Function of Helper T Cells in the Memory CTL-mediated Anti-tumor Immunity

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Abstract To investigate the role of $CD4^+$ helper T (Th) cells in the memory CTL-mediated anti-tumor immunity, the RAG-1 gene knock out mice were adoptively transferred with OT-1 cells to generate the memory CTL, the C57BL/6 mice immunized with the epitope peptide of OVA specific Th cells and with different adjuvants were adoptively transferred with these memory-CTLs, and then the animals were challenged with tumor cells EG7. It was found that although the simple immunization of mice with the epitope peptide of the OVA specific Th cells could generate more effect CTL, but this effect was not so strong enough to resist completely the challenges with tumor cells. Nevertheless, the memory CTL-mediated anti-tumor immune effect required the helps of Th1 and Th2 cells. The cross-regulation between Th1 and Th2 cells seemed to be beneficial for the host to generate more effector CTL for mounting an efficient anti-tumor response. It concluded that the interaction between Th1 and Th2 cells might be more important than the single subset of Th cells in the memory CTL-mediated anti-tumor immune response. More attention should be paid in this regard for the future studies.

Key words: Memory T cells Helper T cells Transgenic CD44

$CD4^+$ T cells are required for the generation and maintenance of cytolytic $CD8^+$ T cells and are essential for the generation of both cellular and humoral immune responses. However, the contribution of $CD4^+$ T cells to the maintenance of anti-tumor immunity is still the subject of intense investigation. Adoptive transfer strategies exploit *in vitro* conditions to activate and expand primed T cells. Although the fate and function of adoptively transferred tumor-specific effector T cells have been extensively studied [1], comparatively little is known concerning the memory T cells *in vivo*. With the introduction of TCR-transgenic mice, this problem has been overcome. Analysis of an Ag-specific T cell response is facilitated by adoptive transfer of number of TCR-transgenic T cells to normal mice and then challenging such animals with the appropriate antigen. This approach allows for direct phenotypic and functional characterization of the responding Ag-specific transgenic T cells during the course of the immune response and avoids the complication inherent in direct antigen stimulation of the TCR-transgenic mouse. More recently, this model has been adapted to study the induction of anti-tumor immunity *in vivo* [2]. However, the contribution of $CD4^+$ T cells to the development, maintenance and turnover of memory CTL-mediated anti-tumor immunity is still unclear. The present study employed this TCR-transgenic strategy to investigate whether memory CTL need $CD4^+$ helper T (Th) cells to obtain complete protection against a solid tumor.

MATERIALS AND METHODS

Peptides and mice

The peptide SIINFEKL (GF257) and ISQAVHAAHAEINEAGR (OVA specific helper T peptide) were specific CTL and helper T epitopes. Control peptide HGSEPCHIHRGKPFQLEAVFEANQNTKTA, GF257 and OVT peptides were synthesized in the Peptide Synthesis Facility at Auspep and highly purified (> 99%) as assessed by HPLC and amino acid analysis. Six- to 12-week-old recipient female mice (C57BL/6, RAG-1 KO) were purchased from the Animal Resource Centre (ARC, Perth, Australia). OT-1 TCR transgenic mice were derived from the $CD8^+$ OVA-specific T cell clone 149.42. EG7 tumor cell line was maintained in complete RPMI media. All mice were maintained under a specific pathogen-free clean conventional animal house at the Princess Alexandra Hospital,

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Adoptive transfer and immunization

Memory CTL generation was based on the methods described [3]. Briefly, lymph node (LN) cells from OT-1 mice were homogenized and washed in PBS. About 5×10^5 of these cells were injected i.v. into RAG-1 KO recipient mice. Recipient mice were immunized 3 d later with 50 μg SIINFEKL peptide in Freund's adjuvant at the base of the tail, the scruff of the neck and the right forepaw. To transfer memory CTL cells into C57BL/6 mice, each recipient received lymph node cells from RAG-1 KO mice immunized with SIINFEKL at least one month before transfer.

