$CD4^+$ T cells stimulate memory $CD8^+$ T cell expansion via acquired pMHC I complexes and costimulatory molecules, and IL-2 secretion

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Abstract: The rapid and efficient expansion of $CD8^+$ memory T cells after the second encounter with a pathogen constitutes a hallmark trait of adaptive immunity. Yet, the contribution of $CD4^+$ T cells to the expansion of memory $CD8^+$ T cells remains the subject of controversy. Here, we show that, antigen-specific $CD4^+$ T cells, once activated by dendritic cells (DC) in vitro, have the capacity to stimulate expansion of memory $CD8^+$ T cells in vivo. The memory $CD8^+$ T cell expansion triggered by active $CD4^+$ T cells are mediated through DC-derived MHC I/peptide complexes and CD80 molecules displayed on the active $CD4^+$ T cells, with the involvement of IL-2 secreted by the active $CD4^+$ T cells. These results highlight a previously undescribed role of active $CD4^+$ T cells in triggering expansion of memory $CD8^+$ T cells. *J. Leukoc. Biol.* 80: 1354–1363; 2006.

Key words: *dendritic cells · CD8 memory · intracellular staining · tetramer staining · flow cytometry*

INTRODUCTION

To generate functional $CD8^+$ memory T (Tm) cells is the goal of vaccinations against infection of virus and intracellular bacteria. After priming, 5% to 10% of the effector $CD8^+$ T cells give rise to long-lived $CD8^+$ Tm cells [1]. These $CD8^+$ Tm cells expand rapidly after the second encounter with a pathogen. $CD4^+$ T cells are essential in the development of CD8 memory as evidenced by recent data showing that $CD8^+$ Tm cells generated in the absence of $CD4^+$ T cells proliferate poorly [2–7] and that the frequency of $CD8^+$ Tm cells are diminished in the $CD4^+$ T cell-absent environment [8]. It has been also proposed that expansion of $CD8^+$ Tm cells is $CD4^+$ T cell dependent, as well [9, 10]. However, in other model systems, it was demonstrated that $CD8^+$ Tm cells proliferate equally in the absence or presence of $CD4^+$ T cells [5, 7]. The mechanism(s) behind the involvement of $CD4^+$ T cells for the mounting of functional CD8 memory still remains mostly unknown. One of the critical questions is whether the "dictation" of CD8 memory development by $CD4^+$ T cells is mediated through direct interaction between $CD4^+$ and $CD8^+$ T cells or via antigen-presenting cells (APC) indirectly. Indeed, recent evidence suggested that a direct interaction between $CD8^+$ T cells and $CD4^+$ T cells through CD40/CD40L is necessary for $CD8^+$ Tm cell development [2], although it was also shown that failure of CD40 expression by $CD8^+$ T cells does not affect the development of functional CD8 memory in other model systems [11–13]. Further, it has been recently reported that $CD4^+$ T cells can directly stimulate differentiation of naïve $CD8^+$ T cells into memory cells through their endogenous MHC class I/peptide (pMHC I) complexes generated by processing exogenously acquired antigen [14]. Hypothetically, direct interactions between $CD4^+$ and $CD8^+$ T cells would be most efficient, if $CD4^+$ T cells possess cell surface molecules characteristic of professional APC and the interaction occurred in an antigen-specific manner.

Recent data have demonstrated that, during the activation of T cells by APCs, an immunological synapse is rapidly formed at the contact sites, which consists of a central cluster of TCR-MHC-peptide complexes and CD28/CD80 surrounded by a ring of engaged accessory molecules, including CD40L/CD40 and complexed lymphocyte function-associated antigen (LFA)-1/CD54 [15, 16]. It has been further demonstrated that $CD8^+$ T cell-APC interactions through the immune synapses can lead to rapid internalization of synapse-composed pMHC I complexes through T cell receptor (TCR)-mediated endocytosis and expression of these APC molecules on the surface of $CD8^+$ T cells by recycling [17, 18]. Similarly, it has been also reported that active $CD4^+$ T cells display APC-derived MHC class II/peptide and CD80 [19–23]. Alternatively, T cells are also able to acquire APC-derived molecules through exosomes shed from APCs [24]. Recently, we have demonstrated, in addition to previously reported immune synapse-composed MHC class II/peptide, CD54 and CD80 [19–23], that $CD4^+$ T cells can also acquire the bystander pMHC I complexes [25].

$CD4^+$ T cells acquire pMHC I complexes and costimulatory molecules during their activation, raising the possibility that active $CD4^+$ T cells have the capacity to directly stimulate the recall responses of $CD8^+$ Tm cells through the interaction

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Received May 10 2006; revised July 4, 2006; accepted July 31, 2006; doi: 10.1189/jlb.0506321.

between active $CD4^+$ T cells and $CD8^+$ Tm cells. To test this, we established a model system in which ovalbumin (OVA)-specific $CD8^+$ T cells were activated in vitro by dendritic cells (DC) pulsed with OVA and then adoptively transferred into naïve mice. The development of CD8 memory was then determined by MHC tetramer staining and intracellular IFN-γ staining. Consecutively, we examined the capacity of active $CD4^+$ T cells to stimulate $CD8^+$ Tm cells and the underlying mechanisms.

MATERIALS AND METHODS

Reagents, antibodies, cell lines, and animals

Ovalbumin (OVA) was obtained from Sigma (St. Louis, MO). The OVA I (SIINFEKL) peptide was synthesized by Multiple Peptide Systems (University of Calgary, Calgary, AB, Canada). Golgistop were purchased from BD-Biosciences (San Diego, CA). The following monoclonal antibodies (mAb) were purchased from BD-Biosciences: FITC-labeled rat anti-mouse CD4 (Clone: GK1.5); FITC-labeled anti-mouse Vβ5.1, 5.2 TCR (Clone: MR9-4); FITC-labeled rat anti-mouse CD11c (Clone: HL3); FITC-labeled rat anti-mouse CD25 (Clone: 7D4); FITC-labeled rat anti-mouse CD40 (Clone: 3/23); FITC-labeled rat anti-mouse CD62L (Clone: MEL-14); FITC-labeled anti-mouse CD69 (Clone: H1.2F3); FITC-labeled rat anti-mouse CD44 (Clone: IM7); FITC-labeled rat anti-mouse CD54 (Clone: 3E2); FITC-labeled anti-mouse CD80 (Clone: 16-10A1); FITC-labeled anti-mouse H-$2K^b$ (Clone: AF6-88.5); FITC-labeled anti-mouse Ia^b (Clone: AF6-120.1); FITC-labeled rat IgG2a (Clone: B39-4); FITC-labeled rat IgG2b (Clone: A95-1); FITC-labeled rat IgM (Clone: R4-22); FITC-labeled mouse IgG1 (Clone: A112-2); FITC-labeled mouse IgG2a (Clone: G155-178); FITC-labeled Hamster IgG (A19-4); biotin-conjugated anti-mouse CD45.1 (Clone: A20); biotin-conjugated mouse IgG2a (G155-178); PE-labeled rat anti-mouse IFN-γ (Clone: XMG1.2); PE-labeled rat IgG1 (R3-34); FITC-labeled rat IgG2a (R35-95). PE-labeled H-$2K^b$/$OVA_{257-264}$ tetramer, FITC-labeled rat anti-mouse CD8 (Clone: KT15), and ECD (phycoerythrin-Texas Red-X)-conjugated streptavidin were obtained from Beckman Coulter (San Diego, CA). FITC-labeled mAb specific for K^b/$OVA_{257-264}$ complexes [26] was gifted by Dr. Germain (NIH, Bethesda, MD). Recombinant mouse granulocyte macrophage colony stimulating factor (GM-CSF), IL-2, and IL4, and CTLA-4/Ig were obtained from R&D systems (Minneapolis, MN). The OVA-transfected tumor cell line EG7 expressing H-$2K^b$ was obtained from American Type Culture Collection (ATCC, Rockville, MD). Female C57BL/6 mice ($CD45.2^+$, B6) were obtained from Charles River Laboratories (St. Laurent, Quebec, Canada). The OVA-specific T cell receptor (TCR) transgenic OT I or OT II mice and $CD54^{-/-}$, $CD80^{-/-}$ as well as H-$2K^{b-/-}$ mice on C57BL/6 background, and B6.SJL-Ptpcra mice ($CD45.1^+$, B6.1) were purchased from the Jackson Laboratory (Bar Harbor, ME). Homozygous OT I/B6.SJL-Ptpcra mice were generated by backcrossing the B6.SJL-Ptpcra mice onto the OT I mice on C57BL/6 background for three generations; OT II/H-$2K^{b-/-}$, OT II/$CD54^{-/-}$, OT II/$CD80^{-/-}$, OT II/IL-$2^{-/-}$, as well as OT II/IFN-$\gamma^{-/-}$ C57BL/B6 mice were generated by backcrossing the designated gene-deficient mice onto OT II background for three generations; homozygosity was confirmed by polymerase chain reaction (PCR), according to Jackson ImmunoResearch Laboratory's protocols. All mice were housed in the animal facility at the Saskatoon Cancer Center and treated according to the Animal Care Committee guidelines of University of Saskatchewan. The mice were used at 8–10 wk of age.

