Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

Interleukin-4-induced loss of CD8 expression and cytolytic function in effector CD8 T cells persists long term *in vivo*

Stuart Olver, Simon H. Apte, Adriana Baz,* Anne Kelso† and Norbert Kienzle‡

The Cooperative Research Centre for Vaccine Technology and the Queensland Institute of Medical Research, Herston, Qld, Australia

doi:10.1111/imm.12068
Received 03 November 2012; revised 07 January 2013; accepted 08 January 2013.
*Present address: CSL Limited, 45 Poplar Rd, Parkville, Vic. 3052, Australia
†Present address: WHO Collaborating Centre for Reference and Research on Influenza, 10 Wreckyn St, North Melbourne, Vic. 3051, Australia
‡Present address: Ingham Institute for Applied Medical Research, Liverpool, NSW 2170, Australia
Correspondence: Dr Anne Kelso, WHO Collaborating Centre for Reference and Research on Influenza, 10 Wreckyn St, North Melbourne, Vic. 3051, Australia.
Email: anne.kelso@influenzacentre.org
Senior author: Dr Norbert Kienzle, email: Norbert.Kienzle@sswahs.nsw.gov.au

Summary

Activation of naive CD8$^+$ T cells in the presence of interleukin-4 modulates their CD8 co-receptor expression and functional differentiation, resulting in the generation of CD8^{low} cells that produce type 2 cytokines and display poor cytolytic and anti-tumour activity. Although this CD8^{low} phenotype becomes stable after about a week and can persist with further stimulation *in vitro*, it is not known whether it can be maintained long term *in vivo*. Here we report that CD8^{low} cells derived from ovalbumin$_{257–264}$-specific T-cell receptor-transgenic CD8$^+$ T cells activated in the presence of interleukin-4 could be detected in the spleen for at least 4 months after adoptive transfer into normal mice. A significant proportion of the long-term surviving cells retained their CD8^{low} phenotype *in vivo* and after clonal re-activation *in vitro*. Although long-term surviving CD8^{low} cells lacked detectable cytolytic activity or perforin expression, they showed some anti-tumour function *in vivo*. The persistence of functional cells with a CD8^{low} phenotype *in vivo* raises the possibility that such cells can contribute to effector or regulatory responses to tumours or pathogens.

Keywords: CD8; cytolytic T lymphocyte; interleukin-4; tumour immunity.

Introduction

Exposure of CD8$^+$ T cells to interleukin-4 (IL-4) during primary polyclonal or antigen-specific activation *in vitro* leads to the generation of effector populations that express the type 2 cytokines IL-4, IL-5 and IL-10 and show markedly reduced expression of CD8α mRNA and CD8$\alpha\beta$ surface protein.[1–4] Compared with conventional CD8^{high} cytolytic T lymphocytes (CTL) activated in the absence of IL-4, these CD8^{low} cells are poorly cytolytic and express reduced levels of interferon-γ (IFN-γ), perforin and granzymes.[1,2,5] CD8^{low} cells with reduced cytolytic potential have also been identified *in vivo* among ovalbumin (OVA)$_{257–264}$-specific T-cell receptor (TCR) transgenic CD8$^+$ T cells from OT-I mice adoptively transferred into RAG-2$^{-/-}$ γc$^{-/-}$ mice and activated with tumour cells co-expressing OVA and IL-4;[4,6] the ability of these cells to control a secondary tumour challenge *in vivo* was also impaired.[6]

We have previously shown that the IL-4-dependent development of CD8^{low} cells occurs by a process of progressive differentiation and commitment: generation of these cells required exposure to IL-4 for the first few days of primary activation but they retained their low CD8 expression and cytolytic activity for many weeks *in vitro*, even when exogenous IL-4 was removed and endogenous IL-4 was neutralized.[4,5] These results suggest that the IL-4-induced down-regulation of CD8α expression is heritable[5] and mechanistically distinct from the transient CD8 down-regulation observed following TCR engagement.[7,8]

It is not known, however, whether IL-4-induced CD8^{low} cells can also persist *in vivo* and, if so, whether they retain or re-acquire any functional capacity, such as cytolytic or anti-tumour activity. Here we have addressed these questions by examining the phenotypic and functional properties of activated CD8^{low} and CD8^{high} cells at periods up to 4 months after adoptive transfer into normal mice.

Materials and methods

Mice

Specific pathogen-free B6.SJL/J-Ptprc^a (CD45.1) and C57BL/6 and C57BL/6-RAG-1$^{-/-}$ mice (Animal Resources Centre, Murdoch, WA, Australia) were used at 6–9 weeks of age. TCR transgenic OT-I (243.2) mice (Dr William Heath, Department of Microbiology and Immunology, The University of Melbourne, Parkville, Vic., Australia) were bred at the Queensland Institute of Medical Research (QIMR). All animal studies were approved by the QIMR Animal Ethics Committee.

Antibodies for fluorescence-activated cell sorting and analysis

Antibodies to CD8α (53-6.7), CD4 (GK1.5), CD62L (MEL-14) and CD45.2 (104) and isotype controls were purchased from BioLegend (San Diego, CA). Antibodies to CD44 (IM7) and an isotype control were obtained from BD Biosciences (San Jose, CA); antibodies to Vα2 (B20.1) and an isotype control were purchased from eBioscience (San Diego, CA). Exclusion of dead cells was based on forward scatter and uptake of propidium iodide (Merck, Darmstadt, Germany).

Naive CD8$^+$ T-cell preparation and activation in vitro

Pooled single cell suspensions from spleen and brachial, axillary and inguinal lymph nodes of OT-1 mice were enriched for leucocytes on a Ficoll–Paque gradient (GE Healthcare, Uppsala, Sweden). In most experiments, CD4$^-$ CD8$^+$ Vα2$^+$ CD44^{low} cells were isolated to > 95% purity using a MoFlo flow cytometer (Beckman Coulter, Galway, Ireland) running SUMMIT Software V4.3 (Dako, Glostrup, Denmark) with or without prior depletion of B cells and MHC Class II$^+$ cells using magnetic beads (QIAGEN, Hilden, Germany). In two experiments, CD8$^+$ cells were positively selected on MACS Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to obtain CD8$^+$ Vα2$^+$ cells of > 90% purity. Naive OT-1 CD8$^+$ T cells were activated by culture at 2×10^4 to 5×10^4/well in six-well plates coated with purified antibodies to CD3ε (145-2C11; 10 μg/ml), CD8α (53.6; 10 μg/ml) and CD11a (I21/7.7; 5 μg/ml) (anti-receptor antibodies) in 8 ml modified Dulbecco's modified Eagle's medium containing 50 μM 2-mercaptoethanol, 216 mg/l L-glutamine, 10% heat-inactivated fetal calf serum, 120 IU/ml human recombinant (r)IL-2 (National Institutes of Health AIDS Research & Reference Reagent Program, Germantown, MD) and the type 2 polarizing stimuli mouse rIL-4 [25 ng/ml IL-4 (ProSpec-Tany TechnoGene, Rehovot, Israel) or 3·3 U/ml baculovirus-derived IL-4][2] and 1 μg/ml anti-IFN-γ antibody (XMG1.2). After 7–8 days, cultured cells were sorted on a MoFlo to obtain Vα2$^+$ CD8^{low} and Vα2$^+$ CD8^{high} cells with purities of > 99% and > 94%, respectively.