Antibodies and flow cytometry

Antibodies to SIINFEKL-MHC-tetramers conjugated with phycoerythrin (tetramer-PE) were purchased from the NIH Tetramer Core Facility (Atlanta GA). Allophycocyanin conjugated CD8 (CD8-APC), CD44 conjugated biotin (CD44-Bio), and streptavidin conjugated fluorescein isothiocyanate (Strep-FITC) were purchased from Pharmingen (San Diego, CA). Recipient mice were sacrificed and single cell suspensions from spleen and lymph nodes were prepared. Cells were stained in the 3 mg/ml anti-FcR in PBS containing BSA and NaN_3 and stained with combinations of the following antibodies. Biotinylated antibodies were revealed by using Strep-FITC. The cells were analyzed by using a Becton Dickinson FACSCaliber with both CELLQUEST (Becton Dickinson) and FLOWJO (TreeStar, San Carlos CA) softwares.

Adjuvant and Th generation

Quil-A was from Iscotec (Sweden Lulea). CFA and IFA were purchased from Sigma Chemical, and algammulin (γ-inulin-adsorbed alum) was kindly provided by Dr. Peter Cooper (Australian National University, Canberra, Australia). Th cells were generated by immunization of OVT peptide mixed with Quil-A, Algammulin or IFA.

ELISPOT assay for CTL

Multiscreen®-HA Opaque sterile Plates were coated overnight with anti-IFN-γ antibody. 1×10^7 splenocytes per ml were prepared according to the standard methods [4]. 1×10^6 cells per well were prepared with or without IL-2 (GibcoBRL Life technologies), then CTL peptide SIINFEKL was added to give a final concentration of 1 and 0.001 μg/ml respectively and incubated at 37 ℃ for 16-20 h. The plates were washed with PBST, added biotinylated anti-IFN-γ (PharMingen) and incubated for 2-4 h at room temperature before Avidin-horse radish peroxidase (Sigma) was added. The substrate of DAB and peroxide urea (Sigma) were added after the plates were washed. The spots number of Elispot was counted under reflected light.

ELISPOT assays for Th cell

The methods were similar to Elispot assays for CTL described above, with a little modification. Briefly, cells were added with or without IL-2 (GibcoBRL Life technologies). OVA specific helper T peptide ISQAVHAAHAEINEAGR was added to give a final concentration of 8 and 4 μg/ml respectively, and incubated at 37 ℃ for 36-40 h. The plates were washed and biotinylated anti-IFN-γ was added. After 2-4 h incubation, Avidin-horse radish peroxidase was added. The substrate of DAB and Peroxide urea (Sigma) was added and the plates were washed. The spots number of Elispot was counted under reflected light.

Tumor challenge experiments

EG7 was cultured in complete medium and harvested two or three times per week. Immunization was performed by injecting 50 μg specific helper T peptide or KLH in incomplete Freund's adjuvant into C57BL/6 mice 7 d before the EG7 cells challenged. RAG KO recipient mice were sacrificed and lymph nodes were homogenized. About 5×10^5 of memory CTL cells were injected i.v. into C57BL/6 mice. At the same day, 3×10^6 EG7 cells were challenged under the scruff of the neck. 8 d later, mice were sacrificed and the tumor was weighted.

Statistical analysis

All results were analyzed with paired *t* test or unpaired *t* test. *P* values < 0.05 were considered statistically significant.

RESULTS

Expansion of OT-1 cells in T/B cell-deficient hosts

Irradiation was a main method used to deplete lymphocytes in homeostatic expansion of naive T cells, which might induce some cytokines production to promote naive and activate T cell survival. To avoid these issues, we transferred OT-1 cells into RAG-1-deficient (RAG KO) mice. More

than 50 % splenocytes of OT-1 mouse express SIINFEKL specific TCR. After adoptive transfer of OT-1 cells to RAG KO mice, at day 14, more than 11 % recipient splenocytes expressed SIINFEKL specific TCR. SIINFEKL specific CTL was detected with Elispot technique at day 14, and about 410 of IFN-γ specific spots were specific for SIINFEKL peptide *in vitro* (Fig. 1a). Similarly, more than 1000 spots were detected at day 42 indicating that the OT-1 cells proliferated in the RAG KO (deficient in both B and T cells) mice. As a control, OT-1 cells were transferred into unmanipulated C57BL/6 mice and a week later SIINFEKL specific CTL was detected (Fig. 1b). The results showed that nearly 1000 spots were detected per 1×10^6 splenocytes, but only a half spots were detected in lymph node cells. The degree of expansion in these experiments was similar or more extensive than that had been seen in irradiated B6 recipients.

