple Peptide Systems (San Diego, CA). Female C57BL/6 mice and OT I and OT II mice having transgenic Vα2Vβ5 TCRs specific for $OVA_{257-264}$ (OVA II) epitope in the context of H-$2K^b$ and $OVA_{323-339}$ epitope in the context of Ia^b[22,23], respectively, and H-$2K^b$, IL-2 and IFN-γ gene knockout (KO) mice on C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, Maine). Homozygous OT II/H-$2K^{b-/-}$, OT II/IL-$2^{-/-}$ and OT II/IFN-$\gamma^{-/-}$ mice were generated by backcrossing the designated gene KO mice onto the OT II background for three generations; homozygosity was confirmed by polymerase chain reaction (PCR) according to Jackson laboratory's protocols. All mice were maintained in the animal facility at the Saskatoon Cancer Center and treated according to Animal Care Committee guidelines of University of Saskatchewan.

Preparation of dendritic cells

Bone marrow-derived dendritic cells (DCs) were generated using GM-CSF and IL-4 as described previously[25]. To generate OVA protein-pulsed DCs, DCs derived from wild-type C57BL/6 mice were pulsed overnight at 37° with 0·1 mg/ml OVA protein (Sigma, St. Louis, MO) and termed as $DC_{OVA.}$ DC_{OVA} were capable of stimulating both OT II $CD4^+$ and OT I $CD8^+$ T cell proliferation *in vitro*[26] indicating that OVA-pulsed DC_{OVA} express both pMHC II and pMHC I complexes, respectively. DC_{OVA} derived from C57BL/6 mice with H-$2K^b$ gene KO were termed as ($K^{b-/-}$)DC_{OVA}.

Preparation of active T cell subsets

OVA-specific $CD4^+$ T and $CD8^+$ T cells were isolated from the spleens and lymph nodes of OT-II and OT-I TCR-transgenic mice, enriched by passage through nylon wool columns (C & A Scientific Inc, Manassas, VA), and then, the $CD4^+$ and $CD8^+$ T cells were further fractionated by negative selection using antimouse CD8 (Ly2) and CD4 (L3T4) paramagnetic beads (DYNAL Inc., Lake Success, NY), respectively, according to the manufacturer's protocols. The OVA-specific T cell subsets (Th1 and Tc1 cells) were further generated by culturing naïve $CD4^+$ and $CD8^+$ T cells (3×10^5 cells/well) with irradiated (4000 rad) DC_{OVA} (2×10^5 cells/well) in 96-well plate in the presence of IL-2 (20 U/ml), IL-12 (5 ng/ml) and anti-IL-4 Ab (5 μg/ml)[22], respectively. *In vitro*-activated Th1 and Tc1 cell subsets were harvested after 4 days' culture and purified using Ficoll-Paque (Sigma, St. Louis, MO) density gradient centrifugation[26] and followed by using CD8- and CD4-microbeads (Milttenyi Biotec, Auburn, CA), respectively[22]. These *in vitro* ($K^{b-/-}$)DC_{OVA}-activated wild-type OT II $CD4^+$ T cells were termed as Th1(pMHC $I^{-/-}$), whereas wild-type DC_{OVA}-activated $CD4^+$ T cells from designated gene-deleted OT II (OT II/$K^{b-/-}$, OT II/IL-$2^{-/-}$ and OT II/IFN-$\gamma^{-/-}$) mice under the same culture conditions were termed as $CD4^+$ Th1($K^{b-/-}$), Th1(IL-$2^{-/-}$) and Th1(IFN-$\gamma^{-/-}$), respectively. Con A-stimulated OT II $CD4^+$ T (Con A-OT II) cells were generated and purified as previously described[22].

Phenotypic characterization of active T cell subsets

The active T cell subsets were stained with a panel of antibodies and analyzed by flow cytometry[27]. Isotype-matched monoclonal Abs with irrelevant specificity were used as controls.

Cytokine secretion

Tc1 and Th1 subsets were re-stimulated by culturing T cells ($0{\cdot}5 \times 10^6$ cells/well) in flat-bottom 96-well plates (Costar Corp, Cambridge, MA) with irradiated (6000 rad) EG7 ($0{\cdot}6 \times 10^5$ cells/well) and OVAII-pulsed irradiated (4000 rad) $LB27_{OVAII}$ ($0{\cdot}6 \times 10^5$ cells/well), respectively[26]. The culture supernatants were harvested at 1 day for measurement of IFN-γ, IL-4, and IL-2 secretion by using enzyme-linked immunosorbent assay (ELISA) kits (Endogen, Woburn, MA). The results were normalized to the recombinant cytokine standard curves.

PMHC I complex transfer assay

Naïve $CD4^+$ T cells (3×10^5 cells/well) derived from OT II mice with H-$2K^b$ gene KO were cultured with irradiated (4000 rad) DC_{OVA} (2×10^5 cells/well) in the presence of IL-2 (20 U/ml), IL-12 (5 ng/ml) and anti-IL-4 Ab (5 μg/ml) for 4 days. The active OT II T cells were analyzed using the antipMHC I Ab by flow cytometry.

Naïve $CD8^+$ T cell proliferation assay

A constant number of naïve OT I $CD8^+$ T cells ($0{\cdot}5 \times 10^5$ cells/well) were cultured with irradiated (4000 rad) stimulators including $CD4^+$ Th1, Th1(pMHC $I^{-/-}$), Th1($k^{b-/-}$) cells and DC_{OVA} ($0{\cdot}4 \times 10^5$ cells/well), respectively, and their 2-fold dilutions. After 48 hr, all wells were pulsed for 12 h with 1 μCi of [^{3}H]-thymidine (Amersham, Arlington Heights, IL) and then harvested onto glass fiber filters, respectively. Thymidine incorporation in each well was determined by liquid scintillation counting[27].

Active Tc1 cell survival analysis

To examine $CD8^+$ Tc1 cell survival in absence of IL-2 stimulation, *in vitro*-activated $CD8^+$ Tc1 cells ($0{\cdot}4 \times 10^5$ cells/well) were incubated with or without irradiated (4000 rads) active $CD4^+$ Th1 cells ($0{\cdot}4 \times 10^5$ cells/well) in RPMI 1640 plus 10% FCS in flat-bottom 96-well plates. To assess $CD8^+$ Tc1 cell apoptosis formation, T cells were harvested after 4 days, stained with FITC-Annexin V (BD Pharmingen) and PE-anti-CD8 Ab, and analyzed by flow cytometry. In some experiments, each of a panel of neutralizing reagents (anti-IL-2 and -IFN-γ Abs) (each 15 μg/ml) was added to the culture system. To examine the effect of acquired pMHC I complexes on $CD8^+$ Tc1 cell survival, Th1(pMHC $I^{-/-}$) cells without acquired pMHC I complexes were used as control cells.