Transfer of CD8^{low} and CD8^{high} cells into host mice

In some experiments, activated OT-1 cells were incubated with 2 μM carboxyfluorescein succinimidyl ester (CFSE) for 7 min, washed and sorted for Vα2$^+$ CFSE$^+$ CD8^{low} and Vα2$^+$ CFSE$^+$ CD8^{high} cells; $0{\cdot}5 \times 10^6$ to 1×10^6 cells were then injected intravenously via the tail vein into RAG-1$^{-/-}$ mice. Splenic leucocytes were analysed on days 1, 2 or 4 using a FACSCalibur with CELLQUEST version 3.1f software (Becton Dickinson, San Jose, CA). In other experiments, $0{\cdot}5 \times 10^6$ to 2×10^6 CD45.2$^+$ OT-1 Vα2$^+$ CD8^{low} or Vα2$^+$ CD8^{high} cells were injected intravenously via the tail vein into B6.SJL/J-Ptprc^a mice (CD45.1); where indicated, control Ptprc^a mice received 0·2 ml saline intravenously. CD45.2$^+$ Vα2$^+$ cells were analysed in the spleen 53–126 days later. In one experiment, adoptively transferred host mice also received 1×10^7 cells of the OVA-expressing EL4 thymoma E.G7-OVA subcutaneously at the base of the tail 7 days before killing. In these instances, the draining inguinal and para-aortic lymph nodes and the spleen were collected.

Clonal activation of long-term donor cells

Pooled spleens from mice that had previously received CD8^{low} or CD8^{high} cells were depleted of B cells, MHC Class II$^+$ cells and CD4$^+$ cells using magnetic beads. Donor CD45.2$^+$ Vα2$^+$ CD44$^+$ cells were then purified twice by flow cytometry to obtain CD8^{low} or CD8^{high} cells and single cells were deposited in wells containing anti-receptor antibodies and IL-2 with or without rIL-4 and anti-IFN-γ antibody (type 2 and neutral conditions, respectively) as described above, with the exception that anti-CD3ε was used at 0·5 μg/ml. Clones were analysed after 9 or 10 days.

Cytolytic activity of long-term donor cells

After magnetic bead depletion as described above, donor CD45.2$^+$ Vα2$^+$ CD44$^+$ cells were purified by flow cytometry to obtain CD8^{low} and CD8^{high} cells for use as effectors in a fluorolysis assay.[9] Briefly, effector cells were incubated for 48 hr with uncoated or SIINFEKL-coated EL4 target cells stably transfected with a plasmid expressing the enhanced green fluorescent protein (EGFP) gene. Target cell lysis was measured by flow cytometry to count the number of propidium iodide-negative EGFP$^+$ cells, standardized to a reference number of fluorochrome-linked beads. Per cent lysis was calculated as follows: [1 – (targets in wells with effectors/targets in wells without effectors)] × 100.

mRNA preparation and real-time PCR analysis

RNA was extracted from $1{\cdot}5 \times 10^3$ purified $CD8^{low}$ or $CD8^{high}$ cells by Nonidet P-40 (Sigma-Aldrich, St Louis, MO) hypotonic lysis and cDNA was prepared as described elsewhere.[10] Duplicate or triplicate cDNA samples were prepared from each cell type and each cDNA sample was quantified in duplicate by real-time PCR using primers and probes as previously described by reference to titrations of cDNA standards of known copy number.[4,6,11] Samples were amplified in a Corbett Rotor-Gene 3000 (QIAGEN, Doncaster, Vic., Australia) with initial heating to 95° for 2 min, followed by 45 cycles of 95° for 5 seconds, and 60° for 30 seconds.

Anti-tumour activity

In some experiments, 1×10^7 E.G7-OVA cells or E.G7-OVA-luc^+ cells (a subclone stably expressing the luciferase gene) were injected subcutaneously at the base of the tail into $Ptprc^a$ mice that had previously been adoptively transferred with $CD8^{low}$ cells, $CD8^{high}$ cells or saline; tumours were excised and weighed 7 days later. In other experiments, a classical Winn assay was performed by injecting C57BL/6 mice ($n = 5$) with 4×10^6 E.G7-OVA tumour cells subcutaneously with saline or 6×10^5 purified primary $CD8^{low}$ or $CD8^{high}$ cells.[12] These CD8 cells were derived from primary OT-I $CD8^+$ cells activated in type 2 conditions for 7 days and then FACS-sorted for high and low CD8 expression. Tumour growth was monitored over 32 days and mice were culled when tumour size exceeded 1 cm^3 in accordance with QIMR animal ethics guidelines.

Statistical analyses

Data were evaluated by unpaired two-tailed *t*-test and Mann–Whitney *U*-test or Log-rank (Mantel–Cox) test (PRISM 4.02 software package, GraphPad Software, San Diego, CA). *P* values are expressed as *0·01–0·05, **0·001–0·01, ***< 0·001.

Results

$CD8^{low}$ cells proliferate and maintain low levels of CD8 expression *in vivo*

Naive TCR-transgenic OT-I $V\alpha2^+$ $CD8^+$ T cells specific for the $OVA_{257–264}$ epitope SIINFEKL were activated *in vitro* with antibodies to CD3ε, CD8 and CD11a (anti-receptor antibodies) and IL-2 in type 2 polarizing conditions. After 1 week, the cells displayed variable levels of surface CD8 that ranged from normal to undetectable, as previously observed in CD8 T cells from wild-type or OT-I mice activated in the presence of IL-4.[2,4,5] To determine whether their altered CD8 expression was stable *in vivo* under conditions in which the cells could proliferate, they were incubated with CFSE and the $V\alpha2^+$ $CFSE^{high}$ cells were separated into $CD8^{low}$ and $CD8^{high}$ cells (Fig. 1a) and adoptively transferred into RAG-$1^{-/-}$ mice. Donor cells were identified in the host spleen 1 or 4 days later by gating on $V\alpha2^+$ cells (Fig. 1b). Most of the $CD8^{low}$ cells retained their low CD8 expression over 4 days *in vivo* despite having undergone multiple rounds of cell division, as indicated by the loss of CFSE. The frequency of donor $CD8^{low}$ cells in spleen expanded about 240-fold, from 0·08% at day 1 to 19·5% at day 4. Most of the surviving donor $CD8^{high}$ cells had also proliferated by day 4 and maintained relatively high CD8 levels; the emergence of some cells expressing very low levels of CD8 in this population may be a result of their prior exposure to IL-4 *in vitro* as previously observed.[5] Similar results were obtained in two other experiments; a fourth independent experiment found low CD8 expression 8 days after adoptive transfer of $CD8^{low}$ cells (data not shown). Parallel experiments in which day 0 CFSE-labelled $CD8^{low}$ and $CD8^{high}$ cells were re-cultured with IL-2 but without anti-receptor antibodies showed that both populations proliferated and maintained their respective CD8 expression profiles over 4 days (data not shown). Collectively these data indicate that OT-I $CD8^{low}$ cells maintained their phenotype during proliferation *in vivo* and *in vitro* for at least 4 days in the absence of high-affinity TCR stimulation.