Dendritic cells (DC)

Bone marrow (BM)-derived DC were generated as described previously [25]. Briefly, BM cells were collected from the femorae and tibiae of normal or designated gene-deleted C57BL/6 mice. The BM cells were depleted of red blood cells with 0.84% ammonium chloride and plated in DC culture medium (Dulbecco's modified Eagle's medium [DMEM] plus 10% FCS, GM-CSF [20 ng/ml], and IL-4 [20 ng/ml]). On day 3, the nonadherent granulocytes and T and B cells were gently removed, and fresh media were added. Two days later, the loosely adherent proliferating DC aggregates were dislodged and replanted. On day 6, the nonadherent DC cells were dendritic cells. These DC were then pulsed with 0.4 mg/ml OVA overnight at 37°C in the presence of LPS (1 μg/ml), then washed extensively [25] and referred to as DC_{OVA}.

OT I $CD8^+$ and OT II $CD4^+$ T cells

Spleens were removed from OT I or OT II C57BL/6 mice and mechanically disrupted to obtain a single-cell suspension. The erythrocytes were lysed using 0.84% ammonium chloride. Naïve T cells were enriched by passage through nylon wool columns (C&A Scientific, Manassas, VA). Naïve OVA-specific $CD8^+$ T cells (OT I mice) or $CD4^+$ T cells (OT II mice) were then purified by negative selection using anti-mouse CD4 (L3T4) or CD8 (Ly2) paramagnetic beads (DYNAL Inc., Lake Success, NY). To generate OVA-specific active $CD8^+$ or $CD4^+$ T cells, naïve $CD8^+$ T cells (2×10^5 cells/ml) from OT I mice or naïve $CD4^+$ T cells from OT II mice were stimulated for 72 h with irradiated (4,000 rads) DC_{OVA} (1×10^5 cells/ml) in the presence of IL-2 (10 U/ml). These in vitro activated $CD8^+$ or $CD4^+$ T cells were separated by Ficoll-Paque (Sigma) density gradient centrifugation and further purified using CD8 or CD4 microbeads (Miltenyi Biotech, Auburn, CA) [25]. The viability of purified T cells was examined by trypan blue exclusion methods. More than 95% purified T cells were alive. In vitro DC_{OVA}-activated $CD4^+$ T cells derived from wild-type OT II mice were referred to Th-APC. In vitro DC_{OVA}-activated $CD4^+$ T cells derived from OT II/IL-$2^{-/-}$ and OT II/IFN-$\gamma^{-/-}$ mice were referred to Th(IL-$2^{-/-}$)-APC and Th(IFN-$\gamma^{-/-}$)-APC, respectively. In vitro ($CD54^{-/-}$)DC_{OVA}- and ($CD80^{-/-}$)DC_{OVA}-activated $CD4^+$ T cells derived from OT II/$CD54^{-/-}$ and OT II/$CD80^{-/-}$ mice were referred to Th($CD54^{-/-}$)-APC($CD54^{-/-}$) and Th($CD80^{-/-}$)-APC($CD80^{-/-}$), respectively. In vitro ($K^{b-/-}$)DC_{OVA}- and ($CD80^{-/-}$)DC_{OVA}-activated $CD4^+$ T cells derived from wild-type OT II mice were referred to Th-APC($K^{b-/-}$) and Th-APC($CD80^{-/-}$).

Phenotypic characterization of DC_{OVA} and DC_{OVA}-activated T cells

For the phenotypic analyses, DC_{OVA} were stained with FITC-labeled mAbs specific for Ia^b, CD11c, CD40, CD54, CD80, and K^b/$OVA_{257-264}$ complexes, respectively. DC_{OVA}-activated $CD4^+$ or $CD8^+$ T cells were also stained with FITC-labeled mAbs specific for CD4, CD8, Vβ5.1, 5.2 TCR, CD25, CD62L, and CD69, respectively, and analyzed by flow cytometry. For analysis of the cytokine profile, T cells were incubated in the presence or absence of 4000 rad-irradiated EG7 tumor cells for 24 h. Culture supernatants were analyzed for secretion of IL-2, IL-4, and IFN-γ using ELISA kits, according to the manufacturer's protocols.

Membrane molecule transfer assays

$CD4^+$ T cells derived from designated gene-deleted OT II mice were stained with mAbs specific for the corresponding molecules and K^b/$OVA_{257-264}$ complexes before or after incubation with DC or DC_{OVA} for 72 h and then analyzed by flow cytometry. In addition, active $CD4^+$ T cells generated by incubation of naïve $CD4^+$ T cells from OT II mice with DC_{OVA} derived from wild-type or H-$2K^{b-/-}$ C57BL/6 mice were analyzed for the display of pMHC I following staining with FITC-labeled mAb specific for K^b/$OVA_{257-264}$ complexes. In another set of experiments, $CD4^+$ T cells from OT II mice were incubated with DC_{OVA} derived from wild-type or $CD80^{-/-}$ C57BL/6 mice for 72 h and then stained with mAb specific for K^b/$OVA_{257-264}$ complexes.

Adoptive transfer and challenge

C57BL/6 mice were injected in tail veins with 5×10^6 in vitro DC_{OVA}-activated OVA-specific $CD8^+$ T cells diluted in PBS. In some cases, mice were challenged with DC_{OVA} or active $CD4^+$ T cells. OVA-specific $CD8^+$ T cells were enumerated using tetramer staining or intracellular cytokine staining.

Tetramer staining

Blood was taken from the tail of mice. Spleens were removed from mice, and spleen cells were separated and depleted of erythrocytes. The blood samples or spleen cells were incubated with 10 μl PE-conjugated H-$2K^b$/$OVA_{257-264}$ tetramer and 1 μl FITC-conjugated anti-CD8 mAb or FITC-conjugated anti-CD44 mAb for 30 min at room temperature. In some cases, the cells were additionally stained with biotin-conjugated anti-CD45.1 mAb, followed by

washes and staining with ECD-conjugated streptavidin. The erythrocytes were then lysed using lysis/fixed buffer (Beckman Coulter). The cells were washed and analyzed by flow cytometry.

Intracellular cytokine staining

Cytokine expression was examined ex vivo in freshly isolated spleen cells. Spleens were removed from mice, and spleen cells were harvested and depleted of erythrocytes. The spleen cells were cultured for 4 h with 2 μM monensin (GolgiStop) in the presence of 2 μM OVA I peptide. After culture, cells were stained with FITC-anti-CD8 mAb. The cells were then fixed, and cell membranes were permeabilized in Cytofix/Cytoperm solution (BD Biosciences) and stained with PE-labeled anti-IFN-γ mAb. Cells were then washed and analyzed using a FACSCalibur.