Tc1 cell tumor infiltration

Detection of tumor infiltration of transferred active $CD8^+$ Tc1 cells *in vivo* was performed as previously described[27]. Briefly, *in vitro* DC_{OVA}-activated $CD8^+$ Tc1 cells (5×10^6 cells/mouse) together with or without *in vitro* DC_{OVA}-activated $CD4^+$ Th1, Th1(pMHC $I^{-/-}$), Th1($K^{b-/-}$), Th1(IL-$2^{-/-}$) and Th1(IFN-$\gamma^{-/-}$) cells (2×10^6 cells/mouse) were i.v. injected into C57BL/6 mice bearing EG7 tumors with ~5 mm in diameter, respectively. At different days subsequent to T cell injection, tumors were removed. Cell suspensions were prepared from these EG7 tumors by mincing them into small pieces and pressing them through a fine mesh[16]. Red cells were lysed by using 0·84% ammonium chloride. T cells were then purified from these cell suspensions by using the CD3 microbeads (Mittenyi Biotec), stained using PE-labeled H-$2K^b$/OVA I tetramer (PE-tetramer) and FITC-labeled anti-CD8 Ab (FITC-CD8) for flow cytometric analysis.

Adoptive Tc1 cell immunotherapy model

Mice (10 per group) received s.c. injections of 1×10^6 EG7 tumor cells in their thighs. At 10–12 days postinoculation, tumors became around 5 mm in diameter. To study the help effect of $CD4^+$ Th1 cells, tumor-bearing mice were injected i.v. with 5×10^6 $CD8^+$ Tc1 cells alone, or in conjunction with different amounts of $CD4^+$ Th1 cells, respectively. To study the molecular mechanism of $CD4^+$ Th1's help effect in $CD8^+$ Tc1-cell therapy, tumor-bearing mice were also injected i.v. with 5×10^6 of $CD8^+$ Tc1 cells in conjunction with 2×10^6 $CD4^+$ Th1(pMHC $I^{-/-}$), Th1($K^{b-/-}$), Th1(IL-$2^{-/-}$) and Th1(IFN-$\gamma^{-/-}$) cells, respectively. Animal mortality and tumor growth or regression were monitored daily for up to 10 weeks; for humanitarian reasons, all mice with tumors that achieved a size of 1·5 cm in diameter were sacrificed.

Assessment of $CD8^+$ Tc1 cell memory responses

Naïve C57BL/6 mice were i.v. injected with active $CD8^+$ Tc1 cells (5×10^6 cells) alone or in conjunction with $CD4^+$ Th1 or Th1(pMHC $I^{-/-}$) cells (2×10^6 cells). Tetramer staining assay was performed to examine the presence of OVA-specific $CD8^+$ memory T (Tm) cells in mouse peripheral blood 3 months after the adoptive Tc1 cell transfer. The mouse tail blood samples were incubated with PE-tetramer, FITC-CD8 and ECD-anti-CD44 (ECD-CD44) Abs (Beckman Coulter, Mississauga, Ontario, Canada). The erythrocytes were then lysed using lysis/fixed buffer (Beckman Coulter) and the samples were analyzed by flow cytometry according to the company's protocol. In one set of experiments, the immunized mice were boosted with i.v. injection of irradiated (4000 rad) DC_{OVA} (1×10^6 cells). Four days after the boost, the mouse tail blood samples were tested for tetramer staining as described above. In another set of experiments, the immunized mice were challenged with s.c. injection of 1×10^6 or 3×10^6 EG7 tumor cells. Animal mortality and tumor growth or regression were monitored daily for up to 10 weeks as described above.

Results

Characterization of active $CD4^+$ Th1 and $CD8^+$ Tc1 cells

Two different T cell subsets ($CD4^+$ Th1 and $CD8^+$ Tc1 effector cells) were generated *in vitro* from OVA-specific TCR transgenic OT II and OT I mice, respectively, as described in Materials and Methods. Both $CD4^+$ and $CD8^+$ T cells displayed their T cell subset marker (CD4 or CD8), $V\alpha2V\beta5^+$ TCR and active T cell markers CD25 and CD69 (Fig. 1a), indicating that they are OVA-specific active $CD4^+$ and $CD8^+$ T cells. They also expressed some MHC class II and CD80 molecules, which may be acquired from DC through synapse-composed molecule internalization/recycling[18,19,22]. In addition, there was no CD11c-positive DC population existing in these purified active T cells (Fig. 1a). This is because that any survival of irradiated DC_{OVA} cells and potential small amount of contamination of spleen DC or B cells with the original naïve OT II $CD4^+$ and OT II $CD8^+$ T cell preparations, which might have picked up OVA peptides from irradiated DC_{OVA} in the culture, would be eliminated by the killing activity of these activated Th and Tc cells expressing perforin[22,27,28]. These activated Th and Tc cells secreted abundant IFN-γ (2·5 ng/ml/10^6 cells/24 h and 3·2 ng/ml/10^6 cells/24 hr) and IL-2 (1·9 ng/ml/10^6 cells/24 h and 0·9 ng/ml/10^6 cells/24 hr), respectively, but very little IL-4 (~50–60 pg/ml/10^6 cells/24 hr) in their culture supernatants (Fig. 1b), indicating that they are type 1 $CD4^+$ Th1 and $CD8^+$ Tc1 cells, respectively.

