European Journal of Immunology

IL-7 is superior to IL-2 for *ex vivo* expansion of tumour-specific CD4$^+$ T cells

Stefano Caserta[1,2], Patrizia Alessi[1], Veronica Basso[1] and Anna Mondino[1]

[1] Program in Immunology and Bio-Immuno-gene therapy of Cancer (PIBIC), Division of Immunology, Transplantation and Infectious Diseases, San Raffaele Scientific Institute, Milan, Italy
[2] International Ph.D. Program in Immunology, Vita-Salute San Raffaele University, Milan, Italy

It is well established that tumours hinder both natural and vaccine-induced tumour-specific CD4$^+$ T-cell responses. Adoptive T-cell therapy has the potential to circumvent functional tolerance and enhance anti-tumour protective responses. While protocols suitable for the expansion of cytotoxic CD8$^+$ T cells are currently available, data on tumour-specific CD4$^+$ T cells remain scarce. We report here that CD4$^+$ T cells sensitized to tumour-associated Ag *in vivo*, proliferate *in vitro* in response to IL-7 without the need for exogenous Ag stimulation and accumulate several folds while preserving a memory-like phenotype. Both cell proliferation and survival accounts for the outgrowth of tumour-sensitized T cells among other memory and naive lymphocytes following exposure to IL-7. Also IL-2, previously used to expand anti-tumour CTL, promotes tumour-specific CD4$^+$ T-cell accumulation. However, IL-7 is superior to IL-2 at preserving lymphocyte viability, *in vitro* and *in vivo*, maintaining those properties, that are required by helper CD4$^+$ T cells to confer therapeutic efficacy upon transplantation in tumour-bearing hosts. Together our data support a unique role for IL-7 in retrieving memory-like CD4$^+$ T cells suitable for adoptive T-cell therapy.

Key words: Adoptive T-cell therapy · Cytokines · T helper cells · Tumour immunology


Supporting Information available online

Introduction

Adoptive cell therapy (ACT) with tumour-specific CD8$^+$ T lymphocytes has become one of the most promising approaches in cancer therapy. Rosenberg *et al.* first demonstrated the possibility to expand tumour-specific cytotoxic CD8$^+$ lymphocytes (CTL) from tumour lesions in high doses of IL-2 [1]. These authors later showed that the state of lymphocyte differentiation, induced in the presence of common γ-chain receptor cytokines, is critical for therapeutic efficacy [2, 3]. In spite of the recognized importance of Ag-specific CD4$^+$ T cells in both adaptive and innate immune responses, their identification remains elusive, and their *in vitro* amplification is hindered by the absence of reliable protocols able to support cell proliferation in the absence of terminal differentiation. While, Ag tumours elicit natural tumour-specific CD4$^+$ T-cell responses [4–10], functional tolerance is eventually observed through the induction of T-cell anergy [11, 12], T-cell depletion [13] or the limitation of the memory repertoire [10, 14, 15]. This is possibly due to Ag persistence, and continual TCR signaling, as in the case of chronic

Correspondence: Dr. Anna Mondino
e-mail: anna.mondino@hsr.it

viral infections [16–18]. In addition, regulatory $CD4^+$ T cells have been frequently found in the case of tumours, often among the tumour-infiltrating lymphocytes, thereby persuading the general interest towards $CD8^+$ CTL. Thus, $CD4^+$ T cells have not been widely exploited in ACT as well as the properties (*i.e.* homing potential, functionality, and survival) that $CD4^+$ T cells might require for successful applications in ACT are much less known than in the case for $CD8^+$ CTL.

A large, still not definitive, amount of literature underline how IL-2, IL-7 and IL-15 play non-redundant roles in shaping the representation of memory cells [19–23]. IL-2 controls T-cell clonal expansion and contraction, and promotes lymphocyte differentiation. IL-2 and IL-15 can also support memory cell division and have been used in combination with Ag-driven stimulation, for the expansion of CTL [24–29]. IL-7 regulates peripheral T-cell homeostasis, and contributes to the generation and long-term survival of both $CD4^+$ and $CD8^+$ memory T lymphocytes *in vivo* [30, 31]. In some cases IL-7 amplifies Ag-driven T-cell responses [32–36], favors the transition of effector to memory cells [31, 37–39], and sustains a slow, homeostatic-like, Ag-independent memory T-cell proliferation [24, 30, 40]. Furthermore, its administration at the time of Ag withdrawal supports memory $CD8^+$ T-cell generation [41], and enhances vaccine-mediated immunity when provided in adjuvant settings [42, 43].

Based on our previous results showing that tumours only allow a limited expansion of effector $CD4^+$ T cells, while hinder both natural and vaccine-induced memory-like cell responses [10, 15], we attempted the *ex vivo* expansion of tumour-specific $CD4^+$ T cells to be used in ACT, using common-γ-chain receptor cytokines. We report the ability of IL-7, rather than IL-2 in expanding tumour-sensitized T cells in short-term cultures, capable of sustaining anti-tumour protection in ACT settings.

Results

IL-7 favors the accumulation of tumour-sensitized $CD4^+$ T cells

We and others previously characterized Ag-specific $CD4^+$ T-cell responses by fluorescent MHC class II/peptide multimer and Ag-specific intracellular cytokine staining in 16.2β mice [10, 44], which express a Tg TCR-β-chain specific for the *Leishmania* receptor for Activated C Kinase (LACK, derived from *Leishmania Major*) Ag coupled to a polyclonal α-chain TCR repertoire. This allows the identification of both naive (~0.5% of $CD4^+$ cells) and memory polyclonal LACK-specific $CD4^+$ T cells. By using this model, we found that TS/A tumours expressing LACK as an intracellular tumour-associated Ag (TS/A-LACK tumour cells) promote the expansion of short-lived LACK-specific effector-like $CD4^+$ T cells, while hinder the accumulation of both natural- and vaccine-induced central memory-like T cells [10, 15]. As IL-7 is known to support memory $CD4^+$ T-cell expansion following Ag withdrawal [41], we asked whether this cytokine could be used in short-term *in vitro* cultures for the expansion of tumour-sensitized $CD4^+$ T cells useful in ACT settings. In agreement with our previous findings, $CD4^+CD44^{high}$ T cells able to bind I-A^d/LACK fluorescent multimers (Fig. 1A) and to secrete IL-2 and/or IFN-γ upon LACK-specific stimulation (Fig. 1B) were sizeable in TS/A-LACK tumour-draining LN (T-dLN) (*i.e.* the most proximal LN to the site of tumour growth) *ex vivo*. These cells

