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RESEARCH ARTICLE

Selective expansion and enhanced anti-tumor effect of antigen-specific CD4$^+$ T cells by retrovirus-mediated IL-15 expression

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ABSTRACT

Mounting evidence has demonstrated that CD4$^+$ T cells play an important role in anti-tumor immune responses. Thus, adoptive transfer of these cells may have great potential for anti-cancer therapy. However, due to the difficulty to generate sufficient tumor-specific CD4$^+$ T cells, the use of CD4$^+$ T cells in tumor therapy is limited. It has been found that IL-15 transfection enhances the proliferation and anti-tumor activity of tumor-specific CD8$^+$ T cells, but the effect of IL-15 transfection on CD4$^+$ T cells remains unknown. Here, the effects of retrovirus-mediated IL-15 expression in Ova-specific CD4$^+$ T cells from Do11.10 mice were evaluated and it was discovered that IL-15 transfected CD4$^+$ T cells expressed both soluble and membrane-bound IL-15. Retrovirus-mediated IL-15 expression led to a selective expansion of antigen-specific CD4$^+$ T cells by inhibiting their apoptosis. *In vivo* IL-15 transfected CD4$^+$ T cells were more effective in suppressing tumor growth than control retroviral vector transfected ones. To ensure the safety of the method, the employment of thymidine kinase gene made it possible to eliminate these transgenic CD4$^+$ T cells following ganciclovir treatment. Together, we show that IL-15 transfection induced a selective expansion of antigen-specific CD4$^+$ T cells *ex vivo* and enhanced their tumor-suppression effects *in vivo*. This has an important significance for improving the efficacy of adoptive T cell therapy.

KEYWORDS CD4$^+$ T cells, retrovirus vector, IL-15, tumor therapy

INTRODUCTION

Adoptive T cell transfer after host preconditioning by lymphodepletion represents an important advance in cancer immunotherapy (June, 2007b; Schumacher and Restifo, 2009), and CD4$^+$ T cells have been identified as potent effectors of this approach (Radfar et al., 2009). However, successful adoptive CD4$^+$ T cell based clinical trials are limited (Gattinoni et al., 2006). The major obstacles to achieving satisfactory therapeutic outcomes include (1) the isolation and expansion of tumor-specific CD4$^+$ T cells are not successful and (2) the prolonged *ex vivo* T cell expansion process with recombinant IL-2 may result in CD4$^+$ T cells reaching replicative senescence (June, 2007a; Leen et al., 2007). Development of strategies to overcome these restrictions could significantly improve the clinical outcome of patients receiving adoptive CD4$^+$ T cell therapy.

The efficacy of adoptive T cell transfer is enhanced by other immunotherapy methods, such as the administration of cytokines (Leen et al., 2007). As an important T cell growth factor, IL-15 has been demonstrated to prolong the persistence of adoptively transferred cytotoxic T cells (CTLs) *in vivo* (Quintarelli et al., 2007). The major effects of IL-15 on T cells include (1) enhanced cytotoxic ability and proliferation of CTLs *in vivo* (Klebanoff et al., 2004), (2) activation of the telomerase and elongation of telomeres (Li et al., 2005), (3) ability to rescue tumor-tolerant T cells for use in adoptive immunotherapy (Teague et al., 2006), and (4) inhibition of activation-induced cell death of T cells (Marks-Konczalik et al., 2000). However, whether and how IL-15 treatment could enhance the anti-tumor activity of adoptively transferred CD4$^+$ T cells *in vivo* is not resolved until now.

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The development of retrovirus vectors has increased the efficiency of T cell engineering (Sharma et al., 1996). Moreover, it has been reported that retrovirus vectors carrying T cell receptors may confer T cell a specific ability to recognize tumors and these engineered T cells could mediate cancer regression in melanoma patients (Abad et al., 2008). It is well know that retrovirus vectors only engineer proliferative T cells (Culver et al., 1992) and thus, tumor specific T cells should be selectively engineered under a condition where these cells are specifically stimulated to proliferate with tumor antigens.

The generation of tumor-specific $CD4^+$ T cells is often a limitation, because the intrinsic population of tumor reactive $CD4^+$ T cells in patients is extremely small and OKT3 mediated or repeated antigen-pulse proliferation may lead to apoptosis in the tumor-reactive $CD4^+$ T cells (Muranski and Restifo, 2009). Developing optimal cell culture systems with the ability to expand antigen-specific $CD4^+$ T cells is extremely urgent.

In this report, an MLV-based retroviral vector pTK-IL15 encoding both human IL-15 and thymidine kinase (a well-known suicide gene) was constructed. Thymidine kinase was used to help eliminate IL-15 transfected $CD4^+$ T cells in order to avoid possible T lymphoma or autoimmune diseases. The results demonstrated that retrovirus-mediated IL-15 expression may represent an optimal cell expansion system with the capacity to generate large amount of tumor-specific $CD4^+$ T cells and enhance their anti-tumor activity *in vivo*.

RESULTS

Construction of IL-15 expression retroviral vector

The retroviral vectors, containing either the "suicide" herpes simplex virus thymidine kinase gene alone (pTK-Con) or together with the human IL-15 gene (pTK-IL15) or the green fluorescent protein gene (pTK-GFP) were constructed according to the schema as shown in Fig. 1A. The $CD4^+$ T cells from T cell receptor (TCR) transgenic mice Do11.10 were activated with the specific antigen ovalbumine and transfected subsequently with the retroviral vectors described above. The expression of human IL-15 and TK in transfected $CD4^+$ T cells was determined through RT-PCR analysis (Fig. 1B). And Fig. 1C shows that human IL-15 protein was also detectable in the culture supernatant of pTK-IL15 transfected $CD4^+$ T cells, but not in the control. The dose of secreted IL-15 was low (50 pg/mL per 10^6 cells) in 48 h as determined by ELISA assay.

$CD4^+$ T cells transfected with IL-15 retroviral vector express intracellular and membrane-bound IL-15

Intracellular IL-15 is reported to participate in signal transduction (Bergamaschi et al., 2008). Figure 2A shows that pTK-IL15 transfected $CD4^+$ T cells with a multiplicity of infection (MOI) of 1 held intracellular IL-15.

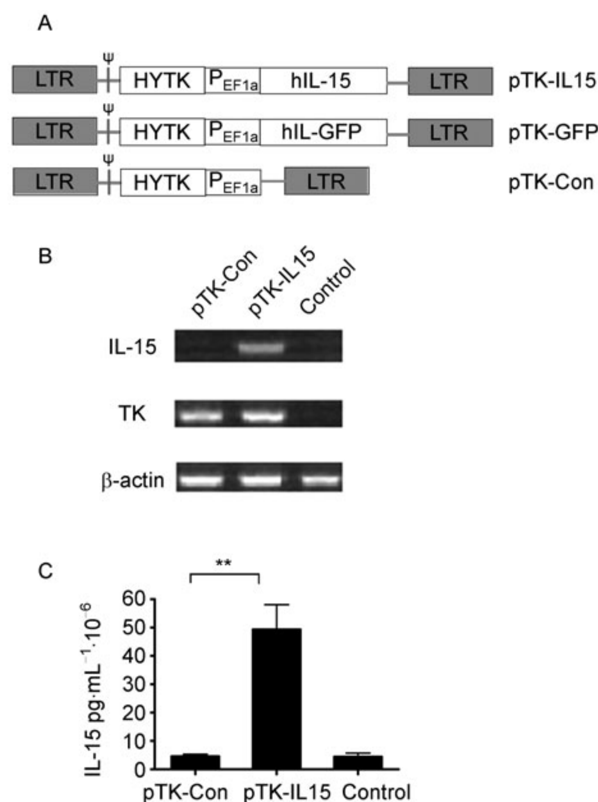

Figure 1. Construction of IL-15 expression retroviral vector. (A) Schema of the retroviral vectors pTK-IL15, pTK-GFP and pTK-Con. LTR, long terminal repeat; HYTK, a fusion gene confers hygromycin B resistance and thymidine kinase activity; P_{EF1a}, the elongation factor 1 promoter; ψ, retroviral packaging signal. (B) RT-PCR to assess mRNA levels of hIL-15 and thymidine kinase. The β-actin gene was used to demonstrate the quality of RNA. Total RNA was isolated from $CD4^+$ T cells transfected with pTK-Con, pTK-IL15 vectors or untreated $CD4^+$ T cells (control). (C) ELISA assay to assess hIL-15 protein level. The culture supernatant of pTK-Con, pTK-IL15 transfected $CD4^+$ T cells or untreated $CD4^+$ T cells was collected. The quantity of hIL-15 was determined in the culture supernatant and measured by specific ELISA. Data are representative of three experiments. ** $p < 0.01$.

IL-15 anchored to the plasma membrane of monocytes or macrophages (so called membrane-bound IL-15) could transmit the signal to neighboring immune cells and induce maturation and proliferation of these cells. Therefore, it is interesting to determine whether pTK-IL15 retroviral vector transfected $CD4^+$ T cells express membrane-bound IL-15. As shown in Fig. 2B, only a small proportion (< 5%) of $CD4^+$ T cells after retroviral transfection expressed membrane-bound IL-15. There are two possible mechanisms to explain this phenomenon: (1) only the small proportion of $CD4^+$ T cells were successfully transfected with pTK-IL15 retroviral vector