Statistical analysis

Data are presented as the means ± SE. The significance of differences was determined by Student's *t*-test using StatView SE Software (Abacus Concepts, Berkeley, CA).

RESULTS

Phenotypic characterization of in vitro-activated OVA-specific T cell subsets

OVA-pulsed DC (DC_{OVA}) displayed Ia^b, CD11c, CD40, CD54, and CD80 (**Fig. 1A**), indicating that they are mature DC. In addition, they also expressed MHC I/peptide (pMHC I) complexes. To generate active OVA-specific $CD4^+$ and $CD8^+$ T cells, naïve $CD4^+$ or $CD8^+$ T cells, isolated from OT II or OT I mice, respectively, were incubated with DC_{OVA} for 72 h. As illustrated in Fig. 1B, both of the two T cell subsets displayed their T cell subset marker (CD4 or CD8) and Vβ5.1, 5.2 TCR. They also displayed CD25, and CD69, but not CD62L, indicating that they were highly activated (Fig. 1B). Following in vitro restimulation with irradiated EG7 tumor cells, both CD4 (Th) and CD8 (Tc) populations secreted abundant IL-2 and IFN-γ, but very little IL-4 in their culture supernatants (Fig. 1C), indicating that these $CD4^+$ and $CD8^+$ populations comprised Th1 and Tc1 phenotype cells, respectively.

$CD4^+$ T cells acquire pMHC I complexes and costimulatory molecules, including CD54 and CD80 during their activation by DC_{OVA}

Next, we examined whether $CD4^+$ T cells acquired surface molecules from DC_{OVA} during their activation. To this end, $CD4^+$ T cells derived from OT II/H-$2K^{b-/-}$, OT II/$CD54^{-/-}$, or OT II/$CD80^{-/-}$ C57BL/6 mice were incubated for 72 h with DC (as control) or DC_{OVA} derived from wild-type C57BL/6 mice. These $CD4^+$ T cells were stained with mAbs specific for H-$2K^b$, $K^b/OVA_{257\text{-}264}$ complexes, CD54, or CD80 before or after incubation with DC or DC_{OVA}. As anticipated, naïve $CD4^+$ T cells did not express the corresponding molecules and pMHC I. $CD4^+$ T cells following incubation with DC for 72 h did not display the corresponding molecules and pMHC I either. In contrast, $CD4^+$ T cells activated by DC_{OVA}, do display the corresponding molecules and pMHC I (**Fig. 2A**), suggesting that DC_{OVA} surface molecules, including H-$2K^b$, CD54, and CD80, as well as pMHC I were transferred to $CD4^+$ T cells during their activation. To further address this issue, active $CD4^+$ T cells (Th-APC) generated by incubation of

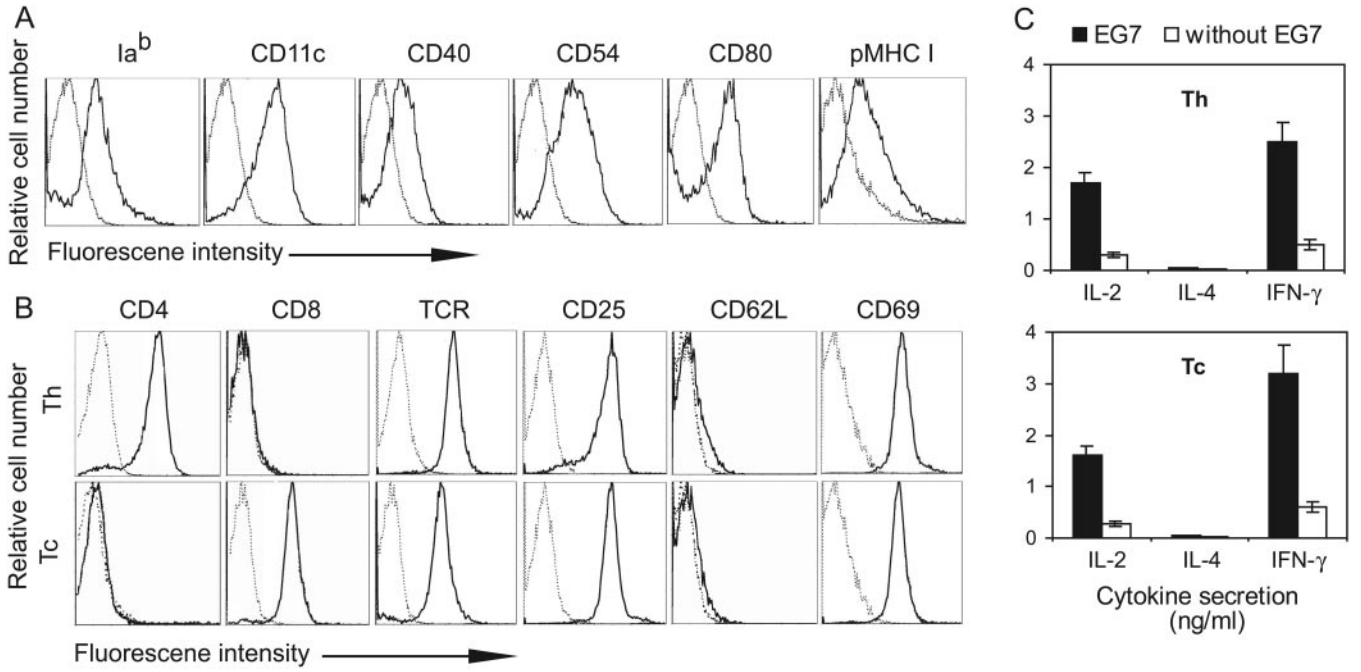

Fig. 1. Phenotypic analysis of OVA-pulsed DC_{OVA} and DC_{OVA}-activated $CD4^+$ and $CD8^+$ T cells. (A) DC_{OVA} were stained using a panel of mAbs (solid lines) specific for Ia^b, CD11c, CD40, CD54, CD80, and $K^b/OVA_{257\text{-}264}$ complexes (pMHC I) or isotype-matched mAbs (dotted lines). (B and C) Naïve $CD4^+$ and $CD8^+$ T cells were separated from spleen cells of OT II and OT I mice, respectively, and then activated in vitro by incubation with DC_{OVA} for 72 h. (B) The activated $CD4^+$(Th) or $CD8^+$(Tc) T cells were stained using a panel of mAbs (solid lines) for analysis of CD4, CD8, Vβ5.1, 5.2 TCR, CD25, CD62L, and CD69, or isotype-matched mAbs (dotted lines). (C) The activated $CD4^+$(Th) or $CD8^+$(Tc) T cells were cultured in the presence (solid bars) or absence (open bars) of irradiated EG7 tumor cells in RPMI 1640 medium containing 10% FCS for 24 h, then the supernatants were harvested and assayed for secretion of IL-2, IL-4, and IFN-γ by ELISA. Data are representative of 2 experiments with 3 mice per group.