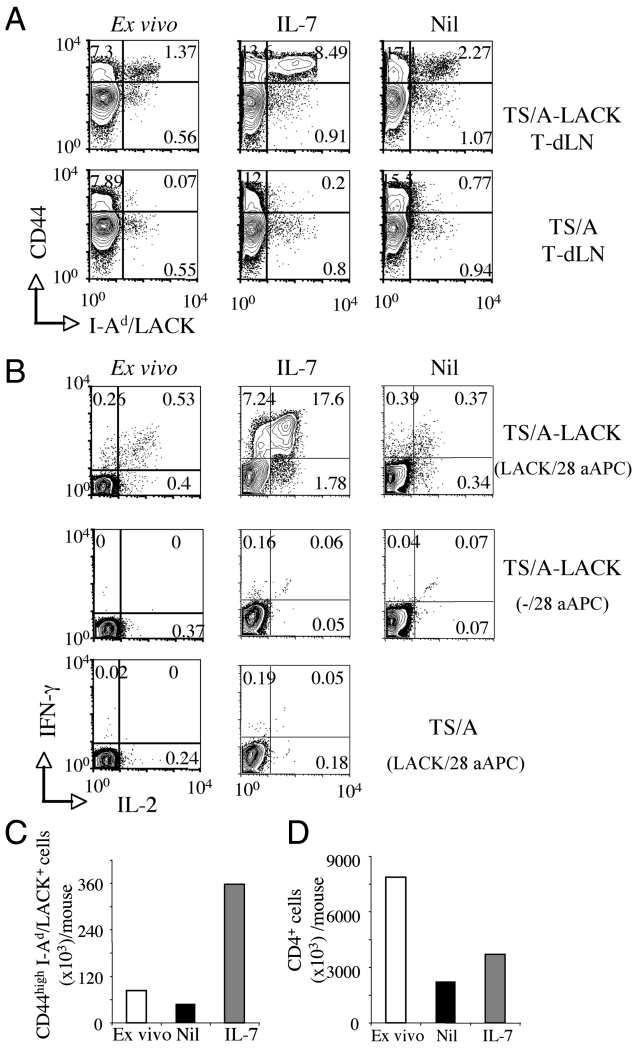

Figure 1. IL-7 favors the accumulation of *in vivo*-primed tumour-specific $CD4^+$ T cells *in vitro*. Briefly, 16.2β mice were challenged with 4×10^5 TS/A-LACK or TS/A (control) tumour cells and sacrificed 21 days later. Cells from pools ($n = 5$) of T-dLN were analyzed *ex vivo* and after 7 days in culture with IL-7. (A) The presence of LACK-specific T cells was examined by flow cytometry using I-A^d/LACK fluorescent multimers. Events are shown after gating on viable $CD4^+$, $B220^-$, $CD8^-$, $CD11b^-$, TO-PRO-3^- cells. The frequency of $CD4^+$ cells in each quadrant is indicated. (B) Cells were stimulated for 5 h with aAPC coated with anti-CD28 mAb only (–/28 aAPC, middle panels) or with I-A^d/LACK molecules and anti-CD28 mAb (LACK/28 aAPC, first and third row panels), fixed, permeabilized, stained with anti-CD4, anti-IL-2 and anti-IFN-γ mAb, and analyzed by flow cytometry. Contour plots show CD4-gated cells. (C and D) The total number of I-A^d/$LACK^+$ $CD44^{high}$ $CD4^+$ cells (C) and I-A^d/$LACK^-$ $CD4^+$ T cells (D) *ex vivo* and following culture in plain medium (Nil) or IL-7, normalized *per* mouse T-dLN. Data are representative of three independent determinations.

were markedly enriched in frequency (Fig. 1A) and total numbers (Fig. 1C) in IL-7-driven and not control (Nil)-cultures. It is worth noting that I-A^d/LACK$^+$, CD4$^+$ T cells accumulated in response to IL-7 in spite of a CD4$^+$ T-cell loss during culture time (Fig. 1D). When quantified in independent experiments, the number of LACK (tumour)-specific cells and in particular of IL-2/IFN-γ-double secreting CD4$^+$ T cells detected in IL-7-driven cultures over control (Nil) cultures was increased by several folds (7.88 ± 0.78 $n = 8$; and 25.3 ± 8.13 $n = 3$, respectively). Memory-like LACK-specific T cells were undetectable in T-dLN of control TS/A tumour-bearing (Fig. 1A and B, lower panels) and tumour-free 16.2β mice (Fig. 2) both *ex vivo* and after IL-7-driven culture. This indicates that *in vivo* Ag sensitization is required for the observed *in vitro* IL-7-driven response.

Both *in vitro* cell division and survival might account for the selective accumulation of LACK-specific lymphocytes in response to IL-7. To analyze proliferation, cells derived from naive (control) and T-dLN, were labeled with CFSE and cultured without (Nil) and with IL-7. In cultures derived from control LN, few CD4$^+$ T cells underwent *in vitro* cell proliferation in the absence of stimulation (Fig. 2A, Nil), while a fraction of cells with a CFSE^{dim} (*i.e.* diminished CFSE content) profile, likely undergoing homeostatic-like cell division, was found in IL-7-driven cultures (Fig. 2A, IL-7), as also described previously [26]. In the case of cultures derived from T-dLN, a sizeable fraction of CD4$^+$ T cells proliferated in the absence of stimulation (Fig. 2B, Nil, hereafter defined as "spontaneous" cell division). LACK-specific IL-2 (not depicted) and IFN-γ-double secreting cells, identified by intracellular cytokine staining, were mostly found among CFSE^{dim} cells, and selectively enriched after exposure to IL-7 (Fig. 2B, IL-7). Similar results were obtained with highly purified (>97%) CD4$^+$ cell cultures. Although spontaneous cell division was no longer detectable in CD4$^+$ cell cultures (Fig. 2C and D, Nil), suggesting that APC might support the *ex vivo* expansion of *in vivo* Ag-sensitized T cells, LACK-specific CFSE^{dim} T cells accumulated in response to IL-7 (Fig. 2D, bottom) to extents comparable to those found in unfractionated T-dLN cultures (compare pie charts in Fig 2B and D). Thus, IL-7-driven *in vitro* expansion of *in vivo* Ag-sensitized memory-like T cells accounts, at least in part, for their selective accumulation.

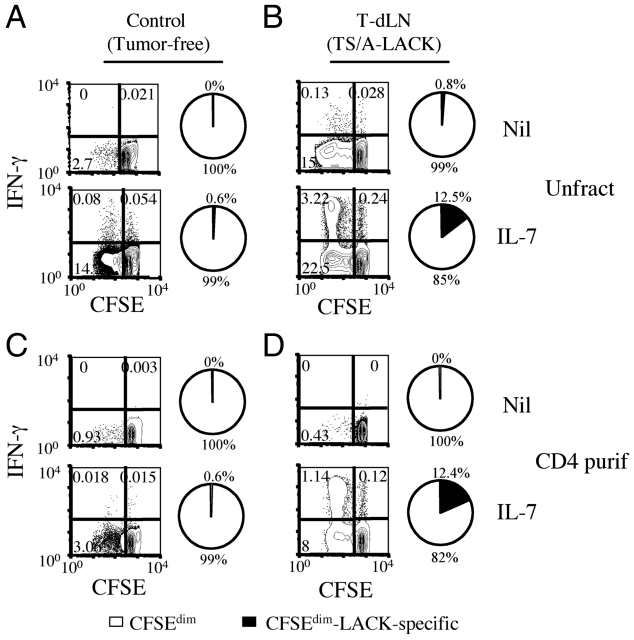

Figure 2. IL-7 sustains the accumulation of CD4$^+$ T cells capable of fast cell division. LN were derived from control, tumour-free (A, C), or TS/A-LACK tumour-bearing (B and D) 16.2β mice ($n = 5$). Pools of unfractionated (A and B) or highly CD4$^+$-purified (>97%) (C and D) cells were CFSE-labeled and cultured for a week without provision of exogenous Ag or cytokines (Nil) or in the presence of optimal IL-7 amounts (IL-7). Cells were stimulated for 5 h with aAPC coated with anti-CD28 mAB (not depicted) or LACK/anti-CD28-coated aAPC and analyzed by flow cytometry as described in Fig. 1. Cytokine secretion in response to anti-CD28-coated aAPC was undetectable and comparable to what is shown in Fig. 1B (second row). Contour plots show CFSE content and IFN-γ production by CD4$^+$ T cells. The frequency of events within each quadrant is depicted. Pie charts depict the relative representation of CFSE^{dim} LACK-specific IFN-γ secreting cells among CFSE^{dim} cells. Data are representative of two independent experiments.

IL-7 and IL-2, but not IL-15 or IL-6 enrich *in vivo* Ag-sensitized T cells in short-term cultures

We further analyzed IL-7-driven cultures derived from T-dLN of BALB/c mice, which have a physiological polyclonal representation of LACK (tumour)-specific naive CD4$^+$ T cells ($\sim 1/10^5$), and additionally compared IL-7 to other cytokines known to play a pro-survival role in T-cell biology. LACK-specific CD4$^+$ T cells were undetectable in tumour-free control BALB/c mice, but measurable in TS/A-LACK tumour-bearing mice by both I-A^d/LACK multimer staining (Fig. 3A) and LACK-specific intracellular cytokine release (Fig. 3B) as published previously [10, 15]. As in the case of 16.2β-derived cultures, LACK-specific cells were markedly enriched in frequency (Fig. 3A and B) and total number (Fig. 3C and D) following IL-7-driven cultures. In addition to IL-7, IL-2 supported the significant accumulation of LACK-specific cells as well, when compared with IL-15 or IL-6 (Fig. 3C–F).

Again, IL-2$^+$ (not depicted) and IFN-γ$^+$ LACK-specific T cells were mainly found among fast dividing CFSE^{dim} cells in IL-7- and also IL-2-driven cultures (Fig. 3G), suggesting that cytokine-driven proliferation of tumour-sensitized LACK-specific T cells contributes to their selective *in vitro* accumulation. Notably, we found that Ag-driven stimulation elicited the expansion of tumour Ag-sensitized LACK-specific CD4$^+$ T cells, but only when provided in minute amounts (Supporting Information Fig. 1), suggesting that currently used expansion methods, heavily relying on efficient Ag-driven stimulation, might not be optimal for the *in vitro* expansion of recently primed T cells.

