TaqMan and northern blot analyses

PolyA[+] RNA and total RNA were extracted from homogenized mice livers using the Fast Track 2.0 (Invitrogen) or the RNeasy (Qiagen) kit. RNA samples were treated with DNase I (Promega) and RNA quality was assessed by gel electrophoresis. cDNA was prepared by reverse transcription of 250 ng mRNA or 500 ng total RNA using the Superscript II enzyme and Oligo dT primer (GIBCO BRL). The resulting cDNAs were amplified using the SYBR green PCR kit and an ABIPRISM 7700 Sequence detector (Perkin Elmer). All mRNA expression data from the TaqMan PCR with reverse transcription was normalized to GAPDH expression in the corresponding sample. Northern blot assays were performed as previously described[25].

Protein analysis

Protein was extracted from frozen liver samples in SDS-urea-lysis buffer and 20 μg of protein were loaded onto a 12% SDS–polyacrylamide gel and blotted onto nitrocellulose membrane. Western blot assays were performed as previously described[25].

Chromatin immunoprecipitation assay

Human hepatoma HepG2 cells were grown to 90% confluence, and the chromatin immunoprecipitation assay was performed as described elsewhere[27]. Specific antibodies against either full-length CREB (253) or the kinase inducible domain (KID) (244) of CREB were used for the immunoprecipitation. Normal rabbit IgG served as a negative control. Precipitated DNA fragments were analysed by PCR amplification using primers directed against the human PGC-1 promoter region, or the GAPDH coding region as negative control.

Plasmids

Expression plasmids pZEO-A-CREB[25] and pcDNA3-PGC-1[28] have been described previously. The liver-specific A-CREB transgene was constructed by inserting A-CREB cDNA into a liver-specific albumin promoter/enhancer plasmid[8]. To construct luciferase expression plasmids −490PEPCK-Luc and −355PEPCK-Luc *Bam*HI/*Bgl*II fragments of the PEPCK promoter region containing 490 or 355 base pairs (bp) of the mouse PEPCK 5′-flanking region, respectively, were cloned into the pGL3 basic luciferase reporter vector (Promega). PGC-1 reporter mPGC-Luc was constructed by inserting a 230-bp fragment of the mouse PGC-1 promoter containing 170 bp of the 5′-flanking region and 68 bp of exon 1 into the pGL3 vector.

Cell culture and transfection assays

Human hepatoma HepG2 cells were transfected using the Lipofectamine 2000 reagent (GIBCO BRL) according to the manufacturer's instructions (500 ng of indicator plasmid per well). Where indicated, expression plasmids encoding A-CREB (pZeo-A-CREB)[25] or PGC-1 (pcDNA3-PGC-1)[28] were cotransfected (50 ng and 800 ng plasmid per well, respectively). Cotransfections were performed with a constant amount of DNA by adding the empty pcDNA3 vector (Invitrogen). Cells were treated with forskolin (10 μM) and/or dexamethasone (100 nM) or insulin (100 nM) for 14 h. Cell extracts were prepared 48 h after transfection and the luciferase assay was performed as described previously[29], normalizing to activity from cotransfected Rous sarcoma virus-β-galactosidase expression plasmid (100 ng plasmid per well).

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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erratum

Roles of tumour localization, second signals and cross priming in cytotoxic T-cell induction

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In this Letter, the first name of Ian Hermans was misspelled as 'Jan'. □

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Roles of tumour localization, second signals and cross priming in cytotoxic T-cell induction

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The vertebrate immune system has evolved to protect against infections that threaten survival before reproduction. Clinically manifest tumours mostly arise after the reproductive years and somatic mutations allow even otherwise antigenic tumours to evade the attention of the immune system[1–3]. Moreover, the lack of immunological co-stimulatory molecules on solid tumours could result in T-cell tolerance[4–8]; that is, the failure of T cells to respond. However, this may not generally apply[9,10]. Here we report several important findings regarding the immune response to tumours, on the basis of studies of several tumour types. First, tumour-specific induction of protective cytotoxic T cells (CTLs) depends on sufficient tumour cells reaching secondary lymphatic organs early and for a long enough duration. Second, diffusely invading systemic tumours delete CTLs. Third, tumours that stay strictly outside secondary lymphatic organs, or that are within these organs but separated from T cells by barriers, are ignored by T cells but do not delete them. Fourth, co-stimulatory molecules on tumour cells do not influence CTL priming but enhance primed CTL responses in peripheral solid tumours. Last, cross priming of CTLs by tumour antigens, mediated by major histocompatibility complex (MHC) class I molecules of antigen-presenting host cells, is inefficient and not protective. These rules of T-cell induction and maintenance not only change previous views but also rationales for anti-tumour immunotherapy[1,2].

Tumours may escape the attention of the immune system and metastasize through various mechanisms, including loss of tumour or MHC antigens and/or the expression of inhibitory molecules[1,2]. However, even antigenic tumours can grow successfully and may persist in a host with an intact, fully functioning immune system[2,3]. This is reminiscent of immunity against pathogens where peripheral, low-level infections coexist with T-cell immunity, resulting in the immune system being incapable of clearing all peripheral infected cells[11]. Failure of immunity against tumours has been explained by deletion of T cells that interact with tumour cells lacking co-stimulatory signals[4–8]. However, a fibrosarcoma was shown not to delete CTLs but rather to prime them directly if tumour cells reached secondary lymphoid tissues early[9,10]. These results were termed as an exception and questioned because antigen-presenting cells (APCs) of the host may have reprocessed tumour antigen onto MHC class I molecules (termed 'cross priming')[12–16]. Such cross priming has been observed *in vitro*, and occasionally also at the peptide level *in vivo*[12,13,17]. The relative role of CTLs versus $CD4^+$ T-helper cells in cross priming *in vivo* remains unclear[17,18].

We studied MC57G (C57BL/6 strain, haplotype $H-2^b$) and D2 (B10.D2, $H-2^d$) fibrosarcoma and EL4 (C57BL/6, $H-2^b$) lymphoma cells transfected with the entire lymphocytic choriomeningitis virus glycoprotein (LCMV-GP) as tumour antigens (MC-GP, D2-GP and EL4-GP)[9,10], and also the Lewis lung carcinoma (LL) (C57BL/6, $H-2^b$) (LL-GP33)[19], EL4 lymphoma (EL4-GP33) and B16.F10 melanoma (C57BL/6, $H-2^b$) (B16-GP33)[20] cell lines transfected with the GP33 D^b epitope. P815 mastocytoma cells (DBA/2, $H-2^d$) were transfected with the entire LCMV nucleoprotein (P815-NP). MC-GP, LL-GP33 and EL4-GP33 cells expressed high levels of MHC class-I D^b, whereas B16-GP33 cells expressed lower levels (Fig. 1A, a). Incubation in 10 U ml^{-1} interferon-γ (IFN-γ) for 48 h increased $H-2D^b$ expression 100-fold (Fig. 1A, c). MC-GP and LL-GP33 cells did not express B7.1, B7.2, lymphocyte function-associated antigen (LFA-1) or intercellular adhesion molecule-1 (ICAM-1) above background levels. B16-GP33 cells expressed ICAM-1 at very low levels (not shown) and EL4-GP33 exhibited B7.1, LFA-1 and ICAM-1, but not B7.2 (Fig. 1A, b). CTL-target-cell qualities of all cell lines were analysed (Fig. 1A, d); they confirmed earlier experiments[10,19,20].

