drawing of evolutionary trees for the Microsoft Windows environment. *Comput. Appl. Biosci.* **10,** 569–570 (1994).

30. Langkjær, R. B. *Re-modelling of the Nuclear and Mitochondrial Genomes during the Evolution of the Saccharomyces Yeasts*. Thesis, BioCentrum-DTU, Technical Univ., Denmark (2002).

Supplementary Information accompanies the paper on *Nature*'s website (**http://www.nature.com/nature**).

Acknowledgements We thank the laboratories of C. Gaillardin and M. Bolotin-Fukuhara for providing *E. coli* clones containing genomic yeast DNA, which were partially sequenced by the Génolevures project. We thank K. H. Wolfe and his co-workers for sharing their manuscripts before publication. Sequencing of *Candida albicans* was accomplished with the support of the NIDR and the Burroughs Wellcome Fund. This work was partially supported by the Danish Research Council.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to J.P. (e-mail: jp@biocentrum.dtu.dk). The analysed gene sequences have the following GenBank accession numbers: AY144796–AY145050.

$CD4^+$ T cells are required for secondary expansion and memory in $CD8^+$ T lymphocytes

Edith M. Janssen*, Edward E. Lemmens*, Tom Wolfe†, Urs Christen†, Matthias G. von Herrath† & Stephen P. Schoenberger*

** Division of Cellular Immunology and † Division of Developmental Immunology, La Jolla Institute for Allergy and Immunology, 10355 Science Center Drive, San Diego, California 92121, USA*

A long-standing paradox in cellular immunology concerns the conditional requirement for $CD4^+$ T-helper (T_H) cells in the priming of cytotoxic $CD8^+$ T lymphocyte (CTL) responses *in vivo*. Whereas CTL responses against certain viruses can be primed in the absence of $CD4^+$ T cells, others, such as those mediated through 'cross-priming' by host antigen-presenting cells, are dependent on T_H cells[1–4]. A clearer understanding of the contribution of T_H cells to CTL development has been hampered by the fact that most T_H-independent responses have been demonstrated *ex vivo* as primary cytotoxic effectors, whereas T_H-dependent responses generally require secondary *in vitro* re-stimulation for their detection. Here, we have monitored the primary and secondary responses of T_H-dependent and T_H-independent CTLs and find in both cases that $CD4^+$ T cells are dispensable for primary expansion of $CD8^+$ T cells and their differentiation into cytotoxic effectors. However, secondary CTL expansion (that is, a secondary response upon re-encounter with antigen) is wholly dependent on the presence of T_H cells during, but not after, priming. Our results demonstrate that T-cell help is 'programmed' into $CD8^+$ T cells during priming, conferring on these cells a hallmark of immune response memory: the capacity for functional expansion on re-encounter with antigen.

After exposure to antigen *in vivo*, $CD8^+$ T-cell responses proceed through an ordered sequence of developmental events. These include an initial expansion phase in which a significant number of cytotoxic effectors are generated, and a subsequent contraction phase in which about 90% of these die, leaving a stable population of memory cells able to mount a rapid secondary response to antigen[5]. Although transition through these stages was thought to be governed by the presence and eventual elimination of antigen, mounting evidence suggests that $CD8^+$ T-cell development is guided by an instructional programme that, once set into motion during priming, is executed independently[6–9]. The role of T_H cells in initiating and promoting the programmed development of $CD8^+$ T cells *in vivo* is poorly understood. Early *in vitro* studies gave rise to models in which help is mediated by paracrine cytokines produced by T_H cells[10]. Recent work, however, has focused on the role of $CD4^+$ T cells in activating antigen-presenting cells (APCs) from an immature state in which they are unable to prime CTLs to a state in which they can do so autonomously[3,11,12]. T_H-independent antigens such as viruses are thought to prime CTLs by achieving a functionally equivalent degree of APC activation as that produced by T_H cells, either through direct infection or provoking inflammatory host responses[13,14]. Although this model provides a possible explanation for the conditional nature of T-cell help for CTL responses, it does not address which aspects of $CD8^+$ development (for example, primary expansion, functional differentiation, or memory) are regulated by $CD4^+$ T cells. We have now investigated this question for both T_H-dependent and T_H-independent CTL responses.

Immunization of C57BL/6 mice with syngeneic human adenovirus type 5 E1-transformed $Tap^{-/-}$ mouse embryo cells ($Tap^{-/-}$ 5E1 MECs) induces a prototypical T_H-dependent, $CD8^+$ T-cell response through cross-priming[15]. Depletion of $CD4^+$ cells before immunization blocks the development of E1B(192–200)-peptide-specific CTLs after secondary *in vitro* re-stimulation 14 days later[3]. To investigate whether the absence of T_H cells abrogates primary expansion, the frequency of interferon-γ (IFN-γ)$^+$ effector $CD8^+$ T cells was monitored directly *ex vivo* at weekly intervals after immunization of intact (wild type) or CD4-depleted mice. The