$CD8^{low}$ cells survive long term *in vivo*

To assess the stability of the $CD8^{low}$ phenotype *in vivo* over longer periods, *in vitro* generated OT-1 $CD8^{low}$ and $CD8^{high}$ cells (Fig. 2a) were adoptively transferred into $CD45.1^+$ congenic host mice in which the donor T cells could be discriminated from endogenous $V\alpha2^+$ cells by their expression of CD45.2. As shown in Fig. 2(b), the median fluorescence intensity (MFI) of CD8 expression by $CD45.2^+$ $V\alpha2^+$ $CD4^-$ $CD44^+$ donor cells in individual spleens of mice that had received $CD8^{low}$ cells 53 days earlier was lower than in mice that had received $CD8^{high}$ cells and a significant subpopulation of the donor $CD8^{low}$ cells was CD8-negative. This experiment (experiment 4a) and others (experiments 1–3) summarized in Table 1 showed that surviving $CD8^{low}$ and $CD8^{high}$ T cells maintained distinct CD8 expression profiles for at least 53–90 days after transfer, although in all experiments there was some convergence of their CD8 expression levels compared with the original transferred populations. Another experiment showed that $CD8^{low}$ cells could be recovered after 126 days (see below, Fig. 3). The majority of recovered splenic $CD8^{low}$ cells and $CD8^{high}$ cells expressed high levels of the activation/memory marker CD44 (means of 94–99% and 92–99% respectively in

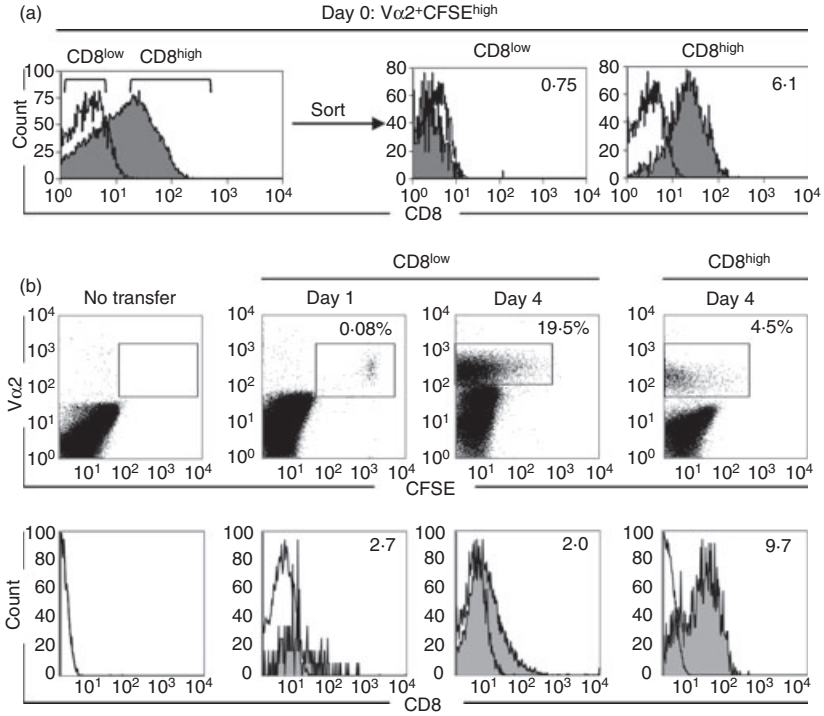

Figure 1. $CD8^{low}$ cells proliferate and maintain low CD8 expression *in vivo*. Naive $CD44^{low}$ $CD4^{-}$ $V\alpha2^{+}$ $CD8^{+}$ cells from OT-I mice were activated with immobilized anti-receptor antibodies, interleukin-2 (IL-2), IL-4 and anti-interferon-γ (IFN-γ) antibody for 7 days *in vitro*, then labelled with CFSE. $V\alpha2^{+}$ $CFSE^{high}$ cells were separated into $CD8^{low}$ and $CD8^{high}$ cells and adoptively transferred into RAG-$1^{-/-}$ mice (day 0). Control mice ('No transfer') received no cells. (a) The left panel shows binding of anti-CD8 antibody (filled histogram) and isotype control antibody (open histogram) and sort gates (horizontal bars) of the activated cells at day 0. The right panels show the CD8 and isotype control profiles of the two populations after sorting; figures within the panels represent CD8 expression levels expressed as CD8 units [ratio of median fluorescence intensity (MFI) of anti-CD8 antibody binding to MFI of isotype control antibody binding]. (b) After 1 or 4 days, donor CD8 T cells were identified in individual spleens by Vα2 expression (rectangles) (upper panels); figures within the panels are the percentage of spleen cells expressing $V\alpha2^{+}$. CD8 and isotype control profiles and relative CD8 expression of the gated donor cells are shown (lower panels).

experiments 1–3). Expression of CD62L was more variable: $CD8^{low}$ cells were 38–62% $CD62L^{+}$ and $CD8^{high}$ cells were 79–85% $CD62L^{+}$ (data not shown).

The effect of tumour challenge on the phenotype of long-term surviving donor cells was tested by injection of cells of the OVA-expressing EL4 tumour line E.G7-OVA 53 days after adoptive transfer and 1 week before analysis. CD8 levels on donor $CD8^{low}$ cells in the spleen and lymph nodes draining the site of tumour injection were significantly lower than on the corresponding donor $CD8^{high}$ cells (Fig. 2c; Table 1, experiment 4b). CD8 levels of both $CD8^{low}$ and $CD8^{high}$ donor cells from tumour-challenged mice were significantly higher than the corresponding group of donor CD8 cells from non-challenged mice (Table 1, compare experiments 4a and b). Although differences were noted between experiments in the absolute numbers of donor $CD8^{low}$ or $CD8^{high}$ cells surviving after adoptive transfer, these differences were not consistently higher for one population than the other (Table 1). We conclude that many $CD8^{low}$ T cells survive and maintain their low CD8 expression long term *in vivo*, even after challenge with an antigen-bearing tumour.

The progeny of many single long-term $CD8^{low}$ cells maintain low CD8 expression

We next investigated whether long-term surviving $CD8^{low}$ cells up-regulate CD8 expression during re-stimulation *in vitro*. To ensure that the cells analysed after expansion were the progeny of long-term surviving $CD8^{low}$ cells rather than contaminating $CD8^{high}$ cells, these analyses were performed by sorting twice followed by single-cell cloning. In independent experiments (Table 1), donor cells were isolated from spleens 53 days (experiment 4a), 90 days (experiment 3) or 126 days (an extension of experiment 3) after adoptive transfer of $CD8^{low}$ and/or $CD8^{high}$ cells and sorted first into $CD8^{low}$ and $CD8^{high}$ populations, respectively, and then a second time to deposit single cells. After this double purification regimen, CD8 levels were similar for day 53 donor cells ($CD8^{low}$ cells, 1·3 CD8 units; $CD8^{high}$ cells, 7·6 CD8

Table 1. Long-term stability of $CD8^{low}$ and $CD8^{high}$ cells *in vivo*

		Primary *in vitro*		Long-term *in vivo*					
Expt	Phenotype	No. transferred cells ($\times 10^{-6}$)[1]	CD8 expression[2]	Tumour[3]	Day *in vivo*	Organ	CD8 expression (n)[4]	P[5]	No. CD8 cells ($\times 10^{-4}$)[6]
1	$CD8^{low}$	2	1·3	–	78	spleen	6·6 ± 1·0 (5)	***	5 ± 3 (5)
	$CD8^{high}$	2	18	–	78	spleen	16 ± 2·9 (5)		16 ± 6 (5)
2	$CD8^{low}$	1	1·6	–	62	spleen	3·0 ± 0·57 (6)	***	5 ± 2 (6)
	$CD8^{high}$	1	30	–	62	spleen	13 ± 1·6 (6)		10 ± 5 (6)
3	$CD8^{low}$	1	0·65	–	90	spleen	2·3 ± 1·1 (3)	*	2·8 ± 0·9 (3)
	$CD8^{high}$	0·5	8·4	–	90	spleen	7·3 (6·8, 7·8)		7·2 ± 3·3 (2)
4a	$CD8^{low}$	0·6	1·7	–	53	spleen	2·2 ± 0·46 (3)[7]	**	0·8 ± 0·2 (3)
	$CD8^{high}$	0·6	21	–	53	spleen	6·0 ± 0·61 (3)[8]		0·4 ± 0·1 (3)
4b	$CD8^{low}$	0·6	1·7	+	60	spleen	5·8 ± 1·2 (3)	***	3·6 ± 1 (3)
						DLN[9]	4·3 ± 0·93 (3)	***	0·7 ± 0·5 (3)
	$CD8^{high}$	0·6	21	+	60	spleen	19 ± 2·0 (3)		0·5 ± 0·4 (3)
						DLN	18 ± 1·0 (3)		0·05 ± 0·07 (3)