We first established that strictly peripheral, extralymphatic tumours are ignored by the immune system. We injected single-cell suspensions or small tumour fragments of $2-5\times10^6$ tumour cells subcutaneously[10]. We then compared tumour growth, CTL induction and migration of the transferred tumour cells to secondary lymphoid organs. Eleven of 20 EL4-GP33 (Fig. 1B, a) pieces grew. All transplanted tumour pieces grew for LL-GP33 (Fig. 1B, e) and B16-GP33; 15 of 20 MC-GP pieces grew (not shown but see ref. 10). Without exception tumour growth correlated with absence of primed CTLs (Fig. 1B, b, d and f). Absence of viable (or polymerase chain reaction (PCR)-detectable[10]) tumour cells in secondary lymphoid organs (Table 1) correlated with absence of primed CTLs (Fig. 1B, b, d and f). In contrast, rejection of EL4-GP33 and MC-GP tumour pieces paralleled induction of GP33-specific CTLs (Fig. 1B, b). Distinct from tumour fragments, MC-GP and EL4-GP33 cell suspensions injected subcutaneously in titrated doses from 10^4 to 10^8 cells were all rejected, but they did prime CTLs (Fig. 1B, a and b). In mice depleted of natural killer and $CD8^+$ T cells, MC-GP and EL4-GP33 tumour cells were detectable by selective culture (or by PCR[10]) in local lymph nodes after injection of cells, but not of tumour fragments (Table 1). Rejection of 50% of EL4-GP33 tumours (Fig. 1B, a) correlated with the fragility of tumour fragments and cells reaching lymphoid organs at variable times: in mice depleted of $CD8^+$ and natural killer cells, EL4-GP33 cells were found in lymph nodes in 1 of 4 mice on day 12 and in 3 of 4 mice on day 16 after transplantation. Notably, 9 of 14 transplanted MC-GP tumour pieces grew in minor histo-incompatible C57BL/10 mice (see Supplementary Information Fig. 1) and 2 of 4 grew in BALB/b mice. Thus, cells that are strictly peripheral failed to induce GP33-specific CTLs even when minor histo-incompatibility antigens provided additional CTL-epitopes and could induce $CD4^+$ T-helper cells.

B16-GP33 and LL-GP33 cells also followed the pattern that

Table 1 Homing of tumour cells to secondary lymphoid organs

Tumour cells or fragments*	Detection of tumour cells†			
	Day 4		Day 8	
	Lymph node	Spleen	Lymph node	Spleen
MC-GP fragment	0/4	0/4	0/4	0/4
MC-GP cells	2/4	3/4	3/4	4/4
EL4-GP33 fragment	0/8	0/8	0/8	0/8
EL4-GP33 cells	0/8	1/4	6/6	4/4
3LL-GP33 fragment	0/4	0/4	0/4	0/4
3LL-GP33 cells	0/8	0/4	0/8	0/4
B16-GP33 fragment	0/4	0/4	0/4	0/4
B16-GP33 cells	0/8	0/4	0/8	0/4

* Cells (2×10^6) or solid tumour fragments (containing $2-5\times10^6$ tumour cells were transplanted to both flanks.
† To avoid immediate rejection mice were $CD8^+$ T-cell-depleted by treatment with 200 μl anti-CD8 antibody (YTS169.4.2) i.p. 3 or 1 days[10] before cell or fragment transfer, and depleted of NK cells by 30 μl of anti-asialo GM1 antibody (Wako Biochemicals) i.v. on 1 day before transfer. Lymph nodes and spleen cell suspensions were cultured in selection medium (G-418). Values indicate positive detection in number of mice over total number tested.

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tumour growth correlated with absence of primed CTLs; $\geq 10^4$ single cells of B16-GP33 and LL-GP33 grew subcutaneously in all animals, as found earlier[19,20] (Fig. 1B, c and e). No viable tumour cells were detected in draining lymph nodes 4–8 days after injection (Table 1) and no CTLs were induced (Fig. 1B, d and f). Growing tumours were not CTL-escape mutants as they expressed D^b-GP33 as targets and were susceptible to CTLs (not shown).

The correlation between tumour growth and, at early time points, absence of viable tumour cells in lymphoid organs and the absence of an anti-tumour CTL response is not called into question by the rejection of a small number of tumour pieces. Sometimes few but sufficient tumour cells may reach draining lymph nodes later, as shown for EL4-GP33. Although this is difficult to quantify, about 10^3 to 10^4 cells (MC-GP, EL4-GP33) seem necessary (Fig. 1D, b). This conclusion is strengthened by experiments with mice lacking all lymph nodes (see below). Notably, in all tumour models, tumour-specific CTLs were neither anergized nor deleted in tumour-bearing mice, as immunization with LCMV (or antigenic dendritic or tumour cells[10]) promptly primed CTLs (Fig. 1B, b, d and f).

CTLs were primed by 1,000 or more live MC-GP and EL4-GP33 (but not by up to 10^6 B16-GP33 and LL-GP33) tumour cells injected directly into the spleen (Fig. 1C, a and b versus c and d, respectively). Instead, B16-GP33 and LL-GP33 cells grew as solid tumours up to a diameter of 2 cm (see also Fig. 2). When tumour cells labelled with fluorescent dye (CFSE) were analysed immunohistochemically during the first four days, EL4-GP33 were diffusely distributed throughout the spleen as single cells (not shown). MC-GP cells were intermixed with $CD8^+$ T cells (Fig. 2a, d, g). In contrast, B16-GP33 (Fig. 2b, e, h) and LL-GP33 tumours (Fig. 2c, f, i) grew solidly with only minimal CTL infiltration. Of note, the LL-GP33 and B16-GP33 cells were surrounded by haemostasis factors (factor VIII-related antigen surrounding B16-GP33 (Fig. 2k)) or collagen (surrounding B16-GP33 (Fig. 2n) and LL-GP33 (Fig. 2o)) when analysed 14 days