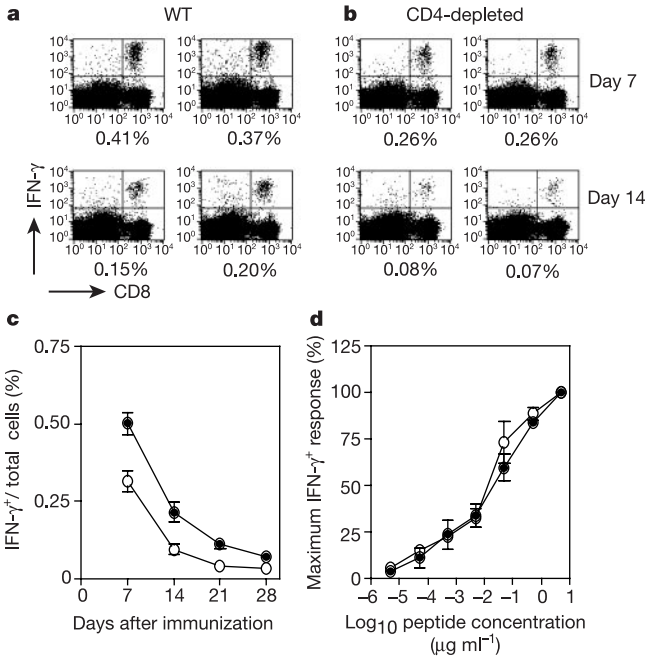

Figure 1 Functional expansion of T-helper (T_H) cell-dependent $CD8^+$ T cells cross-primed in the absence of $CD4^+$ T-cell help. **a**, **b**, The frequency of interferon-γ (IFN-γ)$^+$ E1B(192–200)-specific $CD8^+$ T cells detected in the spleens of $Tap^{-/-}$ Ad5E1-immunized normal (WT) (**a**) or CD4-depleted (**b**) mice on stimulation with E1B(192–200) peptide. (Control peptide-stimulated responses were <0.01% of total splenocytes.) The value under each panel represents the percentage of total cells that are positive for IFN-γ. **c**, Percentage of IFN-γ$^+$ E1B(192–200)-specific $CD8^+$ cells in the spleen of normal (filled circles) and CD4-depleted (open circles) mice at various time points after immunization. **d**, Comparable functional avidity of E1B(192–200)-specific IFN-γ$^+$ $CD8^+$ cells of normal (filled circles) and CD4-depleted (open circles) mice after stimulation with titrated concentrations of E1B(192–200) peptide. Results are displayed as mean ± s.e.m. ($n = 6$) and are representative of three experiments.

absence of $CD4^+$ cells reduces the number of effector $CD8^+$ T cells detected at the day 7 peak of the response by approximately half compared with wild-type mice, and results in a lower per-cell level of IFN-γ production (Fig. 1a, b; see also Supplementary Fig. S1). Despite the difference in peak burst sizes, the rate of subsequent contraction in the two antigen-specific effector populations did not differ significantly over the next 3 weeks (Fig. 1c). Similar data were obtained for E1B-specific $CD8^+$ T cells primed in I-$A\beta^{-/-}$ mice, which lack T_H cells because their APCs do not express major histocompatibility complex (MHC) class II molecules (data not shown)[16]. The reduction in peak burst size is not due to differences in the antigenic responsiveness of the CTLs primed in the presence versus the absence of T_H cells, as the IFN-γ^+ primary effectors in both populations displayed similar functional avidity for their peptide ligand (Fig. 1d; see also Supplementary Fig. S2). Taken together, these data show that 'T_H-dependent' $CD8^+$ T cells can be functionally primed in the absence of T_H cells to generate IFN-γ-producing effectors.

The *in vivo* cytotoxic capacity of $CD8^+$ T cells primed in the absence of T_H cells was assessed within intact mice. This was done by monitoring the specific eradication of an adoptively transferred target population of E1B(192–200)-pulsed splenocytes that had been differentially labelled with carboxy fluorescein succinimidyl ester ($CFSE^{high}$) so as to be distinguishable from a co-transferred reference population ($CFSE^{low}$) pulsed with a control peptide[17]. Figure 2a shows that strong and specific cytotoxic effectors are detectable *in vivo* 7 and 14 days after priming in the absence of T_H cells. These primary CTLs appear to have a transient capacity to generate secondary effectors after re-stimulation at day 7, but not at day 14 (Fig. 2b). In contrast, CTLs primed in the presence of T_H cells are detectable in secondary cultures at days 7 and 14 (Fig. 2b), as well as for several months after priming in wild-type mice (not shown). These results indicate that $CD4^+$ cells are not required for the development of T_H-dependent $CD8^+$ T cells into primary CTLs *in vivo*. The capacity of these CTLs to develop into secondary effectors detectable *in vitro*, however, seems to depend on the length of the interval between priming and re-stimulation, being present at day 7 but absent at day 14 after immunization.

The discrepancy between the presence of primary compared with secondary cytotoxic effectors at days 7 and 14 after immunization