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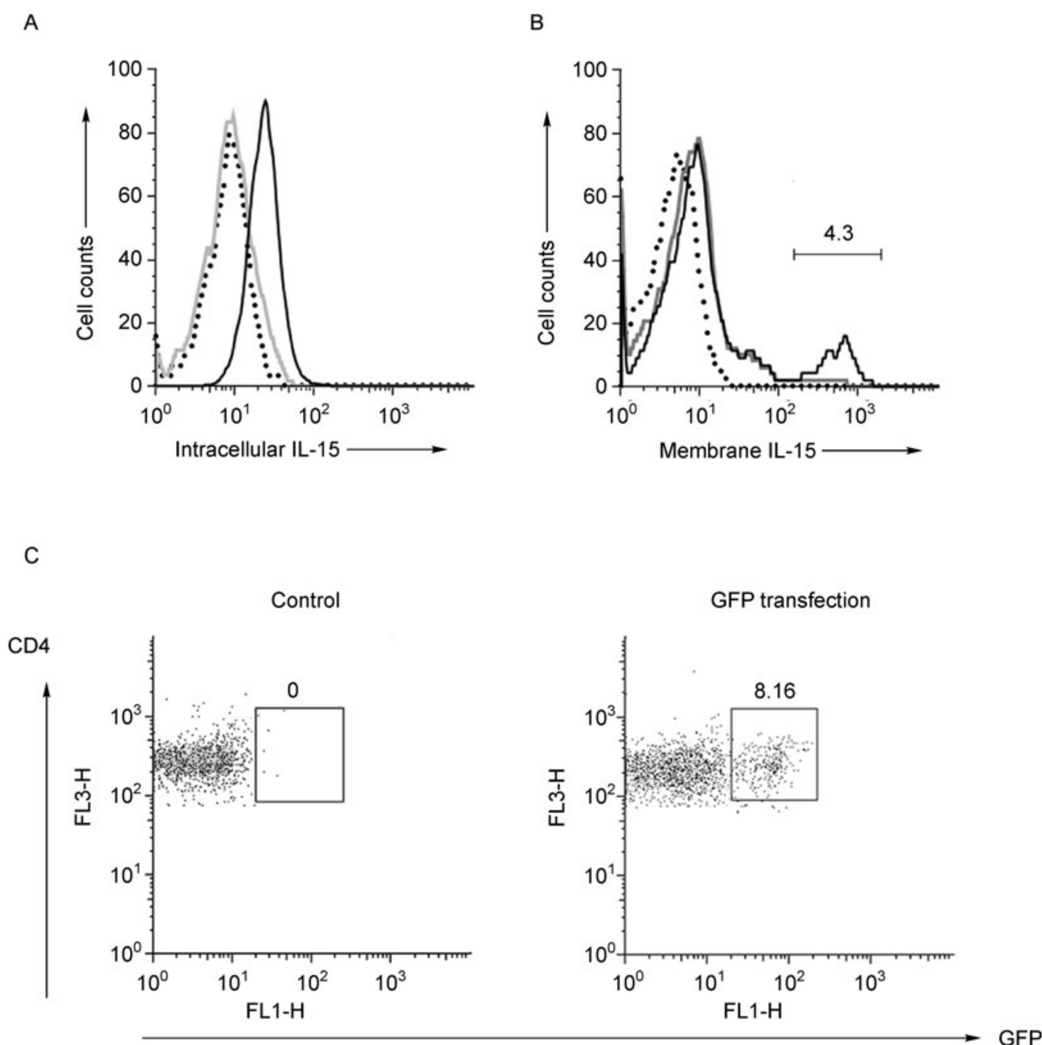

Figure 2. CD4$^+$ T cells transfected with IL-15 retroviral vector express intracellular and membrane-bound IL-15. (A) Expression of intracellular IL-15 by retrovirus transfected CD4$^+$ T cells. Intracellular IL-15 was detected in permeabilized pTK-IL15 transfected CD4$^+$ T cells (solid black line) and pTK-Con transfected CD4$^+$ T cells (solid gray line) by anti-IL15 mAb. The pTK-IL15 transfected CD4$^+$ T cells stained with isotype control mAb were taken as negative control (dotted line). (B) Expression of membrane-bound IL-15 by retrovirus transfected CD4$^+$ T cells. Membrane-bound IL-15 was detected on pTK-IL15 transfected CD4$^+$ T cells (solid black line), pTK-Con transfected CD4$^+$ T cells (solid gray line) by anti-IL15 mAb. The pTK-IL15 transfected CD4$^+$ T cells stained with isotype mAb were taken as negative control (dotted line). The number indicates the percentage for cells positive for PE-labeled IL-15. (C) Transfection efficiency determination by pTK-GFP retroviral vector. Flow-cytometry analysis of GFP expression by pTK-GFP transfected Do11.10 CD4$^+$ T cells and pTK-Con transfected Do11.10 CD4$^+$ T cells (as control). The number above outlined areas indicates the percentage of GFP$^+$ CD4$^+$ T cells. Data are representative of three experiments.

as a result of the low transduction efficiency; (2) some CD4$^+$ T cells transfected with pTK-IL15 did not express membrane-bound IL-15 even if the transfection efficiency of retroviral vector was not low. To resolve this problem, CD4$^+$ T cells were transfected with pTK-GFP retroviral vector to determine the transfection efficiency. As shown in Fig. 2C, the proportion of GFP positive CD4$^+$ T cells after transfection with a MOI of 1 was 8.16% (namely 7.2%± 1.5%), indicating that the transfection efficacy was relatively low under this condition. Since the transfection efficiency could be improved (data not shown) and the low transfection efficiency with a MOI of 1 had significant biological effects, most of the experiments of retroviral vector transfection were done following above protocol.

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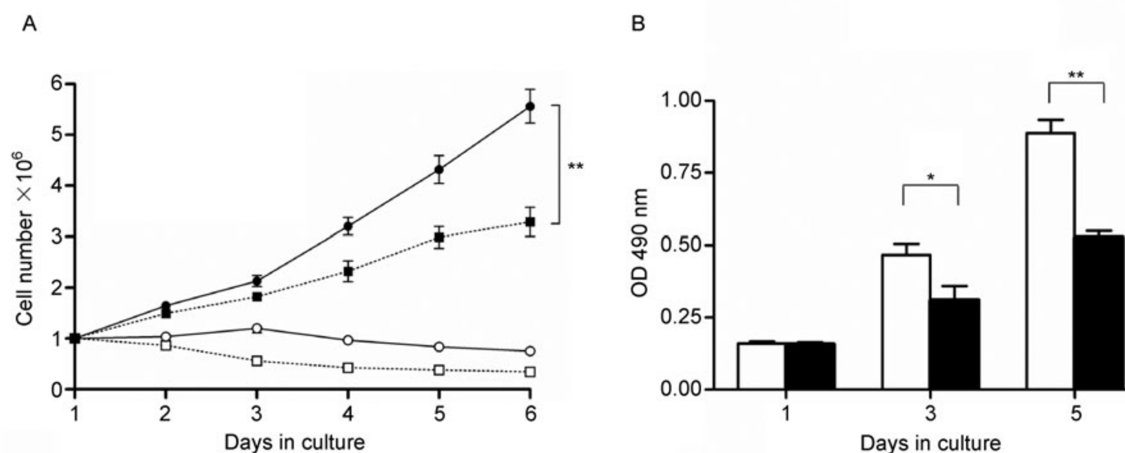

Figure 3. IL-15 modification leads to enhanced CD4⁺ T cell recovery in the presence of IL-2. (A) Cell counting assay to determine the recovery of CD4⁺ T cells. Two days after retroviral vector transfection, CD4⁺ T cells transfected with IL-15 retroviral vector were cultured with recombinant hIL-2 (10 ng/mL) (●) or without hIL-2 (○). And the CD4⁺ T cells transfected with pTK-Con retroviral vector were cultured with hIL-2 (10 ng/mL) (■) or without hIL-2 (□). The number of CD4⁺ T cells was counted every day. (B) MTT assay to determine the recovery of CD4⁺ T cells. The CD4⁺ T cells transfected with pTK-IL15 retroviral vector (open) or pTK-Con retroviral vector (closed) were cultured in 24-well plate at a density of 2×10^6/mL in the presence of hIL-2. The quantity of CD4⁺ T cells was determined by MTT assay. Data are representative of three experiments. * $p < 0.05$, ** $p < 0.01$.

IL-15 modification leads to enhanced CD4⁺ T cell recovery in the presence of IL-2

Soluble IL-15, membrane-bound IL-15 and intracellular IL-15 mediated by pTK-IL15 transfection are speculated to play important roles in survival and expansion of CD4⁺ T cells. However, the exact efficacy of pTK-IL15 transfection on proliferation of CD4⁺ T cells is still not known. To resolve this problem, the transfected CD4⁺ T cells were cultured with recombinant hIL-2 (10 ng/mL) after retroviral vector transfection and the quantity of CD4⁺ T cells was determined by cell counting (Fig. 3A) or an MTT assay (Fig. 3B). The results indicate that IL-15 modification enhanced CD4⁺ T cell recovery in the presence of IL-2 but this efficacy disappeared when transfected CD4⁺ T cells were cultured without IL-2.

Retrovirus-mediated IL-15 expression selectively expands antigen-specific CD4⁺ T cells

IL-15 transfection was reported to enhance the expansion of tumor-specific CD8⁺ T cells. The technical limitations of expanding tumor-specific CD4⁺ T cells currently pose a formidable barrier for conducting successful clinical trials (Muranski and Restifo, 2009). To resolve this problem, retrovirus mediated IL-15 expression was applied in antigen-specific CD4⁺ T cell expansion. Do11.10 CD4⁺ T cells were diluted with non-specific CD4⁺ T cells (from wild type BALB/c mice) in a series of ratios of $1{:}10^2$ to $1{:}10^6$. The mixed CD4⁺ T cells were activated by $Ova_{323–339}$ peptide pulsed APC (antigen-presenting cell) for two days. Following antigen stimulation, T cells were transfected with pTK-IL15 or pTK-Con retrovirus and transfected T cells were cultured in the presence of recombinant hIL-2 (10 ng/mL).

The results elucidated that pTK-IL15 transfection increased the number of antigen-specific CD4⁺ T cells (Fig. 4A) but not non-specific CD4⁺ T cells in dilution ratios of $1{:}10^4$–$1{:}10^6$ (Fig. 4B). In accord with this, the percentage of antigen-specific CD4⁺ T cells increased in dilution ratios of $1{:}10^4$–$1{:}10^6$ (Fig. 4C and 4D). Table 1 shows the change of percentages of antigen-specific CD4⁺ T cells with days of culture. It is concluded that pTK-IL15 transfection selectively expanded antigen-specific CD4⁺ T cells as a result of retroviral vector mediated selective pTK-IL15 transfection on antigen-specific CD4⁺ T cells.