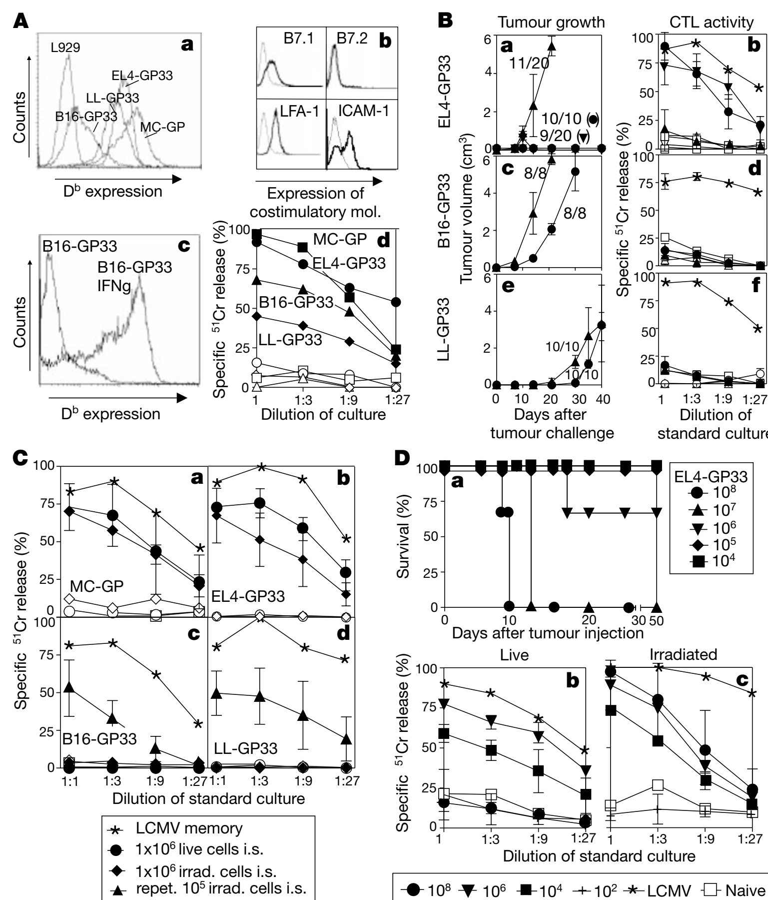

Figure 1 Ignorance, priming and tolerance of tumour-specific T cells. **A**, Characterization of the tumour cell lines. **a–d**, D^b expression (**a**), expression of second signals or adhesion markers on EL4-GP33 cells (**b**), D^b expression on B16-GP33 cells after 10 U IFN-γ for 48 h (**c**), lysis of MC-GP, EL4-GP33, LL-GP33 and B16-BP33 cells by LCMV-GP33-specific CTLs (**d**). Open symbols, untransfected control tumour cells. **B**, Comparison of tumour take versus induction of anti-tumour CTLs. **a–f**, EL4-GP33 (**a**, **b**), B16-GP33 (**c**, **d**) and LL-GP33 (**e**, **f**) single cells injected subcutaneously (filled circles) or implanted as small tumour pieces (filled triangles). Tumour growth (**a**, **c**, **e**) and secondary CTL activity (**b**, **d**, **f**) in mice with growing (filled, normal triangle) or rejected (filled, inverted triangle) tumours (numbers indicate growing tumours over the total number of transferred tumours). Secondary CTL activity against LCMV-GP33 targets after 5 days *in vitro* re-stimulation of spleen cells taken 8 days after injection of the tumour-cell suspension using unpulsed (open symbols) or GP33-pulsed (filled symbols) EL-4 (**d**, **f**) or MC57 (**b**, to avoid cross reactivities on EL4) as target cells. For tumour fragment experiments, CTL activity was similarly assessed at the end of the experiment (after ~20 days for EL-4-GP33 and B16-GP33 (**b**, **d**) and 40 days for LL-GP33 (**f**)). CTL activity is given for each group as mean ± s.d. Some tumour-bearing mice were immunized with LCMV and 8 days later cytotoxicity of GP33 specific CTLs was assessed (asterisks). **C** Secondary CTL responses induced by 10^6 live tumour cells. **a–d**, MC-GP (**a**), EL4-GP33 (**b**), B16-GP33 (**c**) and LL-GP33 (**d**) cells injected directly into the spleen once (circles) and by single (diamonds) or repetitive (3 times on alternating days, CTL analysis 5 days after the last injection) (triangles) injection of irradiated tumour cells (MC-GP, 80 Gy; EL4-GP33, 40 Gy; B16-GP33, 200 Gy; LL-GP33, 100 Gy. LCMV-primed mice (200 PFU LCMV day 60) were used as controls (asterisks). **D,** Survival of C57BL/6 mice given EL4-GP33 lymphoma intravenously (**a**). Titrated numbers of 10^2 to 10^8 live (**b**) or irradiated (40 Gy); (**c**) EL4-GP33 cells were injected intravenously. Eight days later the secondary GP33-specific CTLs were assessed (mean ± s.d. of 3 to 4 mice per group). All experiments were done twice with similar results (see text).

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after intrasplenic injection. In the absence of primed CTLs this suggested that naïve T cells were separated from antigen-expressing tumour cells and therefore were not induced. IFNγ-treated B16-GP33 cells expressing high levels of the D^b epitope (Fig. 1A, c) formed comparably separated tumours (as in Fig. 2h, k, n) and also failed to prime CTLs after splenic injection of 2×10^6 cells. Thus, low immunogenicity of B16-GP33 cells did not correlate with low expression of MHC class I but rather with barrier formation. When these tumour-bearing mice were immunized with 200 plaque-forming units (PFU) of LCMV by intraperitoneal injection, activated CTLs massively infiltrated splenic tumours 6 days later (Fig. 2p, q; MC-GP tumours were rejected at this time and cannot be shown), thereby negating suppression or tumour inaccessibility explanations.