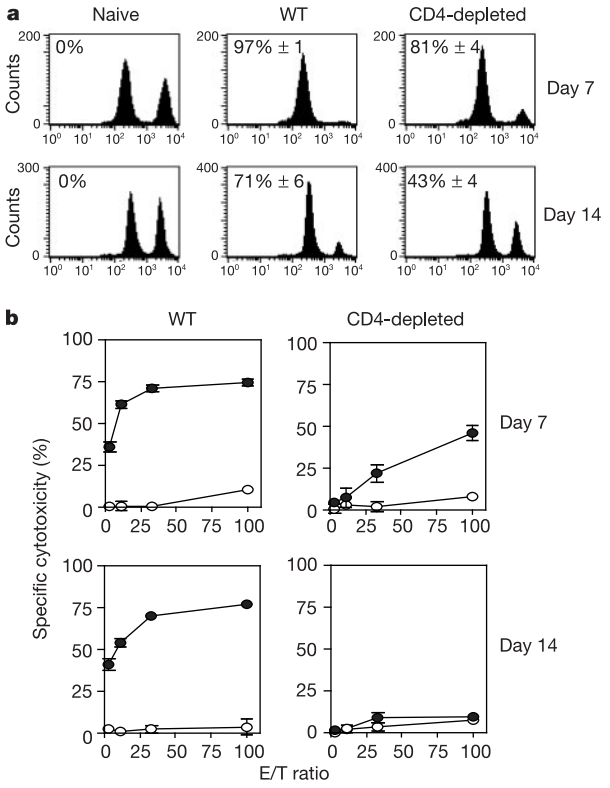

Figure 2 Primary and secondary cytotoxic responses of T_H-dependent cytotoxic T lymphocytes (CTLs). **a**, *In vivo* killing of E1B(192–200)-pulsed target cells labelled with carboxy fluorescein succinimidyl ester ($CFSE^{high}$) in normal or CD4-depleted mice at various times after immunization with $Tap^{-/-}$ 5E1 mouse embryo cells (MECs). The values in the left corner of each panel represent the percentage of specific killing compared with control-pulsed, $CFSE^{low}$-labelled cells (mean ± s.e.m.; $n = 6$). **b**, *In vitro* cytolytic activity of re-stimulated splenocytes from normal or CD4-depleted mice 7 and 14 days after immunization. Various effector/target (E/T) ratios were tested for killing of syngeneic EL-4 cells pulsed with E1B(192–200) peptide (filled circles) or OVA(257–264) peptide (open circles). Values represent mean ± s.e.m. ($n = 6$).

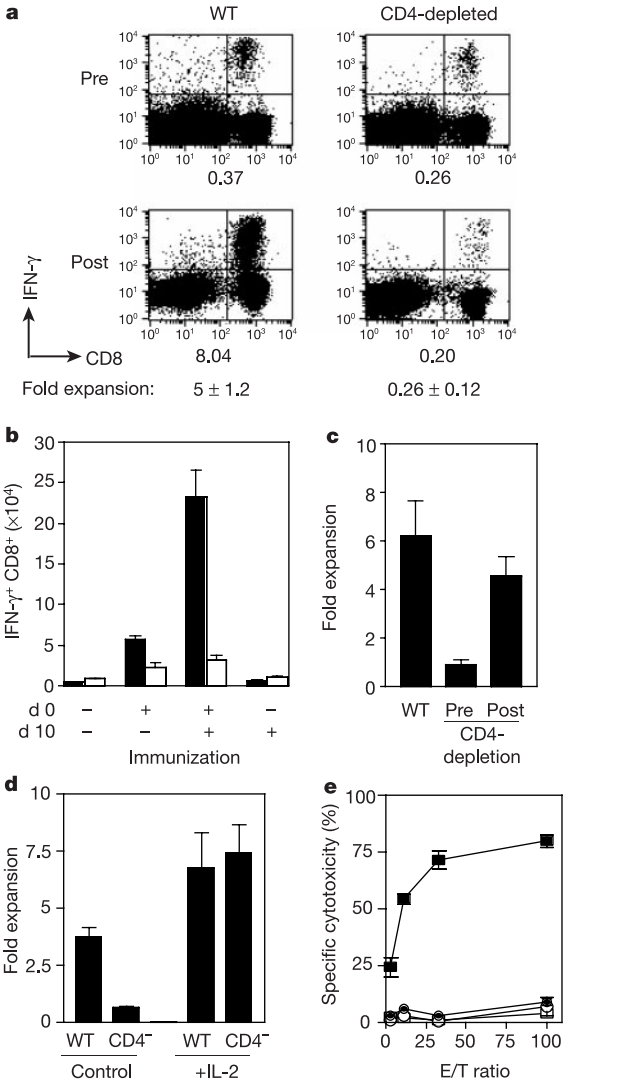

Figure 3 Defective secondary expansion of CTLs cross-primed in the absence of T_H cells. **a**, Per cent of IFN-γ^+ E1B(192–200)-specific CTLs among total splenocytes before and after *in vitro* re-stimulation. The value under each panel represents the per cent of total $CD8^+$ cells that are IFN-γ^+. Fold expansion is calculated from the increase in the absolute number of specific CTLs. **b**, Primary and secondary *in vivo* responses of E1B(192–200)-specific IFN-γ^+ CTLs detected directly in spleens of normal (filled bar) and CD4-depleted (open bar) mice 4 days after secondary (d 10) immunization. **c**, Fold expansion after re-stimulation at day 7 of purified $CD8^+$ T cells from mice depleted of $CD4^+$ T cells before (pre) or after (post) immunization. **d**, Rescue of secondary expansion after re-stimulation by addition of interleukin-2 (IL-2). **e**, Cytotoxic activity of splenocytes from CD4-depleted $Tap^{-/-}$ 5E1 MEC-immunized mice after day 7 of re-stimulation. Squares, IL-2 added to re-stimulation culture; circles, no IL-2 added; filled symbols, EL-4 target pulsed with E1B(192–200) peptide; open symbols, EL-4 pulsed with control peptide.

prompted us to monitor CTL expansion after re-stimulation. This was accomplished by direct *ex vivo* enumeration of the antigen-specific IFN-γ^+ CTLs before *in vitro* re-stimulation and then again six days later. Figure 3a shows that, although a population of E1B-specific effector CTLs is readily detectable at day 7 after immunization in wild-type or CD4-depleted mice, only CTLs primed in the presence of T_H cells are able to expand (by fivefold on average) on secondary encounter with antigen. In contrast, CTLs primed in CD4-depleted mice fail to expand and generally contract after re-stimulation. This same pattern of secondary expansion of 'helped' CTLs from wild-type mice and contraction of 'helpless' CTLs from CD4-depleted mice seen at day 7 was also observed at 14, 21 and 28 days after immunization (Table 1).