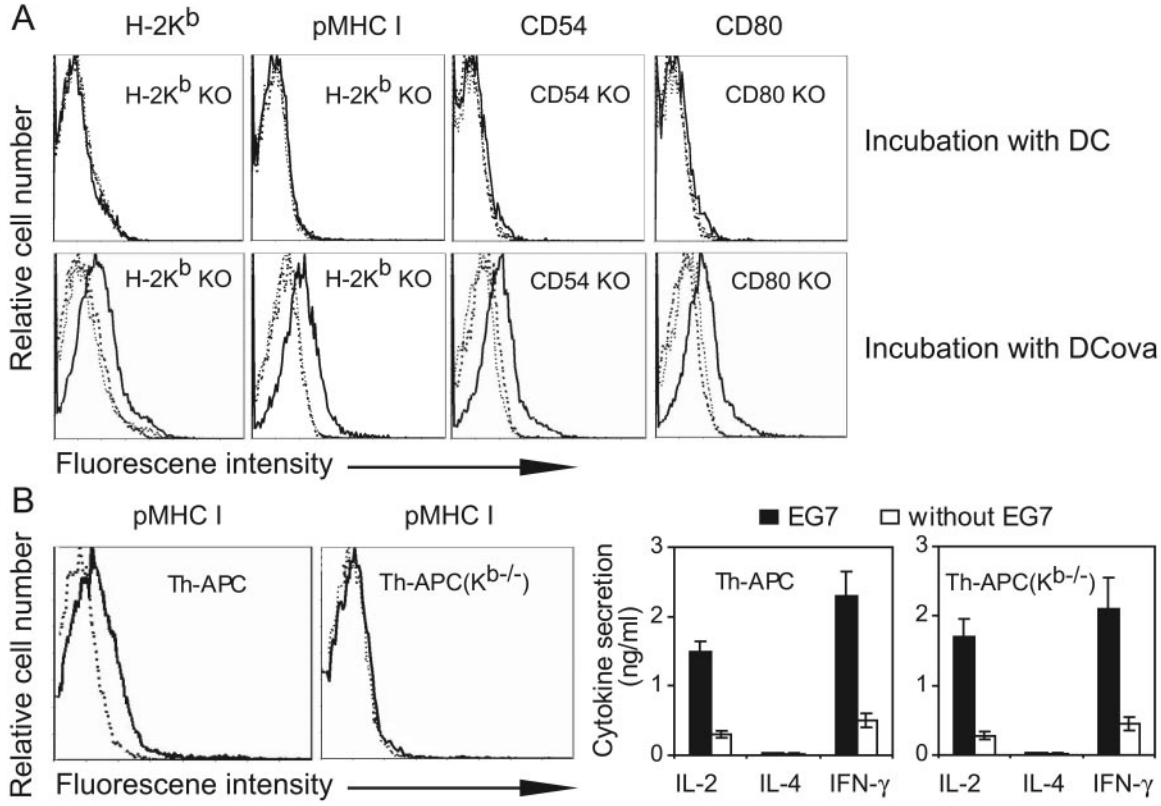

Fig. 2. Transfer of DC membrane molecules to active $CD4^+$ T cells. (A) $CD4^+$ T cells from H-$2K^b$-, CD54- or CD80-deficient OT II mice were analyzed for the display of H-$2K^b$, pMHC I, CD54, or CD80 before (thick dotted lines) or after (thick solid lines) incubation with DC or DC_{OVA} for 72 h. As a control, $CD4^+$ T cells (thin dotted lines) were also stained with isotype-matched mAbs after incubation with DC or DC_{OVA}. (B) Naïve $CD4^+$ T cells from OT II mice were incubated with DC_{OVA} derived from wild-type [Th-APC] or H-$2K^b$ deficient [Th-APC($Kb^{-/-}$)] C57BL/6 mice for 72 h. The activated T cells were analyzed for the displaying of pMHC I complexes following incubation with FITC-labeled mAb specific for $K^b/OVA_{257-264}$ complexes (solid line) or isotope-matched mAb (dotted line). In addition, activated T cells were cultured in the presence (solid bars) or absence (open bars) of irradiated EG7 tumor cells in RPMI 1640 medium containing 10% FCS for 24 h; the supernatants were harvested and assayed for production of IL-2, IL-4, and IFN-γ by ELISA. Data are representative of 2 experiments with 3 mice per group.

naïve $CD4^+$ T cells from OT II mice with DC_{OVA} derived from wild-type C57BL/6 mice and active $CD4^+$ T cells [Th-APC($K^{b-/-}$)] generated by incubation of naïve $CD4^+$ T cells from OT II mice with DC_{OVA} derived from H-$2K^{b-/-}$ C57BL/6 mice were stained with mAb specific for $K^b/OVA_{257-264}$ complexes. Figure 2B shows that Th-APC, rather than Th-APC($K^{b-/-}$), were shown to display pMHC I complexes. These results further confirmed that active $CD4^+$ T cells acquired pMHC I complexes from DC_{OVA} during their activation. Next, we examined the phenotype of Th-APC($K^{b-/-}$). After restimulation with irradiated EG7 tumor cells, Th-APC($K^{b-/-}$), analogous to Th-APC, secreted large amount of IL-2 and IFN-γ, but not IL-4 (Fig. 2B). These results indicated that Th-APC($K^{b-/-}$) were the Th1 phenotype.

$CD8^+$ T cells activated by DC_{OVA} become long-lived memory cells

The major aim of the present study is to assess the potential contribution of active $CD4^+$ T cells to recall responses of $CD8^+$ Tm cells. We initially tried to establish a model system for $CD8^+$ Tm cells. To this end, naive OVA-specific $CD8^+$ T cells isolated from OT I mice were incubated with DC_{OVA} for 72 h, purified using density gradient centrifugation and CD8 microbeads, and then injected i.v. into naïve C57BL/6 mice. Three months later, blood samples were taken and stained for CD8 and $K^b/OVA_{257-264}$ tetramer. As shown in **Fig. 3A**, no tetramer-positive cells were detected in the blood samples of control mice. In contrast, $Tetramer^+$ $CD8^+$ cells were detected in the blood samples of mice injected with active $CD8^+$ T cells 3 mo previously. Double staining also showed that the tetramer-positive cells were CD44 positive (Fig. 3A), suggesting that they are memory cells [27, 28]. Next, we examined the dynamic of tetramer-positive cells. The frequency of tetramer-positive cells was relatively stable during the period from day 30 to 90 after adoptive transfer of the in vitro activated OVA-specific $CD8^+$ T cells (Fig. 3B). Even 6 months after the adoptive transfer, comparable number of OVA-specific $CD8^+$ T cells could still be detected in the peripheral blood (Fig. 3C). Thus, our data showed that OVA-specific $CD8^+$ T cells, after activation by DC_{OVA} in vitro, give rise to long-lived $CD8^+$ Tm cells.

DC-activated $CD4^+$ T cells stimulate expansion of $CD8^+$ Tm cells

It is well established that $CD4^+$ T cell-mediated help is required for mounting a functional memory of $CD8^+$ T cells.

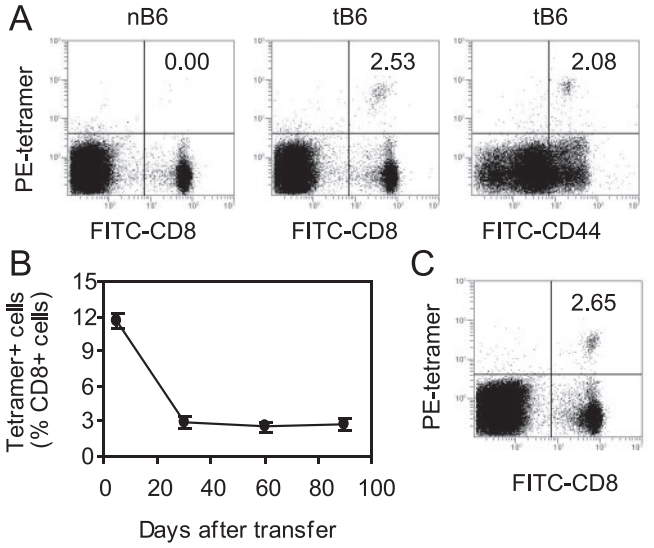

Fig. 3. $CD8^+$ T cells activated by DC in vitro are able to become long-lived memory T cells. (A) 5×10^6 of OVA-specific $CD8^+$ T cells activated by DC_{OVA} in vitro were adoptively transferred to C57BL/6 mice. Three months later, blood samples were taken from the injected mice (tB6), as well as naïve mice (nB6), and double staining for K^b/OVA tetramer and CD8 or CD44 were done to determine the percentage of OVA-specific cells in the total $CD8^+$ population or $CD44^+$ population. (B) 5×10^6 of OVA-specific $CD8^+$ T cells activated by DC_{OVA} in vitro were adoptively transferred into C57BL/6 mice. The frequency of OVA-specific $CD8^+$ T cells in peripheral blood was enumerated at various time points as indicated. (C) 5×10^6 of OVA-specific $CD8^+$ T cells activated by DC_{OVA} in vitro were adoptively transferred to C57BL/6 mice. Six months later, blood samples were taken from the injected mice, and double staining for K^b/OVA tetramer and CD8 were performed to determine the percentage of OVA-specific cells in the total $CD8^+$ population. Data are representative of 2 experiments with 3 mice per group.