Retrovirus-mediated IL-15 expression protects CD4⁺ T cells from apoptosis *in vitro*

There are two possible mechanisms for increasing the number of CD4⁺ T cells by pTK-IL15 transfection: accelerating the proliferation rate or decreasing the apoptosis. The proliferation rate was examined through [^{3}H]-thymidine incorporation analysis. As shown in Fig. 5A, pTK-IL15 transfection did not boost the proliferation rate of CD4⁺ T cells, while great doses of recombinant hIL-15 (from 5 ng/mL to 100 ng/mL) did. This phenomenon is probably associated with the low dose recombinant hIL-15 secreted by pTK-IL15 transfected CD4⁺ T cells. Besides, the proliferation rate of pTK-IL15 transfected CD4⁺ T cells cultured with recombinant hIL-2 (10 ng/mL) did not increase compared to pTK-Con

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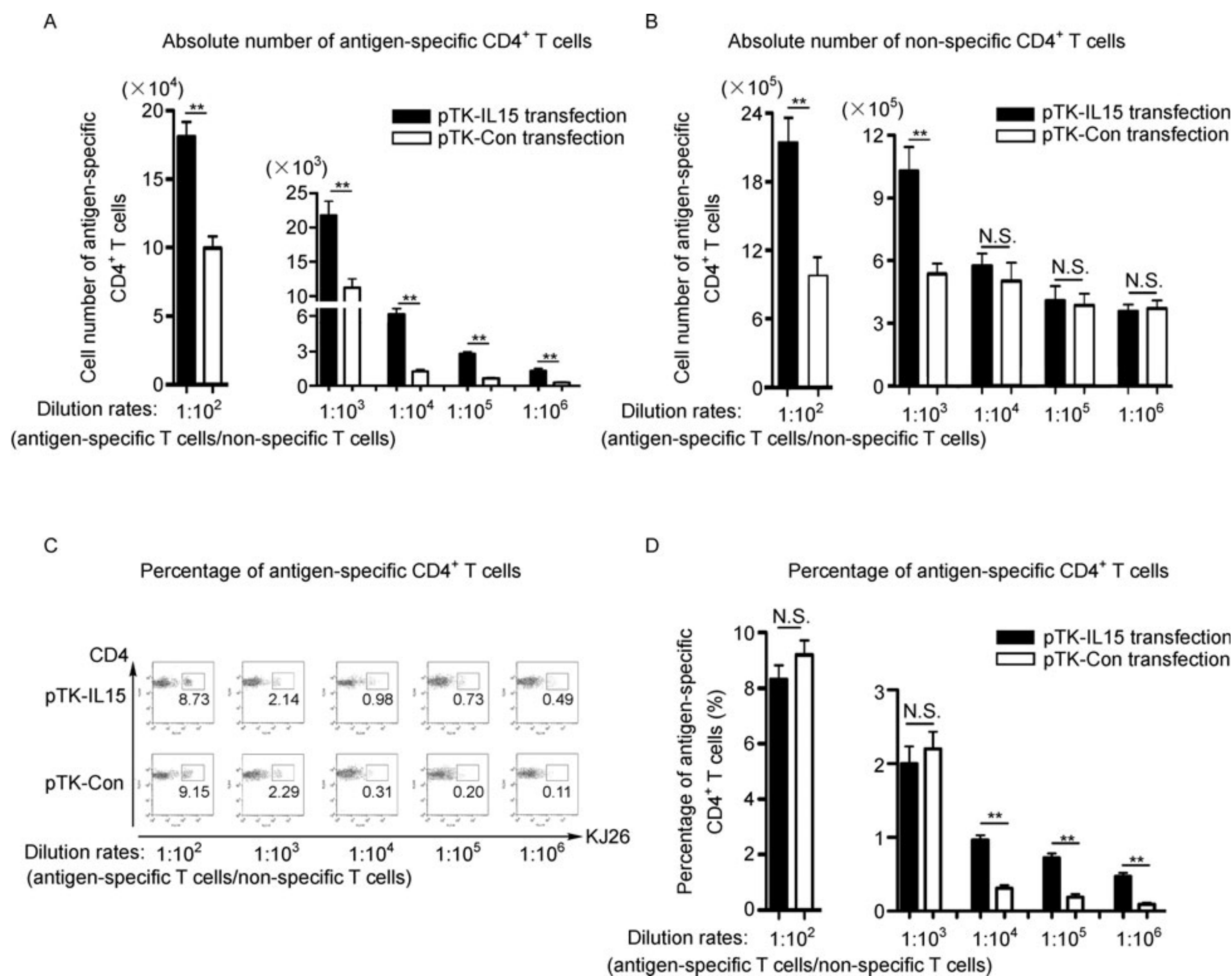

Figure 4. IL-15 selectively expands antigen-specific CD4$^+$ T cells. Do11.10 CD4$^+$ T cells were co-cultured at different ratios (from 1:10^2 to 1:10^6) with non-specific CD4$^+$ T cells. The initial number of non-specific CD4$^+$ T cells was 5 × 10^6 and the number of specific Do11.10 CD4$^+$ T cells was adjusted from 5 × 10^4 to 5 according to indicated ratios. The numbers of living CD4$^+$ KJ26$^+$ T cells (specific CD4$^+$ T cells) and CD4$^+$ KJ26$^-$ (non-specific CD4$^+$ T cells) were confirmed by guava staining assay on 7th day of culture. (A) The numbers of antigen-specific CD4$^+$ T cells. (B) The numbers of non-specific CD4$^+$ T cells. (C) Flow cytometry analysis to determine the percentages of antigen-specific CD4$^+$ T cells. Numbers indicate the percentages of specific CD4$^+$ cells. (D) The percentages of specific CD4$^+$ T cells. Data are representative of four experiments. ** $p < 0.01$.

Table 1 The percentages of antigen-specific CD4$^+$ T cells in T cell culture after retroviral transfection

Days of culture	Transfected T cells	Ratios of antigen-specific CD4$^+$ T cells to non-specific CD4$^+$ T cells				
		1:10^2	1:10^3	1:10^4	1:10^5	1:10^6
1st day	No transfection	1%	0.1%	0.01%	0.001%	0.0001%
3rd day	No transfection	2.81%± 0.65%	0.53%± 0.19%	No data	No data	No data
5th day	pTK-Con	4.32%± 0.92%	1.16%± 0.32%	0.17%± 0.09%	No data	No data
	pTK-IL15	3.85%± 0.43%	1.13%± 0.26%	0.24%± 0.06%	No data	No data
7th day	pTK-Con	9.39%± 0.96%	2.25%± 0.35%	0.31%± 0.09%	0.19%± 0.07%	0.10%± 0.08%
	pTK-IL15	8.53%± 1.35%	2.05%± 0.31%	0.94%± 0.14%	0.72%± 0.08%	0.48%± 0.11%
9th day	pTK-Con	21.71%± 4.38%	5.36%± 1.02%	0.62%± 0.23%	0.45%± 0.17%	0.43%± 0.16%
	pTK-IL15	19.22%± 3.21%	5.05%± 0.94%	2.15%± 0.41%	1.58%± 0.22%	1.14%± 0.24%
11th day	pTK-Con	57.51%± 7.52%	13.07%± 2.21%	1.18%± 0.41%	0.75%± 0.21%	1.06%± 0.18%
	pTK-IL15	54.08%± 8.21%	12.68%± 3.21%	3.94%± 0.81%	2.95%± 0.21%	2.54%± 0.61%

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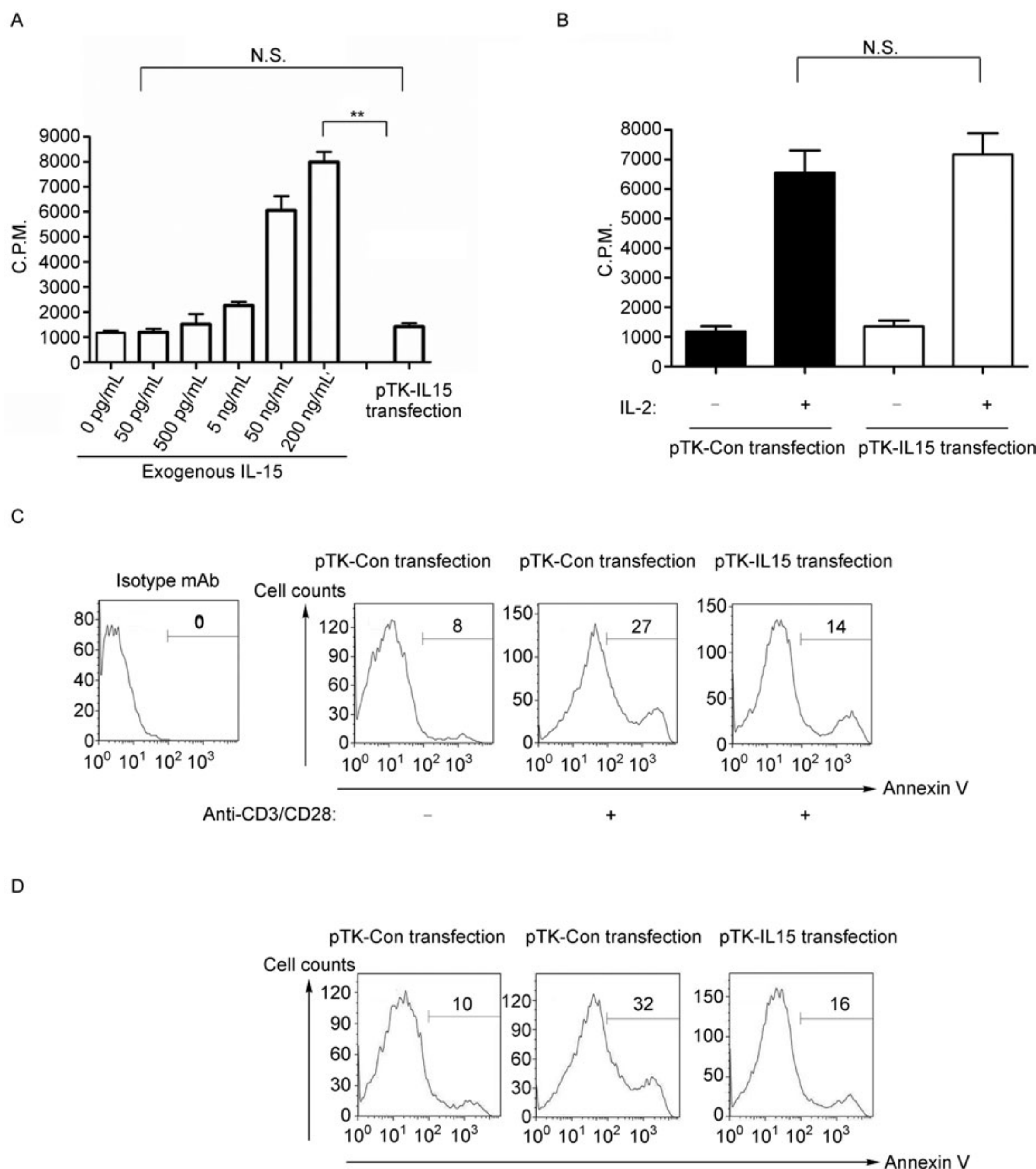