[1]Number of donor $V\alpha2^+$ CD8 T cells transferred per mouse (day 0).
[2]Ratio of MFI of anti-CD8 to isotype control antibody binding to $V\alpha2^+$ CD8 T cells before transfer (day 0).
[3]Where indicated, E.G7-OVA tumour cells were injected 7 days before analysis.
[4]Ratio of MFI of anti-CD8 to isotype control antibody binding on long-term surviving donor $V\alpha2^+$ $CD4^-$ $CD44^+$ $CD45.2^+$ T cells (mean ± SD) (n, number of mice analysed).
[5]P value in a two-tailed t-test of difference in CD8 expression between $CD8^{low}$ and $CD8^{high}$ cells.
[6]Number of long-term surviving donor $CD4^-$ $CD44^+$ $CD45.2^+$ $V\alpha2^+$ T cells (mean ± SD) (n, number of mice analysed).
[7]Two-tailed t-test indicated significant (**P 0·001–0·01) difference in CD8 levels between $CD8^{low}$ cells in spleen in experiment 4a versus 4b.
[8]Two-tailed t-test indicated significant (***$P < 0·001$) difference in CD8 levels between $CD8^{high}$ cells in spleen in experiment 4a versus 4b.
[9]DLN, draining lymph nodes.

units; Fig. 3a), day 90 donor cells ($CD8^{low}$ cells, 1·0 CD8 units; $CD8^{high}$ cells, 10·5 CD8 units) and day 126 $CD8^{low}$ donor cells (2·2 CD8 units). Individual cells were then cloned in neutral or type 2 conditions for 9 or 10 days before analysis of CD8 expression. Secondary culture conditions did not markedly affect cloning efficiencies (see legend to Fig. 3).

Clones derived from single $CD8^{low}$ or $CD8^{high}$ cells displayed a range of CD8 expression profiles; two examples are shown in Fig. 3(b). Among the clones generated in neutral conditions, CD8 levels were biased toward the phenotype of the founder cell and differed significantly between the 21 clones of $CD8^{low}$ and 26 clones of $CD8^{high}$ cells (3·3 versus 21·9, $P < 0·0001$, two-tailed Mann–Whitney U-test) derived from day 53 and day 90 donor cells (Fig. 3c, upper and middle panels). Cloning in type 2 conditions did not significantly alter CD8 expression: clones of $CD8^{low}$ cells grown in neutral ($n = 21$) and type 2 ($n = 22$) conditions displayed similar median CD8 levels (3·3 versus 4·7) and clones of $CD8^{high}$ cells grown in neutral ($n = 26$) and type 2 ($n = 12$) conditions displayed similar median CD8 levels (21·9 versus 24·3). Median CD8 levels were also similar among clones derived from day 126 $CD8^{low}$ cells grown in neutral ($n = 36$) and type 2 conditions ($n = 25$) (12·7 versus 7·7; Fig. 3c, lower panels).

These data suggest that long-term surviving $CD8^{low}$ and $CD8^{high}$ cells have variable potential to alter their CD8 levels following re-activation and expansion *in vitro*. Although some expressed significantly higher or lower levels than their founder cells, on average the distribution of CD8 expression profiles was strongly influenced by founder cell phenotype. The observation that exogenous IL-4 did not significantly reduce CD8 expression among clones derived from $CD8^{low}$ or $CD8^{high}$ cells suggests moreover that the clones were no longer dependent on or responsive to the CD8-modulating effects of this cytokine.

Long-term $CD8^{low}$ cells are poorly cytolytic and express low levels of mRNA for effector molecules

To determine whether the functional properties of $CD8^{low}$ cells were altered by long-term survival *in vivo* in the absence of OVA, $CD8^{low}$ and $CD8^{high}$ cells were isolated 62 days after adoptive transfer (experiment 2, Table 1) and tested for cytolytic function and expression of mRNAs encoding cytolytic molecules and cytokines. For cytolytic assessment we used the fluorolysis assay which detects very small numbers of CTL by measuring the lysis of EGFP-expressing target cells.[9] When assayed *ex vivo*, long-term $CD8^{high}$ cells showed strong specific cytolytic