IL-7-better than IL-2 preserves lymphocyte viability and homing potential

We next investigated the role of IL-7-driven cell survival. Cell recovery was first analyzed. IL-7, but not IL-2 supported a significant higher recovery of both CD4$^+$ (Fig. 4A), and CD4$^+$

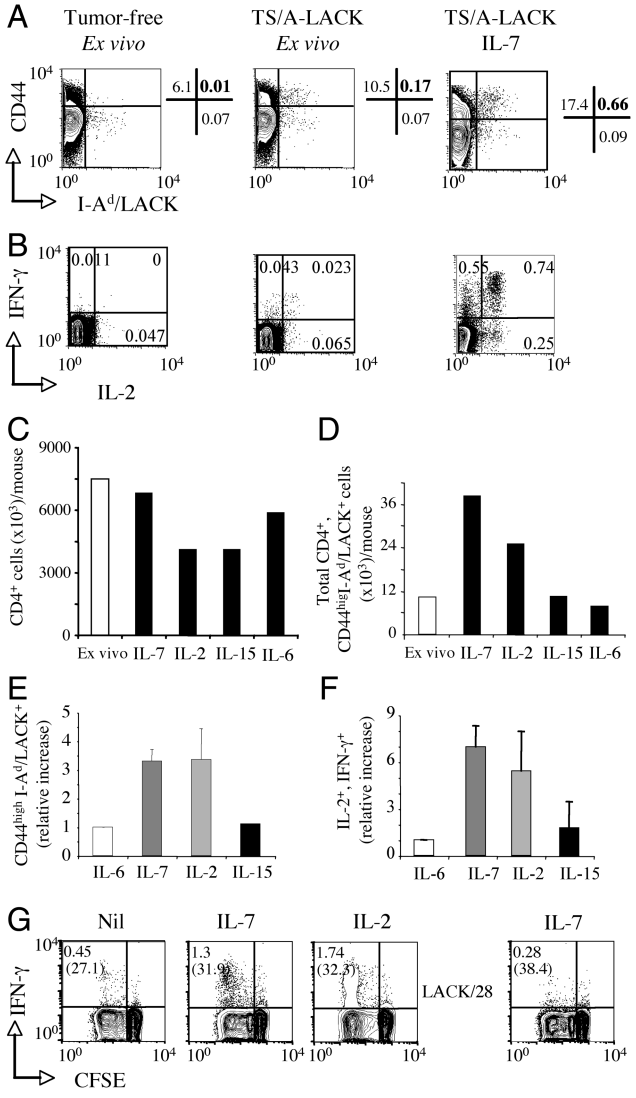

Figure 3. IL-7 and IL-2 enable the detection of rare tumour-specific $CD4^+$ T cells. Pools of LN cells recovered from TS/A-LACK tumour-bearing BALB/c mice ($n = 5$) were analyzed *ex vivo* or after culture with IL-7, IL-2, IL-15 and IL-6 alone (A–F). After 7 days, cells were recovered and surface stained to determine the frequency of Ag-sensitized LACK-specific T cells (A, C and E) as described in Fig. 1A, or exposed to control (not depicted), or LACK-specific stimulation (B and F) to evaluate LACK-specific intracellular cytokine release, as described in Fig. 1B. One of the three representative experiments is shown. (A) Flow-cytometry profiles after gating on viable $CD4^+$, $B220^-$, $CD8^-$, $CD11b^-$, TO-PRO-3^- cells. The frequency of I-A^d/$LACK^+CD44^{high}$ cells among $CD4^+$ cells is indicated in bold. (B) The frequency of cytokine-producing cells among $CD4^+$ T cells. (C and D) The total number of I-A^d/$LACK^-$ $CD4^+$ T cells (C) and I-A^d/$LACK^+CD44^{high}$ $CD4^+$cells (D), normalized *per* mouse T-dLN, in one of the three representative experiment. (E and F) The total number of I-A^d/$LACK^+CD44^{high}$ $CD4^+$ cells (E) and LACK-specific IL-2 and IFN-γ secreting cells (F) in IL-7-cultures was divided by the total number of I-A^d/$LACK^+$ $CD44^{high}$ $CD4^+$ cells and LACK-specific IL-2 and IFN-γ secreting cells in IL-6-driven cultures, which were repeatedly comparable to plain media cultures (data not shown). The relative fold increase ± SD calculated in three independent experiments is depicted. (G) Contour plots depict cytokine production by CFSE-labeled cells cultured for a week in the absence (Nil) and in the presence of IL-7 or IL-2, and stimulated for 5 h with LACK/anti-CD28-coated aAPC or anti-CD28-coated control APC (IL-7 cultured cells are depicted as representative on the right). $CD4^+$ events are depicted.

$CFSE^{dim}$ dividing cells (Fig. 4B) when compared with control (Nil) cultures in several independent experiments. Furthermore, while up to 72% of $CFSE^{dim}$ cells remained viable in IL-7-driven cultures (as determined by exclusion of TO-PRO-3, a dye which labels dead cells, Fig. 4C), only 40% of proliferating cells were viable in IL-2-driven cultures (Fig. 4C). Finally, while the vast majority (82.5%) of IL-7 cultured $CD4^+$ T cells upregulated Bcl-2 expression with respect to medium-cultured cells (Fig. 4D, left, compare thick line to shaded histograms), suboptimal Bcl-2 levels were found in IL-2 cultured cells (Fig. 4D, right). It is worth noting that IL-7 better than IL-2 preserved $CD62L^{high}$ cells (Fig. 4E), while IL-2 mostly enriched cultures cells of $CD44^{high}$ lymphocytes (Fig. 4F). No significant differences were observed in $FOXP3^+$ T-cell representation (not depicted), or CD25, and CD132 expression (Fig. 4F), while CD127 was specifically down-regulated in response to IL-7 (Fig. 4F), as expected [45]. Together, these findings indicate that while both IL-7 and IL-2 sustain the accumulation of *in vivo* primed T cells, IL-7 best preserves lymphocyte viability *in vitro*, and *in vivo* survival (Bcl-2) and LN-homing (CD62L) potential.

IL-7- and not IL-2-cultured $CD4^+$ T cells provide anti-tumour protection in adoptive T-cell therapy

IL-2 and/or IL-2-expanded $CD8^+$ CTL have been previously used in ACT with various degree of success [1]. Having found that IL-7-cultured $CD4^+$ T cells qualitatively differ from those cultured in IL-2, we compared their *in vivo* potential. First we investigated prophylactic settings. $CD4^+$ T cells were purified from IL-7- or IL-2-driven T-dLN culture and adoptively transferred in syngenic mice (5×10^5 *per* mouse). A day later mice were challenged with 4×10^5 TS/A-LACK cells. Results, reported in Fig. 5A indicate that the infusion of IL-7- and not IL-2-cultured $CD4^+$ cells significantly resulted in a considerable delay in tumour development (left), and a survival advantage (right). Therapeutic settings were then analyzed. Mice bearing established TS/A-LACK tumours (10 days are sufficient to reveal an established growing tumour in this model [10]) were subjected to total body irradiation (TBI, 600 rad). This conditioning regimen was employed as it favors ACT [46] and only delays TS/A-LACK tumour growth (Supporting Information Fig. 2). A day after TBI, mice received $CD4^+$ cells (i.v., 2×10^6) purified from IL-7 cultured T-dLN or tumour-free LN cells. In total 20×10^6 syngenic splenocytes derived from tumour-free mice were co-transferred to obviate peripheral radiation-induced lymphopenia and allow proper responses to TS/A-LACK tumours, which requires $CD8^+$ T cells [47]. While IL-7-cultured naive cells failed to support tumour protection, IL-7-cultured T-dLN $CD4^+$ T cells promoted protective responses able to control the growth of TS/A-LACK tumours (Fig. 5B). Up to 60% of these mice remained free of disease by the time control mice had to be sacrificed, and for up to 3 months, and rejected a secondary tumour challenge (data not shown). Additionally, when T-dLN cells derived *ex vivo* were compared with