Figure 5. Retrovirus-mediated IL-15 expression protects CD4$^+$ T cells from apoptosis *in vitro*. (A) IL-15 transfection could not accelerate the proliferation rate of CD4$^+$ T cells *in vitro*. CD4$^+$ T cells transfected with pTK-IL15 were inoculated without hIL-15 while pTK-Con transfected CD4$^+$ T cells were inoculated with different doses of recombinant hIL-15 (from 50 pg/mL to 200 ng/mL) as control. [^{3}H] thymidine incorporation was determined three days later. (B) IL-15 transfection did not accelerate the proliferation rate of CD4$^+$ T cells in the presence of hIL-2. CD4$^+$ T cells transfected with pTK-IL15 and CD4$^+$ T cells transfected with pTK-Con were cultured with hIL-2 (10 ng/mL), while CD4$^+$ T cells cultured without additional cytokine were taken as control. [^{3}H] thymidine incorporation was determined three days later. (C) CD4$^+$ T cells transfected with pTK-IL15 resisted activation-induced cell death (AICD). CD4$^+$ T cells transfected with pTK-IL15 and CD4$^+$ T cells transfected with pTK-Con were cultured for 16 h in the presence of plate-bound anti-CD3 and soluble anti-CD28 without exogenous cytokine. Evaluation of apoptosis through flow cytometry of isolated CD4$^+$ KJ26$^+$ T cells with an anti-Annexin-V mAb. Numbers indicate the percentage of cells positive for Annexin-V. (D) CD4$^+$ T cells transfected with pTK-IL15 resisted AICD in the presence of recombinant hIL-2. CD4$^+$ T cells transfected with pTK-IL15 and CD4$^+$ T cells transfected with pTK-Con were cultured in the presence of anti-CD3 and anti-CD28 with 10 ng/mL recombinant hIL-2 for 16 h. Numbers indicate the percentage of cells positive for Annexin-V. ** $p < 0.01$, * $p < 0.05$ and N.S. ($p > 0.05$)

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transfected $CD4^+$ T cells cultured with hIL-2 as shown in Fig. 5B. It is concluded that high dose recombinant hIL-15 boosted the proliferation rate of $CD4^+$ T cells while pTK-IL15 transfection did not.

The anti-apoptotic effect of IL-15 retroviral vector transfection on $CD4^+$ T cells was examined via the activation-induced cell death (AICD) model *ex vivo*. Figure 5C shows that pTK-IL15 transfection dramatically decreased the apoptotic percentage of $CD4^+$ T cells from 27% to 14% compared to pTK-Con transfection. Recombinant hIL-2 did not abrogate the anti-apoptotic effect of pTK-IL15, and the apoptotic percentage of $CD4^+$ T cells decreased from 32% to 16% (Fig. 5D). Retrovirus mediated IL-15 expression was demonstrated to inhibit the apoptosis of $CD4^+$ T cells to ensure the expansion dominance of pTK-IL15 transfected $CD4^+$ T cells.

Prolonged survival of pTK-IL15 transfected $CD4^+$ T cells *in vivo*

The pTK-IL15 transfection selectively expands antigen-specific $CD4^+$ T cells *in vitro*. To follow the fate of such cells *in vivo*, pTK-IL15 transfected Do11.10 T cells were adoptively transferred into TSA-mOva bearing mice and traced by using specific mAbs against KJ26 and CD4 .

As shown in Fig. 6A, three days after T cell infusion, the proportion of pTK-IL15 transfected $CD4^+$ T cells was higher compared with pTK-Con transfected $CD4^+$ T cells in the spleen (3.5% *vs* 2.1% of the total $CD4^+$ T lymphocytes). But the population of $CD4^+$ T cells was no longer detectable on 45th day after T cell infusion. In order to determine whether the increased percentage of pTK-IL15 transfected $CD4^+$ T cells was due to an increase in the proliferation of $CD4^+$ T cells, BrdU incorporation experiment was performed. After cyclophosphamide-mediated lymphodepletion, T cells were transferred into mice and the proliferation of Do11.10 $CD4^+$ T cells within the spleen was determined. Our data presented that about 10% of the transferred $CD4^+$ T cells in spleen were proliferating. $CD4^+$ T cells transfected with pTK-IL15 did not improve cell proliferating ability compared to pTK-Con transfected $CD4^+$ T cells in the spleen three days after T cell infusion (10.62%± 0.76% *vs* 11.86%± 1.41%; Fig. 6B). The pTK-IL15 transfection was demonstrated to inhibit AICD of $CD4^+$ T cells *ex vivo*, and the anti-apoptotic ability of pTK-IL15 transfected $CD4^+$ T cells *in vivo* was further examined. Figure 6C shows that pTK-IL15 transfected $CD4^+$ T cells resisted apoptosis compared with pTK-Con transfected $CD4^+$ T cells in spleen (25.31%± 4.21% *vs* 36.39%± 5.76%).

The fate of these cells in tumor site of recipient mice was also examined. As shown in Fig. 7A, three days after T cell infusion the frequency of pTK-Con transfected $CD4^+$ T cells was similar to pTK-IL15 transfected $CD4^+$ T cells (7% *vs* 7% of the total $CD4^+$ T lymphocytes). But 45 days after T cell infusion the frequency of pTK-Con transfected $CD4^+$ T cells was about seven-fold lower compared with pTK-IL15 transfected $CD4^+$ T cells at the tumor site (3% *vs* 21% of the total $CD4^+$ T lymphocytes). Sixty days after T cell infusion tumor-bearing mice receiving pTK-Con transfected $CD4^+$ T cells were no longer alive while mice receiving pTK-IL15 transfected $CD4^+$ T cells were. And the percentage of transferred $CD4^+$ T cells in total $CD4^+$ T lymphocytes at tumor site was 7%. Collectively, pTK-IL15 transfected $CD4^+$ T cells infiltrated into tumor site and survived within tumor environment for a long time. The percentage of pTK-IL15 transfected $CD4^+$ T cells was higher than pTK-Con transfected $CD4^+$ T cells within tumor environment. To examine the exact fate of transferred $CD4^+$ T cells at the tumor site, the proliferation and apoptosis of transferred $CD4^+$ T cells were analyzed. The percentage of proliferating T cells in tumor site were about 50% and pTK-IL15 transfected $CD4^+$ T cells did not show elevated proliferating ability compared to pTK-Con transfected $CD4^+$ T cells (46.25%± 6.39% *vs* 52.62%± 6.08%; Fig. 7B). $CD4^+$ T cells transfected with pTK-IL15 resisted apoptosis compared with pTK-Con transfected $CD4^+$ T cells (53.43%± 5.41% *vs* 83.67%± 3.76%, Fig. 7C).

Taking the situations of spleen and tumor site into account, proliferating percentages and apoptosis proportions of transferred $CD4^+$ T cells at tumor site were higher compared with transferred $CD4^+$ T cells in spleen. It is speculated that Ova antigen existing in tumor site but not in spleen led to second antigen activation of transferred T cells, which boosted the proliferation and apoptosis of transferred T cells.

Efficient tumor suppression by pTK-IL15 transfected $CD4^+$ T cells

Although IL-15 transfection had been shown to enhance the antitumor effect of adoptive CTL therapy, the efficacy of pTK-IL15 transfected $CD4^+$ T cells in adoptive tumor therapy *in vivo* is still unknown. To address this, the antitumor effect of these $CD4^+$ T cells *in vivo* was analyzed. Do11.10 $CD4^+$ T cells are able to constrain the growth of Ova-expressing tumor cells such as J558L-mOva plasmacytoma and TS/A-mOva adenocarcinoma. Our data demonstrated that compared with pTK-Con transfected $CD4^+$ T cells, pTK-IL15 transfected $CD4^+$ T cells significantly inhibited the growth of TS/A-mOva adenocarcinoma (Fig. 8A; $p < 0.05$), and efficiently prolonged the survival of tumor-bearing mice (Fig. 8B). Similar results were obtained in the J558L-mOva plasmacytoma model (Fig. 8C and 8D; $p < 0.05$). These findings suggested that pTK-IL15 transfection enhanced the antitumor effect of $CD4^+$ T cells.

$CD4^+$ T cells transfected with pTK-IL15 could be eliminated by GCV treatment

Retrovirus mediated gene modification of $CD4^+$ T cells may result in T cell lymphoma (Hacein-Bey-Abina et al., 2003). To avoid this, pTK-IL15 retrovirus vector carrying the suicide