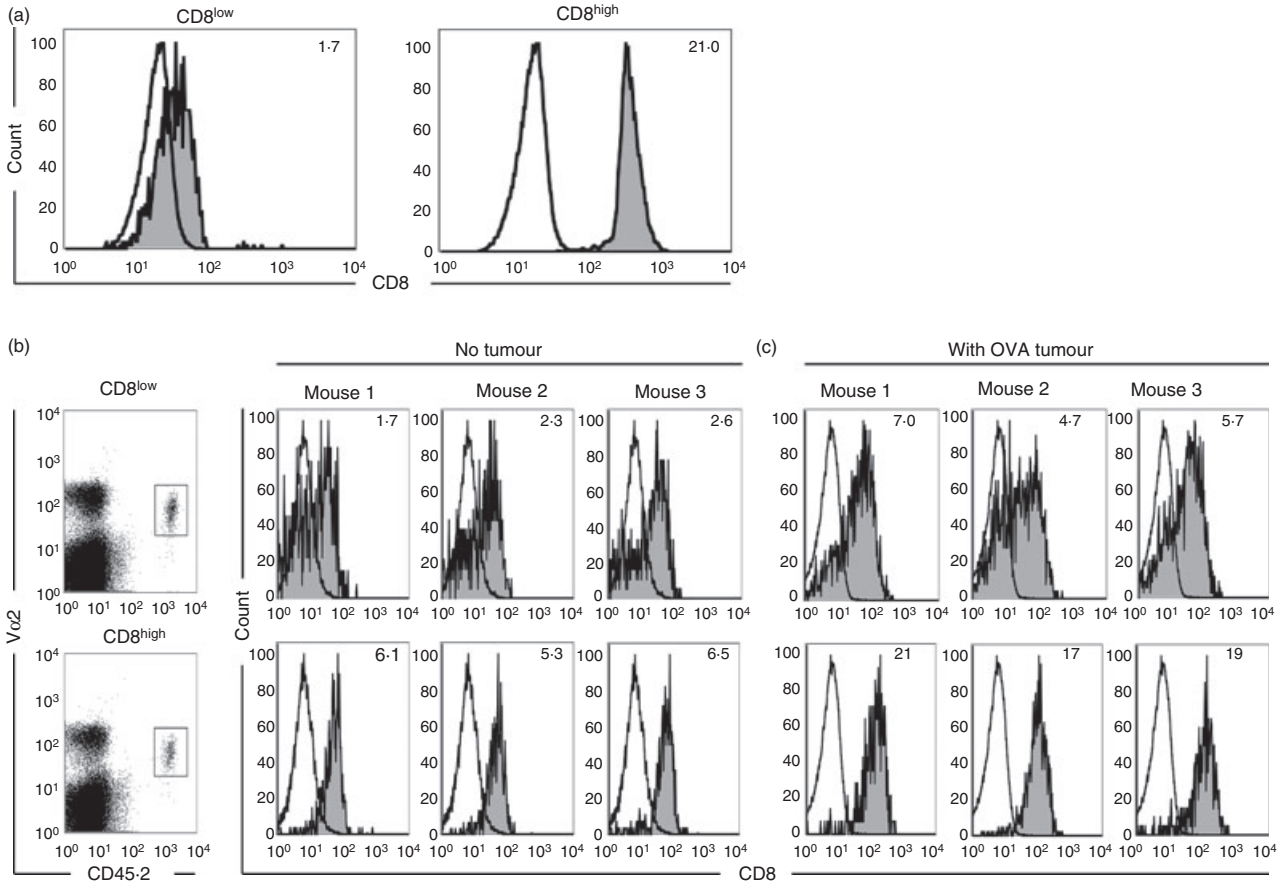

Figure 2. $CD8^{low}$ cells survive long term *in vivo*. *In vitro* activated OT-I $CD45.2^+$ $V\alpha2^+$ CD8 cells were separated into $CD8^{low}$ and $CD8^{high}$ cells and adoptively transferred into $CD45.1^+$ congenic mice (day 0). (a) The panels show binding of anti-CD8 antibody (filled histogram) and isotype control antibody (open histogram) of the sorted cells before transfer; figures within the panels represent CD8 expression levels (see legend to Fig. 1). (b) After 53 days, donor $CD45.2^+$ $V\alpha2^+$ $CD4^-$ $CD44^+$ cells (rectangles) were identified in individual spleens of mice ($n = 3$) that had received $CD8^{low}$ (upper panels) or $CD8^{high}$ (lower panels) cells. CD8 profiles and relative CD8 expression of the gated donor cells are shown; the same isotype control staining of pooled spleens from mice that had received either $CD8^{low}$ or $CD8^{high}$ panels was used. The results are summarized in Table 1 (experiment 4a). (c) At day 53, mice ($n = 3$) that had received $CD8^{low}$ (upper panels) or $CD8^{high}$ (lower panels) cells were challenged subcutaneously with ovalbumin-expressing E.G7-OVA tumour cells. One week later donor cells were analysed as in (b). The results are summarized in Table 1 (experiment 4b).

activity against SIINFEKL peptide-coated target cells whereas $CD8^{low}$ cells displayed negligible activity (Fig. 4a). Similar results were obtained in another experiment with sorted donor CD8 T cells collected after 78 days *in vivo* (experiment 1, Table 1) (data not shown). The cytolytic activity of long-term $CD8^{low}$ and $CD8^{high}$ cells was similar to that of primary $CD8^{low}$ and $CD8^{high}$ cells after activation for 8 days *in vitro* (Fig. 4b). In three other independent experiments, primary $CD8^{low}$ cells showed sixfold to tenfold lower lytic activity per cell than $CD8^{high}$ cells; similar relative activity was observed in conventional ^{51}Cr-release assays with primary $CD8^{low}$ and $CD8^{high}$ cells and SIINFEKL peptide-coated target cells (data not shown). We concluded that long-term surviving $CD8^{low}$ cells retained the poor CTL function typical of *in vitro* activated $CD8^{low}$ cells.

When assayed *ex vivo* 62 days after adoptive transfer, $CD8^{low}$ cells expressed levels of CD8α mRNA that were undetectable by real-time PCR and at least 14-fold lower than those expressed by $CD8^{high}$ cells (Fig. 5). Average CD8α mRNA levels in the $CD8^{high}$ population were about 100-fold lower than those detected in $CD8^{high}$ cells isolated from 7-day primary type 2 cultures (data not shown), suggesting that co-receptor turnover was low in these long-term surviving cells *in vivo*. By contrast, $CD8^{low}$ and $CD8^{high}$ cells expressed similar levels of mRNA for IFN-γ, granzymes A and B and the housekeeping gene β_2-microglobulin. The IL-4, IL-10 and perforin mRNAs were not detected. Similar results were obtained in another experiment with cells assayed 78 days after adoptive transfer (experiment 1, Table 1) (data not shown). The data show that the long-term surviving cells