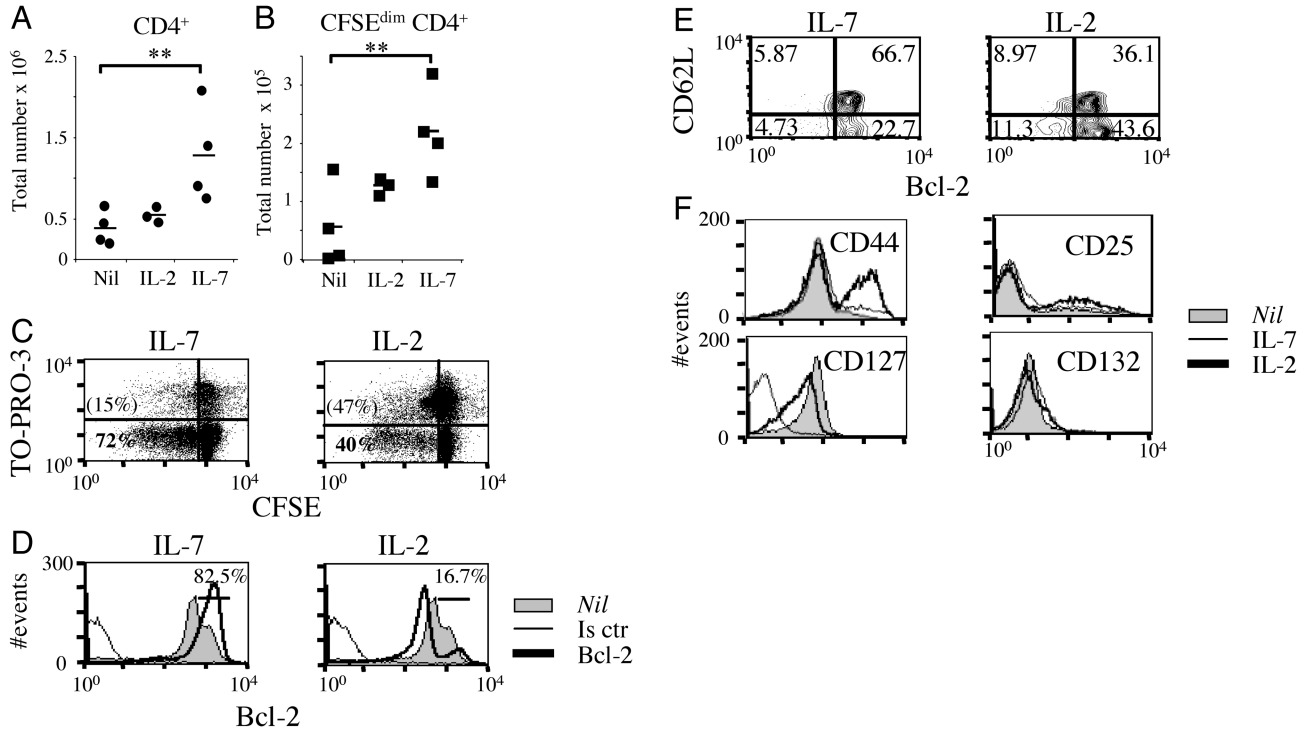

Figure 4. IL-7 is superior to IL-2 in terms of preserving lymphocyte viability, subset representation, and survival and homing potential. Cells from –T-dLN were pooled from TS/A-LACK tumour-bearing BALB/c mice ($n = 10$), labeled with CFSE and cultured for 7 days in the absence (Nil) or in the presence of the indicated cytokines. After 7 days, cells were recovered and analyzed by flow cytometry. CFSE-labeled cultured cells were stained with anti-CD4 mAb and TO-PRO-3 (A–C), or stained with anti-CD4 mAb, anti-CD62L mAb, fixed, permeabilized and stained with anti-Bcl-2 mAb (D–E). (A and B) Absolute numbers of viable $CD4^+$ cells (A), and $CD4^+CFSE^{dim}$ cells (B) were calculated by multiplying the percentage of $CD4^+$ (A) and $CD4^+$ $CFSE^+$ (B) by the viable counts (Trypan Blue exclusion) in three independent experiments. Two-tails Student's *t*-test was used to determine statistical significance (**$p<0.01$). (C) Viability was determined by provision of TO-PRO-3 (a DNA intercalating dye) before cytofluorimetric evaluation. Events are shown after gating on total $CD4^+$ cells. The frequency of overall dead cells, $CD4^+$ TO-PRO-3^+ is reported in brackets. Viable and proliferating, $CFSE^{dim}$ TO-PRO-3^- cell-frequency is also specified (bold). (D) Histograms depict $CD4^+$ T lymphocytes. (Shaded histograms: medium-cultured cells (Nil) stained with anti-Bcl-2 mAb; thin line histograms: cytokine-cultured cells stained with an isotype control mAb; thick line histograms: cytokine-cultured cells stained with anti-Bcl-2 mAb). The frequency of cells expressing optimal Bcl-2 levels among $CD4^+$ cells is reported in IL-7 (left)- and IL-2 (right)-driven cultures. (E) Bcl-2 expression *versus* the LN-homing molecule, CD62L is shown in representative contour plots that are shown after gating on $CD4^+CFSE^{dim}$ cells. The experiment is representative of three independent determinations. (F) Cells were also stained with CD44, CD25, CD127 and CD132 mAb. Histogram plots represent expression of CD44, CD25, CD127 and CD132 on $CD4^+$ T cells recovered from cultures in plain medium (Nil, shaded histograms), IL-7 (thin line histograms) or IL-2 (thick line histograms). Events are shown after gating on viable $CD4^+$ cells.

IL-7-cultured memory cells in similar experiments, we found that IL-7-cultured cells had a superior therapeutic potential than *ex vivo* effectors (Supporting Information Fig. 2, TBI- *ex vivo*/ACT compared to TBI-IL-7/ACT).

To understand why IL-7-cultured $CD4^+$ T cells were superior to IL-2-cultured $CD4^+$ T cells, we compared their *in vivo* behaviors. Naive, IL-7-, and IL-2-cultured T-dLN 16.2β cells were labeled with CFSE and transferred into TS/A-LACK tumour-bearing mice. Tumour distal and proximal LN and the tumour-infiltrating lymphocytes were recovered 48 (data not shown) −72 h after transfer and analyzed by flow cytometry. This time point was chosen to directly address homing, survival and Ag recognition shortly after infusion. The frequency of $CD4^+$, $CFSE^+$ cells within the lymphoid and non-lymphoid tissue was taken as indicative of homing abilities, while $CD4^+$, $CFSE^+$ expressing high levels of CD44 and CD69 was considered as indicative of Ag-driven activation. Mice transplanted with naive and IL-7-cultured cells showed a higher frequency of $CD4^+$, $CFSE^+$ cells in T-dLN when compared with mice transplanted with IL-2 cultured cells (Fig. 6A and B; 6A in brackets). Furthermore, T-dLN of mice transplanted with IL-7-cultured cells revealed higher frequency of recently activated $CD4^+$ T cells ($CD69^{high}$, also $CD44^{high}$) when compared with mice transplanted with IL-2-cultured cells (Fig. 6A and C). It is worth noting that $CD4^+$, $CFSE^+$ $CD44^{high}$, $CD69^{high}$ cells were not detectable in tumour-distal LN (Fig. 6A) or in T-dLN of TS/A-control tumour-bearing mice (not depicted). $CD4^+$, $CFSE^+$ lymphocytes derived from IL-7 cultures also appeared best represented within the tumour (Fig. 6D), but to variable extents among independent experiments. Thus, these data indicate that preserved LN homing, survival and Ag responsiveness in the T-dLN of IL-7 cultured cells best account for their superior therapeutic efficacy (Fig. 5). Together our data suggest that IL-7, rather than IL-2, should be adopted for short-term cultures of T-dLN cells in the generation of $CD4^+$ T lymphocytes optimal for ACT.

