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Autologous cellular vaccine overcomes cancer immunoediting in a mouse model of myeloma

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Summary

In the Sp6 mouse plasmacytoma model, a whole-cell vaccination with Sp6 cells expressing *de novo* B7-1 (Sp6/B7) induced anatomically localized and cytotoxic T cell (CTL) -mediated protection against wild-type (WT) Sp6. Both WT Sp6 and Sp6/B7 showed down-regulated expression of MHC H-2 L^d. Increase of H-2 L^d expression by cDNA transfection (Sp6/B7/L^d) raised tumour immune protection and shifted most CTL responses towards H-2 L^d-restricted antigenic epitopes. The tumour-protective responses were not specific for the H-2 L^d-restricted immunodominant AH1 epitope of the gp70 common mouse tumour antigen, although WT Sp6 and transfectants were able to present it to specific T cells *in vitro*. Gp70 transcripts, absent in secondary lymphoid organs of naive mice, were detected in immunized mice as well as in splenocytes from naive mice incubated *in vitro* with supernatants of CTL-lysed Sp6 cell cultures, containing damage-associated molecular patterns (DAMPs). It has been shown that Toll-like receptor triggering induces gp70 expression. Damage-associated molecular patterns are released by CTL-mediated killing of Sp6/B7-Sp6/B7/L^d cells migrated to draining lymph nodes during immunization and may activate gp70 expression and presentation in most resident antigen-presenting cells. The same could also apply for *Mus musculus* endogenous ecotropic murine leukaemia virus 1 particles present in Sp6-cytosol, discharged by dying cells and superinfecting antigen-presenting cells. The outcome of such a massive gp70 cross-presentation would probably be tolerogenic for the high-affinity AH1-gp70-specific CTL clones. In this scenario, autologous whole-tumour-cell vaccines rescue tumour-specific immunoprotection by amplification of subdominant tumour antigen responses when those against the immune dominant antigens are lost.

Keywords: cancer immunoediting; cellular vaccines; myeloma.

Introduction

There are several mechanisms exploited by tumour cells to evade the host immune response. Antigen loss, down-regulation of MHC-I molecules and lack of co-stimulation may contribute to the failure of endogenous immune responses in tumour control.[1–3] As a result of their inherent genetic instability, tumours can withstand the contrasting actions of the immune system by giving rise to variants of reduced immunogenicity. Once the parental cells are eliminated, a new hierarchy is established among the tumour variant subpopulations, with immunodominant antigens turned off, and formerly immunorecessive epitopes becoming dominant.[4] Alternatively, tumour variants may lose or down-regulate the restricting MHC-I allele while retaining the immunodominant antigen, and so professional antigen-presenting cells (APCs) maintain an immunodominant response to a 'phantom' target instead of more appropriate and effective responses to other antigens.[4,5] The APC-mediated cross-priming of $CD8^+$ T cells usually induces cytotoxic T-cell (CTL) immune responses restricted to immunodominant epitopes.[6] This implies that vaccine strategies of direct priming with autologous or syngeneic antigen-expressing cells may induce a broader repertoire of

tumour-specific CTLs directed also against subdominant epitopes, that may be useful to counterattack tumour immunoescape. Therefore, whole tumour cells remain a potent vehicle for generating anti-tumour immunity, as they express a complex array of target antigens for the immune system, either dominant or sub-dominant, avoiding problems associated with MHC-restricted epitope identification for individual patients. Furthermore, whole cells are relatively simple to propagate and are potentially efficient at contributing to the process of T-cell priming. However, tumour cells are considered poorly immunogenic, mainly because they express self-antigens in a non-co-stimulatory context and they may also induce immune evasion, for instance by surface expression or secretion of immunosuppressive molecules (e.g. programmed cell death-1, transforming growth factor-β).[7,8]

Hence many efforts were made to enhance the immune response against tumour cells: one strategy is represented by the modification of tumour cells to become stimulatory. In fact, the use of whole tumour cell vaccines genetically engineered to express *de novo* cytokines, MHC or co-stimulatory molecules in animal models generated successful immunization against tumours by direct priming of $CD8^+$ T-cell effectors.[9–16]