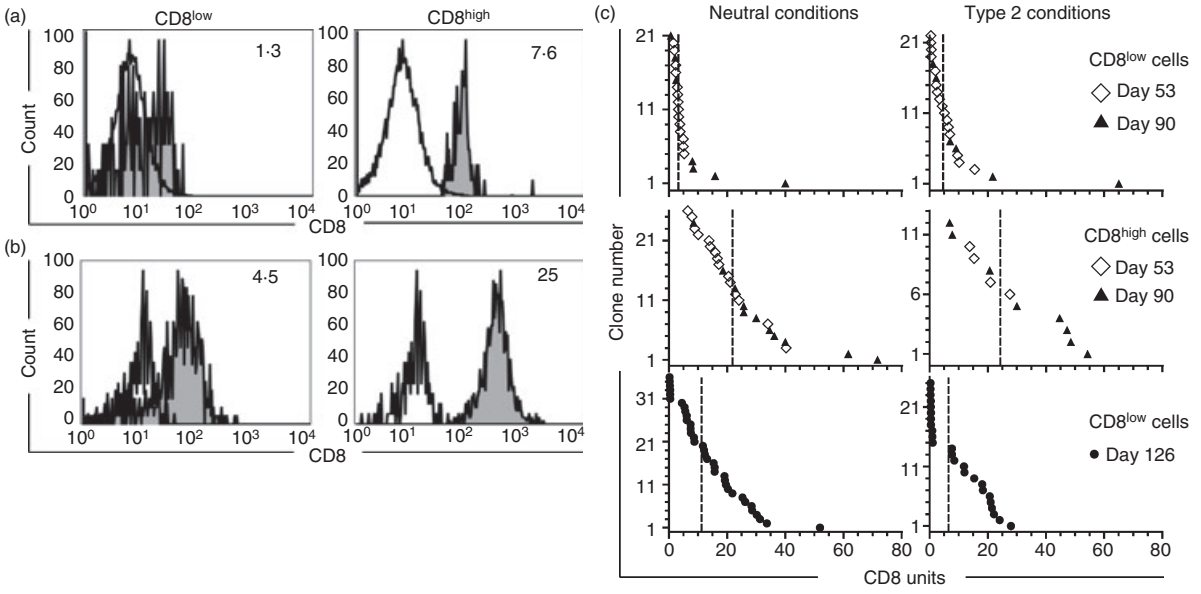

Figure 3. Long-term surviving $CD8^{low}$ cells can maintain low CD8 expression levels during clonal activation *in vitro*. In three experiments (day 53, experiment 4a; day 90 or day 126, experiment 3, Table 1), donor $CD45.2^+$ $V\alpha2^+$ $CD4^-$ $CD44^+$ cells were sorted twice for $CD8^{low}$ or $CD8^{high}$ cells before single cell deposition into wells containing immobilized anti-receptor antibodies, interleukin-2 (IL-2) and neutral or type 2 polarizing conditions. Clones were analysed after 9 or 10 days. (a): The panels show binding of anti-CD8 antibody (filled histogram) and isotype control antibody (open histogram) of the sorted day 53 cells before cloning; figures within the panels represent CD8 expression levels. (b) CD8 expression profiles (filled histogram) and levels (in CD8 units) are shown for two clones of day 126 $CD8^{low}$ cells grown in neutral conditions for 9 days; the same isotype control staining (open histogram) of a pool of clones is shown in both panels. (c) CD8 expression levels are shown for all tested clones derived from cells recovered on day 53, day 90 and day 126. Most clones were estimated by microscopic inspection to contain between 100 and 1000 cells. Each symbol represents a clone; the broken line marks the median CD8 expression level in each set. For each group of clones in each experiment, isotype control staining was carried out using pooled $CD8^{high}$ or $CD8^{low}$ clones from the same group. The following cloning efficiencies were obtained: Day 53: $CD8^{low}$ cells, 20% in neutral conditions, 20% in type 2 conditions; $CD8^{high}$ cells, 30% in neutral conditions, 28% in type 2 conditions. Day 90: $CD8^{low}$ cells, 31% in neutral conditions, 38% in type 2 conditions; $CD8^{high}$ cells, 44% in neutral conditions, 36% in type 2 conditions. Day 126: $CD8^{low}$ cells: 37% in neutral conditions, 45% in type 2 conditions.

had low or undetectable expression levels of all the tested effector molecules; the minor differences observed in the expression of the cytolytic mediators by $CD8^{low}$ and $CD8^{high}$ cells appeared insufficient to account for differences in their cytoloytic activity.

Long-term $CD8^{low}$ cells have protective anti-tumour function *in vivo*

In view of their poor CTL function *ex vivo*, long-term $CD8^{low}$ cells were tested for anti-tumour activity *in vivo*. In two independent experiments, congenic mice received $CD8^{low}$ or $CD8^{high}$ cells or saline; after 53 or 79 days, the mice were challenged with OVA-expressing tumour cells and 7 days later the mice were culled and tumours were excised and weighed. Long-term $CD8^{high}$ cells significantly inhibited tumour weight in both experiments. $CD8^{low}$ cells also reduced tumour weight but the effect was significant in only one of the experiments (Fig. 6a).

In vitro activated primary $CD8^{low}$ or $CD8^{high}$ cells also exhibited anti-tumour activity when they were injected with E.G7-OVA cells into normal mice in a classical Winn assay. Both $CD8^{low}$ and $CD8^{high}$ cells prolonged survival but again $CD8^{low}$ cells were less effective than $CD8^{high}$ cells ($P = 0{\cdot}011$, Log-rank test; Fig. 6b). From this and three other independent Winn assays, we concluded that long-term surviving $CD8^{low}$ cells possessed some anti-tumour function which was weaker than that of $CD8^{high}$ cells.

Discussion

Here we show that CD8 T cells that had down-regulated CD8 during primary activation *in vitro* in the presence of IL-4 could survive for at least 4 months after adoptive transfer into normal mice. These long-term surviving cells on average displayed significantly lower levels of surface CD8 than those recovered after transfer of $CD8^{high}$ cells. The $CD8^{low}$ cells among the long-term survivors of $CD8^{low}$ donor cells expressed markedly lower levels of CD8α mRNA and antigen-specific cytolytic activity than long-term surviving $CD8^{high}$ cells. Moreover, many single long-term surviving $CD8^{low}$ cells gave rise to $CD8^{low}$ clones when restimulated *in vitro* in the absence of

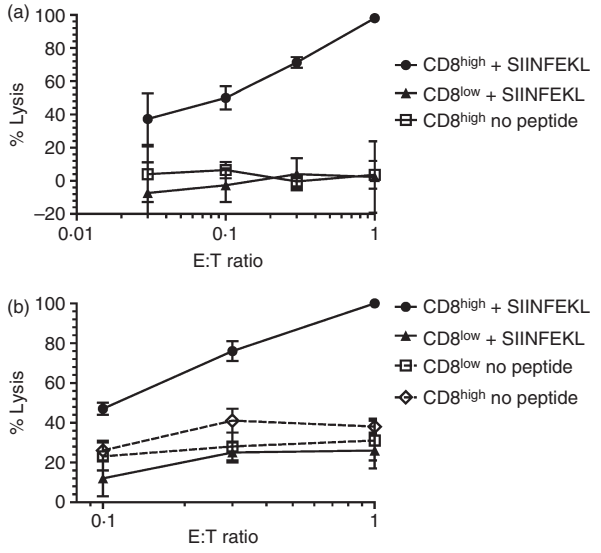

Figure 4. $CD8^{low}$ cells have poor cytolytic function. (a) Donor $CD45.2^+$ $V\alpha2^+$ $CD4^-$ $CD44^+$ cells were identified in spleen 62 days after adoptive transfer and sorted for $CD8^{high}$ and $CD8^{low}$ cells (experiment 2, Table 1). (b) In an independent experiment, primary OT-I CD8 T cells were activated for 8 days *in vitro* and then separated into $CD8^{high}$ and $CD8^{low}$ populations. Effector cells from both regimens were tested for cytolytic activity at the indicated effector to target (E : T) ratios in a fluorolysis assay using enhanced green fluorescent protein-expressing EL4 target cells coated with or without SIINFEKL peptide; the means ± SD of three replicates are shown.

exogenous IL-4. We conclude that some IL-4-induced $CD8^{low}$ cells can retain their phenotypic and functional properties for several months *in vivo* and through multiple subsequent divisions *in vitro*.

We have previously reported that IL-4-induced $CD8^{low}$ cells also retained their $CD8^{low}$ phenotype and impaired cytolytic activity long-term *in vitro*, through many rounds of cell division in the absence of IL-4 and even when endogenous IL-4 was neutralized.[4,5] As for long-term surviving $CD8^{low}$ cells *in vivo* (this study), their $CD8^{low}$ phenotype was associated with reduced CD8α mRNA expression. The heritability of this property suggests regulation by epigenetic mechanisms. Others have demonstrated an association of various epigenetic modifications of the CD8α locus with changes in CD8α gene expression as T cells differentiate from double-negative progenitors in the thymus and then migrate to the periphery,[13–16] as well as with maintenance of CD8 expression following activation of peripheral $CD8^+$ T cells.[17] Work is in progress to determine whether some of the epigenetic modifications observed in naive $CD8^+$ T cells are retained or reversed in $CD8^{low}$ cells *in vitro* or in long-term surviving $CD8^{low}$ cells *ex vivo*.

The initial establishment of the $CD8^{low}$ state depends on TCR stimulation[4] but its maintenance *in vivo* apparently does not require continuing TCR ligation by specific peptide–MHC complexes, consistent with other evidence that neither peptide–MHC nor TCR signalling is needed for memory $CD8^+$ T-cell survival *in vivo* in many systems.[18,19] Maintenance of the $CD8^{low}$ state was observed whether the cells were transferred into RAG-$1^{-/-}$ mice (analysed at 4 or 8 days) in which they underwent homeostatic proliferation, or into wild-type mice (analysed at 53–126 days). We did not examine whether transferred cells proliferated in wild-type mice but Perret and Ronchese[20] detected bromodeoxyuridine uptake by transferred effector $CD8^+$ T cells as late as 83–90 days after adoptive transfer into intact congenic mice. Proliferation itself is insufficient to induce the $CD8^{low}$ state, however, as adoptively transferred naive $CD8^+$ T cells did not down-regulate CD8 during homeostatic proliferation in the absence of antigen for 5 days *in vivo*.[4] Endogenous IL-4 may have helped to maintain the $CD8^{low}$ phenotype *in vivo* but it was notable that long-term surviving $CD8^{low}$ cells themselves did not express detectable IL-4 mRNA when analysed immediately *ex vivo*.