However, it is still controversial whether $CD4^+$ T cell help is essential during generation [3–7], or afterwards for maintenance [8], or expansion [9, 10] of $CD8^+$ Tm cells. Another interesting issue is whether antigen-specific $CD4^+$ T cells contribute to expansion of $CD8^+$ Tm cells through alternative pathways, except CD40/CD40L-mediated help described previously [10]. The preceding results and our recent findings [25] have demonstrated that $CD4^+$ T cells acquire antigen presentation machinery (e.g., pMHC I complexes and costimulatory molecules) from DC during their activation. In theory, if DC do transfer a complete set of antigen-presenting molecules, including pMHC I complexes to $CD4^+$ T cells during the activation of the $CD4^+$ T cells, then these recipient $CD4^+$ T cells might have the capacity to, in turn, present these peptides to $CD8^+$ Tm cells. This prompted us to explore the possibility that activated $CD4^+$ T cells stimulate expansion of $CD8^+$ Tm cells in vivo. As described above, OVA-specific $CD8^+$ T cells that had been activated by DC_{OVA} for 72 h were adoptively transferred into C57BL/6 mice. Three months later, these mice were checked for $K^b/OVA_{257\text{-}264}$ tetramer-positive cells and then, in the same day, injected with Th-APC generated by incubation of naïve $CD4^+$ T cells of OT II mice with DC_{OVA}. As control, some of these mice were injected with DC_{OVA} or PBS only. We are very aware that any contamination of DC_{OVA} in Th-APC population will lead to misleading results in our model system. Therefore, Th-APC were separated by Ficoll-Paque density gradient centrifugation and further purified using CD4 microbeads [25]. There was no $CD11b^+/11c^+$ DC population existing in these purified $CD4^+$ T cells [25]. This is not only due to the purification process but also because any survival of irradiated DC_{OVA} cells and the potential small amount of contamination of spleen DCs or B cells within the original naïve OT II $CD4^+$ T cell preparation, which might have picked up OVA peptides from irradiated DC_{OVA} in the culture, would be eliminated by the killing activity of these activated Th1 cells expressing perforin [25, 29, 30]. The recall responses were examined using tetramer staining and intracellular staining of IFN-γ on day 3 after challenge. Three days after stimulation with DC_{OVA} or Th-APC, there are no or very few cells double-labeled with CD8 and tetramer or intracellular IFN-γ (**Fig. 4A** and **B**) in mice that were not received activated OVA-specific $CD8^+$ T cells, indicating that the primary proliferation of $CD8^+$ T cells is undetectable at that time point. As compared with PBS control, the $CD8^+$ Tm cells expanded significantly after stimulation with DC_{OVA} (Fig. 4A and B). Of importance, the $CD8^+$ Tm cells also proliferated after stimulation with Th-APC. However, the magnitude of the recall response stimulated by Th-APC was significantly smaller than that stimulated by DC_{OVA}.

To further confirm the possibility that the increased frequency of tetramer-positive $CD8^+$ T cells was due to the expansion of $CD8^+$ Tm cells rather than naïve $CD8^+$ T cells, C57BL/6 mice were adoptively transferred with activated OVA-specific $CD8^+$ T cells from OT I mice in B6.SJL-Ptpcra background and challenged 3 mo later with Th-APC. A triple staining for CD8, CD45.1, and tetramer showed that the tetramer-positive $CD8^+$ T cells are $CD45.1^+$ (Fig. 4C), indicating that the expansion of $CD8^+$ Tm cells, but not naïve $CD8^+$ T cells, accounted for the elevated frequency of OVA-specific $CD8^+$ T cells in mice on day 3 after injection with Th-APC.

Acquisition of antigen presentation machinery by $CD4^+$ T cells from DC is the prerequisite for DC-activated $CD4^+$ T cells to stimulate expansion of $CD8^+$ Tm cells

We formulated the hypothesis that the acquisition of antigen presentation machinery by Th-APC would enable them to stimulate the expansion of $CD8^+$ Tm cells. To test this hypothesis, active $CD4^+$ T cells were generated by incubation of naïve OVA-specific $CD4^+$ T cells from OT II mice with DC_{OVA} from designated gene knockout mice, and then used to stimulate $CD8^+$ Tm cells in vivo. To make sure that mice have equal number of $CD8^+$ Tm cells before challenge, double staining for tetramer and CD8 was done for the individual mouse. Double staining for CD8 and tetramer (**Fig. 5A**) or intracellular IFN-γ (Fig. 5B) was also performed 3 days after the challenges to enumerate the relative number (percentage of double-positive cells in total peripheral or spleen $CD8^+$ population; Fig. 5, A and B, left) and absolute number (double-positive cells in the whole spleen; Fig. 5, A and B, right) of OVA-specific $CD8^+$ T cells. Accordingly, the $CD8^+$ Tm cells were expanded after stimulation with DC_{OVA} or Th-APC (Fig. 5, A and B). In contrast, Th-APC($K^{b-/-}$) without acquired pMHC I complexes were unable to stimulate the expansion of $CD8^+$ Tm cells (Fig.