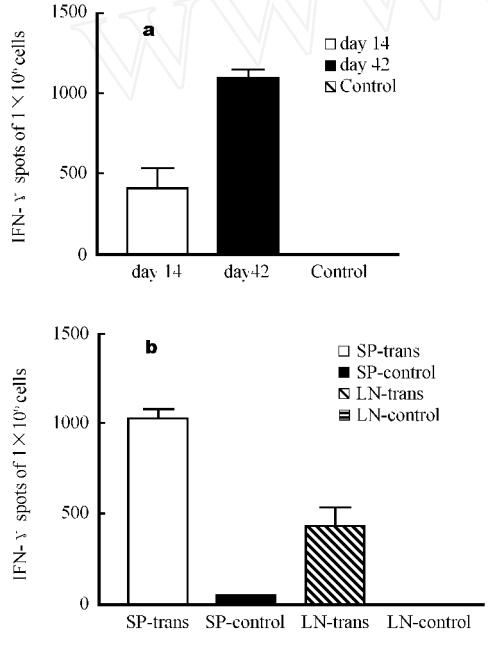

Fig 1. OT-1 cell proliferation in T/B deficient RAG KO mice.

a. Elispot results of RAG KO splenocytes challenged with SIINFEKL which were adoptively transferred OT-1 cells; b. Elispot results of C57BL/6 splenocytes and lymph nodes cells which were adoptively transferred OT-1 cells and SIINFEKL immunization was performed at the same day.

Generation of memory CTL

Memory T cells differ from naive T cells in having previously responded to antigen. For this reason, cellular alterations associated with T cell activation have commonly been examined as potential markers for memory cells. With regard to memory $CD8^+$ T cells, the most relevant molecules are CD44, CD62L and CD45, and the cells expressed $CD44^{high}$ even 10 wk after adoptive transfer. In OT-1 mice, nearly all splenocytes were CD44 positive, but only about 18 % of these cells were CD44 high expression (Fig. 2a). When OT-1 cells were transferred into RAG KO mice, the percentage of CD44 high expression splenocytes were 53.83 % at 14 d after adoptive transfer (Fig. 2b). 21 d later, the percentage of CD44 high expression increased to 96.64 % (Fig. 2c). The SIINFEKL activated OT-1 cells uniformly expressed the characteristic surface phenotype of memory T cells: high levels of CD44 after one month adoptive transfer. They were therefore referred to as memory cells which were specific to SIINFEKL peptide. For all subsequent functional analyses and tumor protection experiments, memory T cells were taken from RAG KO recipients at least 42 d after immunization.

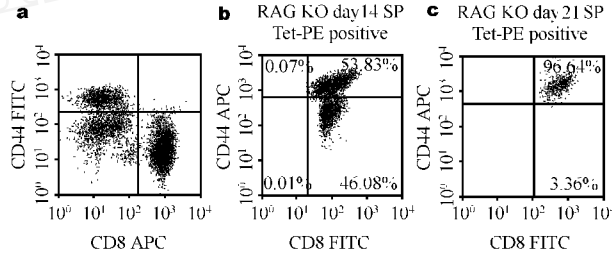

Fig 2. Naive OT-1 cells undergoing SIINFEKL specific-driven proliferation acquire a memory CTL phenotype in RAG KO recipients.

Analysis of expression of $CD8^+$ and $CD44^+$ of OT-1 mouse splenocytes (a), RAG KO mice splenocytes adoptive transfer at day14 (b) and day 21 (c).

Helper T peptide immunization can give rise to more CTL but the host cannot get tumor protection

To ensure that the observed anti-tumor effects were directly mediated by the adoptively transferred T cells, we had to exclude indirect mechanisms mediated via host T or NK cells or host T cells helped by OVA specific Th cells. Unmanipulated and OVT peptide immunized C57BL/6 mice were challenged with EG7 cells. As shown in Fig. 3, unmanipulated and OVT immunized C57BL/6 mice developed complete tumor, while complete tumor protection was observed in CTL transferred and OVT immunized mice and had significance of difference ($P < 0.01$, unpaired t test, Fig. 3a). Also, the splenocytes of above mice were challenged with SIINFEKL to detect specific CTL. The results showed that OVT immunized C57BL/6 mice can generate some CTL against tumor challenge (Fig. 3b). More spots

of IFN-γ were detected in CTL adoptive transfer and OVT immunized mice. A relatively high number of spots were also detected in OVT immunized mice which had significant difference compared to normal C57BL/6 mice.