To avoid cell proliferation and formation of barriers, irradiated cells (MC-GP, 80 Gy; EL4-GP33, 40 Gy; B16-GP33, 200 Gy; LL-GP33, 100 Gy: determined minimal irradiation doses) were injected directly into the spleen. A single injection of $\geq 10^4$ irradiated MC-

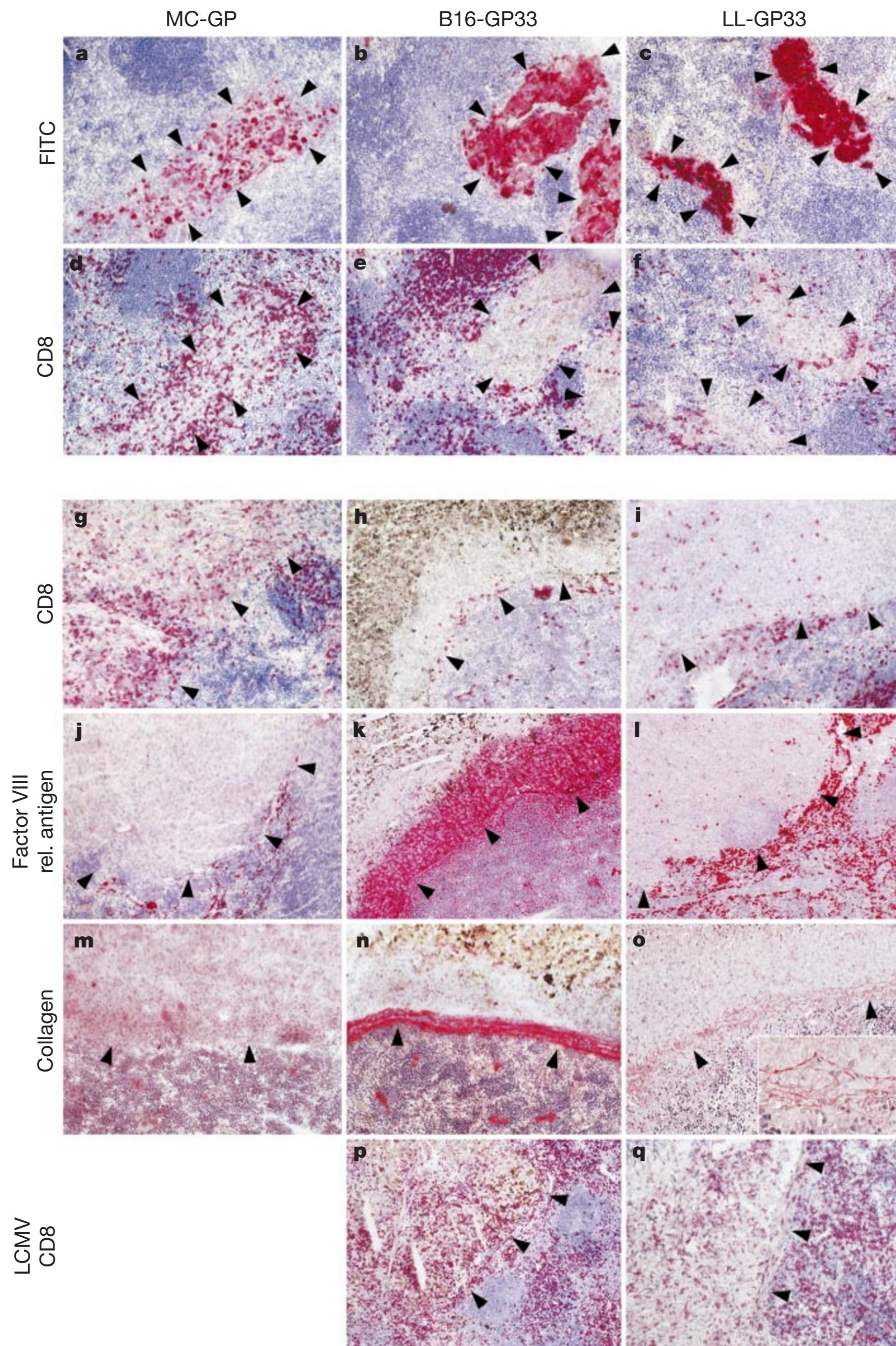

Figure 2 Analysis of tumour growth in lymphoid organs. CFSE-labelled MC-GP, EL4-GP33, B16-GP33 and LL-GP33 cells (10^6) were injected directly into the spleen. **a**–**c**, Anit-fluorescein isothiocyanate (FITC) staining of tumour cells 48 h after injection. **d**–**f**, Distribution of $CD8^+$ T cells assessed by anti-$CD8^+$ staining. **g**–**q**, Fourteen days after injection of B16-GP33 (**h**, **k**, **n**, **p**) and LL-GP33 (**i**, **l**, **o**, **q**), and 8 days after injection of MC-GP (**g**, **j**, **m**) cells, spleens were stained for $CD8^+$ T cells (**g**–**i**), factor VIII-related antigen (**j**–**l**) or collagen (**m**–**o**). Eight days after intrasplenic injection of B16-GP33 (**p**) or LL-GP33 (**q**) tumour cells the recipient mice were infected intravenously with 200 PFU LCMV. After 6 days, the resulting immune response was analysed by anti-$CD8^+$ T-cell staining. One out of three comparable experiments is shown.

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GP or EL4-GP33 cells primed CTLs (Fig. 1C, a, b and D, c). In contrast, one dose of 10^6 irradiated B16-GP33 or LL-GP33 cells alone did not prime CTLs (Fig. 1C, c and d). However, three repetitive intrasplenic injections of 10^5 irradiated tumour cells on alternating days induced GP33-specific CTLs (Fig. 1C, c and d). Thus, compared with MC-GP or EL4-GP33 cells, about 10 times more B16-GP33 or LL-GP33 tumour cells were necessary to induce CTLs, perhaps owing to lower antigen or MHC class I expression (Fig. 1A, a and d). Importantly, 10–1,000 times fewer irradiated, and therefore short-lived, tumour cells (B16-GP33 or LL-GP33) primed naïve CTLs in spleens; whereas huge numbers of living and multiplying but barrier-separated cells in, or tumours strictly outside of, lymphatic organs failed to induce CTLs.

These relatively low numbers ($\geq 10^4$) of CTL-accessible tumour cells in secondary lymphoid organs during minimal periods (4–6 days) seem necessary and sufficient for CTL-induction. The data also indicate that perhaps some widely used tumours (such as B16 and LL multiply passaged *in vivo*) have been selected to evade immunosurveillance by rapidly inducing barriers that isolate them from T cells. Such mechanisms may be one basis of metastatic tumour growth in lymphoid tissues.