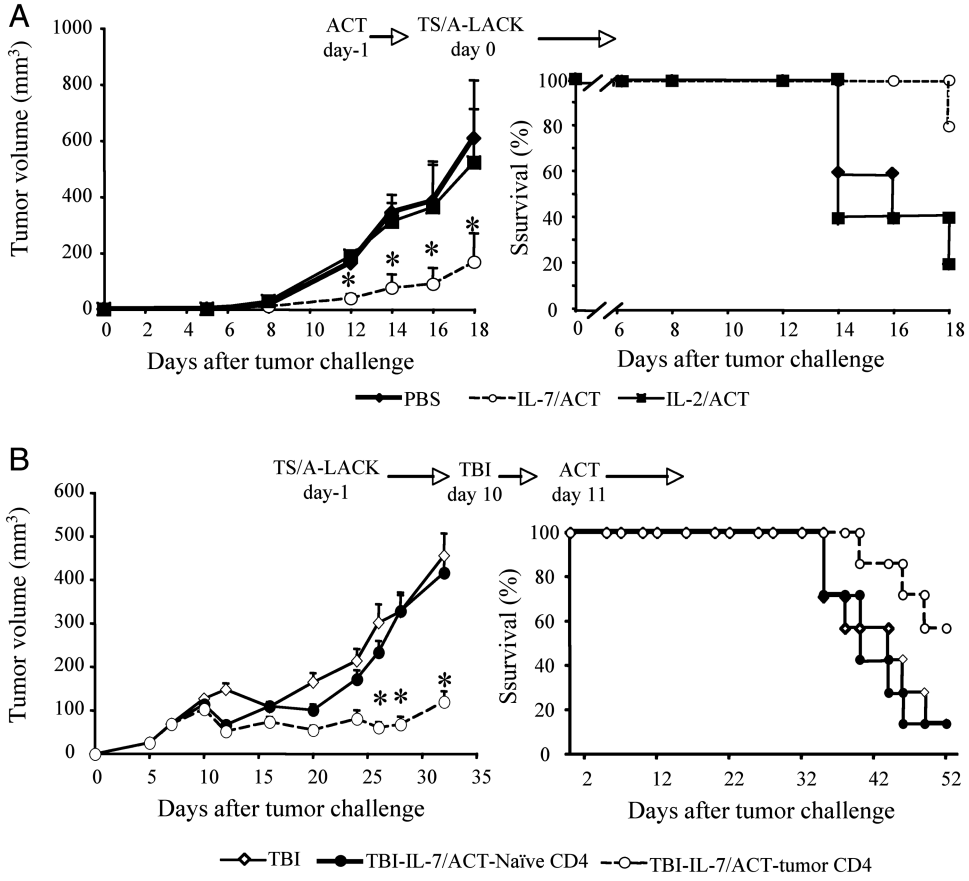

Figure 5. IL-7, but not IL-2-cultured CD4$^+$ T cells confer anti-tumour protection. (A) Cells were derived from the draining LN of BALB/c mice-bearing TS/A-LACK established tumours (day 21) and cultured for 7 days in IL-7 or IL-2. CD4$^+$ T cells were then purified by negative selection and transferred (5×10^5 *per* mouse) by i.v. injection in BALB/c mice (day −1). A day later, control (PBS) and ACT mice were challenged with 4×10^5 TS/A-LACK tumour cells (s.c., in the inguinal flank, day 0). Data are representative of two independent experiments. (B) LN cells were derived from TS/A-LACK tumour-bearing or tumour-free, naive BALB/c mice and cultured in IL-7. CD4$^+$ T cells were then purified by negative selection and transferred (2×10^6 *per* mouse), together with 20×10^6 syngeneic splenocytes derived from tumour-free mice, by i.v. injection in BALB/c mice-bearing 10-day-old TS/A-LACK tumours subjected to TBI 24 h before (IL-7/ACT-tumour CD4, open circles; IL-7/ACT-naïve CD4, closed circle). Data are representative of three independent experiments. In (A) and (B) (left) the average tumour volume (mm^3) ± SD is depicted (seven mice/group). Statistical significance among PBS and IL-7/ACT in A, and among mice undergoing TBI only or TBI and ACT with T-dLN cultured cells (TBI-IL-7/ACT-tumour CD4) in (B) is depicted (Two-tails Student's *t*-test, $^*p<0.05$). In (A) and (B) (right), survival in the different groups is depicted as Kaplan–Mayer plots.

Discussion

A general role of IL-7 in allowing the proliferation of memory T cells has been widely recognized in the past years [23, 48]. However for the first time, we report that recently Ag-sensitized CD4$^+$ T cells, such as the ones found in the T-dLN, outperform other memory cells in their capability to respond to IL-7 and as a result selectively accumulate in short-term cultures.

The specific enrichment of tumour Ag-sensitized T cells was best explained by their propensity to proliferate and survive *in vitro*. In our cultures, CD4$^+$ T cells derived from T-dLN, but not control LN underwent several cell division cycles in the absence of exogenous cytokine or Ag provision. This might suggest that recent tumour Ag encounter *in vivo* might instructs T cells for subsequent cell division, or that residual Ag carry-over or yet-to-be defined accessory signals provided within the culture support their *in vitro* expansion. The finding that spontaneous cell division was no longer detected in CD4$^+$ purified T-cell culture and that anti-MHC class II mAb efficiently prevented spontaneous cell division in T-dLN (data not shown) supports the second possibility. In response to IL-7, a higher fraction of the cells underwent *in vitro* cell division, and lymphocyte viability and survival potential (Bcl-2 levels) were increased when compared to Nil and IL-2-driven cultures. Thus, we propose that both cell division and lymphocyte survival account for the IL-7-driven selective accumulation of tumour Ag-sensitized T cells in unfractionated and highly purified CD4$^+$ T-dLN cultures, and that these cells might be intrinsically sensitive to IL-7. *Ex vivo* analysis of LACK-specific T cells in T-dLN indicated preserved expression of CD127 (Supporting Information Fig. 3), known to be down-regulated following TCR engagement, and quickly re-expressed following Ag withdrawal [49]. CD127 was down-regulated in IL-7-cultures, as expected [45]. It is worth noting that LACK-specific T cells were best retrieved by the use of 50–200 ng/mL of IL-7 (data not shown), a concentration well above that sustaining cell survival

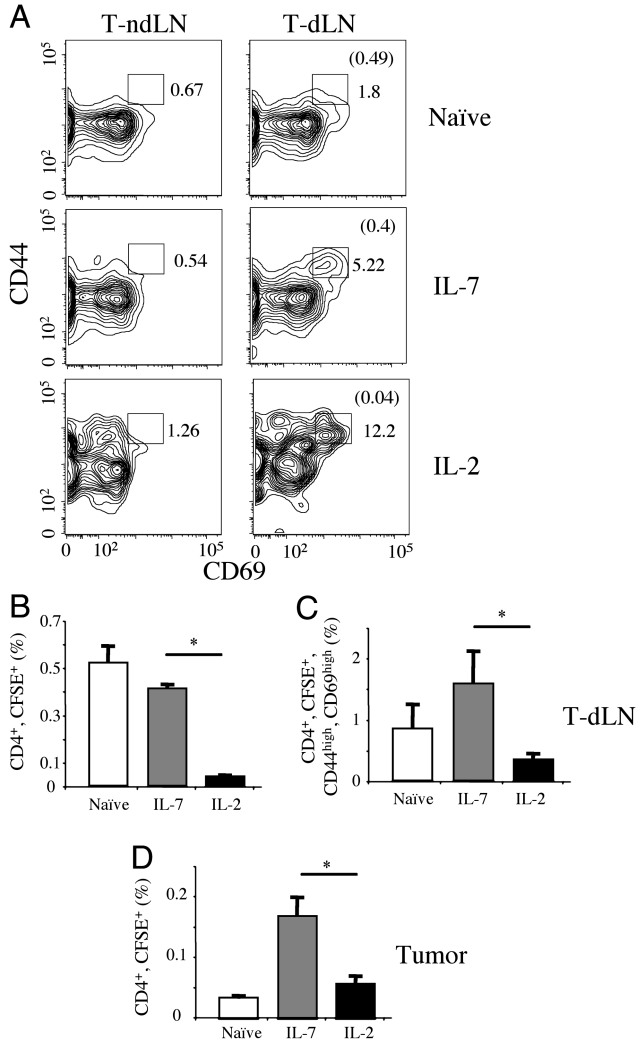

Figure 6. IL-7 better than IL-2 preserves *in vivo* LN homing and Ag responsiveness. Cells were derived from the draining-LN of 16.2β mice-bearing TS/A-LACK established tumours (day 10) and cultured for 7 days in IL-7 or IL-2. LN cells derived from tumour-free mice (Naïve) were used as controls. Cells were labeled with CFSE and 2×10^6 $CD4^+$ cells were transferred into BALB/c mice with established TS/A-LACK (d10) tumours. Mice were sacrificed 48 (data not shown) and 72 h later, and tumour non-draining LN, T-dLN and the tumour recovered and analyzed by flow cytometry for the presence of $CD4^+$, $CFSE^+$ cells to assess homing. (A) CD44 and CD69 expression based on gaiting viable $CD4^+$, $CFSE^+$ lymphocytes. Percentages refer to: $CD4^+$, $CFSE^+$ cells (in brackets) and $CD4^+$, $CFSE^+$, $CD69^{high}$, $CD44^{high}$ within viable lymphocytes. (B–D) Data show mean ± SD (three mice/group) of $CD4^+$, $CFSE^+$ (B, D), $CD4^+$, $CFSE^+$ $CD69^{high}$, $CD44^{high}$ (C) cells within T-dLN (B and C) and the tumour (D). Differences among mice infused with IL-7- and IL-2-cultured cells were analyzed by two-tails Student's *t*-test, $^*p<0.05$. Data depicted in (A)–(D) are representative of two independent experiments.