All of these aspects were investigated in the plasmacytoma-derived Sp6 tumour of the mouse BALB/c strain to obtain a protective immunization protocol against the challenges of wild-type tumour cells (WT Sp6): *de novo* expression of the B7-1 co-stimulatory molecule after transfection of the coding cDNA (Sp6/B7) inhibited tumour growth *in vivo* independently of the injection site.[17] However, a CTL-dependent memory immune response protective against WT Sp6 was obtained only when Sp6/B7 was injected subcutaneously (s.c.). In addition, the antigen dose regulated the anatomical extension of protection, the lower vaccine dose conferring protection limited to challenge s.c. in the same anatomical quarter as the immunization.[17] This marked dose-dependent immunogenicity of the Sp6 tumour system led us in the present work to investigate the exploitation of immunoescape mechanisms: WT Sp6 and Sp6/B7 showed in fact a down-regulated cell surface expression of the MHC-I H-2 L^d molecule, still maintaining normal expression levels of H-2 K^d and D^d. In the BALB/c genetic background, H-2 L^d is the restriction element presenting the immunodominant epitopes of the two commonest mouse tumour-associated antigens gp70 and P1A.[18,19] Gp70 is a gene product of the *Mus musculus* endogenous ecotropic murine leukaemia virus 1 (Mu-MLV-1), expressed in a variety of mouse tumour cell lines of different H-2 haplotypes.[18,20] Genome sequences of the gp70-expressing Mu-MLV-1 virus are present throughout the mouse genome[21] and gp70 expression can be induced by Toll like receptor (TLR) triggering.[22,23] The AH1 peptide is the immunodominant, H-2 L^d-restricted CTL epitope of gp70 in several tumour models, such as CT26 colon adenocarcinoma,[18] CSM4 sarcoma[24] and TS/A mammary adenocarcinoma.[25] The P1A antigen, silent in normal tissues except for male germ cells, is activated in a variety of tumours (MAGE-type tumour antigens),[26] e.g. P815 mastocytoma,[19,27] J558 plasmacytoma[27] and Meth A fibrosarcoma.[28]

Although WT Sp6 and Sp6/B7 were able to present the gp70 antigen to specific T-cell lines in *in vitro* assays, the very low expression of H-2 L^d on the cell surface led us to hypothesize that an increase of H-2 L^d expression, by improving the H-2 L^d-mediated antigen presentation of tumour immunodominant epitopes, would raise the Sp6-specific CTL response. Hence, we transfected WT Sp6 and Sp6/B7 cells with the H-2 L^d-specific cDNA. Sp6/L^d and Sp6/B7/L^d cells showed higher lysis susceptibility to gp70-specific T-cell lines than WT Sp6 and Sp6/B7 *in vitro*. *In vivo*, enhanced H-2 L^d expression lowered the vaccine dose required to induce systemic protection. However, the immune response elicited by immunization with the autologous, B7-1-positive cellular vaccine, either Sp6/B7 or Sp6/B7/L^d, was never gp70-specific.

Materials and methods

Cell lines and transfections

Wild-type Sp6 is an IgM anti-trinitrophenol-secreting hybridoma, derived from somatic cell fusion between the BALB/c-derived mouse P3/X63-Ag8 plasmacytoma (H-2^d genotype) and spleen cells from a BALB/c mouse immunized with trinitrophenylated derivative of lipopolysaccharide.[29] The WT Sp6 cells were chosen for both the present work and a previous study,[17] among several plasmacytomas and hybridomas of the mouse BALB/c background, because of their ability to be transfected and to maintain the transfected genes in a permanent, integrated form. Wild-type Sp6 cells are highly tumorigenic when injected s.c. into a syngeneic BALB/c mouse strain.[17,29] P815 is a mouse mastocytoma-derived cell line of the DBA/2 mouse strain,[30] expressing the P1A tumour antigen. TS/A is a mouse adenocarcinoma cell line derived from a spontaneous mammary cancer of the BALB/c mouse.[31] CT26, a BALB/c carcinogen-induced, undifferentiated colon carcinoma, was previously described.[32] Bulk-AH1 CTL line was obtained by continued AH1 peptide mixed leucocyte-peptide culture (MLPC) re-stimulations of spleen cells from a TS/A-interferon-α (IFN-α) immunized BALB/c mouse rejecting TS/A tumour.[25] The $P1A_{35–43}$-specific LDA5 CTL clone was obtained from spleen cells of a BALB/c mouse immunized with the pBKCMV-P1A plasmid, by several *in vitro* stimulations with syngeneic splenocytes pulsed with $P1A_{35–43}$ peptide and after limiting-dilution cloning.[25] The 293L^d cell line is a human embryonal kidney cell line stably

transfected with pL^d.444 plasmid, which expresses the H-2 L^d class I molecule.[25]

All cells were cultured in RPMI-1640 medium (Gibco Invitrogen Corporation, San Diego, CA) supplied with 10% fetal bovine serum (FBS; Euroclone, Pavia, Italy) and glutamine 1 mM (Biochrom AG, Berlin Germany), at 37° in 5% CO_2 in a humidified incubator.