Immunosurveillance of tumours spreading diffusely within lymphoid tissues was studied using T-cell lymphomas EL4-GP33 and mastocytomas P815-NP injected intravenously. Attempts to intravenously inject $\geq 10^6$ MC-GP or LL-GP33 cells failed owing to embolisms. After an intravenous injection of $> 10^6$ EL4-GP33 cells, hosts died within 9–18 days (Fig. 1D, a). About 10^4 to 10^6 live or $\geq$ 10^4 irradiated EL4-GP33 cells (40 Gy) primed GP33-specific CTLs (Fig. 1D, b and c). Injection of high doses ($\geq 10^7$) of irradiated, but not of live, EL4-GP33 tumour cells induced CTLs (Fig. 1D, b and c). Similarly, $\geq 10^4$ irradiated P815-NP cells primed CTLs in DBA/2 mice, whereas $> 10^6$ live P815-NP cells induced CTL unresponsiveness as assessed by *in vitro* re-stimulation (not shown). However, excessive tumours in lymphoid organs might mask CTL induction

Table 2 Anti-LCMV protection in mice primed with 10^7 or 10^5 EL4-GP33 cells

Immunization	Cell dose	LCMV titre (log_{10})* Spleen	LCMV titre (log_{10})* Liver
–	10^5	3.2 ± 0.2†‡	<1.7†§
–	10^7	4.6 ± 0.4 NS	3.7 ± 0.8 NS
200 PFU LCMV (0)	10^7	<1.7§	<1.7§
200 PFU LCMV (5)	10^7	<1.7§	<1.7§
2×10^6 Vacc-YN4 (14)	10^7	<1.7§	<1.7§
–	0	5.0 ± 0.5‖	3.6 ± 0.2‖
200 PFU LCMV (14)	0	<1.7§	<1.7§

Numbers in parentheses indicate the day of immunization before tumour challenge. NS, not significant.
* Mice were infected 12 days after tumour injection with 10^4 PFU LCMV i.v. and LCMV titres per organ were assessed 4 days later. Results are given as mean ± s.d. of 3–5 animals per group. The experiment was repeated twice with comparable results.
† LCMV-WE replicates to higher titres in spleen than in liver. Overall 100-fold titre reduction by EL4-GP33-priming caused titres to drop in the liver below detection level, whereas in the spleen LCMV was still detectable in a plaque-forming assay.
‡ Students *t*-test (versus untreated mice (‖)); $P < 0.5$.
§ Students *t*-test (versus untreated mice (‖)); $P < 0.001$.
‖ Untreated mice infected with 10^4 PFU LCMV.

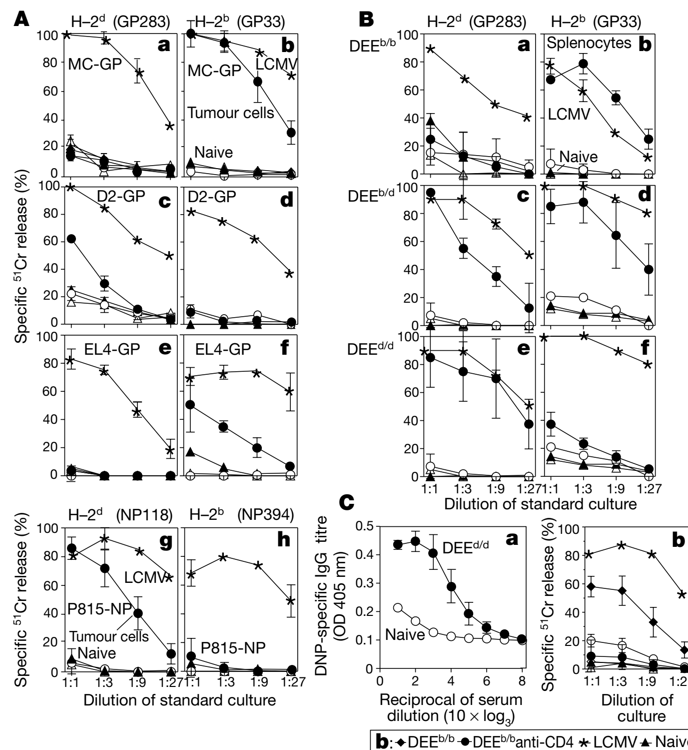

Figure 3 Antigen presentation for an anti-tumour immune response. **A**, CTL induction by 5×10^6 MC-GP (**a**, **b**), D2-GP (**c**, **d**) or EL4-GP cells (**e**, **f**) injected intraperitoneally into CB6 (C57BL/6 H–2^b × BALB/c H–2^d) F_1 animals . On day 14 we carried out re-stimulation *in vitro* on GP283-labelled (H–2^d) or on GP33-labelled (H-2^b) F_1 splenocytes for 5 days: cytotoxicity assay against GP283-labelled P815 (H–2^d) (**a**, **c**, **e**) or GP33-labelled EL4 (H–2^b) (**b**, **d**, **f**) targets. Using the same protocol 5×10^6 P815-NP cells (**g**, **h**) were injected into CB6 (C57BL/6 H–2^b × BALB/c H–2^d) F_1 mice: *in vitro* restimulation with peptide on F_1 cells and Cr-release assay against NP118-labelled (H–2d; **g**) or NP394-labelled (H–2b; **h**) target cells. **B**, CTL induction by 2×10^6 transgenic LCMV-GP-expressing splenocytes of DEE$^{b/b}$ (**a**, **b**), DEE$^{b/d}$ (**c**, **d**) or DEE$^{d/d}$ (**e**, **f**) mice 14 days after intravenous injection into CB6 mice. Re-stimulation and ^{51}Cr release assay as in **A**. **C**, T-helper cell induction. **a**, CB6 (H–2^d × H–2^b) mice treated with 2×10^6 DEE$^{d/d}$ splenocytes intravenously and 12 days later challenged with 20 μg of DNP coupled to P13 (helper epitope GP60–80 in H–2^b (ref. 23). Anti-DNP IgG titres were measured by ELISA on day 7. LCMV-primed (60 days, 10^2 PFU LCMV intravenously) mice served as positive controls. **b**, C57BL/6 mice either depleted of $CD4^+$ T cells (filled circle) (day 3 and day 1, 200 μl anti-$CD4^+$ antibody intraperitoneal injection) or left untreated (filled diamond). Mice were then treated with 2×10^6 DEE$^{b/b}$ cells injected intraperitoneally and the resulting CTL response was analysed after *in vitro* re-stimulation.

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by competitive interference during *in vitro* re-stimulation and/or cytotoxicity assays. Therefore, the GP33-specific CTL response of lymphoma-bearing mice *in vivo* was evaluated by measuring strictly CTL-dependent LCMV-titre control. C57BL/6 mice primed with 10^5 EL4-GP33 cells injected intravenously eliminated the lymphoma (6 of 6 survivors) and controlled an LCMV infection below detectable levels (Table 2). In contrast, mice treated with 10^7 live EL4-GP33 cells could not control LCMV 14 days later and 6 of 6 died with tumours. Unresponsiveness was specific and not caused by generalized tumour growth. Mice primed against D^b-NP396 with vaccinia virus expressing LCMV-NP (Vacc-YN4; Table 2) 14 days before intravenous injection of 10^7 EL4-GP33 cells could control an LCMV infection. Control mice primed with LCMV 5 days before were all protected against both 10^7 EL4 lymphoma cells (day 0) and a subsequent LCMV infection (day 7). Thus, increased LCMV-GP-specific CTL frequencies prevented induction of specific unresponsiveness. These results extend earlier studies[21] showing antigen-specific tolerance of $CD4^+$ T cells to systemic tumours.