Next, we investigated whether the helpless CTLs similarly failed to undergo secondary expansion *in vivo* by monitoring the number of E1B-specific IFN-γ^+ effector CTLs in wild-type or CD4-depleted mice that had received either a single immunization 14 days earlier (d 0), or a second immunization 10 days afterwards (d 10). Although E1B-specific primary effectors are induced in both wild-type and CD4-depleted mice, only CTLs primed in the presence of T_H cells are able to expand *in vivo* on re-challenge (Fig. 3b). We next investigated whether the presence of T_H cells during *in vitro* re-stimulation was required for secondary expansion of helped CTLs. This was accomplished by depleting $CD4^+$ cells either before or after immunization and monitoring secondary expansion of purified $CD8^+$ CTLs following re-stimulation. Whereas depletion of $CD4^+$ cells before priming yields primary effector CTLs that fail to undergo secondary expansion, depletion of CD4 cells three days after priming has no such effect (Fig. 3c). These results indicate that the capacity for secondary expansion is programmed and becomes cell-autonomous after priming in the presence of T_H cells. The helpless CTLs are not irretrievably consigned to defective secondary expansion and cytotoxicity, however, as providing exogenous interleukin-2 (IL-2) at re-stimulation restores both of these functions (Fig. 3d, e). Taken together, these data demonstrate that T_H cells are required for secondary expansion of CTLs *in vitro* and *in vivo*, and that CTLs are endowed with this capacity during priming.

Our combined results indicate that for T_H-dependent responses, $CD4^+$ cells are required to confer secondary, rather than primary, CTL expansion. To investigate whether this is also true for prototypical T_H-independent CTL responses, we monitored the primary and secondary $CD8^+$ responses induced by infection with lymphocytic choriomeningitis virus (LCMV). Wild-type, CD4-depleted, or I-A$\beta^{-/-}$ mice were infected with LCMV (Armstrong), and the resulting LCMV GP(33–41)-peptide-specific CTL response was quantified physically, using D^b/GP(33–41) tetramers, and functionally, using intracellular detection of IFN-γ by GP(33–41)-specific D^b- and K^b-restricted CTLs[18,19]. Although the magnitude of the GP(33–41)-specific response and cytotoxic function between the three groups was equivalent when measured 7 days after infection (data not shown), the GP(33–41)-specific CTLs primed in mice lacking T_H cells were reduced in number and produced less IFN-γ than wild-type controls when measured at day 28 (Fig. 4a, b). Nonetheless, both the I-A$\beta^{-/-}$ and CD4-depleted mice contained a large number of antigen-specific CTLs at day 28, of the order of 1–1.5% of total $CD8^+$ cells. On re-stimulation by LCMV-infected or GP(33–41)-pulsed peritoneal I-A$\beta^{-/-}$ macrophages expressing high levels of CD80 and CD86 (data not shown), only CTLs primed in the presence of T_H cells are able to undergo secondary expansion (Fig. 4c, d; see also Supplementary Fig. S3). These results demonstrate that, as with T_H-dependent responses, T_H-independent LCMV-specific CTLs primed in the absence of $CD4^+$ cells also fail to expand on re-encounter with antigen.

Our results provide new insight into the nature of T-cell help by demonstrating that T_H cells are absolutely required for secondary, but not primary, CTL expansion. The progressive differentiation of lymphocytes involving initial CD4-independent and CD4-dependent events is reminiscent of the function performed by T_H cells in endowing B cells with the capacity for isotype switching[20]. When considered in terms of how T_H-dependent CTLs have traditionally been defined (that is, as lytic effectors after *in vitro* re-stimulation) our findings can explain a number of previous observations, including why the requirement for T_H cells has traditionally been seen as 'conditional'. When primed in the absence of T_H cells, antigens that induce a modest CD8 response will fail to generate cytotoxic effectors detected through standard *in vitro* cytoxicity assays, which rely on secondary expansion during re-stimulation. Antigens able to induce a large clonal expansion, in contrast, will yield CTL responses that are detectable directly *ex vivo* as well as in secondary cultures. Exceptions to this, as our study reveals, occur when a lymphocyte population containing a sufficiently large number of primary effectors are placed into *in vitro* culture. These can survive during re-stimulation and, despite failing to undergo secondary expansion, can nonetheless exhibit activity in subsequent *in vitro* cytotoxicity assays (Fig. 2a, b). This finding offers an explanation for the apparent dependence of transgenic CTLs on T-cell help on their precursor frequency in adoptive transfer experiments[21]. Conversely, the modest (average fivefold) expansion of re-stimulated CTLs and the relatively high sensitivity threshold of *in vitro* lysis assays may explain why $CD8^+$ T cells primed in the presence of T_H cells may nonetheless fail to be detected months after primary antigenic challenge[12]. The fact that added IL-2 was able to mask the secondary expansion defect in primary effectors suggests that re-stimulation protocols that feature addition of this

Table 1 **Secondary CTL expansion**

Day	Fold expansion	
	WT mice	CD4-depleted mice
7	5.1 ± 0.9	0.3 ± 0.3
14	4.7 ± 0.8	0.3 ± 0.3
21	4.0 ± 1.0	0.1 ± 0.1
28	5.0 ± 1.9	0.1 ± 0.1

Interferon-γ-positive (IFN-γ^+), E1B(192–200)-specific cytotoxic T lymphocytes (CTLs) were monitored before and after *in vitro* re-stimulation of splenocytes from wild-type (WT) and CD4-depleted mice at various times after immunization with $Tap^{-/-}$ 5E1 mouse embryo cells (MECs), as described in the Methods. The fold expansion is calculated from the increase in the absolute number of specific CTLs and represents the average of $n = 3$ mice. Similar results were observed in three separate experiments.