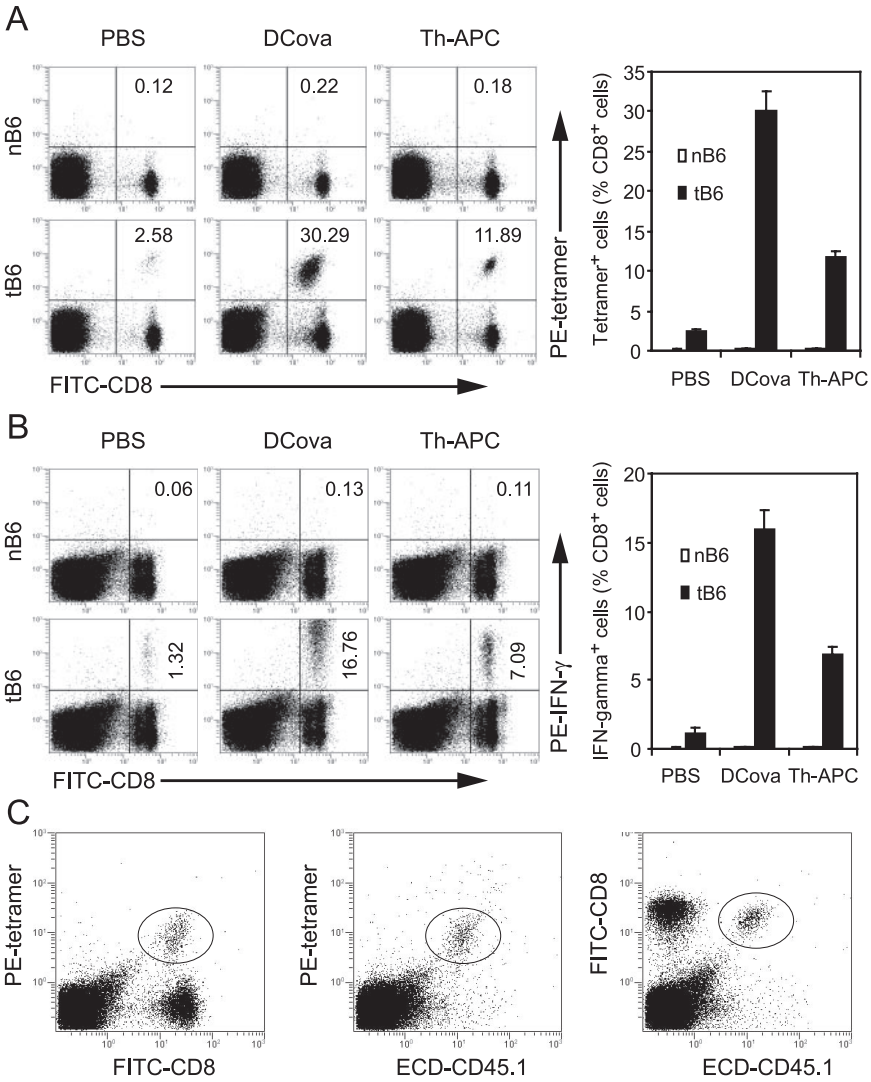

Fig. 4. Expansion of $CD8^+$ Tm cells stimulated by DC-activated $CD4^+$ T cells. Mice (tB6) were injected with 5×10^6 OVA-specific $CD8^+$ T cells activated by DC_{OVA} in vitro. Three months later, these mice, as well as naïve mice (nB6), were challenged with DC_{OVA}, active $CD4^+$ T cells (Th-APC) or PBS only as control. Blood samples were taken from the mice and double staining for K^b/OVA tetramer, and CD8 was performed to determine the percentage of OVA-specific $CD8^+$ T cells in the total $CD8^+$ population (A). Spleen cells were cultured in the presence of OVA I peptide for 4 h and then double staining for CD8 and IFN-γ were done to quantitate the percentage of IFN-γ-producing $CD8^+$ T cells in the total $CD8^+$ population (B). In another set of experiments, C57BL/6 mice were injected with active OVA-specific $CD8^+$ T cells from OT I mice in B6.SJL-Ptpcra background. Three month later, the mice were challenged with Th-APC. Triple staining for K^b/OVA tetramer, CD8 and CD45.1 were performed (C). Data are representative of 2 experiments with 3 mice per group.

5,A and B), indicating that acquisition of pMHC I complexes by active $CD4^+$ T cells is the prerequisite for them to activate the $CD8^+$ Tm cells. Activated $CD4^+$ T cells [Th($CD80^{-/-}$)-APC($CD80^{-/-}$)] without endogenous and acquired CD80 molecules, generated by incubation of $CD4^+$ T cells from OT II/$CD80^{-/-}$ mice with DC_{OVA} from CD80-deficient C57BL/6 mice, failed to activate the $CD8^+$ Tm cells (data not shown). More importantly, activated $CD4^+$ T cells [Th-APC($CD80^{-/-}$)] with endogenous, but without acquired CD80 molecules, generated by incubation of $CD4^+$ T cells from wild-type OT II mice with DC_{OVA} from CD80-deficient C57BL/6 mice, also failed to activate the $CD8^+$ Tm cells (Fig. 5, A and B), suggesting that acquisition of CD80 molecules by active $CD4^+$ T cells is essential for them to stimulate the $CD8^+$ Tm cells. However, active $CD4^+$ T cells [Th($CD54^{-/-}$)-APC($CD54^{-/-}$)], generated by incubation of $CD4^+$ T cells from OT II/$CD54^{-/-}$ mice and DC_{OVA} from CD54-deficient C57BL/6 mice, are able to elicit comparable proliferation of the $CD8^+$ Tm cells as compared with Th-APC (Fig. 5, A and B), suggesting that carrying of CD54 molecules by Th-APC is not essential for Th-APC to stimulate expansion of $CD8^+$ Tm cells. Interestingly, although activated $CD4^+$ T cells [Th($IL\text{-}2^{-/-}$)-APC], generated by incubation of $CD4^+$ T cells from IL-2 deficient OT II mice and DC_{OVA} from C57BL/6 mice, have also the capacity to stimulate the $CD8^+$ Tm cells, the magnitude of the recall responses was significantly reduced as compared with that induced by Th-APC (Fig. 5, A and B), implicating that IL-2 might be involved, to some extent, in the process of stimulation of $CD8^+$ Tm cells. In contrast, secretion of IFN-γ by Th-APC is not essential for them to trigger expansion of $CD8^+$ Tm cells, as evidenced by the fact that active $CD4^+$ T cells [Th(IFN-$\gamma^{-/-}$)-APC], generated by incubation of $CD4^+$ T cells from IFN-γ deficient OT II mice and DC_{OVA} from C57BL/6 mice, induce almost equal expansion of $CD8^+$ Tm cells as compared with Th-APC (Fig. 5, A and B).

To exclude the possibility that the inability of Th-APC($CD80^{-/-}$) to stimulate expansion of $CD8^+$ Tm cells is due to less activated status of the $CD4^+$ T cells, less acquisition of pMHC I, or impaired ability to secrete IL-2, we compared the expression of CD69 and pMHC I on Th-APC and Th-APC($CD80^{-/-}$), as well as the secretion of IL-2 by them after restimulation with irradiated EG7 tumor cells. As shown in Fig. 5C, the intensity of CD69 and pMHC displayed on Th-APC and Th-APC($CD80^{-/-}$) was comparable. As well, Th-APC and Th-APC($CD80^{-/-}$) secreted comparable amount of IL-2 after restimulation. These results demonstrated that