and homeostatic cell division. We speculate that recent Ag encounter might reduce IL-7 receptor expression, but concomitantly render the cells more susceptible to local secretion, possibly allowing the generation and survival of central memory-like T cells.

IL-7 favored the proliferation/survival of *in vivo* primed tumour-specific $CD4^+$ T cells in the absence of further T-cell differentiation even in conditions in which they were found in very low frequencies *ex vivo*, and by that their identification and enumeration. This might have direct implication for all those clinical conditions relying on T-cell detection for diagnosis and prognosis. Furthermore, the finding that IL-7 elicits the expansion of tumour-specific T cells, in the absence of exogenous Ag provision provides a suitable alternative for all those situations for which the Ag remains to be identified.

In the model system adopted in this study, IL-7-expanded $CD4^+$ T cells were capable of supporting the development of protective anti-tumour immunity, while IL-2 cultured cells were not. To date, the clinical efficacy of ACT, although proven in recent clinical trails [1], remains limited to a fraction of the patients. This might be due to limitations imposed by current strategies used to isolate tumour-specific lymphocytes, and insufficient $CD4^+$ T-cell help. Indeed, present ACT strategies mostly exploit Ag in combination with IL-2-driven T-cell stimulation, which favor production of effector $CD8^+$ T cells lacking long-term survival [36, 50]. In agreement, we found that IL-2, although capable of sustaining the proliferation/accumulation of *in vivo* Ag-sensitized $CD4^+$ T cells without the need of exogenous Ag provision, did not promote viability/survival and LN homing to similar extents as IL-7. Alongside with effector cytokine production (IL-2 and IFN-γ secretion), IL-7-cultured $CD4^+$ T cells maintained a less differentiated phenotype than IL-2 cultured $CD4^+$ T cells, allowing superior persistence and LN homing when infused in tumour-bearing hosts. We found that replenishing the cultures with IL-7 after 7 days further promoted the accumulation of tumour-specific T cells (data not shown). At present it remains to be determined whether longer cultures will also preserve the poorly differentiated state of the cells, which might be critical for the *in vivo* efficacy.

Most recently, IL-7 was also shown to promote the *ex vivo* expansion of $CD8^+$ T cells [51, 52]. This together with the notion that less differentiated T-cell subsets might be more potent than fully differentiated effector cells [53], suggest that IL-7 should be preferred to IL-2 when attempting the *ex vivo* expansion of $CD4^+$ and $CD8^+$ T cells to be used in anti-tumour ACT. TS/A-LACK cells do not express MHC class II molecules, and their parental cell line TS/A relies on $CD8^+$ T cells to be rejected [47]. We speculate that IL-7-expanded cells following ACT by migrating to T-dLN and to the tumour might provide help to $CD8^+$ T cells and other immune effectors and by that favor tumour rejection. Thus, together with the finding that IL-7 and IL-15 drives the *in vitro* generation of self-renewing central memory human T lymphocytes [54], our data support the use of IL-7 for the identification and the expansion of clinically relevant T-cell subsets.

Materials and methods

Mice and tumour cells

Eight-week-old BALB/c mice were purchased from Charles River (Charles River Italia, Milan, Italy). 16.2β Tg mice were bred in the Institute-specific pathogen-free facility. T lymphocytes

derived from 16.2β mice express a Tg TCR β-chain specific for an I-A^d-restricted peptide (LACKp, FSPSLEHPIVVSGSWD) derived from the *Leishmania Major*-derived Ag, LACK [10, 44].

TS/A and TS/A-LACK tumour cells were described previously [10, 47, 55]. Briefly, TS/A-LACK tumour cells express the LACK Ag as intracellular protein (*i.e.* as a model tumour-associated Ag) and do not express MHC class II. Exponentially growing TS/A-LACK tumour cells were subcutaneously injected (4×10^5 cells/mouse, 100 μL PBS) in syngeneic mice (BALB/c), resulting in established solid tumours by day 10 [10]. Mice were sacrificed 21 days after tumour-cell injection to obtain T-dLN. At least five mice *per* group were pooled for immunological studies, and seven *per* group in ACT experiments. All the *in vivo* studies were approved by the Ethical Committee of San Raffaele Scientific Institute (Milan, Italy) and performed according to its guidelines.

Mouse T-cell primary cultures

Tumour-free and/or tumour-bearing mice were sacrificed and the axillary, brachial and inguinal LN was surgically excised. Single-cell suspensions were obtained and cultured in 24-well plates at the density of 4–5 $\times 10^6$/mL in complete medium (RPMI-5% FBS, 100 U/mL penicillin, 100 U/mL streptomycin, and 2.5×10^{-5}M 2-ME, Invitrogen Life Technology, Milano, Italy) in the absence or in the presence of recombinant mouse IL-7 (50–100 ng/mL), IL-2 (20 ng/mL), IL-6 (45 ng/mL), or IL-15 (100 ng/mL) (Peprotech). When required, cells were labeled with the fluorescent dye CFSE at the final concentration of 1 μM, according to manufacturer instructions. $CD4^+$ T cells were purified by magnetic beads (Dynal, Invitrogen)-assisted negative depletion of MHC class II^+, $CD8^+$ cells. $CD4^+$ T-cell purity was evaluated by flow cytometry, and proved to be higher than 97%.

Flow cytometry analysis

I-A^d/LACK fluorescent multimer staining was performed and with PE- or PerCP-labeled anti-CD4, anti-CD25, anti-CD44, and anti-CD62L mAb and with allophycocyanin-labeled anti-CD8a, anti-CD11b, and anti-B220 mAb (BD, Pharmingen) as described previously [10]. TO-PRO-3 (1 nM final concentration; Molecular Probes, Invitrogen) was added to the sample just before flow cytometric analyses to discriminate viable and dead cells. $CD8a^+$, $CD11b^+$, $B220^+$, and TO-PRO-3^+ cells were excluded by electronic gating during the acquisition. Typically, 1–3 $\times 10^5$ $CD4^+$ or 10^3 $CD4^+$ I-A^d/$LACK^+$ events were acquired using an FACS Calibur flow cytometer (BD). Intracellular Bcl-2 staining was performed as described previously [56].

LACK-specific stimulation in cytokine release assays

LACK-specific artificial APC (LACK aAPC) were prepared as described previously [57] by coating 5-μm polystyrene sulfate latex beads (Invitrogen) with I-A^d/LACK dimers (20 μg/mL) and anti-CD28 mAb (37.51; 2 μg/mL). Control aAPC were prepared by coating beads with anti-CD28 mAb only (−/28 aAPC). Cytokine production in response to LACK aAPC was comparable to that induced by LACK peptide-pulsed syngeneic splenocytes (data not shown). Thus, stimulation provided by LACK aAPC was referred to LACK-specific re-stimulation, while activation by CD28 aAPC was referred to as control. Typically, cells and aAPC (1:1 ratio) were cultured for 5 h at 37°C. Intracellular IL-2 and IFN-γ content (mAb were from BD) were determined as described previously [57]. In total 50 to 100 $\times 10^3$ $CD4^+$ events were generally collected in the lymphocyte gate on a FACS Calibur. The total number of Ag-specific IL-2^+/IFN-γ^+ T cells was determined by multiplying the percentage as detected in flow-cytometry analyses by the total number of Trypan Blue-negative LN cells. Cytokine release induced by control aAPC remained within background levels (Fig. 1B, second row) and was subtracted from LACK-induced release in all bar graphs.