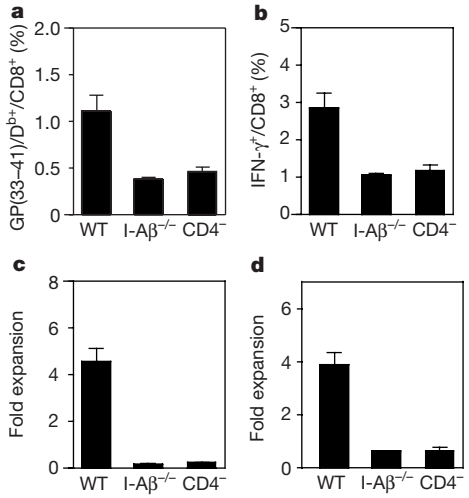

Figure 4 Defective secondary expansion of T_H-independent $CD8^+$ T cells primed in the absence of $CD4^+$ T-cell help. **a**, **b**, The frequency of GP(33–41)-specific T cells detected in total $CD8^+$ splenocytes of mice 28 days after lymphocytic choriomeningitis virus (LCMV) infection as determined by GP(33–41)/D^b tetramers (**a**) and IFN-γ production (**b**) after stimulation with GP(33–41) peptide. **c**, **d**, The fold expansion of GP(33–41)-specific $CD8^+$ T cells on secondary *in vitro* stimulation with I-A$\beta^{-/-}$ (MHC class $II^{-/-}$) macrophages that were infected with LCMV (**c**) or pulsed with GP(33–41) peptide (**d**).

cytokine, including limiting dilution assays, may greatly overestimate the frequency of 'memory' CTLs primed under various conditions[22]. Furthermore, our results also suggest that the attrition of CTLs primed in the absence of T_H cells, as observed for LCMV-specific CTLs in Fig. 4a, b, may result from contraction after encounter with persistent antigen rather than an intrinsic lack of CTL survival[23].

The ability of peripheral T cells to expand on secondary encounter with antigen is a crucial element of immune memory and protective immunity[5,6,24]. Our results indicate that helped CTLs are endowed with this capacity at a discreet step during their primary activation, and can express this memory phenotype as primary effectors as well as during and after their subsequent contraction phase. Our findings also suggest that a re-examination of the terms 'T_H-dependent' and 'T_H-independent' as applied to memory CTLs is needed, as many of the common methods used to monitor their physical or functional presence in short-term assays may instead detect primary effectors incapable of further clonal expansion[22,25]. This may be especially important in clinical settings where accurate monitoring of 'memory phenotype' CTLs is desired, such as in therapeutic vaccination strategies. Finally, our data indicate that the instructional programme guiding CTL development is not invariant and can be modified to include or exclude specific functional capacities. Using T-cell help as a starting point, future efforts will be directed towards understanding how the signals that are transmitted to CD8 T cells are integrated into the particular differentiation programme executed. Such information may increase our ability to manipulate effectively CTL responses in infectious disease, cancer and autoimmunity. □

Methods

Mice and cell lines

C57BL/6J, B6.SJL-P*tpcr*^{a} (B6/SJL) and B6.SJL/I-A$\beta^{-/-}$ (all H–2^b) mice were purchased from The Jackson Laboratory. Mice were maintained by in-house breeding at the La Jolla Institute for Allergy and Immunology, and were maintained under specific pathogen-free conditions in accordance with guidelines by the Association for assessment and Accreditation of Laboratory Animal Care International.

Tap$^{-/-}$ mouse embryo cell lines (MECs) expressing human adenovirus type 5 early region 1 (Ad5E1) were produced by transfection of both C57BL/6 Tap$^{+/+}$ and Tap$^{-/-}$ MEC lines and have been described previously[15]. The EL-4 thymoma and MC57 fibroblasts were purchased from the American Type Culture Collection. MECs were cultured in DMEM medium, and EL-4 and MC57 cells were cultured in IMDM medium. All media were supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 20 U ml^{-1} penicillin and 20 μg ml^{-1} streptomycin.

Immunizations and antibody treatment

Mice were immunized subcutaneously in the right flank with 1×10^7 irradiated (3,000 R) Tap$^{-/-}$ 5E1 MECs, or were inoculated intraperitoneally with 1×10^5 plaque-forming units of LCMV (Armstrong strain). Depletion of CD4$^+$ cells *in vivo* was performed by intraperitoneal administration of 150 μg GK1.5 antibody on the first three days before immunization and every third day thereafter[26]. In parallel experiments, GK1.5 was administered 3 days after immunization for 3 consecutive days.

Isolation of CD8$^+$ T cells

CD8$^+$ T cells were purified from the spleens and lymph nodes of previously immunized mice by antibody-directed complement lysis using antibodies to CD4 (GK1.5), class II MHC (M5/114, Y17 and CA-4.A12), B220 (RA3.6.B2), CD11b (M1/70), dendritic cells (33D1), natural killer cells (PK136) and heat-stable antigen (JIID), as described previously[7]. The resulting cells were greater than 95% pure CD8$^+$ cells, and contained less than 0.1% CD4$^+$ T cells, as demonstrated by fluorescence-activated cell sorting (FACS) analysis.