The WT Sp6 cells were transfected by electroporation with the full-length cDNAs coding for the mouse co-stimulatory molecule B7-1 and/or the H-2 L^d MHC-I molecule and with the corresponding plasmid vectors without inserts, as previously described.[17] H-2 L^d-encoding cDNA was subcloned in the p.444 plasmid vector, containing the neomycin resistance gene[25] or in the pcDNA3.1 plasmid vector (Invitrogen Corporation, San Diego, CA), containing the hygromycin resistance gene. Transfections were performed by electroporation with a Bio-Rad apparatus using 5 μg of DNA added to 4×10^6 cells resuspended in complete medium, using 0·2-mm cuvettes, at 250 V, 250 μF. Selection of transfectants was carried out by growing cells in the presence of 500 μg/ml G418 (Geneticin G418 sulphate, Invitrogen Corporation) and/or of 500 μg/ml hygromycine (Calbiochem-Novabiochem Corporation, Darmstadt, Germany), and later by immunofluorescence analysis for *de novo* expression of B7-1 and H-2 L^d. Transfectants were indicated as follows: (i) Sp6/B7 (transfected with pSRα-Neo/B7), expressing *de novo* B7-1 co-stimulatory molecule; (ii) Sp6/L^d (transfected with pL^d.444), expressing *de novo* L^d molecule; (iii) Sp6/B7/L^d (transfected with pSRα-Neo/B7 and pcDNA3.1/L^d), expressing *de novo* B7-1 and L^d.

Growth assays with wild-type and transfected Sp6 tumour cells

Growth assays with oligoclonal populations of WT and transfected tumour cells were carried out as described by Chignola *et al.*[33] Briefly, cells were plated at 10 000 cells/well in 96-well flat-bottomed culture plates in 200 μl RPMI-1640 + 10% FBS and grown at 37° in 5% CO_2 in a humidified atmosphere without medium changes. To monitor cell growth, 100 μl of the cell suspension was diluted in 400 μl PBS and counted in a cytofluorimeter every 8 hr (Epics XL, Coulter, Hialeah, FL). Dead cells and debris were discarded from the analysis by computerized gating based on the light scattering properties of the analysed cells. Forward angle and 90° light scattering parameters did not change significantly throughout the culture period. Under these culture conditions, tumour cells grew following an initial mono-exponential growth phase followed by a stationary growth phase.[33] The growth rates of analysed cells were calculated for the mono-exponential growth phase by linear least-square fitting of measured data of cell number versus time plotted in a semi-log plot. In fact, the following relationship describing the exponential growth of tumour cells holds:

$$\log [N(t)/N(0)] = k_p\, t$$

where $N(t)$ is the number of cells at time t, $N(0)$ is the initial number of cells and k_p is the growth rate. In a semi-log plot the growth rate k_p is therefore the slope of the straight line best approximating experimental data. The doubling time of exponentially growing tumour cells could then be derived as follows:

$$\text{Doubling Time (DT)} = \log 2/k_p$$

Immunofluorescence analysis

Sp6 cells and their transfected derivatives were analysed by indirect immunofluorescence and cytofluorimetry on an Epics XL apparatus (Coulter, Hialeah, FL) with the following monoclonal antibodies: 34.1.2S, specific for mouse H2-K^d MHC-I molecules; 28.14.8S, specific for mouse H-2 L^d MHC-I molecules; 34-5-8S, specific for mouse H-2 D^d MHC-I molecules; 25.9.17, specific for mouse I-$A^{b,d}$ MHC-II molecules; K22.42.2, specific for mouse I-$E^{b,d}$ MHC-II molecules; 16-10A1, specific for mouse B7-1 (CD80) co-stimulatory molecule; GL-1, specific for mouse B7-2 (CD86) co-stimulatory molecule; BE29G1, specific for mouse intercellular adhesion molecule 1 (ICAM-1). The secondary antibodies were goat anti-mouse IgG, $F(ab')_2$ FITC-IgG conjugate (Instrumentation Laboratory, Milan, Italy) for anti-MHC-I and -II, mouse anti-hamster IgG FITC-IgG conjugate for anti B7-1, goat anti-rat IgG-FITC conjugate for anti B7-2 and anti-ICAM-1 primary antibodies (BD-Pharmingen, Milan, Italy).