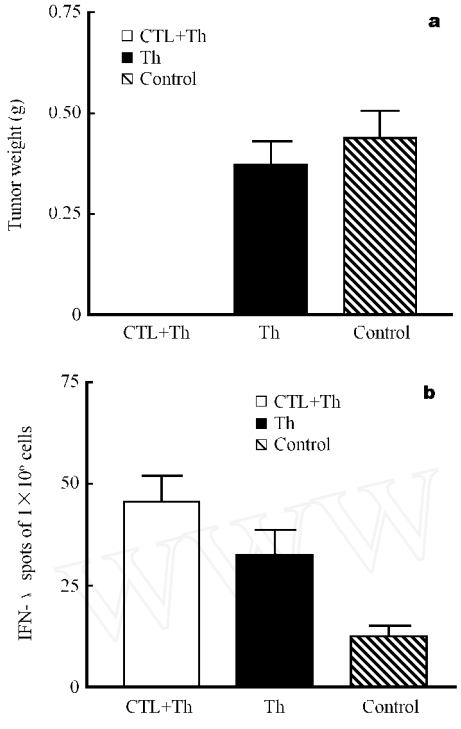

Fig 3. OVA specific helper T peptide immunization could help host generate more CTL but the host cannot get tumor protection.

a. Tumor challenge experiment; b. Elispot results challenged with SIINFEKL [$P < 0.001$ CTL + Th ($n = 10$) vs Control ($n = 5$), $P < 0.05$ Th ($n = 10$) vs Control ($n = 5$), unpaired t test; $P < 0.05$ CTL + Th vs Th paired t test].

Memory CTL needs both Th1 and Th2 cells to get complete tumor protection

To investigate whether a Th1 or Th2 response plus memory CTL could prove the development of specific CTL-mediated protection, C57BL/6 mice were simultaneously immunized with OVT/IFA, OVT/Quil-A, or OVT/algammulin a week before memory CTL adoptive transfer. It was very important to find that mice immunized with OVT/IFA had nearly complete tumor protection compared to those which only immunized with OVT/Quil-A or OVT/algammulin. Splenocytes of these mice were used to detect specific CTL and Th1 cells challenged with SIINFEKL and OVT respectively. It was very interesting to find that OVT/Quil-A immunized mice had the most IFN-γ spots among experimental groups and significant difference compared to other groups when challenged with SIINFEKL peptide, indicating that Th1 cells could generate obviously more CTL (Fig. 4b). On the other hand, we might draw another conclusion that the interaction between Th1 and Th2 cells could help the host generate more specific Th cells when Th1 cell were detected with OVT peptide (Fig. 4c).

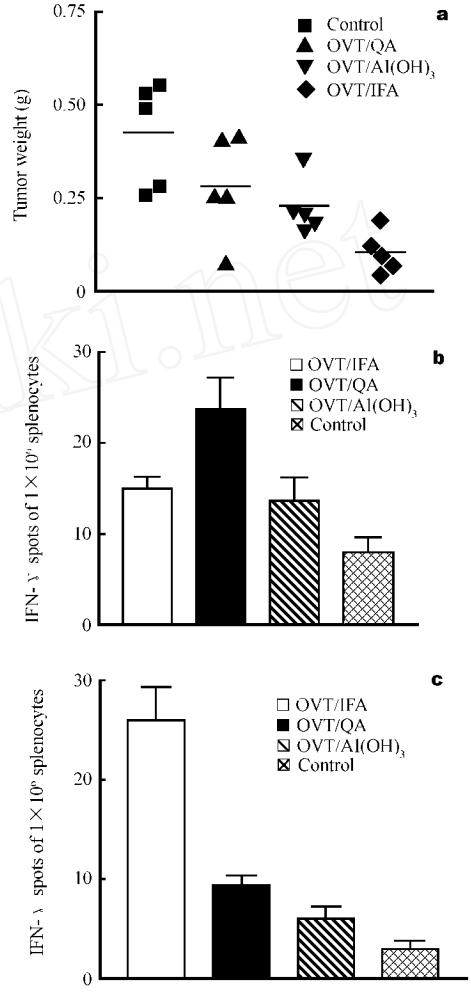

Fig 4. Memory CTL needs both Th1 and Th2 cells to get complete tumor protection.