In vitro re-stimulations

For E1B-specific responses, splenocytes (or purified CD8$^+$ T cells) from Tap$^{-/-}$ 5E1 MEC-immunized mice were collected at different time points after immunization, and stimulated for 6 days *in vitro* with irradiated syngeneic Tap$^{+/+}$ Ad5E1 MECs (10:1 ratio). In parallel cultures, 10 ng ml^{-1} IL–2 (Cetus) was added. For LCMV-specific responses, splenocytes derived from LCMV-infected mice were stimulated for 7 days *in vitro* at a 10:1 ratio with irradiated (3,000 R) syngeneic I-A$\beta^{-/-}$ thioglycollate-induced macrophages infected with LCMV or pulsed with GP(33–41) peptide (KAVYNFATC; 0.5 μg ml^{-1}). At the end of all re-stimulation cultures, viable cells were collected by Ficoll gradient (Lympholyte-M; Cedarlane Laboratories).

Enumeration of specific CD8$^+$ T cells

Directly *ex vivo*, after CD8 purification or after *in vitro* re-stimulation, cells were incubated for 5 h with either the E1B(192–200) peptide (VNIRNCCYI; 0.5 μg ml^{-1}), GP(33–40) (KAVYNFATC; 0.2 μg ml^{-1}) or OVA(257–264) (SIINFEKL; 0.2–0.5 μg ml^{-1}) in the presence of brefeldin A. Surface staining for CD8 and intracellular cytokine staining for IFN-γ and tumour necrosis factor-α were performed using a Cytofix/Cytoperm Kit (Pharmingen) according to the manufacturer's directions. D^b/GP(33–41) tetramer staining was performed as described previously[19]. The fold expansion of specific CD8$^+$ cells was calculated by dividing the absolute number of IFN-γ$^+$ CD8$^+$ cells after *in vitro* culture by the absolute number of IFN-γ$^+$ CD8$^+$ cells at the start of the culture.

Cytotoxicity assays

In vivo cytolytic activity was determined using B6.SJL spleen cells differentially labelled with the fluorescent dye CFSE[17]. The cells labelled with CFSE^{high} were used as targets and pulsed with E1B(192–200) peptide (0.5 μg ml^{-1}; 90 min at 37 °C, 5% CO_2), whereas the cells labelled with CFSE^{low} were pulsed with OVA(257–264) (0.5 μg ml^{-1}) to serve as the internal control. Peptide-pulsed target cells were extensively washed to remove free peptide and then co-injected intravenously in a 1:1 ratio to previously immunized B6 mice. Sixteen hours later, spleens were removed and the ratio of CFSE^{low}/CFSE^{high} cells was determined by flow cytometry. The values in the upper left corner of each panel represents the per cent loss of E1B(192–200)-pulsed (CFSE^{high}) target cells.

In vitro cytolytic activity of re-stimulated splenocytes of Ad5E1 MEC-immunized mice was evaluated by a JAM test as previously described[27], using [^{3}H]thymidine-labelled EL-4 cells loaded with E1B(192–200) peptide or OVA(257–264) peptide. Specific killing was calculated as follows: (spontaneous c.p.m − experimental c.p.m.) × 100/spontaneous c.p.m., where c.p.m. is counts per minute. LCMV-specific cytolytic activity was assessed in a standard 5-h ^{51}Cr release assay on LCMV-infected and uninfected MC57 cells, and plotted as a percentage of the maximum lysis after subtracting the nonspecific lysis (always less than 10%).

Data analysis

Unless stated otherwise, data are expressed as mean ± standard error of the mean, and evaluated using an analysis of variance followed by a Dunnett test. A probability value of $P < 0.05$ was considered statistically significant.

Received 3 December 2002; accepted 23 January 2003; doi:10.1038/nature01441.
Published online 9 February 2003.