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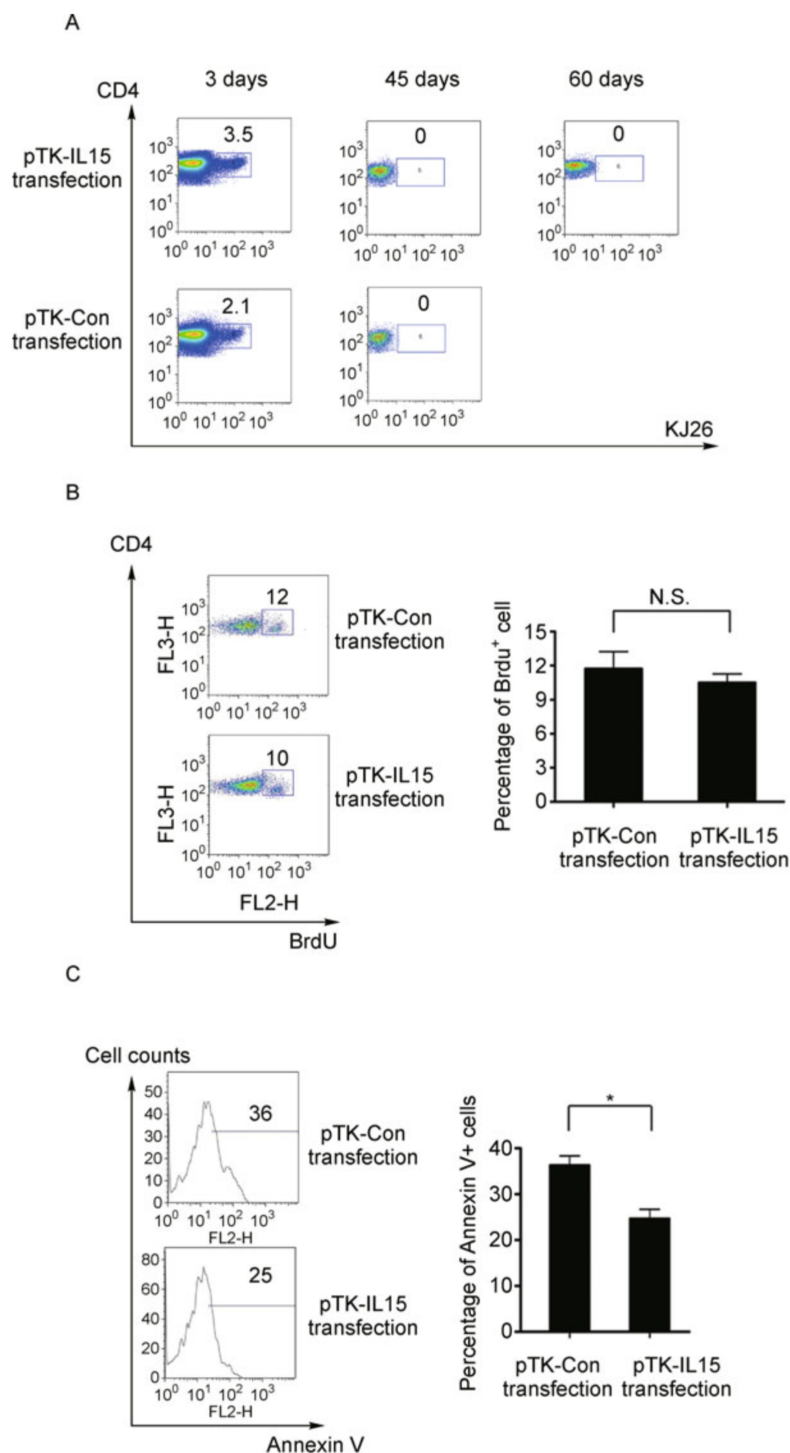

Figure 6. Prolonged survival of pTK-IL15 transfected CD4$^+$ T cells in spleen. (A) Detecting the transferred CD4$^+$ T cells in spleen. Retroviral vector transfected CD4$^+$ T cells were injected i.v. into TS/A-mOva bearing BALB/c mice and lymphocytes from spleen of the recipient mice were analyzed 3 days, 45 days and 60 days after T cell transfer. Transferred CD4$^+$ T cells were traced by KJ26 mAb. The dot plots were gated on total CD4$^+$ T lymphocytes and the numbers represented the percentages of the CD4$^+$ KJ26$^+$ population. (B) Proliferation of transferred CD4$^+$ T cells in spleen. Continuous treatment with BrdU was initiated after T cell infusion and lasted for 3 days before the harvesting of spleens. CD4$^+$ KJ26$^+$ T cells were gated and the numbers represented the percentages of the BrdU$^+$ population. (C) Apoptosis of transferred CD4$^+$ T cells in spleen. Evaluation of apoptosis through flow cytometry of isolated CD4$^+$ KJ26$^+$ T cells in spleen with an anti-Annexin-V mAb 3 days after T cell infusion. Numbers indicate the percentage of cells positive for Annexin-V. Data from one representative experiment out of four are shown. N.S. $p > 0.05$, * $p < 0.05$.

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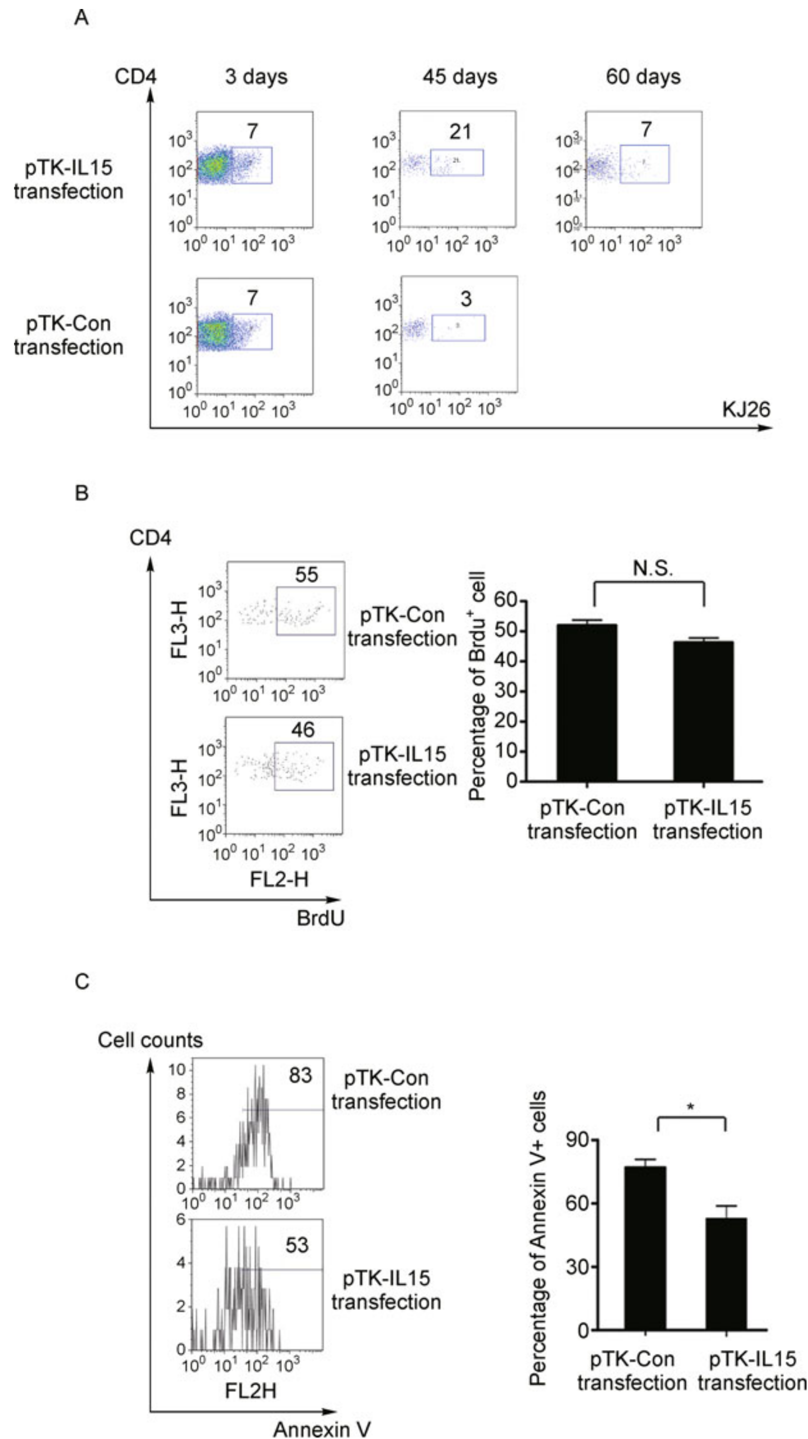

Figure 7. Prolonged survival of pTK-IL15 transfected CD4⁺ T cells in tumor. (A) Detecting the transferred CD4⁺ T cells in tumor. Retroviral vector transfected CD4⁺ T cells were injected i.v. into TS/A-mOva bearing BALB/c mice and lymphocytes from tumor of the recipient mice were analyzed three days, 45 days and 60 days after transfer. Transferred CD4⁺ T Cells were traced by KJ26 mAb. The dot plots were gated on total CD4⁺ T lymphocytes and the numbers represented the percentages of the outlined CD4⁺ KJ26⁺ population. (B) Proliferation of transferred CD4⁺ T cells in tumor. Continuous treatment with BrdU was initiated after adoptive CD4⁺ T cells transfer and lasted for three days before the harvesting of tumors. CD4⁺ KJ26⁺ T cells were gated and the numbers represented the percentages of the outlined BrdU⁺ population. (C) Apoptosis of transferred CD4⁺ T cells in spleen. Evaluation of apoptosis through flow cytometry of isolated CD4⁺ KJ26⁺ T cells in spleen with an anti-Annexin-V mAb three days after T cell infusion. Numbers indicate the percentage of cells positive for Annexin-V. Data from one representative experiment out of four are shown. N.S. $p > 0.05$, $* \ p < 0.05$.

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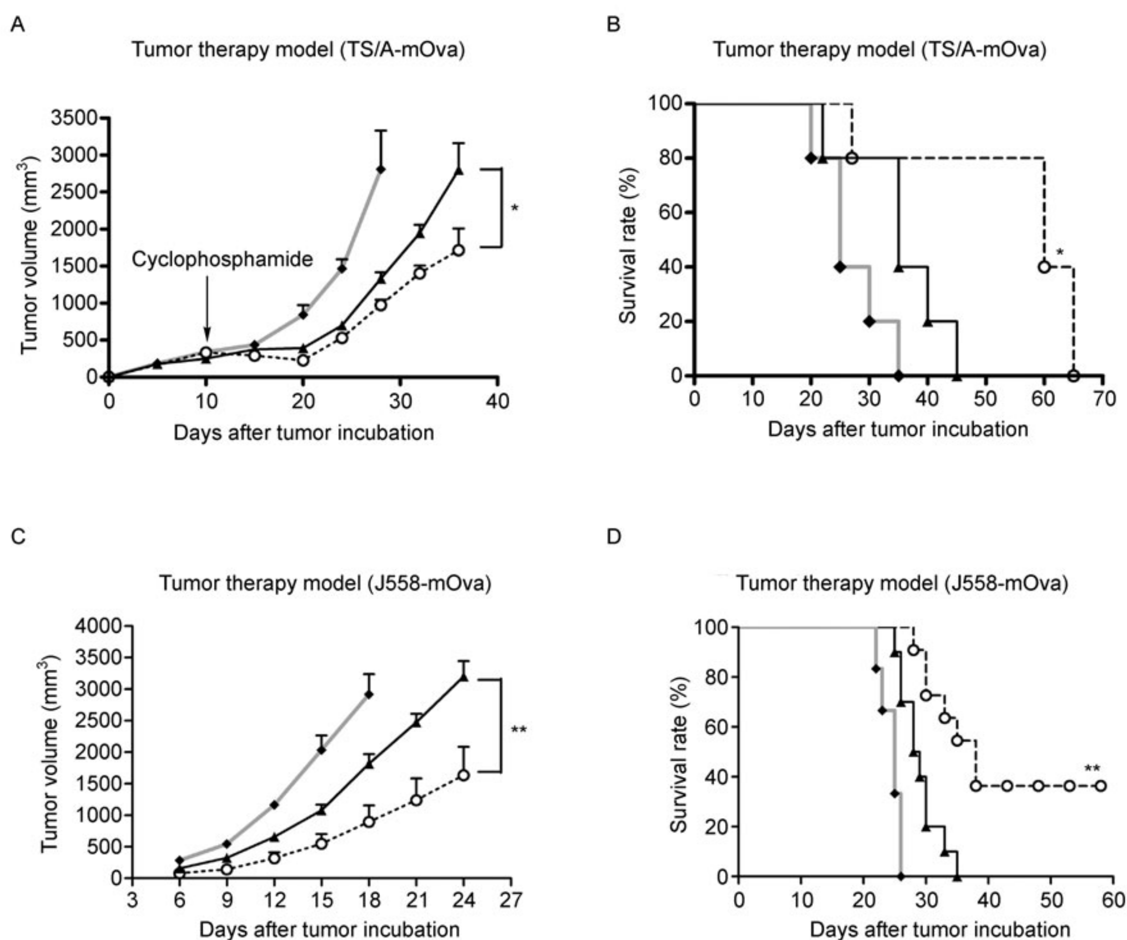