a. Tumor challenge experiment of OVT immunization and memory CTL adoptive transfer ($n = 5$, each group; $P < 0.05$, control vs OVT/algammulin; $P < 0.11$ control vs OVT/IFA; $P < 0.05$ OVT/Quil-A vs OVT/IFA; $P < 0.01$, OVT/algammulin vs OVT/IFA; paired t test); b. Elispot results challenged with SIINFEKL. ($P < 0.01$, OVT/Quil-A vs control; $P < 0.01$, OVT/IFA vs control; $P < 0.05$, OVT/IFA vs OVT/Quil-A; paired t test). c. Elispot results challenged OVT peptide ($P < 0.001$, OVT/IFA vs OVT/Quil-A; $P < 0.0001$, OVT/IFA vs OVT/algammulin; $P < 0.0001$, OVT/IFA vs control; $P < 0.05$, OVT/Quil-A vs OVT/algammulin; $P < 0.001$, OVT/Quil-A vs control; paired t test).

DISCUSSION

OT-1 cells transferred to RAG$^{-/-}$ mice and immunized with minimal CTL epitope peptide acquire memory CTL

The OT-1 TCR utilizes a Vα2Vβ5 heterodimer to recognize a peptide derived from OVA (OVAp) presented by H2-K^b. Cells harvest from the spleen and lymph nodes of OT-1 mice were > 75 % $Vα2^+$ $Vβ5^+$ $CD8^+$ by flow cytometric analysis. Using OT-1 specific SIINFEKL tetramer-PE by flow cytometric analysis, we showed that the splenocytes of OT-1 mice were 48.44 % positive for $Vα2^+$ $Vβ5^+$ $CD8^+$. After adoptive transfer of lymph node cells into RAG KO mice, at day 14, the splenocytes of recipient were still 4.33 % positive of $Vα2^+$ $Vβ5^+$ $CD8^+$. Cho et al [5] described a system for generating large numbers of memory $CD8^+$ T cells, in which naive CD8 T cells from 2C TCR transgenic mice on the recombination activating gene $(RAG)^{-1-/-}$ background were transferred into syngeneic $RAG\text{-}1^{-/-}$ recipients lacking their own lymphocytes. The recipients were then immunized with a potent antigenic peptide. One month or more after immunization, the surviving 2C cells expressed the cell surface markers and functional properties of memory CD8 T cells. Our data showed that in OT-1 mice, nearly all splenocytes were CD44 positive, but only about 18 % of these cells were $CD44^+$ high expression (Fig. 2a). When adoptive transfer of OT-1 cells into RAG KO mice, the percentage of CD44 high expression splenocytes was increased to a relatively high level and maintained until day 42. All these cells acquired the characteristic surface phenotype of memory cells and could be rapidly induced to express IFN-γ within 42 d of transfer.

Without helper T peptide immunization, the adoptive transfer of memory CTL cannot lead to tumor elimination

Compared to naive $CD8^+$ T cells, memory cells divided after a shorter lag time, had an increased division rate, a lower loss rate, and showed more rapid and efficient differentiation to effector functions [6]. Lymphocytic choriomeningitis virus memory mice contained two distinct memory populations: one was predominantly located in the spleen and exerting rapid effector function, and the other was found in the spleen and the lymph nodes, which had lost immediate effector function. This finding suggests that two types of memory CTL exist: $CD44^{high}$ $CD62L^-$ and $CD44^{high}$ $CD62L^+$ memory phenotypes. *In vitro*, CTL reached a $CD44^{high}$ $CD62L^-$ memory phenotype after 6-10 cell divisions and required restimulation to exert effector function. The $CD62L^-$ effector memory CTL population accounts for rapid effector function found in LCMV memory mice. Conversely, the $CD62L^+$ memory CTL representing the major population in the lymph nodes did not display rapid effector function. Franco et al [7] reported that the epitope affinity for MHC class Ⅰ determines helper requirement for CTL priming. The data presented here suggest that although helper T peptide immunization gave rise to more CTL than non-immunization animals, the numbers of CTL which generated by helper T peptide immunization earlier were not enough to get tumor protection. Memory CTL need $CD4^+$ Th cells especially specific Th cells to kill the tumor cells and to complete tumor regression which indicated that OVA specific MHC class Ⅰ restricted SIINFEKL epitope affinity for MHC class Ⅰ determined Th cells requirements for memory CTL priming [4]. Class Ⅰ restricted CTL responses, the highest affinity peptides (those with an IC_{50} < 50 nM) were invariably immunogenic when used together with a helper T epitope to immunize mice. On the other hand, peptides with a binding affinity in the range of 50-500 nM were inconsistently immunogenic, with only 10 %-50 % of peptides being capable of priming animals. Peptides with binding affinities higher than 500 nM were rarely found to be immunogenic. The binding affinity of a peptide epitope for the class Ⅰ MHC restriction element was a key factor in determining helper independence. Furthermore, depending on the nature of the epitope, efficient help may be provided by anti-CD40 treatment and not by coimmunization with a helper epitope or *vice versa* suggesting that dependency on help was not simply due to a single requirement such as CD40-mediated signaling of APCs. Consistent with the concept of multiple pathways for the generation of help was the recent report [8] that demonstrated the presence of at least three different types of signaling pathways involved in the elicitation of help for a CTL response. These were a CD40-dependent pathway leading to APC conditioning, a CD40-independent conditioning pathway and a soluble CD4 -derived cytokine pathway.