$CD4^+$ Th1 cells acquire pMHC I complexes by DC activation

We have previously shown that active $CD4^+$ Th cells can acquire DC molecules such as MHC I and II and costimulatory molecules by DC activation[22]. In this study, we demonstrated that active $CD4^+$ Th1 cells expressed large amount of H-$2K^b$ and some pMHC I complexes detected by the antipMHC I Ab (Fig. 1c). To confirm the acquisition of pMHC I complexes, we used $CD4^+$ T cells derived from OT II mice with H-$2K^b$ gene KO to rule out the possibility of its endogenous H-$2K^b$ picking up OVA I peptide released from DC_{OVA} in the culture. As shown in Fig. 1(d), naïve $CD4^+$ Th1($K^{b-/-}$) cells themselves did not display any endogenous H-$2K^b$ expression, but the active $CD4^+$ Th1($K^{b-/-}$) cells derived from naïve $CD4^+$ Th1($K^{b-/-}$) cells activated by DC_{OVA} did express both H-$2K^b$ and pMHC I. As a control, the active $CD4^+$ Th1(pMHC $I^{-/-}$) cells derived from activation of naïve OT II $CD4^+$ T cells with ($K^{b-/-}$)DC_{OVA} did express H-$2K^b$, but did not express pMHC I (Fig. 1c), indicating that the pMHC I complexes on active $CD4^+$ Th1 cells are acquired from DC_{OVA}, but not derived from uptaking the DC_{OVA}-derived OVA protein and self-loading OVA I peptide onto the self H-$2K^b$ molecules. In addition, the active $CD4^+$ Th1(pMHC $I^{-/-}$) cells with acquired pMHC I complexes displayed a similar cytokine profiles as the active $CD4^+$ Th1 cells with acquired pMHC I complexes (Fig. 1b). Therefore, these two types of $CD4^+$ Th1 cells became useful reagents in studying the role of acquired pMHC I complexes in delivery of $CD4^+$ Th1's effect to $CD8^+$ Tc1 cells *in vivo*.

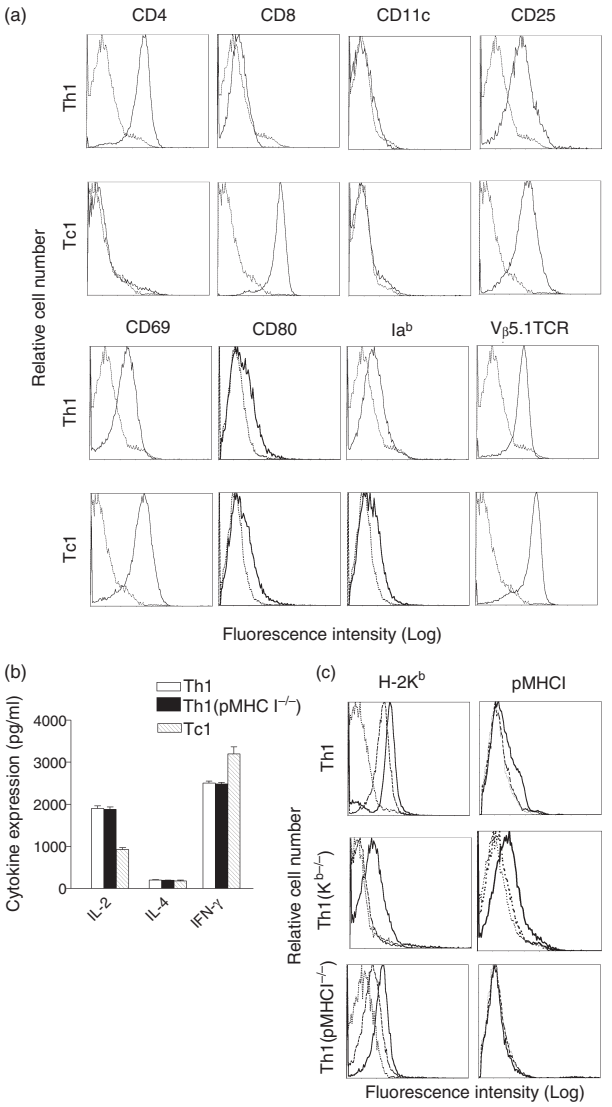

Figure 1. Phenotypic characterization of *in vitro* DC_{OVA}-activated $CD4^+$ Th1 and $CD8^+$ Tc1 cells. (a) The *in vitro* DC_{OVA}-activated $CD4^+$ Th1 and $CD8^+$ Tc1 cells as described in Materials and Methods were stained using a panel of Abs for analysis of CD4, CD8, CD11c, CD25, CD69, CD80, Ia^b and Vβ5·1TCR (solid lines). The isotype-matched irrelevant Abs were used as controls (dotted lines). (b) The supernatants of these $CD4^+$ Th1, Th1(pMHC $I^{-/-}$) and $CD8^+$ Tc1 cells were assayed for IFN-γ, IL-4 and IL-2 secretion by ELISA, respectively. The values presented represent the means of triplicate cultures from three distinct experiments. (c) Transfer of H-$2K^b$ and pMHC I molecules onto $CD4^+$ Th1 cells by DC activation. The active $CD4^+$ Th1 or $CD4^+$ Th1($K^{b-/-}$) cells (solid lines) derived from activation of naïve OT II $CD4^+$ T cells or $CD4^+$ T($K^{b-/-}$) cells of OT II/H-$2K^{b-/-}$ mice by DC_{OVA}, and the original naïve $CD4^+$ T or $CD4^+$ T($K^{b-/-}$) cells (thick dotted lines) as well as the active $CD4^+$ Th1(pMHC $I^{-/-}$) cells (solid lines) derived from activation of naïve OT II $CD4^+$ T cells by ($K^{b-/-}$)DC_{OVA}, and the original naïve OT II $CD4^+$ T cells (thick dotted lines) were stained with a panel of Abs for H-$2K^b$ and pMHC I and analyzed by flow cytometry. The isotype-matched irrelevant Abs were used as controls (thin dotted lines). One representative experiment of three in the above different experiments is shown.