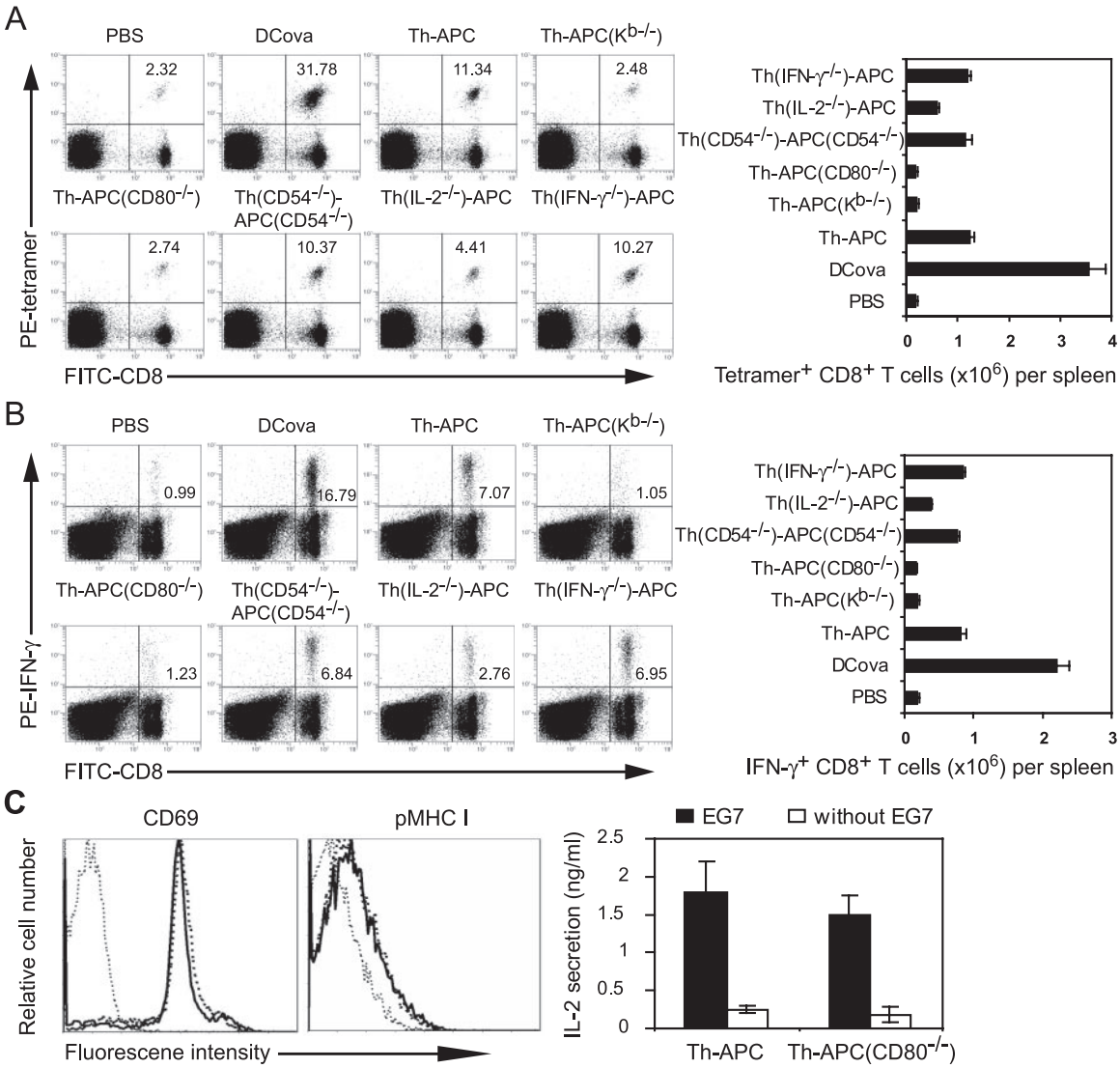

Fig. 5. Expansion of $CD8^+$ Tm cells elicited by DC-activated $CD4^+$ T cells were mediated by their acquired pMHC I complexes and CD80 from DC, with the involvement of IL-2 secreted by the active $CD4^+$ T cells. (A and B) Mice were injected with OVA-specific $CD8^+$ T cells activated by DC_{OVA} in vitro, rested for three months, and then challenged with DC_{OVA}, Th-APC, Th-APC($K^{b-/-}$), Th-APC($CD80^{-/-}$), Th($CD54^{-/-}$)-APC($CD54^{-/-}$), Th($IL\text{-}2^{-/-}$)-APC, Th($IFN\text{-}\gamma^{-/-}$)-APC, or PBS only as control. Blood samples and spleen cells were collected for double staining for K^b/OVA tetramer and CD8. The frequency (A, left) of OVA-specific $CD8^+$ T cells in total peripheral $CD8^+$ population or the absolute number of OVA-specific $CD8^+$ T cells (A, right) in the whole spleen were enumerated. Spleen cells were cultured for 4 h in the presence of OVA I peptide and then stained for CD8 and IFN-γ. The frequency (B, left) of IFN-γ-producing $CD8^+$ T cells in total spleen $CD8^+$ population and absolute number (B, right) of IFN-γ-producing $CD8^+$ T cells in the whole spleen were quantitated. Data are representative of two experiments with four mice per group. (C) $CD4^+$ T cells from OT II mice were incubated with DC_{OVA} derived from wild-type (solid lines) or $CD80^{-/-}$ C57BL/6 (thick dotted lines) mice for 72 h and then stained with mAb specific for CD69 or $K^b/OVA_{257\text{-}264}$ complexes, as well as isotype-matched mAbs (thin dotted lines) (left). In addition, the activated $CD4^+$ T cells were cultured in the presence (solid bars) or absence (open bars) of irradiated EG7 tumor cells in RPMI 1640 medium containing 10% FCS for 24 h; the supernatants were harvested and analyzed for IL-2 secretion (right). Data are representative of 2 experiments with 3 mice per group

Th-APC and Th-APC($CD80^{-/-}$) are comparable in activation status, acquisition of pMHC I, and IL2-secretion.

The preceding results have indicated that both pMHC I complexes and CD80 molecules acquired by Th-APC are essential for stimulation of the expansion of $CD8^+$ Tm cells. It has been reported that recall responses can be optimized by the costimulation between memory T cells and APC, although recall responses are less dependent on costimulatory signals as compared with primary responses [31, 32]. In our model system, the relatively weak signal 1 (acquired pMHC I complexes) provided by Th-APC to $CD8^+$ Tm cells might account for the necessity of CD80 costimulation during the interaction between Th-APC and $CD8^+$ Tm cells. Indeed, it has been proposed that CD80 costimulation is essential for APC to stimulate expansion of $CD8^+$ Tm cells if the antigen density is too low [32]. To further address this issue, DC derived from CD80 deficient or wild-type C57BL/6 mice were pulsed for 1 h with OVA I peptide at various concentrations and then used to challenge the mice that had been injected with in vitro DC_{OVA}-activated $CD8^+$ T cells 3 mo previously. As shown in **Fig. 6**, CD80-deficient DC pulsed with OVA I peptide at a concentration of 1 μM failed to stimulate the expansion of $CD8^+$ Tm

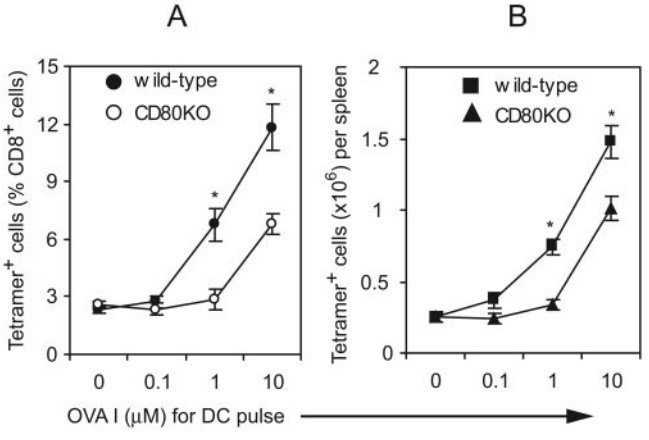

Fig. 6. CD80 costimulation is required for DC to stimulate expansion of $CD8^+$ Tm cells when antigen concentration is low. OVA-specific $CD8^+$ T cells (5×10^6) activated in vitro by DC_{OVA} were adoptively transferred to C57BL/6 mice. Three months later, the mice were challenged with DC derived from wild-type or $CD80^{-/-}$ C57BL/6 mice. The DC used for challenge were pulsed for 1 h with OVA I peptide at various concentrations, as indicated. The frequency (A) and absolute number (B) of OVA-specific $CD8^+$ T cells in peripheral blood or spleen were enumerated using double staining for K^b/OVA tetramer and CD8. Data are presented as the means ± SE (Student *t*-test, $*P < 0.05$). The results presented are representative of 2 separate experiments with 3 mice per group.

cells. In contrast, pulsed with OVA I at this concentration, DC derived from wild-type mice do have the capacity, to some extent, to elicit the expansion of $CD8^+$ Tm cells. Although pulsing with higher concentrations enable CD80-deficient DC to activate $CD8^+$ Tm cells, the magnitude of recall responses [both relative number (Fig. 6A) and absolute number (Fig. 6B) of OVA-specific $CD8^+$ T cells] stimulated by CD80-deficient DC is significantly smaller as compared with that triggered by wild-type DC (Fig. 6). These results are consistent with previous reports [32] and support our hypothesis.

DISCUSSION

It has been previously shown that Th-APC acquire antigen-presenting machinery (i.e., MHC II-peptides complexes and costimulatory molecules) from APCs during their activation [17, 20–23, 33]. In our previous study [25] and our present study, we demonstrated that pMHC I complexes are transferred from APCs to Th-APC as well. In addition, $CD4^+$ T cells, analogous to professional APC, are able to generate endogenous pMHC I complexes by processing exogenously acquired antigen [14]. The Th-APC that carries antigen-presenting machinery may potentially activate other naïve or memory T cells in an antigen-specific manner. Indeed, it has been demonstrated that Th-APC or active $CD8^+$ T cells are able to serve as APC to stimulate other naïve T cells [14, 17, 21–23]. In the present study, we focused on addressing whether expansion of $CD8^+$ Tm cells can be elicited by Th-APC-carrying antigen presenting machinery. Our data indicated that Th-APC, after acquisition of antigen-presenting machinery, do have the ability to stimulate recall responses of $CD8^+$ Tm cells.