Memory CTL needs both Th1 and Th2 cells to get complete tumor protection

T cells can differ in their cytokine profile. The development of Th1- and Th2-like cells is defined by the microen-

vironmental milieu, e.g., IL-12 fosters Th1 responses, whereas IL-4 and IL-10 favor Th2 responses. In general, Th1-like responses are thought to be associated with tumor regression, whereas Th2-like responses are correlated with increased suppressor activity. CD40/CD154 interactions are essential for the Th1/Th2 differentiation. Although retrovirally transduced mouse dendritic cells require $CD4^+$ T cell help to elicit anti-tumor immunity, divergent roles for $CD4^+$ T cells in the priming and effector/memory phases of adoptive immunotherapy were also reported [7]. To investigate the necessary of Th1 and Th2 in memory CTL mediated-anti-tumor, the helper T peptides are immunized with different adjuvants to give rise to different Th subsets. Our data show that although Th1 or Th2 cells can give partly tumor protection associated with adoptive transfer of memory CTL, the complete tumor regression needs both subsets of Th cells. While there is no doubt that re-immunization will drive memory T cells to proliferate, the rate of division amongst memory T cells under resting conditions is much slower than that typically observed during immune responses. In fact, most of these cytokines failed to stimulate cell division when added alone to purified T cells. The exception is IL-15, which induced strong proliferation of $CD44^{high}$ $CD8^+$ T cells implying that IL-15 is an important regulator of memory T cell turnover *in vivo* in human as well as in mice. IFN-γ induced by both IL-12 and IL-18 is also able to stimulate IL-15 expression by which indicated that IL-15 acts as the final common effector $CD8^+$ molecule in the *in vivo* induction of memory $CD8^+$ T cells turnover by each of the above cytokines. Th1 TCC can secret the cytokines as IL-2, IL-12, IFN-γ, then causing IL-15 which turnover memory CTL into activated CTL and complete tumor protection.

Our data showed that pre-existing Th2 cells associated with adoptive transfer memory CTL can get tumor elimination in part. Meanwhile pre-existing Th1 and Th2 cells can get complete tumor elimination when adoptive transfer memory CTL. IL-12 induced stable priming for IFN-γ production during differentiation of Th cells and transient IFN-γ production in established Th2 cell clones. A novel adoptive tumor immunotherapy model was developed using OVA-specific Th1 and Th2 cells and an OVA gene-transfected tumor and demonstrated that both antigen-specific Th1 and Th2 cells had strong anti-tumor activity *in vivo* with distinct mechanisms [9]. IL-4 was found to decrease the basal expression of the IL-2 receptor β subunit utilized by IL-15, and had no effect on the expression of the β 1 chain of the IL-12 receptor which suggested that the existence of a distinct cross-talk between IL-4 and IL-15 or IL-12 signaling pathways during the regulation of human non-major histocompatibility complex-restricted cytotoxicity [10]. The cross-regulation between Th1 and Th2 subsets was beneficial for mounting an efficient anti-tumor response. In this study we reported that memory CTL need both subsets of Th cells to eliminate completely tumor challenge which implied that interaction between Th1 and Th2 cells should be given more attention in memory CTL-mediated anti-tumor study.

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