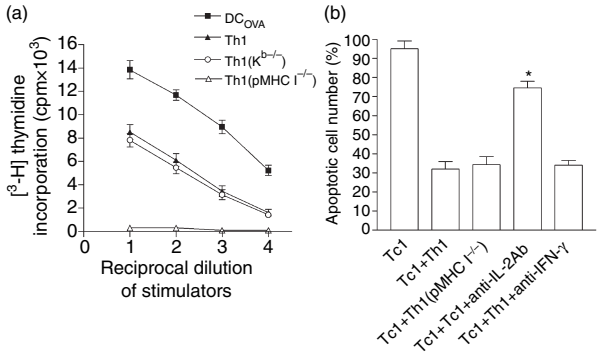

Figure 2. Functional characterization of *in vitro* DC_{OVA}-activated $CD4^+$ OT II Th1 cells. (a) Naïve OT I $CD8^+$ T cell proliferation assay. Varying numbers of stimulators including the irradiated $CD4^+$ Th1, Th1($K^{b-/-}$), Th1(pMHC $I^{-/-}$) cells and DC_{OVA} were cocultured with a constant number of naïve OT I $CD8^+$ T cells. After two days, the proliferative responses of the $CD8^+$ T cells were determined by ^{3}H-thymidine uptake assays. (b) Apoptosis formation assay. Active $CD8^+$ Tc1 cells were harvested 4 days after *in vitro* DC_{OVA} activation, and cultured with or without $CD4^+$ Th1 cells in the medium without IL-2 for 4 days. T cells were harvested, stained with FITC-Annexin V and PE-anti-CD8 Ab, and analyzed by flow cytometry. In some experiments, each of a panel of neutralizing reagents (anti-IL-2 and -IFN-γ Abs) (each 15 μg/ml) was added to the culture system. Th1(pMHC $I^{-/-}$) cells without acquired pMHC I complexes were used as control cells in the above assay. $^*P < 0{\cdot}05$ (Student *t*-test) vs. cohorts of Th1 plus Tc1 cells. One representative experiment of two in the above different experiments is shown.

$CD4^+$ Th1 cells stimulate naïve $CD8^+$ T cell proliferation via acquired pMHC I complexes

We have previously shown that active $CD4^+$ Th cells with acquired DC molecules can stimulate naïve $CD8^+$ T cell proliferation and activation[22]. To confirm that the stimulation of naïve $CD8^+$ T cells is mediated via acquired pMHC I on $CD4^+$ Th1 cells, we repeated T cell proliferation assays using $CD4^+$ Th1(pMHC $I^{-/-}$) cells with similar cytokine profiles [secreting IFN-γ (~2·6 ng/ml/10^6 cells/24 hr) and IL-2 (~2·2 ng/ml/10^6 cells/24 hr)] as $CD4^+$ Th1 cells, but without acquired pMHC I complexes. As shown in Fig. 2(a), the positive control DC_{OVA} strongly induced OT I cell proliferation. Active $CD4^+$ Th1 cells with acquired DC molecules also stimulated proliferation of naïve OT I $CD8^+$ T cells, but in a less extent, possibly due to its less costimulatory molecules compared with DC_{OVA}. Interestingly, $CD4^+$ Th1(pMHC $I^{-/-}$) cells without acquired pMHC I complexes failed in stimulation of naïve $CD8^+$ T cell proliferation, whereas $CD4^+$ Th1($K^{b-/-}$) cells lacking self H-$2K^b$, but with acquired pMHC I complexes had similar effect as $CD4^+$ Th1 cells, thus confirming that activation of naïve $CD8^+$ OT I T cells is critically mediated via acquired pMHC I complexes.

$CD4^+$ Th1 cells prolong active $CD8^+$ Tc1 cell survival *in vitro* via IL-2 secretion

T cell death or apoptosis formation can be derived from lymphokine withdrawal[29]. As shown in Figs 2(b) and 95% of active $CD8^+$ Tc1 cells expressed Annexin V (early apoptosis marker) in medium without IL-2 after 4 days incubation. The $CD8^+$ Tc1 cell apoptosis formation dramatically dropped to only 32% in presence of $CD4^+$ Th1 cells, indicating that $CD4^+$ Th1 cells can inhibit apoptosis formation of active Tc1 cells. $CD4^+$ Th1(pMHC $I^{-/-}$) with similar level of IL-2 secretion, even without acquired pMHC I, can still prolong $CD8^+$ Tc1 survival *in vitro*. Interestingly, our data showed that Th1's protection was significantly reduced ($P < 0{\cdot}05$) in presence of anti-IL-2 Ab, but not in presence of anti-IFN-γ Ab, indicating that Th1's help effect is mediated via its IL-2 secretion.

$CD4^+$ Th1 cells promote $CD8^+$ Tc1 cell tumor infiltration *in vivo*

We next tested whether $CD4^+$ Th1 cells can promote $CD8^+$ Tc1 cell tumor infiltration *in vivo* by flow cytometry. Our data showed that the amount of transferred $CD8^+$ Tc1 cells reached peak at 3 days in tumors, but dramatically dropped to a minimal level of detection at 5 days subsequent to T cell injection (Fig. 3a). At day 3 subsequent to Tc1 cell injection, the transferred OVA-specific $CD8^+$ Tc1 cells detected in tumors of mice with injection of $CD8^+$ Tc1 and $CD4^+$ Th1 cells is 0·52% of the total $CD8^+$ T cells, which is around 3-folds more than that (0·16%) detected in tumors of mice with injection of $CD8^+$ Tc1 cells alone (Fig. 3b). These results clearly indicate that OVA-specific $CD4^+$ Th1 cells can also promote tumor infiltration of OVA-specific $CD8^+$ Tc1 cells *in vivo*. In addition, the numbers of OVA-specific $CD8^+$ Tc1 cells detected in EG7 tumors reduced by nearly 42% and 60% in the mice with cotreatment of Th1(IL-$2^{-/-}$) and Th1(pMHC $I^{-/-}$) cells, respectively, but were not affected with Th1(IFN-$\gamma^{-/-}$) and Th1($K^{b-/-}$) cells (Fig. 3c). Our data thus indicate that the degree of $CD8^+$ Tc1 cell tumor infiltration is greatly affected by the helper cytokine IL-2, but not IFN-γ, and critically dependent on the OVA-specific delivery to $CD8^+$ Tc1 cells *in vivo* via acquired pMHC I complexes on active $CD4^+$ Th1 cells.