Cross processing of tumour antigens by host APCs through the class I MHC pathway (cross priming) has been postulated as a necessary and efficient mechanism to induce a protective CTL response[12–16]. We tested cross priming with our panel of LCMV-NP- and LCMV-GP-expressing tumours or GP-expressing splenocytes. CB6 (BALB/c × C57BL/6) F_1 and B6D2 (C57BL/6 × B10.D2) F_1 mice were immunized with the various tumour cells used previously; in addition we used EL4-GP, transfected with the entire GP that was derived independently from the EL4-GP33 line.

MC-GP ($H{-}2^b$) cells induced a potent $H{-}2^b$-GP33- (Fig. 3A, b) and $H{-}2^b$-GP276-restricted (not shown) CTL response in $H{-}2^b \times H{-}2^d$ F_1 mice, but no $H{-}2^d$-GP283-specific CTLs (Fig. 3A, a). EL4-GP ($H{-}2^b$) induced $H{-}2^b$-GP33- but not $H{-}2^d$-restricted CTLs (Fig. 3A, e and f). In contrast, despite lower LCMV-GP expression when compared with MC-GP, D2-GP primed $H{-}2^d$-GP283-restricted but not $H{-}2^b$-GP33-restricted CTLs (Fig. 3A, c versus d). Similarly, P815-NP ($H{-}2^d$) primed $H{-}2^d$ NP118-specific but not $H{-}2^b$ NP394-specific CTLs (Fig. 3A, g and h). Tumour cells (10^7) freeze thawed or heated at 42 °C, injected subcutaneously or intraperitoneally once or four times every second day, did not prime CTLs, but replicating or irradiated tumour cells induced CTLs directly and efficiently; therefore cross priming was undetectable. However, cross priming seems more efficient with antigen-expressing splenocytes than with tumour cells[16]. Therefore, LCMV-GP-expressing transgenic C57BL/6 ($H{-}2^b$, DEE) mice[22] were bred with B10.D2 mice to obtain $DEE^{b/d}$ F_1 and $DEE^{d/d}$ F_2 offspring. Immunization of $H{-}2^b \times H{-}2^d$ F_1 mice with 2×10^6 $DEE^{b/b}$ or $DEE^{d/d}$ splenocytes induced CTLs restricted only to $H{-}2^b$-GP33 (Fig. 3B, a and b), or only $H{-}2^d$-GP283 (Fig. 1B, e and f), respectively. Control F_1 mice immunized with $DEE^{b/d}$ had $H{-}2^d$- and $H{-}2^b$-restricted CTLs (Fig. 3B, c and d). Thus, GP-expressing splenocytes induced CTLs directly, but cross priming was undetectable.

Inefficient cross priming could be due to inefficient uptake by host APCs, or could reflect a relatively strict separation of MHC class I from class II pathways of antigen presentation or an unlikely selected 'avoidance of cross priming' by viral antigens. To test for conventional APC peptide presentation of exogenous cell debris through MHC class II, CB6 mice were immunized with 2×10^6 $DEE^{d/d}$ splenocytes (or GP-expressing tumour cells; not shown). Twelve days later, these mice were challenged with the defined $H{-}2^b$ T helper epitope P13 (GP60-80) coupled to 2,4-dinitrophenol (DNP)[23]. $DEE^{d/d}$-primed, but not naïve, CB6 mice mounted DNP-specific immunogloublin-γ (IgG) titres at day 7 (Fig. 3C, a). Therefore, LCMV-GP from $DEE^{d/d}$ splenocytes was presented efficiently by MHC class II but not class I molecules of host APCs. Importantly, anti-CD4 treatment of mice revealed the necessity for $CD4^+$ T-helper cells for direct $CD8^+$ T-cell priming by all tumour cells (not shown) and DEE splenocytes (Fig. 3C, b). Therefore, antigen processing by host APCs seems necessary for

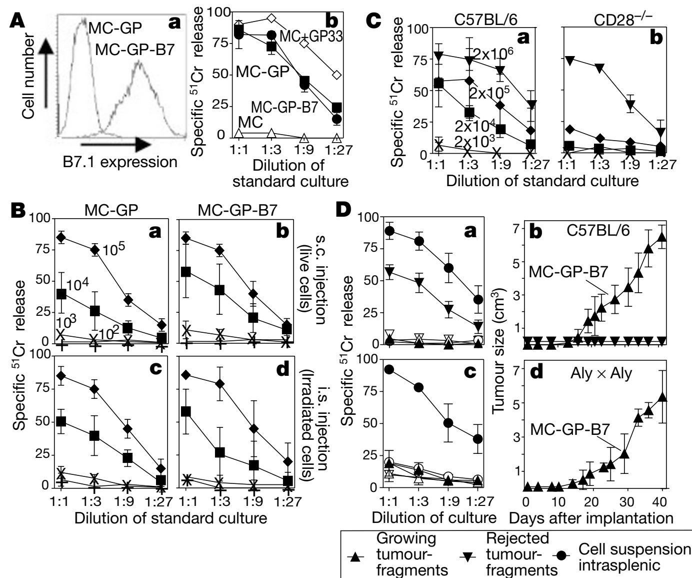

Figure 4 Role of B7 in the induction of an anti-tumour CTL response. **A**, MC-GP and MC-GP-B7 cells tested for B7.1 expression by FACS (**a**) and with or without peptide labelling (**b**) (GP33) as target cells in CTL assays. **B**, Immunogenicity of MC-GP versus MC-GP-B7 cells. **a**–**d**, 10^2 to 10^5 live (**a**, **b**) or irradiated (**c**, **d**) (80 Gy) MC-GP or MC-GP-B7 cells injected directly into the spleen in C57BL/6 mice; 8 days later secondary GP33-specific CTLs were tested. **C**, MC-GP priming in $CD28^{-/-}$ mice. **a**, **b**, Titrated numbers of MC-GP cells injected intraperitoneally into C57BL/6 (**a**) or $CD28^{-/-}$ (**b**) mice and tested for primed CTLs as in **A**. **D**, CTL induction and tumour growth in C57BL/6 and Aly × Aly (C57BL/6 mice lacking all lymph nodes) recipients of tumour pieces. **a**–**d**, Secondary CTL activity (**a**, **c**) and tumour growth (**b**, **d**) presented as either growing (filled, normal triangle) or rejected (filled, inverted triangle) tumours. Secondary CTL responses in all mice at the end of the experiment (day 40) as in **A**. In addition, 10^6 MC-GP-B7 tumour cells were injected directly into the spleen of C57BL/6 (filled circles; **a**) and Aly x Aly mice (filled circles; **c**); 8 days later the secondary GP33-specific CTL activity was assessed as in **A**. Open symbols, unlabelled targets; filled symbols, GP33-labelled targets. Mean ± s.d. of 3–5 animals per group; 2–3 experiments with similar results.