Figure 8. Efficient tumor suppression by pTK-IL15 transfected CD4⁺ T cells. (A) The growth of TS/A-mOva adenocarcinoma in mice infused with transfected CD4⁺ T cells. Tumor-bearing mice were infused by an i.v. adoptive transfer of 5 × 10^6 pTK-IL15 transfected CD4⁺ T cells (○, *n* = 5) or pTK-Con transfected CD4⁺ T cells (▲, *n* = 5) or just PBS (♦, *n* = 5) as control. Shown here are mean tumor volumes with SDs. Tumor sizes of TS/A-mOva bearing mice were monitored for over 70 days after tumor incubation. (B) The survival of TS/A-mOva adenocarcinoma bearing mice. (C) The growth of J558L-mOva plasmacytoma in mice infused with transfected CD4⁺ T cells. Tumor-bearing mice were infused by an i.v. adoptive transfer of pTK-IL15 transfected CD4⁺ T cells (○, *n* = 10) or pTK-Con transfected CD4⁺ T cells (▲, *n* = 10) or just PBS (♦, *n* = 6) as control. Tumor growth was followed. Shown here are mean tumor volumes plus SDs after tumor cell inoculation. (D) The survival of J558L-mOva plasmacytoma bearing mice. All data shown are representative of three independently performed experiments. ** $p < 0.01$ and * $p < 0.05$.

gene thymidine kinase (TK) was used to modify T cells (Black et al., 1996). In the J558-mOva tumor model, tumor growth was completely suppressed after infusion of pTK-IL15 transfected T cells in partial experimented animals. And these mice had been monitored for another 15 months and no T lymphoma was observed. To be sure that ganciclovir (GCV) could eliminate pTK-IL15 transfected $CD4^+$ T cells *in vivo*, 15 mg/kg GCV was injected once a day for 3 days after T cells infusion. Our results proved that GCV treatment efficiently eliminated both pTK-IL15 transfected $CD4^+$ T cells and pTK-Con transfected $CD4^+$ T cells *in vivo* (Fig. 9A and 9B). This enhances the safety of using pTK-IL15 transfected $CD4^+$ T cell in clinical therapy.

DISCUSSION

For the first time, we demonstrated here that retrovirus mediated IL-15 expression induced a selective expansion of antigen-specific $CD4^+$ T cells *ex vivo* and enhanced their tumor-suppression effect *in vivo*. This finding may have important significance for the improvement of T cell therapy against cancer.

Major obstacles for efficient adoptive T cell therapy are: (1) the difficulty in preparing enough tumor-specific T cells over a short time (Schumacher and Restifo, 2009) and (2) immune-negative factors existing within “tumor environment” prevent the survival of transferred T cells (Willimsky and Blankenstein,

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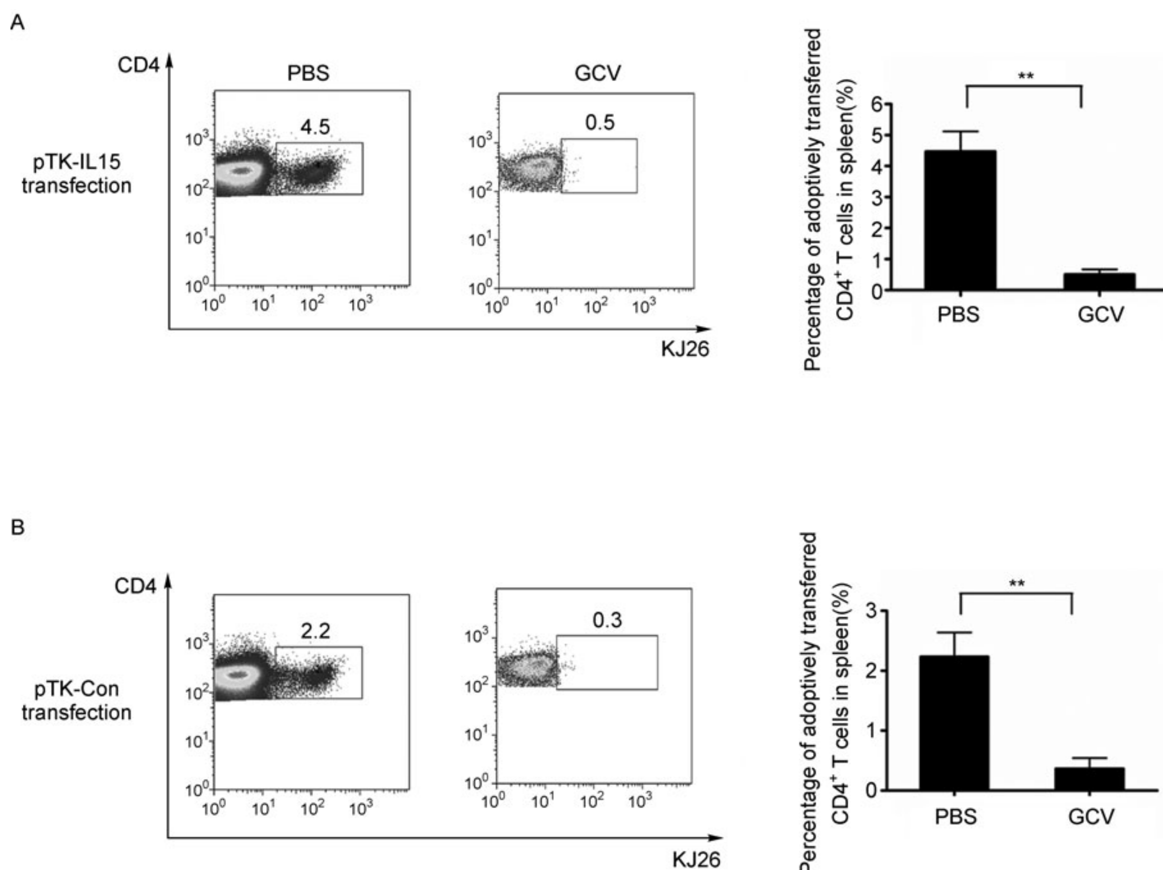

Figure 9. CD4$^+$ T cells transfected with pTK-IL15 could be eliminated by GCV treatment. After infusion with pTK-IL15 transfected CD4$^+$ T cells or pTK-Con transfected CD4$^+$ T cells, mice were treated with GCV or PBS (as control) every day. Three days later, splenocytes were isolated and transferred CD4$^+$ T cells were detected by CD4 & KJ26 double staining. (A) Detecting the pTK-IL15 transfected CD4$^+$ T cells in GCV treated mice. CD4$^+$ T cells were gated and the numbers represented the percentages of the outlined CD4$^+$ KJ26$^+$ population. (B) Detecting the pTK-Con transfected CD4$^+$ T cells in GCV treated mice. CD4$^+$ T cells were gated and the numbers represented the percentages of the outlined CD4$^+$ KJ26$^+$ population. All data shown are representative of three independently performed experiments. ** $p < 0.01$.

2005). There are currently no forms of FDA-approved T cell therapy available for cancer, because no randomized clinical trials have been able to demonstrate the anti-tumor efficacy of adoptive T cell therapy for cancers other than melanoma (June, 2007a).

To ensure the source of the adoptively transferred T cells, two major strategies have emerged: naturally-occurring T cells and TCR/CAR (chimeric antigen receptor) genetically engineered T cells (Cox et al., 1994; Dunbar et al., 1999; Schumacher, 2002). For the first strategy, tumor-reactive T cells are scarce in cancer patients, and repetitive antigen-pulse leads to the apoptosis of tumor-reactive T cells. Thus, the generation of T cell lines and T cell clones is a long and cumbersome process (Disis et al., 2002). Therefore, optimization of protocols for generation of tumor-reactive T cells for adoptive cell therapy is essential.

In this study, pTK-IL15 retroviral vector was used to enhance the antigen-specific CD4$^+$ T cell expanding efficiency *ex vivo*. It demonstrated that retroviral mediated IL-15 expression selectively expands antigen-specific CD4$^+$ T cells *ex vivo*. By improving the proportion of antigen-specific T cells, retrovirus mediated IL-15 expression facilitates the generation of tumor-specific T cell clones and shortens the time required for preparing enough tumor-reactive T cells. Furthermore, pTK-IL15 transfected CD4$^+$ T cells resisted apoptosis after infused into tumor-bearing host and exerted potent anti-tumor effect *in vivo*. CD4$^+$ T cells transfected with pTK-IL15 had a significant advantage in tumor immunotherapy over pTK-Con transfected CD4$^+$ T cells. Besides, some important points of our findings should be paid attention to.