$CD4^+$ Th1 cells enhance adoptive $CD8^+$ Tc1 cell therapy *in vivo*

To assess $CD4^+$ Th1's help effects *in vivo*, we conducted animal studies using *in vitro*-activated $CD8^+$ Tc1 cells for the passive transfers in combination with different amounts of *in vitro*-activated $CD4^+$ Th1 cells. As shown in Fig. 4, $CD4^+$ Th1 cells alone did not induce any tumor growth reduction. A combined adoptive T-cell therapy of $CD8^+$ Tc1 (5×10^6 cells per mouse) with $CD4^+$ Th1 (2×10^6 cells per mouse) resulted in regression of all 10 established EG7 tumors (~5 mm in diameter), indicating that OVA-specific $CD4^+$ Th1 cells can greatly enhance $CD8^+$ Tc1-cell immunotherapy. The therapeutic efficiency

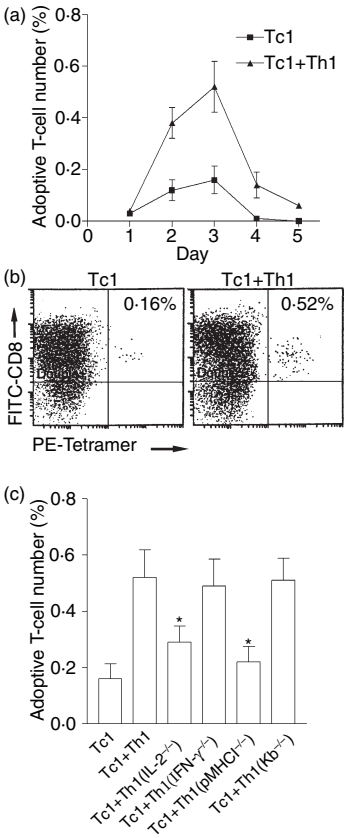

Figure 3. Enumeration of OVA-specific $CD8^+$ Tc1 cells in tumors. Active $CD8^+$ Tc1 cells (5×10^6 cells/mouse) alone or together with *in vitro*-activated Th1 cells (2×10^6 cells/mouse) were i.v. injected into EG7 tumor-bearing C57BL/6 mice. (a) Tumors were removed for preparation of cell suspensions as described in Materials and Methods at different days subsequent to T cell injection. T cells were purified from the cell suspensions by using anti-CD3 Ab-coated magnetic beads and analyzed by flow cytometry using PE-tetramer and FITC-labeled anti-CD8 Ab. (b) The tumor-bearing mice were injected i.v. with 5×10^6 Tc1 cells alone or in conjunction with 2×10^6 Th1 cells. Tumors were removed for preparation of cell suspansions 3 days after T cell injection. Plots display the percentages of FITC-CD8 and PE-tetramer positive $CD8^+$ Tc1 cells in the total $CD8^+$ T cell population. (c) The tumor-bearing mice were injected i.v. with 5×10^6 Tc1 cells in conjunction with 2×10^6 Th1, Th1(IL-$2^{-/-}$), Th1(IFN-$\gamma^{-/-}$), Th1(pMHC $I^{-/-}$) and Th1($K^{b-/-}$) cells. Tumors were removed for preparation of cell suspensions 3 days after T cell injection. $^*P < 0{\cdot}05$ vs. cohorts of injection of Tc1 plus Th1 cells (Student's *t*-test). Data shown are representative of three separate experiments with three to five mice per experimental group.

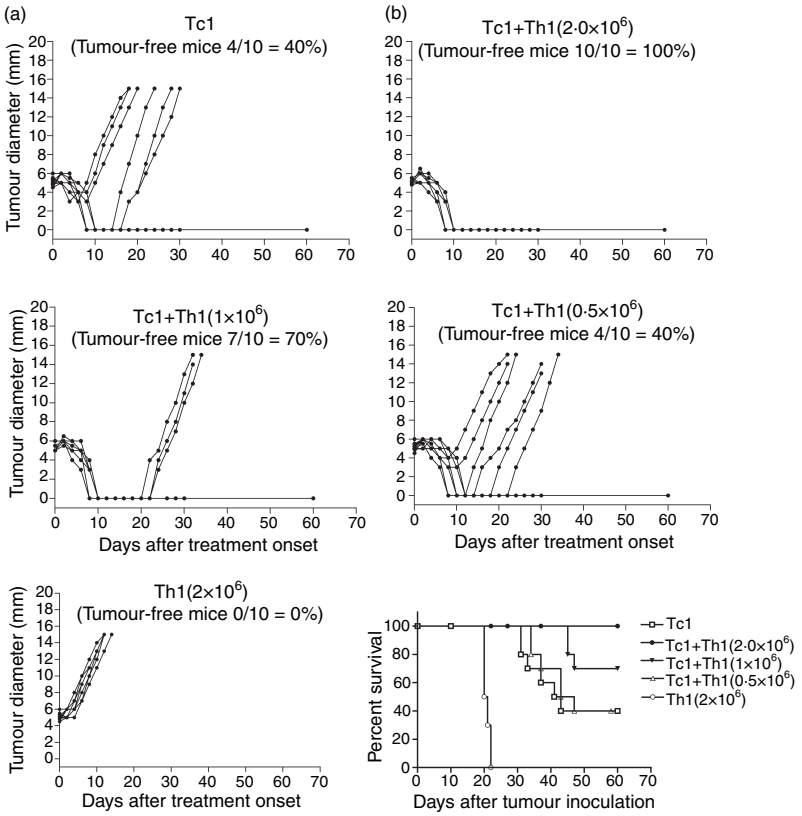

Figure 4. Impact on tumor growth and mortality rates of cotransfer of Tc1 with Th1 cell subset in adoptive T-cell immunotherapy of established tumors. Mice bearing established (i.e. ~5 mm in diameter) EG7 tumors were given i.v. injections of 5×10^6 $CD8^+$ Tc1 in conjuction with different amounts of $CD4^+$ Th1 cells. (a) Tumor growth was monitored and the tumor size (diameter) measured daily using an engineering caliper. The evolution of the tumors in individual mice is depicted, as are the fractions of mice in each treatment group that were tumor-free at 60 days post-treatment. (b) The readout in this figure represents the long-term mortality among the animals, as determined by daily assessments across 60 days post-treatment. The data closely mirror that in (a) with a conclusion that the combined $CD4^+$ Th1 and $CD8^+$ Tc1 cell immunotherapy provides a significant therapeutic advantage over the single use of Tc1 cells alone. One representative experiment of two is shown.

is dose-dependent, since $CD8^+$ Tc1 cells (5×10^6 cells per mouse) in conjunction with less $CD4^+$ Th1 cells (1×10^6 and $0{\cdot}5 \times 10^6$ cells per mouse) only cured 7/10 (70%) and 4/10 (40%) tumor bearing mice, respectively.