1. Buller, R. M., Holmes, K. L., Hugin, A., Frederickson, T. N. & Morse, H. C. III Induction of cytotoxic T-cell responses *in vivo* in the absence of CD4 helper cells. *Nature* **328,** 77–79 (1987).
2. Rahemtulla, A. *et al.* Normal development and function of CD8$^+$ cells but markedly decreased helper cell activity in mice lacking CD4. *Nature* **353,** 180–184 (1991).
3. Schoenberger, S. P., Toes, R. E., van der Voort, E. I., Offringa, R. & Melief, C. J. T-cell help for cytotoxic T lymphocytes is mediated by CD40–CD40L interactions. *Nature* **393,** 480–483 (1998).
4. Bennett, S. R., Carbone, F. R., Karamalis, F., Miller, J. F. & Heath, W. R. Induction of a CD8 + cytotoxic T lymphocyte response by cross-priming requires cognate CD4 + T cell help. *J. Exp. Med.* **186,** 65–70 (1997).
5. Sprent, J. & Surh, C. D. T cell memory. *Annu. Rev. Immunol.* **20,** 551–579 (2002).
6. Ahmed, R. & Gray, D. Immunological memory and protective immunity: understanding their relation. *Science* **272,** 54–60 (1996).
7. van Stipdonk, M. J., Lemmens, E. E. & Schoenberger, S. P. Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nature Immunol.* **2,** 423–429 (2001).
8. Kaech, S. M. & Ahmed, R. Memory CD8 + T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nature Immunol.* **2,** 415–422 (2001).
9. Badovinac, V. P., Porter, B. B. & Harty, J. T. Programmed contraction of CD8(+) T cells after infection. *Nature Immunol.* **3,** 619–626 (2002).
10. Keene, J. & Forman, J. Helper activity is required for the *in vivo* generation of cytotoxic T lymphocytes. *J. Exp. Med.* **155,** 768–782 (1982).
11. Bennett, S. R. *et al.* Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* **393,** 478–480 (1998).
12. Ridge, J. P., Di Rosa, F. & Matzinger, P. A conditioned dendritic cell can be a temporal bridge between a CD4$^+$ T-helper and a T-killer cell. *Nature* **393,** 474–478 (1998).
13. Matzinger, P. Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* **12,** 991–1045 (1994).
14. Lanzavecchia, A. Immunology. Licence to kill. *Nature* **393,** 413–414 (1998).
15. Schoenberger, S. P. *et al.* Cross-priming of CTL responses *in vivo* does not require antigenic peptides in the endoplasmic reticulum of immunizing cells. *J. Immunol.* **161,** 3808–3812 (1998).
16. Grusby, M. J., Johnson, R. S., Papaioannou, V. E. & Glimcher, L. H. Depletion of CD4 + T cells in major histocompatibility complex class II-deficient mice. *Science* **253,** 1417–1420 (1991).
17. Oehen, S. & Brduscha-Riem, K. Differentiation of naive CTL to effector and memory CTL: correlation of effector function with phenotype and cell division. *J. Immunol.* **161,** 5338–5346 (1998).
18. Hudrisier, D., Oldstone, M. B. & Gairin, J. E. The signal sequence of lymphocytic choriomeningitis virus contains an immunodominant cytotoxic T cell epitope that is restricted by both H-2D(b) and H-2K(b) molecules. *Virology* **234,** 62–73 (1997).
19. Busch, D. H., Pilip, I. M., Vijh, S. & Pamer, E. G. Coordinate regulation of complex T cell populations responding to bacterial infection. *Immunity* **8,** 353–362 (1998).
20. Parker, D. C. T cell-dependent B cell activation. *Annu. Rev. Immunol.* **11,** 331–360 (1993).
21. Mintern, J. D., Davey, G. M., Belz, G. T., Carbone, F. R. & Heath, W. R. Cutting edge: precursor frequency affects the helper dependence of cytotoxic T cells. *J. Immunol.* **168,** 977–980 (2002).
22. Di Rosa, F. & Matzinger, P. Long-lasting CD8 T cell memory in the absence of CD4 T cells or B cells. *J. Exp. Med.* **183,** 2153–2163 (1996).
23. Klenerman, P., Hengartner, H. & Zinkernagel, R. M. A non-retroviral RNA virus persists in DNA form. *Nature* **390,** 298–301 (1997).
24. Kaech, S. M., Wherry, E. J. & Ahmed, R. Effector and memory T-cell differentiation: implications for vaccine development. *Nature Rev. Immunol.* **2,** 251–262 (2002).
25. Suresh, M. *et al.* Role of CD28-B7 interactions in generation and maintenance of CD8 T cell memory. *J. Immunol.* **167,** 5565–5573 (2001).
26. Dialynas, D. P. *et al.* Characterization of the murine T cell surface molecule, designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human Leu-3/T4 molecule. *J. Immunol.* **131,** 2445–2451 (1983).

27. Matzinger, P. The JAM test. A simple assay for DNA fragmentation and cell death. *J. Immunol. Methods* **145,** 185–192 (1991).

Supplementary Information accompanies the paper on *Nature*'s website (http://www.nature.com/nature).

Acknowledgements This work was supported by grants from the National Institutes of Health, the American Cancer Society, and CaP CURE to S.P.S. The authors thank G. Palioungas for technical assistance and the staff of the LIAI animal facility for animal care.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to S.P.S. (e-mail: sps@liai.org).

STAT3 signalling is required for leptin regulation of energy balance but not reproduction

Sarah H. Bates*, Walter H. Stearns*, Trevor A. Dundon*, Markus Schubert*, Annette W. K. Tso*, Yongping Wang†, Alexander S. Banks*, Hugh J. Lavery*, Asma K. Haq* Eleftheria Maratos-Flier*, Benjamin G. Neel†, Michael W. Schwartz‡ & Martin G. Myers Jr*

** Research Division, Joslin Diabetes Center, and † Cancer Biology Program, Beth Israel Deaconess Medical Center and Harvard Medical School, 1 Joslin Place, Boston, Massachusetts 02215, USA*
‡ Harborview Medical Center, University of Washington, Seattle, Washington 98122, USA

Secretion of leptin from adipocytes communicates body energy status to the brain by activating the leptin receptor long form (LRb). LRb regulates energy homeostasis and neuroendocrine function; the absence of LRb in *db*/*db* mice results in obesity, impaired growth, infertility and diabetes[1–4]. Tyr 1138 of LRb mediates activation of the transcription factor STAT3 during leptin action[5–8]. To investigate the contribution of STAT3 signalling to leptin action *in vivo*, we replaced the gene encoding the leptin receptor (*lepr*) in mice with an allele coding for a replacement of Tyr 1138 in LRb with a serine residue (*lepr*^{S1138}) that specifically disrupts the LRb–STAT3 signal. Here we show that, like *db*/*db* mice, *lepr*^{S1138} homozygotes (*s*/*s*) are hyperphagic and obese. However, whereas *db*/*db* mice are infertile, short and diabetic, *s*/*s* mice are fertile, long and less hyperglycaemic. Furthermore, hypothalamic expression of neuropeptide Y (NPY) is elevated in *db*/*db* mice but not *s*/*s* mice, whereas the hypothalamic melanocortin system is suppressed in both *db*/*db* and *s*/*s* mice. LRb–STAT3 signalling thus mediates the effects of leptin on melanocortin production and body energy homeostasis, whereas distinct LRb signals regulate NPY and the control of fertility, growth and glucose homeostasis.