First of all, retrovirus-mediated IL-15 expression endowed the CD4$^+$ T cells to express membrane-bound IL-15. Membrane-bound IL-15 triggers IL-15 signal *in Trans* to neighboring immune cells (Burkett et al., 2004) and it is significant to enhance the immune-adjuvant function of CD4$^+$ T cells. It has been reported that membrane-bound IL-15 promotes the development and maintain the homeostasis of memory CTLs and NK cells (McGill et al., 2010). Under

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physiological condition, membrane-bound IL-15 is not detected unless IL-15 and IL-15Rα are coordinately expressed by the same immune cells (Burkett et al., 2004). Naturally, $CD4^+$ T cells express IL-15Rα but not IL-15 and hold no membrane-bound IL-15 (Cosman et al., 1995). Therefore, upon retroviral modification, the $CD4^+$ T cells express both soluble and membrane-bound IL-15. These lead to enhanced expansion of gene-modified T cells as well as bystander cells such as CTLs and NK cells.

Secondly, retrovirus mediated IL-15 expression induced a selective expansion of antigen-specific $CD4^+$ T cells. The retroviral vector selectively transfects the proliferating cells (Culver et al., 1992). In our experiment, only Do11.10 $CD4^+$ T cells were pulsed to proliferation under $Ova_{323–339}$ peptide stimulation and these antigen-specific $CD4^+$ T cells were selectively transfected by IL-15 retroviral vector. The transfected $CD4^+$ T cells expressed membrane-bound IL-15 (Fig. 2) and could provide IL-15 signal to neighboring T cells. This signal inhibited the apoptosis of transfected antigen-specific $CD4^+$ T cells and also some neighboring non-specific $CD4^+$ T cells or IL-15 dependent cells. Therefore, to be more precisely, pTK-IL15 transfection increased the proportion of antigen-specific $CD4^+$ T cells. As the percentage of transfected specific T cells was great in the ratios of $1:10^2$ to $1:10^3$, a large number of non-specific T cells received IL-15 signal from pTK-IL15 transfected antigen-specific T cells. The increase in non-specific $CD4^+$ T cells was proportional to the increase in antigen-specific $CD4^+$ T cells. Besides, the number of non-specific T cells was far greater than antigen-specific T cells in dilution ratios of $1:10^4$ to $1:10^6$. The increase of non-specific $CD4^+$ T cells was insignificant (Fig. 4). Fortunately, natural tumor-specific $CD4^+$ T cells present at very low frequencies and the frequency of tumor-specific $CD4^+$ T cells in responding melanoma patients ranged from 0.0037% to 0.080% with a median of 0.024% of the $CD4^+$ cell population (Ladekarl et al., 2004). So the dilution ratios of $1:10^4$–$1:10^6$ are rational but ratios of $1:10^2$–$1:10^3$ are not. Moreover, fewer pTK-IL15 transfected $CD4^+$ T cells would express less soluble IL-15 and membrane-bound IL-15. So these cells would not induce severe side effect *in vivo*.

Thirdly, pTK-IL15 transfected $CD4^+$ T cells efficiently suppressed tumor growth *in vivo* (Fig. 8). Tumor-specific $CD4^+$ T cells perform anti-tumor effect through multiple mechanisms: (1) $CD4^+$ T cells directly recognize and eliminate MHCII-positive tumor cells (Quezada et al., 2010; Xie et al., 2010); (2) $CD4^+$ T cells secrete IFN-γ to inhibit the angiogenesis and suppress the growth of tumor (Qin and Blankenstein, 2000); (3) $CD4^+$ T cells promote the proliferation, activation and maturation of CTLs, NK cells and macrophages to enhance the anti-tumor immune responses (Toes et al., 1999; Cohen et al., 2000). IL-15 transduction prolonged the survival of antigen-specific $CD4^+$ T cells *in vivo*. These in turn activated CTLs, NK cells and macrophages to enhance multiple anti-tumor reactions.

Finally, IL-15 retroviral vector carries suicide gene to ensure the clinical safety. In our model, adoptive pTK-IL15 transfected T cells mainly bear two aspects of adverse effect: (1) Infused $CD4^+$ T cells damage the normal tissue. Partial T cells used in adoptive T cell therapy recognize the tumor-associated antigen but not tumor-specific antigen, which means normal tissues could not avoid being injured (Phan et al., 2003). For example, vitiligo occurred in some melanoma patients receiving adoptive T cell therapy (Dudley et al., 2002). (2) Retrovirus vector modification may induce insertion mutation and lead to T lymphoma. To avoid above side effects, TK was constructed into retroviral vector and GCV treatment could efficiently eliminate the cells expressing TK. As a chemical drug, GCV has been used in phase III clinical trials and holds no obvious adverse reaction to patients (Rainov, 2000). Importantly, our results proved that pTK-IL15 transfected T cells could be simply eliminated by GCV with no obvious side effects *in vivo* (Fig. 9).

In a word, this work shows potent effect of selectively expanded antigen-specific $CD4^+$ T cells upon pTK-IL15 transfection and indicates that retrovirus-mediated IL-15 expression enhanced the feasibility and efficiency of adoptive $CD4^+$ T cell therapy.

MATERIALS AND METHODS

Plasmid construction and retrovirus production

The full-length human IL15 gene was cloned by PCR from a plasmid obtained from InVivogene (San Diego, CA). The human IL15 gene encodes the isoform with a 48 AA signal peptide. Tgls (+)-hytk vector was a gift from the lab of Dr. Robert Overell. IL-15 and GFP gene were cloned into tgls (+)-hytk vector to acquire recombinant retrovirus vector pTK-IL15 through restriction site *Bam*HI, while pTK-Con vector was taken as a control vector (Fig. 1A). Retrovirus packaging vector pCL-10A1 was obtained from Imgenex (San Diego, CA) and is a MuLV-based vector. The retroviral supernatant was prepared as following. Briefly, 293T cells were cotransfected with pCL-10A1 and pTK-IL15/pTK-Con vectors using Lipofectamine 2000 (Invitrogen, San Diego, CA) in accordance with the manufacturer's instruction. The quantity of pTK-IL15/pTK-Con is 10% of pCL-10A1. And the stable retrovirus packaging cell clones were screened by 200 μg/mL hygromycin B (Roche, Indianapolis, IN) resistance. The supernatants containing retrovirus were collected and infiltrated (0.45 μm pore size, Millipore, Bedford, MA).

Mice and tumor models

All mice were maintained in a specific pathogen-free environment at the Institute of Biophysics, Chinese Academy of Sciences, Beijing. Sex and age-matched mice were used with the approval of appropriate authorities. The wild type BALB/c mice were purchased from Weitonglihua.

For adoptive T cell transfer experiments, tumor cells were s.c. inoculated at the flank of BALB/c mice, at 2.5×10^6 J558L-mOva or 1.0×10^5 TS/A-mOva. For mice bearing J558L-mOva, 5×10^6 $CD4^+$ T

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cells were i.v. injected via tail veil into each mouse 24 h after tumor inoculation. For mice bearing TS/A-mOva, cyclophosphamide was i. p. administrated into each mouse at a dose of 15 mg/kg 10 days later after tumor inoculation. And 48 h after cyclophosphamide administration, 5×10^6 $CD4^+$ T cells were i.v. injected via tail veil into each mouse. The tumor growth would be monitored every two days.

Reagents, peptides, antibodies and flow cytometry

The cells were labeled in preparation for immunofluorescence and were analyzed using flow cytometry for the expression of cell surface and/or internal molecules. The antibodies were diluted in PBS with 2% FCS (HyClone, Logan, UT). The samples were analyzed on a FACSCalibur cytometer (BD Biosciences, Franklin Lakes, NJ).The $OVA_{323–339}$ (ISQAVHAAHAEINEAGR), Percp-CD4 (BD Pharmingen Franklin Lakes, NJ), FITC-KJ26 (Biolegend, San Diego, CA), PE-AnnexinV (Biolegend, San Diego, CA), and PE-IL15 (R&D Systems, Minneapolis, MN) were used for analysis.

Preparation of antigen-presenting cells

Splenocytes were isolated from the spleen of wild type BALB/c mice. The fresh working solution of 25 μg/mL mitomycin C was prepared in culture medium. 1×10^7 splenocytes were treated in 500 μL mitomycin C working solution for 30 min at 37°C.

Generation and transduction of $CD4^+$ T cells

Do11.10 $CD4^+$ T cells which recognized OVA peptide 323–329 were purified from the spleen of Do11.10 mice (Jackson Laboratory, Maine, USA) using CD4 beads (Miltenyi Biotec, Heidelberg, Germany). For transfection, these T cells were plated at 2×10^6 cell/well in 24-well plates and stimulated with $Ova_{323–339}$ peptide pulsed 1×10^7 APC/well (antigen-presenting cells) for 2 days. Then the proliferating T cells were incubated with retroviral supernatants for 6 h. Then the transfected $CD4^+$ T cells were incubated in 10 ng/mL recombinant hIL-2 or 50 ng/mL recombinant hIL-15 (R&D system, Minneapolis, MN). The release of IL-15 by control and transfected $CD4^+$ T cells was measured in the culture supernatant by specific ELISAs (BD Biosciences, Franklin Lakes, NJ).

[^{3}H] thymidine incorporation assay

For the ^{3}H-thymidine incorporation assay, $CD4^+$ T cells were seeded into a 96-well plate at a density of 1×10^5 cells/mL. After 6 h, 1 uCi/well of [^{3}H] thymidine (Perkinelmer, Waltham, MA) was added and incubated during the last 18 h in a 5% CO_2 humidified atmosphere at 37°C. After washing the cells twice with cold PBS, the cells were counted in a scintillation counter (Perkinelmer). Each concentration point was repeated in triplicate wells, and at least three independent experiments were performed. The results are expressed as the standard error of the mean.

Enzyme-linked immunospot assay

Cytokine concentrations in culture supernatants were measured using a human IL-15 ELISA kit (BD Bioscience, Franklin Lakes, NJ) in accordance with the manufacturer's instructions.

Western blot analysis

The protein samples were run on sodium dodecyl sulfate (SDS)-10% polyacrylamide gels and transferred to nitrocellulose membranes. The membrane was blocked in Superblock blocking buffer, incubated with the appropriate antibody and subsequently incubated with a secondary antibody conjugated to horseradish peroxidase. The immunoreactive bands were visualized with an ECL system (Bio-Rad, Hercules, CA).