Targeted delivery of Th1's IL-2 to $CD8^+$ Tc1 cells *in vivo* via acquired pMHC I

To study the mechanism of Th1's effect, mice bearing tumors were i.v. injected with 5×10^6 Tc1 cells in conjunction with 2×10^6 $CD4^+$ Th1(IL-$2^{-/-}$) and $CD4^+$ Th1(IFN-$\gamma^{-/-}$) cells with respective cytokine deficiency. As shown in Fig. 5, the Th1's help effect was substantially lost in mice with administration of $CD4^+$ Th (IL-$2^{-/-}$) cells, but not affected in mice injected with $CD4^+$ Th1(IFN-$\gamma^{-/-}$) cells, indicating that the Th1's help effect is mainly dependent upon its IL-2, but not IFN-γ. $CD4^+$ Th1($K^{b-/-}$) cells with acquired pMHC I complexes had similar help effect as $CD4^+$ Th1 cells. Interestingly, the $CD4^+$ Th1's help effect was completely lost in mice with administration of $CD4^+$ Th1(pMHC $I^{-/-}$) cells with similar cytokine profiles as $CD4^+$ Th1, but without acquired pMHC I complexes, indicating the critical importance of acquired pMHC I complexes in targeted delivery of $CD4^+$ Th1's helper cytokine IL-2 to $CD8^+$ Tc1 cells *in vivo*.

$CD4^+$ Th1 cells promote $CD8^+$ Tc1 cell memory responses *in vivo*

We next assessed Th1's effect on $CD8^+$ Tm cell responses by tetramer staining assays. Active $CD8^+$ Tc1 cells can become long-lived memory Tm cells after adoptive transfer *in vivo*[30]. Three months after adoptive transfer of 5×10^6 active $CD8^+$ Tc1 cells, there were 1·32% OVA-specific $CD8^+$ T cells with CD44 expression (Tm marker)[31] in the total host $CD8^+$ T cell population (Fig. 6a), indicating that they became $CD8^+$ Tm cells. The number of detected OVA-specific $CD8^+$ Tm cells increased nearly by 3-folds to 3·86% in mice with transfer of both $CD8^+$ Tc1 and $CD4^+$ Th1 cells, indicating that $CD4^+$ Th1 cells greatly promote $CD8^+$ Tc1 cell memory responses. In the mice with transfer of $CD8^+$ Tc1 and $CD4^+$ Th1(pMHC $I^{-/-}$) cells, however, the number of detected OVA-specific $CD8^+$ Tm cells remained the same as that in the mice with transfer of $CD8^+$ Tc1 cells alone, indicating that the acquired pMHC I complexes play an important role in targeting Th1's effect to the promotion of $CD8^+$ Tc1 cell memory responses *in vivo*. These $CD8^+$ Tm cells still remained functional because they could be stimulated for expansion (~10 folds) by DC_{OVA} stimulation (Fig. 6b). Interestingly, transfer of $CD8^+$ Tc1 cells alone can completely protect 8/8 mice from a challenge of 1×10^6 EG7 tumor cells, but only 4/8 mice from a challenge of

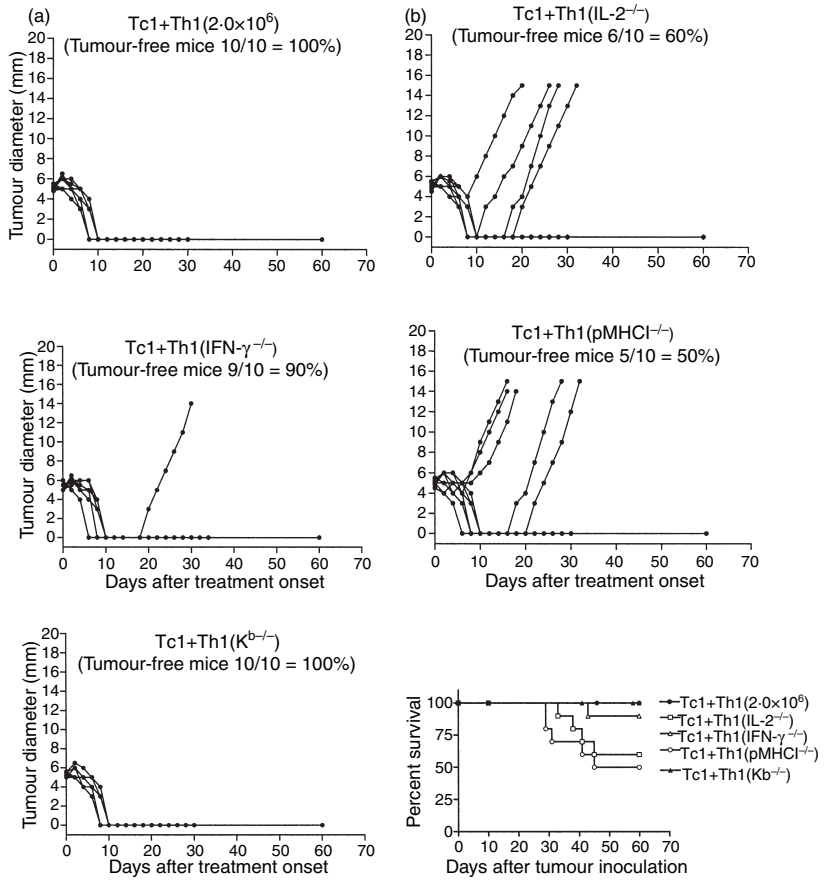

Figure 5. Animal study using $CD4^+$ Th1 cells with respective gene deficiency. The mice bearing tumors were injected i.v. with 5×10^6 of Tc1 cells in conjunction with 2×10^6 Th1, Th1(IL-$2^{-/-}$), Th1(IFN-$\gamma^{-/-}$), Th1($K^{b-/-}$) and Th1(pMHC $I^{-/-}$) cells. Tumor growth was monitored and the tumor size (diameter) measured daily using an engineering caliper. The evolution of the tumors in individual mouse is depicted, as are the fractions of mice in each treatment group that were tumor-free at 60 days post-treatment.

3×10^6 EG7 tumor cells (Fig. 6c). However, all (8/8) mice with transfer of both $CD8^+$ Tc1 and $CD4^+$ Th1 cells survived even with a challenge of 3×10^6 EG7 tumor cells, indicating that $CD4^+$ Th1 cells can promote $CD8^+$ Tc1 cell memory responses leading to an enhanced antitumor immunity.