To test the requirement for LRb–STAT3 signalling in the physiological functions of leptin, we generated the *lepr*^{S1138} mutant by homologous gene targeting (Fig. 1a). LRb^{S1138}, the protein encoded by *lepr*^{S1138}, fails to activate STAT3 although it is expressed normally on the cell surface and mediates other leptin signals normally (Fig. 1b, c)[8]. We used these targeted 129Sv embryonic stem cell clones to produce chimaeric animals, which were crossed with C57Bl/6 mice to generate heterozygous 'knock-in' mice expressing LRb^{S1138} in a manner identical to wild-type LRb. Interbreeding of heterozygous animals yielded wild-type (+/+), heterozygous (*s*/+) and homozygous (*s*/*s*) mice for study.

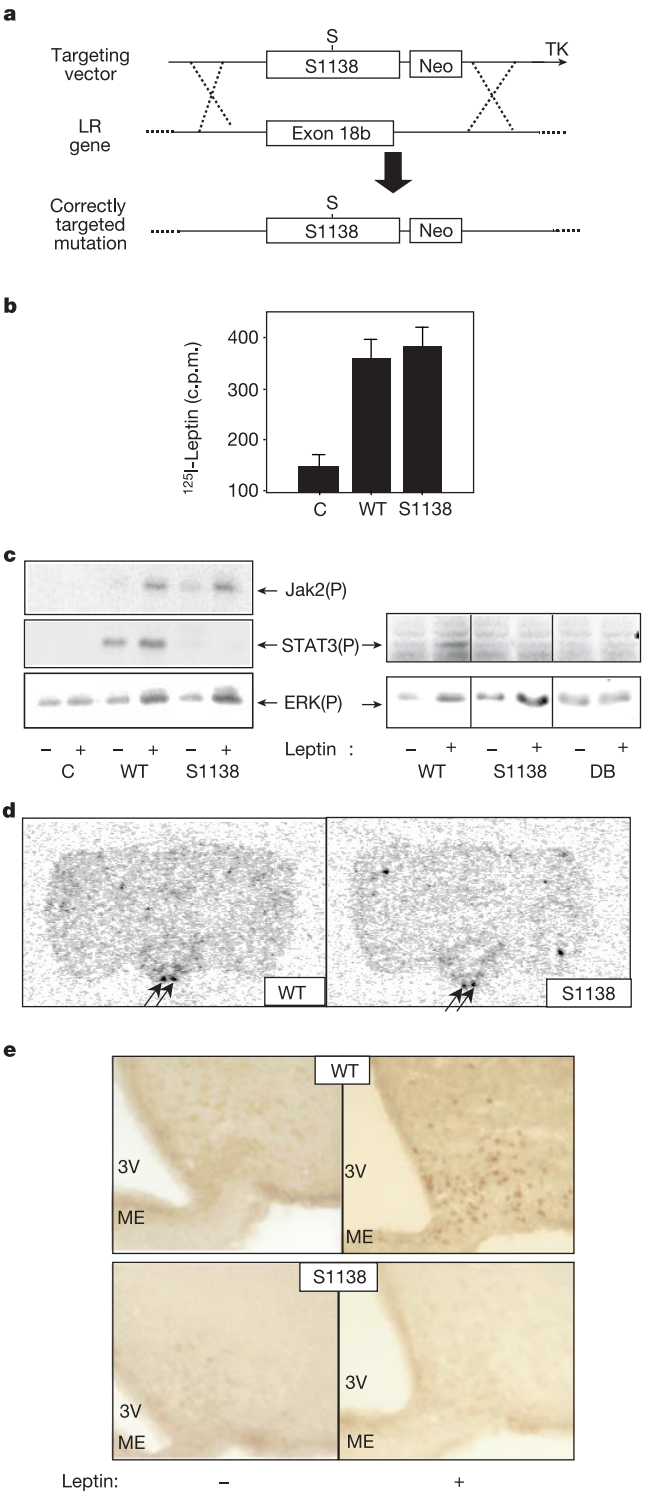

Figure 1 Generation of mice expressing LRb^{S1138}. **a**, Diagram of gene-targeting strategy to replace the LRb-specific exon 18b with the mutant exon S1138 (S; Tyr 1138 → Ser). **b**, Cell-surface ^{125}I-leptin binding by LRb^{S1138} in COS-7 cells (C), expressing LRb (WT) or LRb^{S1138} (S1138). **c**, Signalling by LRb^{S1138} in HEK-293 cells (left panels) and hypothalamus (right panels). Shown are STAT3, Jak2 and ERK phosphorylation after treatment with leptin (+) or vehicle (−). **d**, Distribution of LRb and LRb^{S1138} expression by *in situ* hybridization with LRb-specific probes (see Supplementary Information). LRb probe hybridization is indicated (arrows). **e**, Leptin-stimulated nuclear translocation of STAT3 in the hypothalamus. Hypothalamic sections from wild-type or S1138 mice treated with saline (−) or leptin (+) were immunostained for STAT3 (see Supplementary Information). Nuclear STAT3 is visible as distinct darkly staining nuclei. Third ventricle (3V) and median eminence (ME) are indicated. Mice: +/+ (wild type; WT), *s*/*s* (S1138), *db*/*db* (DB).