While long-term surviving $CD8^{low}$ cells displayed significantly lower levels of surface CD8 than those recovered after transfer of $CD8^{high}$ cells, median expression levels were reproducibly higher than those of the starting population. This partial reacquisition of CD8 *in vivo* could reflect the absence of the down-regulating signal provided by IL-4 and/or exposure to an up-regulating signal. A strong candidate for the latter is IFN-γ. We have reported elsewhere that IFN-γ and IL-4 exert opposing effects on CD8 expression during primary T-cell activation *in vitro* and *in vivo* such that, for example, IL-4-mediated CD8 down-regulation was enhanced in IFN-γ-deficient compared with wild-type $CD8^+$ T cells.[4,6] In addition, both $CD8^{low}$ and $CD8^{high}$ cells expressed somewhat higher levels of CD8 following OVA tumour challenge *in vivo*, raising the possibility that specific TCR stimulation led to partial recovery of CD8 expression (e.g. by inducing IFN-γ production) and/or selective recruitment or expansion of cells with higher CD8 levels. Further work will be necessary to determine whether IFN-γ can reverse the effects of IL-4 in cells that have already down-regulated CD8 mRNA and protein expression.

It was also notable that the cells that survived long-term after adoptive transfer of $CD8^{low}$ cells displayed some anti-tumour activity *in vivo*, albeit weaker than that of long-term surviving $CD8^{high}$ cells. Cells that retained a $CD8^{low}$ phenotype long-term *in vivo* lacked cytolytic function and expressed perforin and granzyme mRNA levels near or below the threshold of detection, at least before re-exposure to antigen on tumour challenge. It is not known whether tumour clearance was mediated by these persisting non-cytolytic $CD8^{low}$ cells (perhaps via IFN-γ) or by cells that reacquired some cytolytic function during the preceding weeks or following challenge. Over-

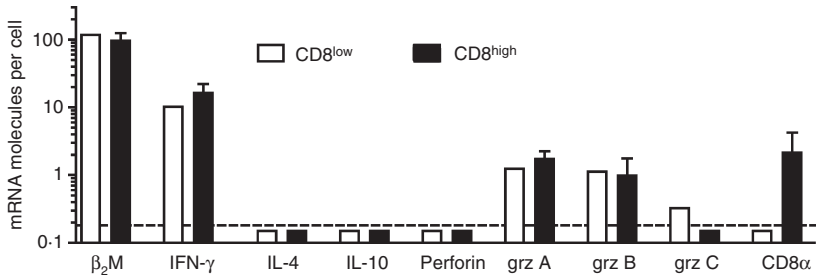

Figure 5. Long term CD8^{low} cells have low CD8α mRNA expression. Donor CD45.2$^+$ Vα2$^+$ CD4$^-$ CD44$^+$ cells were identified in spleen 62 days after adoptive transfer and sorted for CD8^{high} and CD8^{low} cells (experiment 2, Table 1). The indicated effector cell populations were assayed for expression of mRNAs encoding β_2-microglubulin (β_2M), cytokines [interferon-γ (IFN-γ), interleukin-4 (IL-4), IL-10], cytolytic molecules (perforin, granzyme A, granzyme B, granzyme C) and CD8α by real-time PCR; the means, and where applicable ± SD, of two or three replicate cDNA samples, each calculated from duplicate PCR runs, are shown. The broken line indicates the threshold of detection.

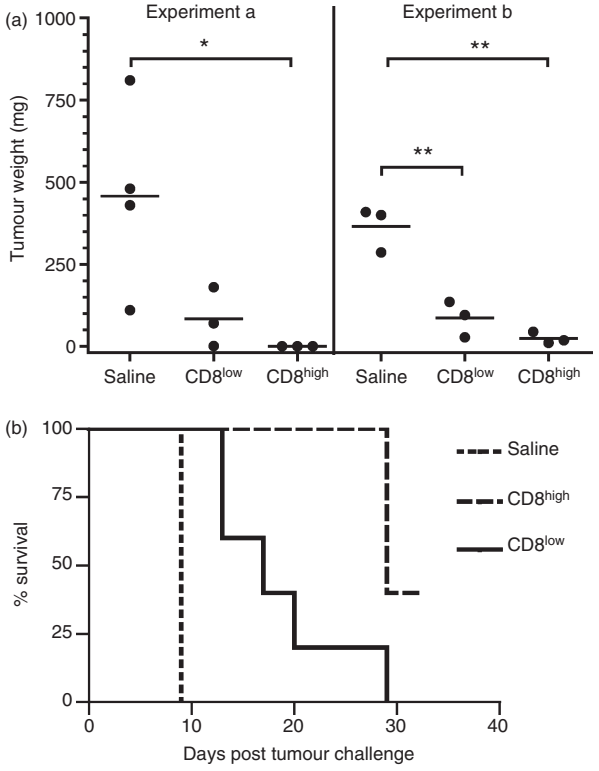

Figure 6. CD8^{low} cells have protective anti-tumour function. (a) In two independent experiments mice adoptively transferred with CD8^{low} or CD8^{high} cells or saline were injected with E.G7-OVA tumour cells at day 53 (experiment a) or with E.G7-OVA-luc$^+$ tumour cells at day 79 (experiment b). Tumour weights were measured 7 days later. Experiment a corresponds to day 60 (experiment 4b, Table 1) and experiment b to day 86 long-term CD8 donor cells. Each data point represents one mouse and horizontal lines indicate the mean of each group; groups were compared by unpaired *t*-test. (b) CD8^{low} or CD8^{high} cells, purified from primary *in vitro* activated OT-I CD8 T cells, or saline were co-injected with E.G7-OVA tumour cells into normal mice (five mice per group). Tumour growth was monitored over 32 days and mice were culled when tumour size exceeded 1 cm^3. Representative results of one of three similar experiments are shown.

all, however, the data are consistent with a number of earlier studies showing that IL-4 modulates anti-tumour function in CD8 T cells, in some cases activating alternative pathways of tumour clearance.[6,21–25]

Given that functionally similar CD8^{low} cells can be generated in response to antigen under certain circumstances *in vivo*,[4,6] the ability of transferred CD8^{low} cells to persist for months *in vivo* raises the question whether this ever occurs under physiological conditions. Those cells expressing the lowest levels of CD8 identified here would be phenotypically indistinguishable from some other CD4$^-$ CD8$^-$ αβ T cells which can be found at low frequency in normal mice and humans and which have been ascribed regulatory function in a variety of conditions.[26,27] In the future, identification of epigenetic modifications may enable distinction of IL-4-down-regulated CD8^{low} cells from double-negative cells of other origins. Importantly, the cells we have described are not functionally inert. Although their sensitivity to peptide–MHC may be reduced in the absence of the CD8 co-receptor,[4] they retain the capacity to proliferate and synthesize (predominantly type 2) cytokines *in vitro* and, as shown here, they exert some anti-tumour activity *in vivo*. Understanding their induction, regulation and persistence may therefore contribute to mapping the full range of differentiation pathways available to naive CD8$^+$ T cells during immune responses *in vivo*.