Discussion

The cooperative role of $CD4^+$ T cells has been extensively reported for $CD8^+$ CTLs in tumor eradication in animal models[11,15,16]. However, the potential immune mechanisms underlying enhancement of $CD4^+$ Th1 cells in adoptive $CD8^+$ Tc1-cell therapy have not been well elucidated. In this study, we developed a model system with a defined tumor Ag OVA using the OVA-transfected EG7 tumor cells and the OVA-specific TCR transgenic OT I and OT II mice with class I and II specificities, respectively[23,24]. These transgenic mice provide a virtually monoclonal source of T cells with known specificity, where the respective role of OVA-specific $CD4^+$ Th1 cells in OVA-specific $CD8^+$ Tc1 cell-mediated antitumor immunity can thus be feasibly evaluated. In previous animal tumor models using TCR transgenic $CD8^+$ T cells, the treatment of only lung tumor metastasis or solid tumors in small palpable size has been investigated[5,6]. In clinical settings, however, patients usually have well-established tumors. In this study, we demonstrated that a combined adoptive transfer of $CD8^+$ Tc1 cells with $CD4^+$ Th1 cells resulted in regression of well-established tumors (5 mm in diameter), which more mimics the clinical situations, in all 10/10 mice, compared to only 40% curing rate when using adoptive transfer of $CD8^+$ Tc1 cells alone, indicating the critical help effect of $CD4^+$ Th1 cells in $CD8^+$ Tc1-cell immunotherapy of established solid tumors. Active $CD8^+$ Tc1 cells can become long-lived $CD8^+$ Tm cells after adoptive transfer *in vivo*[30]. In this study, we also demonstrated that $CD4^+$ Th1 cells greatly promoted $CD8^+$ Tc1 cell memory responses by 3-folds. Interestingly, mice with transfer of $CD8^+$ Tc1 cells alone can only protect 50% (4/8) of them from a challenge of 3×10^6 EG7 tumor cells, whereas all (8/8) mice with transfer of both $CD8^+$ Tc1 and $CD4^+$ Th1 cells survived, indicating that $CD4^+$ Th1 cells can promote $CD8^+$ Tc1 cell memory responses leading to an enhanced antitumor immunity.

Interleukin-2 (IL-2) support of transferred Tc cells *in vivo* can be critical to T-cell therapeutic efficiency. IL-2 administration after T cell transfer was shown to augment the antitumor therapy and increase transferred Tc cell persistance[32,33]. The potential mechanism by which IL-2 treatment may augment adoptive Tc cell therapy *in vivo*

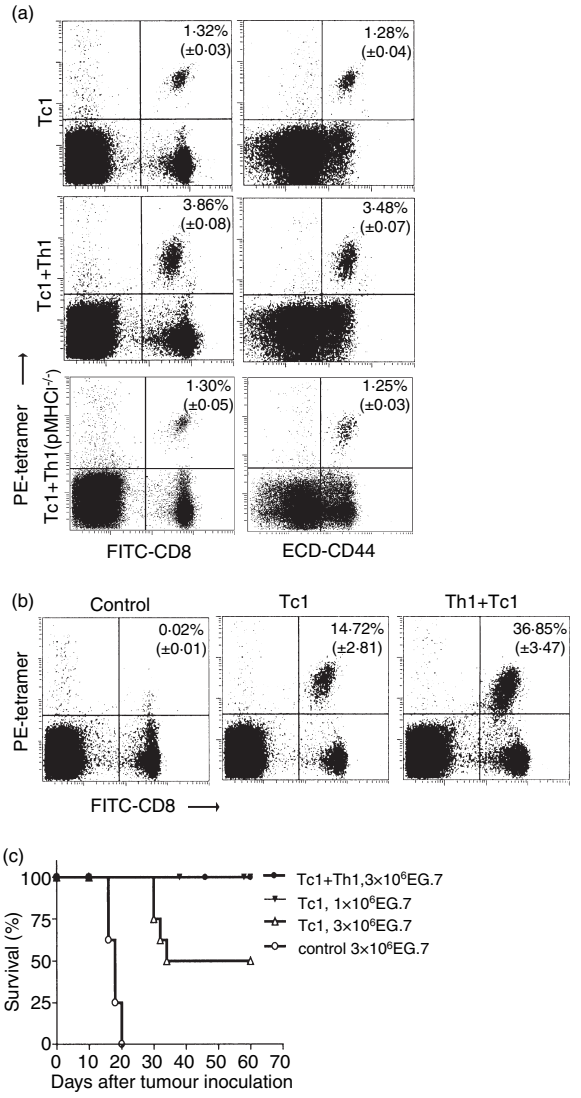

Figure 6. $CD4^+$ Th1 cells promote active $CD8^+$ Tc1 cell memory responses. (a) *In vitro* DC_{OVA}-activated $CD8^+$ Tc1 cells can become long-lived memory T cells. *In vitro* DC_{OVA}-activated $CD8^+$ Tc1 cells (5×10^6 cells/mouse) alone or in conjuction with active $CD4^+$ Th1 or Th1(pMHC $I^{-/-}$) cells (2×10^6 cells/mouse) were i.v. injected into C57BL/6 mice. Three months later, tail blood samples were taken from the mice, and a triple staining for PE-K^b/OVA tetramer, FITC-anti-CD8 and ECD-anti-CD44 Abs were conducted to determine the percentage of OVA-specific triple staining-positive $CD8^+$ T cells in the total $CD8^+$ population indicated in each right upper plot. The T cell population with PE-tetramer and FITC-CD8 double positivity was also the respective population with PE-tetramer and ECD-CD44 double positivity by flow cytometric analysis. (b) Three months later, the immunized mice were boosted by i.v. injection of 1×10^6 irradiated DC_{OVA}. Four days after the boost, tail blood samples were taken from the boosted mice, and a double staining for PE-K^b/OVA tetramer and FITC-anti-CD8 Ab were conducted to determine the percentage of OVA-specific double staining-positive $CD8^+$ T cells in the total $CD8^+$ population indicated in each right upper plot. Naive C57BL/6 mice were also boosted with DC_{OVA} and used as control. (c) Three months later, the immunized mice were also s.c. challenged with 1×10^6 or 3×10^6 EG7 tumor cells. Mouse survival was monitored daily. The results presented are representative of two separate experiments with 4 mice (a & b) or 8 mice (c) per group.