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induction of limiting T help, which is necessary for direct CTL priming by tumour cells. These findings confirm, extend and may explain both earlier experiments and some discrepancies on cross priming *in vivo*; they imply a limiting role for T-helper cells during priming *in vivo*[18] but not for *in vitro* secondary CTL responses[13,17].

The two-signal hypothesis[4–8] proposes that tumours without B7 or other co-stimulatory signals not only fail to induce CTLs but delete them. It predicts that strictly extra-lymphatic antigenic tumours expressing B7 should induce CTLs. We tested this hypothesis with MC-GP cells additionally transfected with B7.1, MC-GP-B7 (Fig. 4A, a) or with MC-GP cells without B7.1 in C57BL/6 mice possessing or lacking CD28 (ref. 24), the ligand for B7.1. MC-GP and MC-GP-B7 were comparably lysed by LCMV-specific CTLs (Fig. 4A, b). At least 10^4 live or irradiated MC-GP (Fig. 4B, a) or MC-GP-B7 (Fig. 4B, b) cells injected subcutaneously or into the spleen, respectively, were necessary to induce CTLs. To exclude small proliferation differences, CTL induction was compared after injection of irradiated MC-GP or MC-GP-B7 cells (80 Gy) subcutaneously or directly into the spleen. As with irradiated MC-GP cells, one dose of 10^7 irradiated MC-GP-B7 cells injected subcutaneously did not prime CTLs; however, $\geq 10^4$ irradiated MC-GP (Fig. 4B, c) or MC-GP-B7 cells (Fig. 4B, b) did prime CTLs when injected intrasplenically (see also Supplementary Information Fig. 2).

The overall role of B7–CD28 interactions in lymphoid organs was analysed 8 days after priming of CD28$^{-/-}$ mice with different numbers of MC-GP cells. CD28$^{-/-}$ mice generated CTLs; however, 100 times more MC-GP or MC-GP-B7 cells (not shown) injected intraperitoneally were necessary (Fig. 4C, a and b) compared with C57BL/6 mice. Taken together, B7 on tumour cells did not enhance induction of CTLs, whereas lack of CD28–B7 interactions in host lymphatic organs impaired CTL priming in a dose-dependent manner.

How then can other[5–7] beneficial effects of B7 on tumour rejection be explained? MC-GP and MC-GP-B7 cell suspensions injected subcutaneously induced CTLs (Fig. 4B, a and b) and no tumours grew. In contrast, small MC-GP-B7 tumour pieces transplanted subcutaneously grew; both MC-GP (not shown, but similar to Fig. 4D, b) and MC-GP-B7 (Fig. 4D, b) reached sizes of up to 5–7 cm^3 after 30–40 days and did not prime CTLs (Fig. 4D, a). As shown above (Fig. 1B), mice that rejected tumours always exhibited primed CTLs independently of B7 expression on the tumour (Fig. 4D, a and b); again, all growing tumours expressed D^b-GP33 and B7 (not shown).

To avoid the migration of tumour cells to lymph nodes completely, cell suspensions or small tumour fragments were given to alymphoplastic (Aly × Aly) (C57BL/6) mice that lack lymph nodes but possess lymphatic vessels, spleen and normal CTLs[25]. In contrast to controls, not only fragments but also cell suspensions of B7$^-$ or B7$^+$ tumours injected subcutaneously grew in Aly × Aly mice (suspensions (2×10^6 cells): MC-GP 6 of 6 but also 10^4 cells formed tumours, MC-GP-B7 12 of 12; fragments: MC-GP 6 of 6, MC-GP-B7 8 of 8) and failed to prime splenic CTLs (Fig. 4D, c and d). Strictly peripheral MC-GP-B7 cells did not prime CTLs because they could not reach absent lymph nodes in Aly × Aly mice. Aly × Aly mice with growing tumours did generate CTLs when treated intrasplenically with 2×10^6 MC-GP-B7 cells (Fig. 4D, c), and could reject tumours when transfused with sufficient activated specific CTLs (Table 3). Thus CTLs were not rendered tolerant in tumour-bearing Aly × Aly mice; tumours were accessible and not generally suppressive. Even allogenic melanoma fragments (K1735, H–2^k) transfected or not with B7.1 (ref. 5) grew subcutaneously in Aly × Aly mice (10 of 10 K1735-B7 tumours grew) and no allospecific CTLs were induced; no (n = 8) tumours grew in C57BL/6 mice. Thus, an allogenic B7$^+$ tumour strictly outside of lymphoid organs failed to prime CTLs.

To explain earlier documented benefits of B7 expression[5–7] on immune rejection of tumours, we tested whether B7 expression on strictly peripheral tumours could enhance or maintain CTLs that had been already induced in lymphatic organs[26,27]. MC-GP and MC-GP-B7 pieces were transplanted subcutaneously into the contralateral flank of the same T- and B-cell-deficient Rag-1$^{-/-}$ mice—the tumours grew to a comparable size (5–7 mm) by day 10 after transplantation. At this time point, 5×10^7 GP33-specific acute day 8 LCMV immune spleen cells were transfused into tumour-bearing Rag-1$^{-/-}$ mice. Only 1 out of 7 MC-GP but 6 of 7 MC-GP-B7 tumours were controlled in the same recipient mice (Table 3).

Immunohistology showed similar CD4$^+$ T-cell, B-cell or CD11$^+$ APC infiltration in B7$^+$ versus B7$^-$ tumours (not shown). In contrast, CD8$^+$ T-cell infiltration in MC-GP tumours was more extensive in connective tissue around blood vessels and around the border of solid tumour tissue, but they infiltrated MC-GP-B7 tumours efficiently (Fig. 5).