An MHC-I tetramer-based cytofluorimetric analysis was performed to detect $CD8^+$ T cells expressing gp70-specific T-cell receptors, using fluorescent Pro5 MHC class I Pentamer loaded with H-2L^d $gp70_{423–431}$ peptide (Proimmune, Oxford, UK). Staining was performed according to the manufacturer's instructions.

In vivo experiments

BALB/c mice (BALB/cByJIco; Charles River Italia, Calco, Lecco, Italy) were bred at our colony at the University of Verona. Three-month-old male and/or female mice, syngeneic with Sp6 cells (H-2^d) were used. Mice were injected s.c. and intraperitoneally (i.p.) with $0{\cdot}5 \times 10^6$ and 5×10^6 tumour cells resuspended in 0·2 ml or 0·5 ml of PBS, respectively. The dose of $0{\cdot}5 \times 10^6$ WT Sp6 cells was the lowest s.c. dose to be tumorigenic in 100% of syngeneic animals and gave rise to s.c. solid tumours that became palpable within 10 days following injection. Tumours grew locally and in a non-metastatic fashion up to an average dimension of 1–2 cm in diameter at the 20th day after injection. Injection i.p. of WT

Sp6 cells always resulted in ascite-solid tumours growing and expanding to colonize the lymphoid tissue of the peritoneal cavity by about 15 days after injection. γ-ray-irradiated CT26 tumour cells were injected s.c. into naive mice (1×10^6 cells in 0·2 ml PBS), as described previously.[34] Three weeks later mice were killed, and spleen and lymph node specimens were collected and used as positive control in the tetramer staining and ELISPOT functional assays described below.

All animals bearing tumours were killed when the subcutaneously growing tumour mass reached a maximum diameter of 2 cm (volume = 4·187 cm^3), or became ulcerated. If signs of pain and fatigue became evident earlier in the animals, they were killed immediately. The Italian Ministry of Health, Decree n. 14/2012B, approved all the *in vivo* experiments.

In vivo depletion of $CD4^+$ and $CD8^+$ T-cell subsets

Four groups of 12 BALB/c mice were treated by i.p. injection with 0·5 ml of concentrated hybridoma culture supernatant of GK1.5 anti-mouse CD4 [American Type Culture Collection (ATCC), Rockville, MD], or 2.43 anti-mouse CD8 (ATCC), or both of them, or with complete medium (RPMI-1640 + FBS 10% + glutamine 1 mM) every 3 days for about 1 month, starting 10 days before injection of 5×10^6 Sp6/B7 cells. Highly concentrated supernatants of hybridoma culture were obtained by means of INTEGRA CELLline system flasks (Integra Biosciences Celbio S.r.l., Milan Italy). Treatment conditions were experimentally determined on sample groups of mice analysing the spleen-derived T $CD8^+$ and T $CD4^+$ cell numbers at different times since the beginning of treatment, by direct immunofluorescence and FACS analysis. The following FITC-conjugated monoclonal antibodies were used: rat anti-mouse CD4 monoclonal antibody clone GK1.5, rat anti-mouse CD8 monoclonal antibody clone 53-6.7, rat anti-mouse CD3 molecular complex clone 17A2, all purchased from BD Pharmingen (San Diego, CA).

Animals depleted of the T-lymphocyte subsets (T $CD4^+$, T $CD8^+$ or both T $CD4^+$ and T $CD8^+$) were immunized by injecting s.c. 5×10^6 Sp6/B7 cells. Among these groups, tumour-free mice that rejected Sp6/B7 were challenged s.c. with $0{\cdot}5 \times 10^6$ of WT Sp6 cells in the same anatomical quarter as the immunizing injection, without performing any further treatment with GK1.5 antibody.