The underlying mechanisms responsible for stimulation of $CD8^+$ Tm cells by Th-APC have been elucidated in the current study. We found that Th-APC($K^{b-/-}$) failed to activate the $CD8^+$ Tm cells. As Th-APC($K^{b-/-}$) do not hold pMHC I complexes, which are considered as the first signal for T cell activation [34, 35], they cannot serve as APC to stimulate $CD8^+$ Tm cells. Thus, the acquisition of pMHC I complexes by $CD4^+$ T cells is essential for their function as APC to stimulate $CD8^+$ Tm cells. The inability to stimulate recall responses of $CD8^+$ Tm cells by Th-APC($CD80^{-/-}$) suggested that acquisition of CD80 molecules from DC is essential for Th-APC to activate $CD8^+$ Tm cells. This was further confirmed by the observation that blocking of CD80 on Th-APC using CTLA-4/Ig led to the inability of Th-APC to stimulate the recall responses of $CD8^+$ Tm cells (data not shown). It has been reported that costimulation provided by CD80 is required for recall responses of memory cells in some circumstances [36], particularly in the case of lower antigen concentrations [32], although it is well known that recall responses are less dependent on costimulation as compared with primary responses [31, 32]. In the current study, we showed that CD80 costimulation is crucial for stimulating $CD8^+$ Tm cells in the case of lower concentrations of antigens, supporting previous reports [31, 32, 36]. One of the explanations for the necessity of CD80 during the activation of $CD8^+$ Tm cells stimulated by Th-APC is that the first signal delivered by acquired pMHC I complexes is relatively weak, compared with DC. Thus, it is conceivable that Th-APC need both signal 1 and signal 2 (e.g., CD80 costimulation) to activate $CD8^+$ Tm cells.

Our data presented in the current study, which shows that Th-APC secrete a great amount of IL-2 and IFN-γ, raise the question regarding whether IL-2 and IFN-γ secreted by Th-APC are involved in the stimulation of expansion of $CD8^+$ Tm cells, as a direct delivery of immunity-potentiating cytokines to $CD8^+$ T cells by Th-APC via an immunological synapse would certainly be an efficient way for facilitating $CD8^+$ T cell expansion. To this end, we assessed the capacity of Th($IL\text{-}2^{-/-}$)-APC and Th($IFN\text{-}\gamma^{-/-}$)-APC to activate $CD8^+$ Tm cells in the current study. It is of interest to note that IL-2, but not IFN-γ, secreted by Th-APC is also involved, to some extent, in the expansion of $CD8^+$ Tm cells. It was reported that, during T cell activation, the signal 2 mediated by costimulation can lead to IL-2 production [34, 37, 38]. In particular, the development of primary CD8 responses has been proven to be supported by $CD4^+$ T cells via producing cytokines, notably IL-2 [39, 40]. In our model system, although not an absolute necessity, IL-2 secretion by Th-APC appeared to optimize the expansion of $CD8^+$ Tm cells stimulated by Th-APC.

The interplay among APC, $CD4^+$ T cells, and $CD8^+$ T cells leading to the activation and proliferation of $CD8^+$ naïve or $CD8^+$ Tm cells has been extensively studied, and the understanding of how these cells interact will assist the development of future vaccination strategies. Mounting evidences have demonstrated that $CD4^+$ T cells are critically involved in the development of $CD8^+$ Tm cells [2–10, 14]. Once $CD8^+$ Tm cells are generated, $CD4^+$ T cell are not essential for expansion of the $CD8^+$ Tm cells [5, 7]. Nevertheless, it was also reported that antigen-specific $CD4^+$ T cells are required to activate $CD8^+$ Tm cells via a yet still unknown mechanism(s)

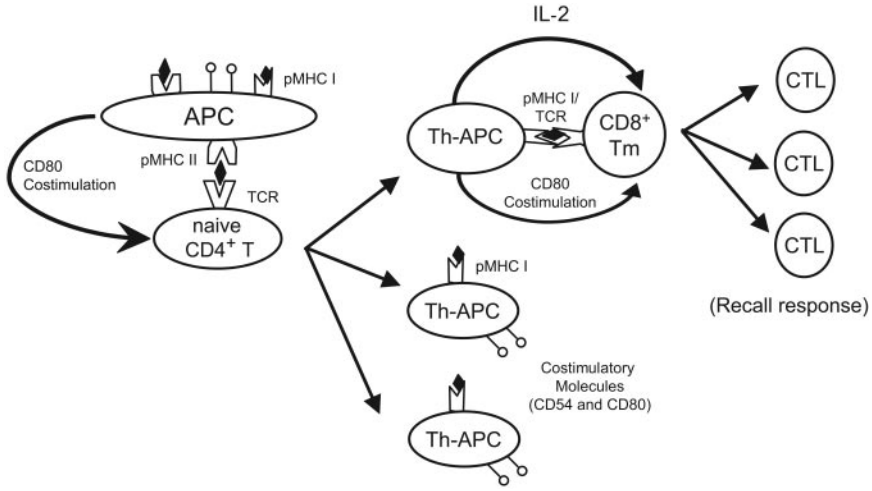

Fig. 7. The dynamic model of the interaction between active $CD4^+$ Th and $CD8^+$ Tm cells. Active $CD4^+$ T cells derived from stimulation of naïve $CD4^+$ T cells acquire pMHC I and costimulatory molecules from APC during their activation and act as APC (Th-APC). The $CD4^+$ Th-APC then stimulate $CD8^+$ Tm cell expansion via their acquired pMHC I complexes and CD80 molecules and IL-2 secretion.

[9]. A recent report also showed that the magnitude of recall responses of $CD8^+$ Tm cells was diminished in the absence of $CD4^+$ T cells [10], indicating that $CD4^+$ T cells might, to some extent, contribute to the expansion of $CD8^+$ Tm cells in some model systems. Of interest, CD40L is also required for stimulation of expansion of $CD8^+$ Tm cells [10], implying that $CD4^+$ T cells might be involved in recall responses of $CD8^+$ Tm cells through CD40/CD40L-mediated "licensing" of APC or even CD40-CD40L interaction between $CD4^+$ and $CD8^+$ Tm cells [2]. Thus, it is highly likely that one of the mechanisms underlying the importance of $CD4^+$ T cells for recall responses of $CD8^+$ Tm cells is that $CD4^+$ T cells provide help to APC or even directly to $CD8^+$ T cells through CD40L signaling. Our findings that active $CD4^+$ T cells have the ability to stimulate the expansion of $CD8^+$ Tm cells through acquired pMHC I complexes and costimulatory molecules might implicate another potential mechanism behind the importance of $CD4^+$ T cells for recall responses of $CD8^+$ Tm cells. However, it is of importance to realize that the frequency of recall $CD8^+$ Tm cells stimulated by active $CD4^+$ T cells was much smaller than that stimulated by DC pulsed with protein, raising the possibility that the recall responses of $CD8^+$ Tm cells induced by APCs overwrite those induced by active $CD4^+$ T cells in physiological situation, which might explain the fact that CD4 deficiency does not significantly affect expansion of $CD8^+$ Tm cells described previously [5, 7, 41]

In summary, we showed that $CD4^+$ T cells acquire pMHC I and costimulatory molecules including CD54 and CD80 from DC during their activation by DC. The activated $CD4^+$ T cells are able to stimulate $CD8^+$ Tm cell expansion via their acquired pMHC I complexes and CD80 molecules. Also, IL-2 secreted by the active $CD4^+$ T cells is involved, to some extent, in the stimulation of $CD8^+$ Tm cells (**Fig. 7**). Thus, our results demonstrate a novel role of active $CD4^+$ T cells in triggering expansion of $CD8^+$ Tm cells.

ACKNOWLEDGMENTS

This work was supported by research grants from the Canadian Institute of Health Research (MOP 63259 and 67230) to J. Xiang. We are grateful to Mark Boyd for his assistance with the FACS analyses.

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