Statistical analyses

Statistical analyses were performed using unpaired two-tailed Student's *t*-test. Statistical significance: $p<0.05$.

Acknowledgements: The authors are grateful to PIBIC members (San Raffaele Scientific Institute, Milan) and Professor Zamoyska, Dr. Kassiotis, and Dr. Seddon (National Institute for Medical Research, London) for critical suggestions. This work was supported by grants from the European Community (contract LSHC-CT-2005-018914 "ATTACK"), Ministero della Salute, Progetto Integrato (PIO) 2006, Associazione Italiana Ricerca sul Cancro (AIRC), and Ministero dell'Istruzione, dell'Università e della Ricerca, Fondo per gli Investimenti della Ricerca di Base (RBNE017B4C_006). S.C. was supported by the International Ph.D. Program in Basic and Applied Immunology (Vita-Salute San Raffaele University, Milan, Italy).

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Rosenberg, S. A., Restifo, N. P., Yang, J. C., Morgan, R. A. and Dudley, M. E.,** Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nat. Rev. Cancer* 2008. **8**: 299–308.

2 **Gattinoni, L., Klebanoff, C. A., Palmer, D. C., Wrzesinski, C., Kerstann, K., Yu, Z., Finkelstein, S. E. *et al.*,** Acquisition of full effector function *in vitro* paradoxically impairs the *in vivo* antitumor efficacy of adoptively transferred CD8(+) T cells. *J. Clin. Invest.* 2005. **115**: 1616–1626.

3 **Klebanoff, C. A., Gattinoni, L., Torabi-Parizi, P., Kerstann, K., Cardones, A. R., Finkelstein, S. E., Palmer, D. C. *et al.*,** Central memory self/tumor-

reactive CD8+T cells confer superior antitumor immunity compared with effector memory T cells. *Proc. Natl. Acad. Sci. USA* 2005. **102**: 9571–9576.

4 **Topalian, S. L.,** MHC class II restricted tumor antigens and the role of CD4+ T cells in cancer immunotherapy. *Curr. Opin. Immunol.* 1994. **6**: 741–745.

5 **Hung, K., Hayashi, R., Lafond-Walker, A., Lowenstein, C., Pardoll, D. and Levitsky, H.,** The central role of CD4(+) T cells in the antitumor immune response. *J. Exp. Med.* 1998. **188**: 2357–2368.

6 **Pardoll, D. M. and Topalian, S. L.,** The role of CD4+T cell responses in antitumor immunity. *Curr. Opin. Immunol.* 1998. **10**: 588–594.

7 **Marzo, A. L., Kinnear, B. F., Lake, R. A., Frelinger, J. J., Collins, E. J., Robinson, B. W. and Scott, B.,** Tumor-specific CD4(+) T cells have a major "post-licensing" role in CTL mediated anti-tumor immunity. *J. Immunol.* 2000. **165**: 6047–6055.

8 **Klein, L., Trautman, L., Psarras, S., Schnell, S., Siermann, A., Liblau, R., von Boehmer, H. and Khazaie, K.,** Visualizing the course of antigen-specific CD8 and CD4 T cell responses to a growing tumor. *Eur. J. Immunol.* 2003. **33**: 806–814.

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

10 **Benigni, F., Zimmermann, V. S., Hugues, S., Caserta, S., Basso, V., Rivino, L., Ingulli, E., Malherbe, L. *et al.*,** Phenotype and homing of CD4 tumor-specific T cells is modulated by tumor bulk. *J. Immunol.* 2005. **175**: 739–748.

11 **Staveley-O'Carroll, K., Sotomayor, E., Montgomery, J., Borrello, I., Hwang, L., Fein, S., Pardoll, D. and Levitsky, H.,** Induction of antigen-specific T cell anergy: an early event in the course of tumor progression. *Proc. Natl. Acad. Sci. USA* 1998. **95**: 1178–1183.

12 **Shrikant, P., Khoruts, A. and Mescher, M. F.,** CTLA-4 blockade reverses CD8+T cell tolerance to tumor by a CD4+T cell- and IL-2-dependent mechanism. *Immunity* 1999. **11**: 483–493.

13 **Hernandez, J., Aung, S., Redmond, W. L. and Sherman, L. A.,** Phenotypic and functional analysis of CD8(+) T cells undergoing peripheral deletion in response to cross-presentation of self-antigen. *J. Exp. Med.* 2001. **194**: 707–717.

14 **Wick, M., Dubey, P., Koeppen, H., Siegel, C. T., Fields, P. E., Chen, L., Bluestone, J. A. and Schreiber, H.,** Antigenic cancer cells grow progressively in immune hosts without evidence for T cell exhaustion or systemic anergy. *J. Exp. Med.* 1997. **186**: 229–238.

15 **Zimmermann, V. S., Casati, A., Schiering, C., Caserta, S., Michelini, R. H., Basso, V. and Mondino, A.,** Tumors hamper the immunogenic competence of CD4+T cell-directed dendritic cell vaccination. *J. Immunol.* 2007. **179**: 2899–2909.

16 **Fuller, M. J. and Zajac, A. J.,** Ablation of CD8 and CD4 T cell responses by high viral loads. *J. Immunol.* 2003. **170**: 477–486.

17 **Harari, A., Petitpierre, S., Vallelian, F. and Pantaleo, G.,** Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: changes after antiretroviral therapy. *Blood* 2004. **103**: 966–972.

18 **Pantaleo, G. and Harari, A.,** Functional signatures in antiviral T-cell immunity for monitoring virus-associated diseases. *Nat. Rev. Immunol.* 2006. **6**: 417–423.

19 **Jelley-Gibbs, D. M., Dibble, J. P., Filipson, S., Haynes, L., Kemp, R. A. and Swain, S. L.,** Repeated stimulation of CD4 effector T cells can limit their protective function. *J. Exp. Med.* 2005. **201**: 1101–1112.

20 **Dooms, H. and Abbas, A. K.,** Control of CD4+T-cell memory by cytokines and costimulators. *Immunol. Rev.* 2006. **211**: 23–38.

21 **Stockinger, B., Bourgeois, C. and Kassiotis, G.,** CD4+memory T cells: functional differentiation and homeostasis. *Immunol. Rev.* 2006. **211**: 39–48.

22 **Swain, S. L., Agrewala, J. N., Brown, D. M., Jelley-Gibbs, D. M., Golech, S., Huston, G., Jones, S. C.,** CD4+T-cell memory: generation and multi-faceted roles for CD4+T cells in protective immunity to influenza. *Immunol. Rev.* 2006. **211**: 8–22.

23 **Caserta, S. and Zamoyska, R.,** Memories are made of this: synergy of T cell receptor and cytokine signals in CD4(+) central memory cell survival. *Trends Immunol.* 2007. **28**: 245–248.

24 **Unutmaz, D., Pileri, P. and Abrignani, S.,** Antigen-independent activation of naive and memory resting T cells by a cytokine combination. *J. Exp. Med.* 1994. **180**: 1159–1164.

25 **Kanegane, H. and Tosato, G.,** Activation of naive and memory T cells by interleukin-15. *Blood* 1996. **88**: 230–235.

26 **Geginat, J., Sallusto, F. and Lanzavecchia, A.,** Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. *J. Exp. Med.* 2001. **194**: 1711–1719.

27 **Tan, J. T., Ernst, B., Kieper, W. C., LeRoy, E., Sprent, J. and Surh, C. D.,** Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+cells but are not required for memory phenotype CD4+cells. *J. Exp. Med.* 2002. **195**: 1523–1532.

28 **Jaleco, S., Swainson, L., Dardalhon, V., Burjanadze, M., Kinet, S. and Taylor, N.,** Homeostasis of naive and memory CD4+T cells: IL-2 and IL-7 differentially regulate the balance between proliferation and Fas-mediated apoptosis. *J. Immunol.* 2003. **171**: 61–68.

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

30 **Seddon, B. and Zamoyska, R.,** TCR and IL-7 receptor signals can operate independently or synergize to promote lymphopenia-induced expansion of naive T cells. *J. Immunol.* 2002. **169**: 3752–3759.