Determination of transgenic $CD4^+$ T cells *in vivo*

BALB/c mice were treated with 0.8 mg/mL BrdU (Sigma-Aldrich, San Diego, CA) in their drinking water as described before (Judge et al., 2002). BrdU solution was prepared in sterile water, protected from light exposure, and changed daily. During the continuous labeling phase, BALB/c mice infused with IL-15 transfected $CD4^+$ T cells received BrdU up to 3 days. As a control, BALB/c mice infused with pTK-Con transfected $CD4^+$ T cells were treated with BrdU in parallel. On the day of the assay, splenocytes or tumor-infiltrated lymphocytes from these mice were used as positive BrdU staining control. BrdU intracellular staining was performed. In each staining experiment, Percp-CD4 and FITC-KJ26 were used to define the transferred $CD4^+$ T cells.

Validation of the suicide gene thymidine kinase

To evaluate the functionality of the suicide gene, mice bearing the IL-15 transfected with hytk vector were treated with 50 mg/kg GCV every day for 3 days. The GCV treatment was initiated 1 day after adoptive immunotherapy. The percentage of transferred $CD4^+$ T cell was detected by fluorescence-activated cell sorting (FACS).

Statistical analysis

The data were analyzed using Fisher's exact test and two-tailed unpaired Student's *t*-test. Means ± SD are presented. $p < 0.05$ was considered statistically significant.

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ABBREVIATIONS

AICD, activation-induced cell death; APC, antigen-presenting cell; CTLs, cytotoxic T lymphocytes; GCV, ganciclovir; GFP, green fluorescent protein; MOI, multiplicity of infection; TK, thymidine kinase

REFERENCES

Abad, J.D., Wrzensinski, C., Overwijk, W., De Witte, M.A., Jorritsma, A., Hsu, C., Gattinoni, L., Cohen, C.J., Paulos, C.M., Palmer, D.C., *et al.* (2008). T-cell receptor gene therapy of established tumors in a murine melanoma model. J Immunother 31, 1–6.

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Bergamaschi, C., Rosati, M., Jalah, R., Valentin, A., Kulkarni, V., Alicea, C., Zhang, G.M., Patel, V., Felber, B.K., and Pavlakis, G.N. (2008). Intracellular interaction of interleukin-15 with its receptor alpha during production leads to mutual stabilization and increased bioactivity. J Biol Chem 283, 4189–4199.

Black, M.E., Newcomb, T.G., Wilson, H.M., and Loeb, L.A. (1996). Creation of drug-specific herpes simplex virus type 1 thymidine kinase mutants for gene therapy. Proc Natl Acad Sci U S A 93, 3525–3529.

Burkett, P.R., Koka, R., Chien, M., Chai, S., Boone, D.L., and Ma, A. (2004). Coordinate expression and trans presentation of interleukin (IL)-15Ralpha and IL-15 supports natural killer cell and memory CD8 + T cell homeostasis. J Exp Med 200, 825–834.

Cohen, P.A., Peng, L., Plautz, G.E., Kim, J.A., Weng, D.E., and Shu, S. (2000). CD4 + T cells in adoptive immunotherapy and the indirect mechanism of tumor rejection. Crit Rev Immunol 20, 17–56.

Cosman, D., Kumaki, S., Ahdieh, M., Eisenman, J., Grabstein, K.H., Paxton, R., DuBose, R., Friend, D., Park, L.S., Anderson, D., *et al.* (1995). Interleukin 15 and its receptor. Ciba Found Symp 195, 221–229, discussion 229–233.

Cox, A.L., Skipper, J., Chen, Y., Henderson, R.A., Darrow, T.L., Shabanowitz, J., Engelhard, V.H., Hunt, D.F., and Slingluff, C.L. Jr. (1994). Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. Science 264, 716–719.

Culver, K.W., Ram, Z., Wallbridge, S., Ishii, H., Oldfield, E.H., and Blaese, R.M. (1992). In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. Science 256, 1550–1552.

Disis, M.L., Gooley, T.A., Rinn, K., Davis, D., Piepkorn, M., Cheever, M.A., Knutson, K.L., and Schiffman, K. (2002). Generation of T-cell immunity to the HER-2/neu protein after active immunization with HER-2/neu peptide-based vaccines. J Clin Oncol 20, 2624–2632.

Dudley, M.E., Wunderlich, J.R., Robbins, P.F., Yang, J.C., Hwu, P., Schwartzentruber, D.J., Topalian, S.L., Sherry, R., Restifo, N.P., Hubicki, A.M., *et al.* (2002). Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science 298, 850–854.

Dunbar, P.R., Chen, J.L., Chao, D., Rust, N., Teisserenc, H., Ogg, G. S., Romero, P., Weynants, P., and Cerundolo, V. (1999). Cutting edge: rapid cloning of tumor-specific CTL suitable for adoptive immunotherapy of melanoma. J Immunol 162, 6959–6962.

Gattinoni, L., Powell, D.J. Jr, Rosenberg, S.A., and Restifo, N.P. (2006). Adoptive immunotherapy for cancer: building on success. Nat Rev Immunol 6, 383–393.

Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M.P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C.S., Pawliuk, R., Morillon, E., *et al.* (2003). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 302, 415–419.

Judge, A.D., Zhang, X., Fujii, H., Surh, C.D., and Sprent, J. (2002). Interleukin 15 controls both proliferation and survival of a subset of memory-phenotype CD8(+) T cells. J Exp Med 196, 935–946.

June, C.H. (2007a). Adoptive T cell therapy for cancer in the clinic. J Clin Invest 117, 1466–1476.

June, C.H. (2007b). Principles of adoptive T cell cancer therapy. J Clin Invest 117, 1204–1212.

Klebanoff, C.A., Finkelstein, S.E., Surman, D.R., Lichtman, M.K., Gattinoni, L., Theoret, M.R., Grewal, N., Spiess, P.J., Antony, P.A., Palmer, D.C., *et al.* (2004). IL-15 enhances the in vivo antitumor activity of tumor-reactive CD8 + T cells. Proc Natl Acad Sci U S A 101, 1969–1974.

Ladekarl, M., Agger, R., Fleischer, C.C., Hokland, M., Hulgaard, E.F., Kirkin, A., von der Maase, H., Petersen, M.S., Rytter, C., Zeuthen, J., *et al.* (2004). Detection of circulating tumor lysate-reactive CD4 + T cells in melanoma patients. Cancer Immunol Immunother 53, 560–566.

Leen, A.M., Rooney, C.M., and Foster, A.E. (2007). Improving T cell therapy for cancer. Annu Rev Immunol 25, 243–265.

Li, Y., Zhi, W., Wareski, P., and Weng, N.P. (2005). IL-15 activates telomerase and minimizes telomere loss and may preserve the replicative life span of memory CD8 + T cells in vitro. J Immunol 174, 4019–4024.

Marks-Konczalik, J., Dubois, S., Losi, J.M., Sabzevari, H., Yamada, N., Feigenbaum, L., Waldmann, T.A., and Tagaya, Y. (2000). IL-2-induced activation-induced cell death is inhibited in IL-15 transgenic mice. Proc Natl Acad Sci U S A 97, 11445–11450.

McGill, J., Van Rooijen, N., and Legge, K.L. (2010). IL-15 trans-presentation by pulmonary dendritic cells promotes effector CD8 T cell survival during influenza virus infection. J Exp Med 207, 521–534.

Muranski, P., and Restifo, N.P. (2009). Adoptive immunotherapy of cancer using CD4 (+) T cells. Curr Opin Immunol 21, 200–208.

Phan, G.Q., Yang, J.C., Sherry, R.M., Hwu, P., Topalian, S.L., Schwartzentruber, D.J., Restifo, N.P., Haworth, L.R., Seipp, C.A., Freezer, L.J., *et al.* (2003). Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci U S A 100, 8372–8377.

Qin, Z., and Blankenstein, T. (2000). $CD4^+$ T cell—mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells. Immunity 12, 677–686.

Quezada, S.A., Simpson, T.R., Peggs, K.S., Merghoub, T., Vider, J., Fan, X., Blasberg, R., Yagita, H., Muranski, P., Antony, P.A., *et al.* (2010). Tumor-reactive CD4 (+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. J Exp Med 207, 637–650.

Quintarelli, C., Vera, J.F., Savoldo, B., Giordano Attianese, G.M., Pule, M., Foster, A.E., Heslop, H.E., Rooney, C.M., Brenner, M.K., and Dotti, G. (2007). Co-expression of cytokine and suicide genes to enhance the activity and safety of tumor-specific cytotoxic T lymphocytes. Blood 110, 2793–2802.

Radfar, S., Wang, Y., and Khong, H.T. (2009). Activated CD4 + T cells dramatically enhance chemotherapeutic tumor responses in vitro and in vivo. J Immunol 183, 6800–6807.

Rainov, N.G. (2000). A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. Hum Gene Ther 11, 2389–2401.

Schumacher, T.N. (2002). T-cell-receptor gene therapy. Nat Rev Immunol 2, 512–519.

Schumacher, T.N., and Restifo, N.P. (2009). Adoptive T cell therapy of cancer. Curr Opin Immunol 21, 187–189.

Sharma, S., Cantwell, M., Kipps, T.J., and Friedmann, T. (1996).

 © Higher Education Press and Springer-Verlag Berlin Heidelberg 2011

Efficient infection of a human T-cell line and of human primary peripheral blood leukocytes with a pseudotyped retrovirus vector. Proc Natl Acad Sci U S A 93, 11842–11847.

Teague, R.M., Sather, B.D., Sacks, J.A., Huang, M.Z., Dossett, M.L., Morimoto, J., Tan, X., Sutton, S.E., Cooke, M.P., Ohlén, C., *et al.* (2006). Interleukin-15 rescues tolerant CD8 + T cells for use in adoptive immunotherapy of established tumors. Nat Med 12, 335–341.

Toes, R.E., Ossendorp, F., Offringa, R., and Melief, C.J. (1999). CD4 T cells and their role in antitumor immune responses. J Exp Med 189, 753–756.

Willimsky, G., and Blankenstein, T. (2005). Sporadic immunogenic tumours avoid destruction by inducing T-cell tolerance. Nature 437, 141–146.

Xie, Y., Akpinarli, A., Maris, C., Hipkiss, E.L., Lane, M., Kwon, E.K., Muranski, P., Restifo, N.P., and Antony, P.A. (2010). Naive tumor-specific CD4 (+) T cells differentiated in vivo eradicate established melanoma. J Exp Med 207, 651–667.

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