by promoting the survival and proliferation of transferred Tc cells[34,35]. Although the systemic use of IL-2 could enhance T-cell therapy efficiency in mouse models, the results from clinical trials in treatment of melanomas and renal cell carcinomas were very disappointed, showing no improvement in T-cell therapeutic efficiency[36,37]. In addition, the systemic administration of IL-2 also induced severe side-effects such as the vascular leak syndrome[38]. An alternative is the use of $CD4^+$ Th cells providing IL-2 help to $CD8^+$ Tc cells[13]. However, the molecular mechanism by which the $CD4^+$ Th cell's IL-2 is specifically delivered to $CD8^+$ Tc cells *in vivo* is unclear. T cell death or apoptosis formation can be derived from lymphokine withdrawal[29]. In this study, we demonstrated that $CD4^+$ Th1 cells can reduce apoptosis formation of active $CD8^+$ Tc1 cells by 3-folds in the culture medium without IL-2 stimulation, indicating that this help effect may be mediated via its secreted helper cytokine IL-2. In addition, we also demonstrated that (i) the $CD4^+$ Th1 cells greatly augmented the *in vivo* $CD8^+$ Tc1 cell-therapy in treatment of established tumors, and (ii) the Th1's help effect was substantially lost in mice with administration of $CD4^+$ Th1(IL-$2^{-/-}$) cells, indicating that the *in vivo* Th1's help effect is dependent upon its IL-2.

T cell-to-T cell (T-T) Ag presentation, dependent upon $CD4^+$ T cells first acquiring MHC and costimulatory molecules from APCs, and then becoming Th-APCs with the capacity in stimulation of other T cells, is increasingly attracting attention[39,40]. Recently, Brandes *et al.* have shown that active human T cells expressed a similar phenotype as APC and can act as Th-APCs in stimulation of CTL responses after directly pulsed with soluble Ag[41]. Although these active $CD4^+$ T cells pulsed with Ag can act as T-APCs in stimulation of CTL responses, they are different from the concept of $CD4^+$ Th-APCs as recently described[22]. We have recently demonstrated that *in vitro* DC-activated $CD4^+$ T cells with acquired DC membrane molecules by DC activation can act as Th-APCs in stimulation of CTL responses and antitumor immunity[22] and $CD4^+$ Th(pMHC $I^{-/-}$) cells without acquired pMHC I complexes lost its antitumor protection (data not shown). In addition, we also found that *in vivo* DC-activated $CD4^+$ T cells can also acquire APC membrane molecules by DC activation, and induce antitumor immunity (data not shown), indicating the physiological significance of APC membrane molecule acquisition of $CD4^+$ T cells *in vivo*. Kennedy *et al.* have also demonstrated that $CD4^+$ T cells can acquire APC membrane molecules *in vivo*, and induce memory CTL responses[42] further confirming the

physiological significance of the above concept. In this study, in addition to the acquired costimulatory molecules[22] we further demonstrated that $CD4^+$ T cells acquired pMHC I complexes by using a specific antibody recognizing pMHC I complexes. More importantly, for the first time, we elucidated the molecular mechanism on targeted delivery of $CD4^+$ T cell help to active $CD8^+$ Tc cells *in vivo* by using the recently established control $CD4^+$ Th1(pMHC $I^{-/-}$) cells. We showed that it is the acquired pMHC I complex on active $CD4^+$ Th1 cells that mediate the specific delivery of the $CD4^+$ Th cytokine IL-2 to $CD8^+$ Tc1 cells *in vivo*, leading to an enhanced Tc1-cell immunotherapy of established EG7 solid tumors. In addition, we also found that the DC_{OVA}-activated $CD4^+$ Th1 cells with acquired pMHC I complexes were able to stimulate naive $CD8^+$ T cell proliferation *in vitro* and *in vivo*, and induce antitumor immunity[22] whereas $CD4^+$ Th1(pMHC $I^{-/-}$) cells with the same cytokine profiles as $CD4^+$ Th1 cells, but without acquired pMHC I complexes completely lost its stimulatory effect (data not shown), thus confirming the importance of acquired pMHC I complexes in targeting $CD4^+$ Th1's helper effects to both naïve or active $CD8^+$ Tc cells *in vivo*.

The importance of successful Tc-cell immunotherapy of Tc-cell tumor infiltration is increasingly being recognized[2,26,43]. The systemic administration of IL-2 has been shown to enhance $CD8^+$ T cells tumor infiltration[10]. However, the severe side-effects[38] derived from the systemic administration of IL-2 limit its use in clinic trials. It has also been shown that the transferred $CD8^+$ T cells infiltrated into tumors only in presence $CD4^+$ T cell help by immunohistochemical analysis[16]. In this study, we kinetically and quantitatively examined $CD8^+$ Tc1 cell tumor infiltration by flow cytometric analysis. Our data showed that a 3-fold more of the transferred OVA-specific $CD8^+$ Tc1 cells can be detected in EG7 tumors of mice with treatment of both $CD4^+$ Th1 and $CD8^+$ Tc1 cells, indicating that $CD4^+$ Th1 cells can significantly promote $CD8^+$ Tc1 cell tumor infiltration. Interestingly, we also clearly demonstrated that the *in vivo* $CD4^+$ Th1 cell's helper effect in promoting $CD8^+$ Tc1 cell tumor infiltration is mediated by its IL-2. More importantly, we further demonstrated that the critical role of acquired pMHC I complexes on $CD4^+$ Th1 cells in targeting its helper effect in promoting $CD8^+$ Tc1 cell tumor infiltration. In addition, some other factors may also be involved in enhanced $CD8^+$ Tc1 cell tumor infiltration, including (i) an augmented $CD8^+$ Tc1 cell pool in the mice, thereby allowing for enhanced $CD8^+$ T cell infiltration of tumors, and (ii) a prolonged survival of tumor infiltrating $CD8^+$ Tc1 cells in tumors. In addition, some other mechanisms may also be involved. For example, it has been reported that the costimulatory signals[41,42,44] and MHC class II molecules[45] were involved in survival of the responding cells such as T cells. Therefore, the active Th1 cells with acquired CD80 and pMHC II may also provide its helper effects to active Tc1 cells *in vivo* through these mechanisms.

Taken together, our results provide clear evidence that $CD4^+$ Th1 cells are capable of enhancing adoptive $CD8^+$ Tc1-cell immunotherapy by prolonging $CD8^+$ Tc1 cell survival *in vitro* and promoting $CD8^+$ Tc1 cell tumor localization and memory responses *in vivo*. The $CD4^+$ Th1's effect is mainly mediated by its own helper cytokine IL-2 specifically targeted to $CD8^+$ Tc1 cells *in vivo* via acquired pMHC I complexes. Therefore, these results will have important implications for designing adoptive T-cell immunotherapy protocols in treatment of established solid tumors.

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