31 **Seddon, B., Tomlinson, P. and Zamoyska, R.,** Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells. *Nat. Immunol.* 2003. **4**: 680–686.

32 **Cohen, P. A., Kim, H., Fowler, D. H., Gress, R. E., Jakobsen, M. K., Alexander, R. B., Mule, J. J. *et al.*,** Use of interleukin-7, interleukin-2, and interferon-gamma to propagate CD4+T cells in culture with maintained antigen specificity. *J. Immunother.* 1993. **14**: 242–252.

33 **Jennes, W., Kestens, L., Nixon, D. F. and Shacklett, B. L.,** Enhanced ELISPOT detection of antigen-specific T cell responses from cryopreserved specimens with addition of both IL-7 and IL-15 – the Amplispot assay. *J. Immunol. Methods* 2002. **270**: 99–108.

34 **Jackson, H. M., Dimopoulos, N., Chen, Q., Luke, T., Yee Tai, T., Maraskovsky, E., Old, L. J. *et al.*,** A robust human T-cell culture method suitable for monitoring CD8+and CD4+T-cell responses from cancer clinical trial samples. *J. Immunol. Methods* 2004. **291**: 51–62.

35 **Chen, H. W., Liao, C. H., Ying, C., Chang, C. J. and Lin, C. M.,** *Ex vivo* expansion of dendritic-cell-activated antigen-specific CD4+T cells with anti-CD3/CD28, interleukin-7, and interleukin-15: potential for adoptive T cell immunotherapy. *Clin. Immunol.* 2006. **119**: 21–31.

36 **Liu, S., Riley, J., Rosenberg, S. and Parkhurst, M.,** Comparison of common gamma-chain cytokines, interleukin-2, interleukin-7, and interleukin-15 for the *in vitro* generation of human tumor-reactive T lymphocytes for adoptive cell transfer therapy. *J. Immunother.* 2006. **29**: 284–293.

37 **Li, J., Huston, G. and Swain, S. L.,** IL-7 promotes the transition of CD4 effectors to persistent memory cells. *J. Exp. Med.* 2003. **198**: 1807–1815.

38 **Kondrack, R. M., Harbertson, J., Tan, J. T., McBreen, M. E., Surh, C. D. and Bradley, L. M.,** Interleukin 7 regulates the survival and generation of memory CD4 cells. *J. Exp. Med.* 2003. **198**: 1797–1806.

39 **Kaech, S. M., Tan, J. T., Wherry, E. J., Konieczny, B. T., Surh, C. D. and Ahmed, R.,** Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat. Immunol.* 2003. **4**: 1191–1198.

40 **Geginat, J., Campagnaro, S., Sallusto, F. and Lanzavecchia, A.,** TCR-independent proliferation and differentiation of human CD4+T cell subsets induced by cytokines. *Adv. Exp. Med. Biol.* 2002. **512**: 107–112.

41 **Nanjappa, S. G., Walent, J. H., Morre, M. and Suresh, M.,** Effects of IL-7 on memory CD8 T cell homeostasis are influenced by the timing of therapy in mice. *J. Clin. Invest.* 2008. **118**: 1027–1039.

42 **Pellegrini, M., Calzascia, T., Elford, A. R., Shahinian, A., Lin, A. E., Dissanayake, D., Dhanji, S. *et al.*,** Adjuvant IL-7 antagonizes multiple cellular and molecular inhibitory networks to enhance immunotherapies. *Nat. Med.* 2009. **15**: 528–536.

43 **Colombetti, S., Levy, F. and Chapatte, L.,** IL-7 adjuvant treatment enhances long-term tumor antigen-specific CD8+T-cell responses after immunization with recombinant lentivector. *Blood* 2009. **113**: 6629–6637.

44 **Malherbe, L., Filippi, C., Julia, V., Foucras, G., Moro, M., Appel, H., Wucherpfennig, K. *et al.*,** Selective activation and expansion of high-affinity CD4+T cells in resistant mice upon infection with Leishmania major. *Immunity* 2000. **13**: 771–782.

45 **Swainson, L., Verhoeyen, E., Cosset, F. L. and Taylor, N.,** IL-7R alpha gene expression is inversely correlated with cell cycle progression in IL-7-stimulated T lymphocytes. *J. Immunol.* 2006. **176**: 6702–6708.

46 **Gattinoni, L., Finkelstein, S. E., Klebanoff, C. A., Antony, P. A., Palmer, D. C., Spiess, P. J., Hwang, L. N. *et al.*,** Removal of homeostatic cytokine sinks by lymphodepletion enhances the efficacy of adoptively transferred tumor-specific CD8+T cells. *J. Exp. Med.* 2005. **202**: 907–912.

47 **Rosato, A., Santa, S. D., Zoso, A., Giacomelli, S., Milan, G., Macino, B., Tosello, V. *et al.*,** The cytotoxic T-lymphocyte response against a poorly immunogenic mammary adenocarcinoma is focused on a single immunodominant class I epitope derived from the gp70 Env product of an endogenous retrovirus. *Cancer Res.* 2003. **63**: 2158–2163.

48 **Bradley, L. M., Haynes, L. and Swain, S. L.,** IL-7: maintaining T-cell memory and achieving homeostasis. *Trends Immunol.* 2005. **26**: 172–176.

49 **Hammerbeck, C. D. and Mescher, M. F.,** Antigen controls IL-7R alpha expression levels on CD8 T cells during full activation or tolerance induction. *J. Immunol.* 2008. **180**: 2107–2116.

50 **Gett, A. V., Sallusto, F., Lanzavecchia, A. and Geginat, J.,** T cell fitness determined by signal strength. *Nat. Immunol.* 2003. **4**: 355–360.

51 **Le, H. K., Graham, L., Miller, C. H., Kmieciak, M., Manjili, M. H. and Bear, H. D.,** Incubation of antigen-sensitized T lymphocytes activated with bryostatin 1+ionomycin in IL-7+IL-15 increases yield of cells capable of inducing regression of melanoma metastases compared to culture in IL-2. *Cancer Immunol. Immunother.* 2009. **58**: 1565–1576.

52 **Kittipatarin, C. and Khaled, A. R.,** *Ex vivo* expansion of memory CD8 T cells from lymph nodes or spleen through *in vitro* culture with interleukin-7. *J. Immunol. Methods* 2009. **344**: 45–57.

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

54 **Kaneko, S., Mastaglio, S., Bondanza, A., Ponzoni, M., Sanvito, F., Aldrighetti, L., Radrizzani, M. *et al.*,** IL-7 and IL-15 allow the generation of suicide gene-modified alloreactive self-renewing central memory human T lymphocytes. *Blood* 2009. **113**: 1000–1015.

55 **Lollini, P. L., de Giovanni, C., Eusebi, V., Nicoletti, G., Prodi, G. and Nanni, P.,** High-metastatic clones selected *in vitro* from a recent spontaneous BALB/c mammary adenocarcinoma cell line. *Clin. Exp. Metastasis* 1984. **2**: 251–259.

56 **Korsmeyer, S. J., Shutter, J. R., Veis, D. J., Merry, D. E. and Oltvai, Z. N.,** Bcl-2/Bax: a rheostat that regulates an anti-oxidant pathway and cell death. *Semin. Cancer. Biol.* 1993. **4**: 327–332.

57 **Caserta, S., Alessi, P., Guarnerio, J., Basso, V. and Mondino, A.,** Synthetic CD4+T cell-targeted antigen-presenting cells elicit protective antitumor responses. *Cancer Res.* 2008. **68**: 3010–3018.

Abbreviations: **aAPC:** artificial APC · **ACT:** adoptive cell therapy · **LACK:** Leishmania receptor for activated C kinase · **TBI:** total body irradiation · **T-dLN:** tumour-draining LN

Full correspondence: Dr. Anna Mondino, Division of Immunology, Transplantation and Infectious Diseases, San Raffaele Scientific Institute, Milan, Italy Via Olgettina, 58, 20132 Milan, Italy
Fax: +22-643-4844
e-mail: anna.mondino@hsr.it

Current Addresses: Dr. Stefano Caserta, The University of Edinburgh, Edinburgh, EH9 3JT, UK
Dr. Patrizia Alessi, Department of Biomedical Sciences and Biotechnology, University of Brescia Medical School, 25123 Brescia, Italy

Received: 17/7/2009
Revised: 24/9/2009
Accepted: 12/11/2009