Our findings suggest that strictly extra-lymphatic peripheral tumours are ignored by CTLs and do not delete them. Perhaps rare tumours that reach lymphatic organs early are rejected before they become clinically evident. Once a tumour has reached a certain size and effector CTLs get induced late, the outcome reflects a race of

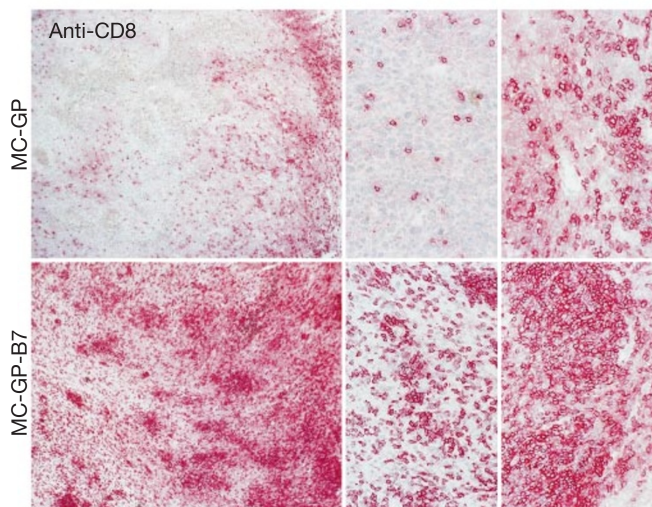

Figure 5 Immunohistochemistry of CTL-infiltrated MC-GP and MC-GP-B7 tumours. Tumour fragments of MC-GP and MC-GP-B7 tumours implanted subcutaneously each on one flank of Rag-1$^{-/-}$ mice. Ten days later 5×10^7 day-8 LCMV effector cells (splenocytes of C57BL/6 mice infected 8 days previously with 200 PFU LCMV) were adoptively transferred into tumour-bearing recipients. Ten days later, tumours were removed and immunohistology was performed with anti-CD8. Magnification: ×50, left panels middle panels (middle of the tumour); ×200, middle panels (middle of the tumour) and right panels (at the tumour border). One out of five comparable histological sections for each tumour is shown.

Table 3 Control of B7$^+$ and B7$^-$ tumours by transferred specific CTLs

Recipient	Tumour transplant*	LCMV CTLs†	Controlled/tested‡
Aly × Aly	MC-GP	–	0/6
Aly × Aly	MC-GP	$3 \times 2 \times 10^7$	7/8
Rag-1$^{-/-}$	MC-GP	–	0/6
Rag-1$^{-/-}$	MC-GP-B7	–	0/6
Rag-1$^{-/-}$	MC-GP	5×10^7	1/7
Rag-1$^{-/-}$	MC-GP-B7	5×10^7	6/7

*Tumours were transplanted subcutaneously as solid fragments of MC-GP and MC-GP-B7 into contralateral flanks of the same recipient mouse 14 days before adoptive transfer.
† Splenocytes of C57BL/6 mice immunized with 200 PFU LCMV (8 days previously) and transferred (i.v.) to mice bearing tumours ≥0.5 cm. Tumour control in Aly × Aly mice after repetitive (3 times in 3-day intervals) transfer of 2×10^7 effector cells.
‡ Tumour control was defined as complete rejection or stable tumour size for the next 21 days.

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growing tumour masses and limited effector cells versus tumour mutant selection. One extreme form of tumour selection in the host is probably illustrated here by lymphohaemopoietic tumours where exhaustive induction and deletion of CTLs correlated with the tumour success.

Our results seem to contradict the fact that late lymph-node metastases usually signal failure of immunosurveillance[1,2]. We show that very early trapping of tumour cells in secondary lymphoid organs is beneficial as it induces CTLs to reject tiny tumours early, at a time when selection of immune evasion mutants is improbable.

In contrast to earlier studies[12–16] our analyses show direct, specific CTL induction by signal 2 negative tumour cells without demonstrable cross presentation through MHC class I when analysed for peptide specificity *ex vivo* or protection *in vivo*. Notably, direct CTL induction depended on immune $CD4^+$ T-helper-cell activity[18,28]. As fibroblasts and epithelial cells are MHC class II-negative, antigen presentation to $CD4^+$ cells depends on and is performed conventionally by host APCs. Perhaps some results that have not defined the cross-priming peptide specificity may indicate T-helper cell involvement during priming *in vivo*[17,28]. Cross priming has been documented *in vitro*[14] and *in vivo* with ovalbumin and viral antigens[12,13,17] but its role in CTL-mediated protection is unclear. Our study suggests that such a process seems inefficient unless special tricks are used[12–15,17]. Only additional studies will show whether antigen studied under special conditions or with normal or very high CTL frequencies reveal generalizable versus rare mechanisms of cross priming of CTL induction or tolerization *in vivo*[12–15,17].

CTL induction in secondary lymphoid tissues, while enhanced by CD28, was independent of B7.1 on tumour cells. Thus CD28–B7 co-stimulation acts in bystander fashion in lymphatic organs but improved or maintained CTL effectors in $B7^+$ peripheral solid tumours—other results are compatible with our findings[26,27]. Use of transgenic T cells may yield uninterpretable results because their high frequency enhances bystander induction or activation in secondary lymphoid organs, faking peripheral B7-driven induction[29]. Our results also document that neither $B7^+$ nor $B7^-$ strictly extra-lymphatic tumours can induce or delete T cells, negating predictions of the two-signal hypothesis. Therefore, T-cell deletion by tumours is probably not through negative signals but more likely represents 'over-induction', suggested for $CD4^+$ T cells[21] or here for CTLs. An involvement of B7 on natural killer cells[30] was not analysed here, but B7-independent growth kinetics and the lack of natural killer control of MC-GP tumours[10] renders this possibility unlikely.

This study suggests a number of apparently simple possibilities to improve the overall unfavourable balance between peripheral solid tumours and the host. They all depend on tumour loads being as small as possible at an early time of T-cell induction in lymphoid organs to avoid immune escape, and also on the maintenance of great numbers of activated T cells. □

Methods

Mice and *in vivo* experiments

C57BL/6, lymph-node-deficient Aly×Aly[25] and DEE mice[22] (LCMV-GP transgenic) on a pure C57BL/6 background were bred locally. We obtained $CD28^{-/-}$ mice[24] from the Basel Institute for Immunology. Tumour size was assessed twice a week and the animals were killed when the tumour reached $6\,cm^3$. Tumour volume was calculated by the formula $V = \pi \times abc/6$, where a, b and c are the orthogonal diameters.

Tumour cell lines, cell analysis, histology and CTL assays

The MC57-GP[9,10], LL-GP33 (ref. 19) and B16-GP33 (ref. 20) cell lines were described earlier. A part of the LCMV-GP (residues 1–60) as well as the entire LCMV-GP and LCMV-NP were subcloned from the pLCMV-GP and pLCMV-NP plasmids, respectively, into a CMV-driven eukariotic expression vector containing the geneticin resistance gene. Co-transfection[9] of MC-GP with a plasmid encoding the murine B7.1 (CMV promotor) and a hygromycin-resistance gene (pX343) (Hygromycin B $150\,\mu g\,ml^{-1}$; Fluka Chemie AG) by calcium phosphate co-precipitation. The K1735 $B7.1^+$ or $B7.1^-$ cell lines were a gift of J. P. Allison[5].

All FACS antibodies were from PharMingen. Histochemistry was performed as described[25]. Histological collagen staining was done with sirius red. *In vitro* re-stimulation of LCMV GP- or NP-specific or of allospecific spleen cells has been reported previously[25].

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