Acknowledgements

We thank Dr William Heath for the generous gift of mice, Grace Chojnowski and Paula Hall for assistance with cell sorting, Suzanne Cassidy and staff from the QIMR animal facility for animal husbandry, and Penny Groves for technical contributions. The following reagent (human rIL-2) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health: human rIL-2 from

Dr Maurice Gately, Hoffmann-LaRoche (Nutley, NJ). This work was supported by grants from the National Health and Medical Research Council of Australia, the Cancer Council Queensland and the Cooperative Research Centre for Vaccine Technology. The Cooperative Research Centre for Vaccine Technology was established and supported under the Australian Government's Cooperative Research Centres Programme. S.H.A. was supported by an Australian Postgraduate Award and the Basil Shaw Fellowship of the Australian Rotary Health Research Fund.

Disclosures

The authors state that there are no financial or commercial conflicts of interest.

References

1 Erard F, Wild MT, Garcia-Sanz JA, Le Gros G. Switch of CD8 T cells to noncytolytic $CD8^-CD4^-$ cells that make TH2 cytokines and help B cells. *Science* 1993; **260**:1802–5.

2 Kienzle N, Buttigieg K, Groves P, Kawula T, Kelso A. A clonal culture system demonstrates that IL-4 induces a subpopulation of noncytolytic T cells with low CD8, perforin, and granzyme expression. *J Immunol* 2002; **168**:1672–81.

3 Kemp RA, Backstrom BT, Ronchese F. The phenotype of type 1 and type 2 $CD8^+$ T cells activated *in vitro* is affected by culture conditions and correlates with effector activity. *Immunology* 2005; **115**:315–24.

4 Apte SH, Baz A, Groves P, Kelso A, Kienzle N. Interferon-γ and interleukin-4 reciprocally regulate CD8 expression in $CD8^+$ T cells. *Proc Natl Acad Sci U S A* 2008; **105**:17475–80.

5 Kienzle N, Olver S, Buttigieg K, Groves P, Janas ML, Baz A, Kelso A. Progressive differentiation and commitment of $CD8^+$ T cells to a poorly cytolytic $CD8^{low}$ phenotype in the presence of IL-4. *J Immunol* 2005; **174**:2021–9.

6 Apte SH, Groves P, Olver S, Baz A, Doolan DL, Kelso A, Kienzle N. IFN-γ inhibits IL-4-induced type 2 cytokine expression by CD8 T cells *in vivo* and modulates the antitumor response. *J Immunol* 2010; **185**:998–1004.

7 Viola A, Salio M, Tuosto L, Linkert S, Acuto O, Lanzavecchia A. Quantitative contribution of CD4 and CD8 to T cell antigen receptor serial triggering. *J Exp Med* 1997; **186**:1775–9.

8 Xiao Z, Mescher MF, Jameson SC. Detuning CD8 T cells: down-regulation of CD8 expression, tetramer binding, and response during CTL activation. *J Exp Med* 2007; **204**:2667–77.

9 Kienzle N, Olver S, Buttigieg K, Kelso A. The fluorolysis assay, a highly sensitive method for measuring the cytolytic activity of T cells at very low numbers. *J Immunol Methods* 2002; **267**:99–108.

10 Doyle AG, Buttigieg K, Groves P, Johnson BJ, Kelso A. The activated type 1-polarized $CD8^+$ T cell population isolated from an effector site contains cells with flexible cytokine profiles. *J Exp Med* 1999; **190**:1081–92.

11 Olver S, Groves P, Buttigieg K, Morris ES, Janas ML, Kelso A, Kienzle N. Tumor-derived interleukin-4 reduces tumor clearance and deviates the cytokine and granzyme profile of tumor-induced $CD8^+$ T cells. *Cancer Res* 2006; **66**:571–80.

12 Winn HJ. Immune mechanisms in homotransplantation. II. Quantitative assay of the immunologic activity of lymphoid cells stimulated by tumor homografts. *J Immunol* 1961; **86**:228–39.

13 Carbone AM, Marrack P, Kappler JW. Demethylated CD8 gene in $CD4^+$ T cells suggests that $CD4^+$ cells develop from $CD8^+$ precursors. *Science* 1988; **242**:1174–6.

14 Pestano GA, Zhou Y, Trimble LA, Daley J, Weber GF, Cantor H. Inactivation of misselected CD8 T cells by CD8 gene methylation and cell death. *Science* 1999; **284**:1187–91.

15 Bilic I, Koesters C, Unger B, Sekimata M, Hertweck A, Maschek R, Wilson CB, Ellmeier W. Negative regulation of CD8 expression via *Cd8* enhancer-mediated recruitment of the zinc finger protein MAZR. *Nat Immunol* 2006; **7**:392–400.

16 Harker N, Garefalaki A, Menzel U, Ktistaki E, Naito T, Georgopoulos K, Kioussis D. Pre-TCR signaling and CD8 gene bivalent chromatin resolution during thymocyte development. *J Immunol* 2011; **186**:6368–77.

17 Hassan H, Sakaguchi S, Tenno M *et al. Cd8* enhancer *E8I* and Runx factors regulate CD8α expression in activated $CD8^+$ T cells. *Proc Natl Acad Sci U S A* 2011; **108**:18330–5.

18 Sprent J, Cho JH, Boyman O, Surh CD. T cell homeostasis. *Immunol Cell Biol* 2008; **86**:312–19.

19 Leignadier J, Hardy MP, Cloutier M, Rooney J, Labrecque N. Memory T-lymphocyte survival does not require T-cell receptor expression. *Proc Natl Acad Sci U S A* 2008; **105**:20440–5.

20 Perret R, Ronchese F. Effector $CD8^+$ T cells activated *in vitro* confer immediate and long-term tumor protection *in vivo*. *Eur J Immunol* 2008; **38**:2886–95.

21 Rodolfo M, Zilocchi C, Accornero P, Cappetti B, Arioli I, Colombo MP. IL-4-transduced tumor cell vaccine induces immunoregulatory type 2 CD8 T lymphocytes that cure lung metastases upon adoptive transfer. *J Immunol* 1999; **163**:1923–8.

22 Kemp RA, Ronchese F. Tumor-specific Tc1, but not Tc2, cells deliver protective antitumor immunity. *J Immunol* 2001; **167**:6497–502.

23 Dobrzanski MJ, Reome JB, Dutton RW. Role of effector cell-derived IL-4, IL-5, and perforin in early and late stages of type 2 CD8 effector cell-mediated tumor rejection. *J Immunol* 2001; **167**:424–34.

24 Dobrzanski MJ, Reome JB, Hollenbaugh JA, Dutton RW. Tc1 and Tc2 effector cell therapy elicit long-term tumor immunity by contrasting mechanisms that result in complementary endogenous type 1 antitumor responses. *J Immunol* 2004; **172**:1380–90.

25 Baschuk N, Utermöhlen O, Gugel R *et al.* Interleukin-4 impairs granzyme-mediated cytotoxicity of Simian virus 40 large tumor antigen-specific CTL in BALB/c mice. *Cancer Immunol Immunother* 2007; **56**:1625–36.

26 Thomson CW, Lee BP, Zhang L. Double-negative regulatory T cells: non-conventional regulators. *Immunol Res* 2006; **35**:163–78.

27 Duncan B, Nazarov-Stoica C, Surls J, Kehl M, Bona C, Casares S, Brumeanu TD. Double negative ($CD3^+$ 4^- 8^-) TCR$\alpha\beta$ splenic cells from young NOD mice provide long-lasting protection against type 1 diabetes. *PLoS ONE* 2010; **5**:e11427.