In vitro functional assays

The cytotoxicity of spleen-derived cells was measured in a 4-hr ^{51}Cr-release assay, performing both mixed leucocyte tumour cultures (MLTCs) and MLPCs, according to established protocols[35–37] and described also by some of the authors of the present manuscript.[17,25,37] In MLTCs, splenocytes were re-stimulated *in vitro* with γ-irradiated tumour cells. Briefly, 5×10^6 spleen cells were co-cultured with $0{\cdot}5 \times 10^6$ γ-irradiated (6000 cGy) tumour cells in 2 ml of complete medium/well of a 24-well tissue culture plate (Greiner Bio-One, Frickenhausen Germany). Wild-type Sp6 and/or Sp6/L^d cell targets were labelled with ^{51}Cr by incubating aliquots of 4×10^6 cells for 1 hr at 37° with 50 μCi ^{51}Cr sodium chromate (PerkinElmer Life Sciences Inc. Boston, MA).[17] For MLPC experiments, the lyophilized H-2 L^d-restricted peptides corresponding to amino acids 423–431 of the gp70 env product (SPSYVYHQF; AH1 peptides, $gp70_{423–431}$), amino acids 35–43 of the P1A protein (LPYLGWLVF, $P1A_{35–43}$) and amino acids 876–884 of the β-gal protein (TPHPARIGL) (> 95% pure; Technogen, Naples, Italy), were dissolved in DMSO (stock solution at 10 mM; Sigma Aldrich, Milan, Italy) and stored at −80° before use.[25] For MLPC stimulation, peptides were diluted in complete medium and added to splenocyte cultures at a final concentration of 1 μM, in 10 ml of Dulbecco's modified Eagle's medium containing 10% FBS and maintained in a 25-cm^2 culture flask (Greiner Bio-One).[37] After 5 days of incubation at 37° in 5% CO_2[17,25,36] cells were collected and tested for their lytic activity in a ^{51}Cr-release assay at different effector/target ratios.[17,25,34] Bulk-AH1 CTL line[25] and LDA5 CTL clone[25] were cultured in flat-bottomed 24-well plates (Falcon; Becton Dickinson, Lincoln Park, NJ) in the presence of 3×10^6 syngeneic splenocytes pulsed with $gp70_{423-431}$ or $P1A_{35-43}$ peptide (1 μM) respectively, in 2 ml of Dulbecco's modified Eagle's medium containing 10% FBS and supplemented with 20 IU/ml recombinant interleukin-2 (courtesy of Euro Cetus-Chiron, Milan, Italy).[25] Peptide pulsing of target cells was performed by incubating 10^6/ml ^{51}Cr-labelled target cells with relevant peptides (1 μM final concentration) for 30 min at 37°. Labelled target cells were then co-cultured for 4 hr with spleen-derived effector cells.

Irradiated tumour cells (in MLTCs) or peptides (in MLPCs) were used to stimulate, in a 5-day co-culture, the splenic tumour-specific and antigen-specific $CD8^+$ T-cell population of the central memory, induced by the previous subcutaneous immunization. Following this 5-day stimulation, enough $CD8^+$ T memory cells will become 'armed' CTL and could be detected in standard ^{51}Cr-release assays.

For MHC-I subset-restricted blocking of cytotoxic cell function, labelled target cells were incubated for 30 min at 4° with anti-H-2 L^d (30-5-7S, HB-31; ATCC),[38] anti-H-2 K^d (20-8-4S, HB-11; ATCC),[39] anti-H-2 D^d (34-5-8S, HB-102; ATCC),[40] at the final concentration of 10 μg/ml before addition to effectors.[41]

To evaluate whether the HMGB1 damage-associated molecular pattern (DAMP) molecule directs gp70 expression, naive spleen cells were cultured for 48 hr with

HMGB1 or BSA as unrelated protein at a concentration of 100 ng/ml, or with $gp70_{423–431}$ or β-$gal_{876–884}$ peptides at 1 μg/ml. Then cells were washed and co-cultured with bulk AH1 CTL clone in a mouse IFN-γ ELISPOT assay. ELISPOT was performed as previously described.[42] Briefly, 1×10^6 spleen cells were plated in duplicate in 96-well MAIP plates (Merck-Millipore, KGaA, Darmstadt, Germany) pre-coated with anti-mouse IFN-γ antibody (R4-6A2, BD Pharmingen). Cells were incubated for 20 hr at 37° with 1 μg/ml solution of $gp70_{423–431}$ or β-$gal_{876–884}$ peptides. Anti-CD3 and anti-CD28 were used as positive controls at 5 μg/ml. Plates were washed and incubated for 12 hr at 4° with 50 μl/well of biotin-conjugated rat anti-mouse IFN-γ (R4-6A2; BD Pharmingen). After washes, streptavidin-alkaline phosphatase conjugate (BD Biosciences-Pharmingen; catalogue no. 554065) was added for 30 min. Plates were developed by adding nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Pierce Thermo Fisher Scientific, Rockford, IL), washed and air-dried. Spots were counted using an automated ELISPOT reader (AID-Autoimmun Diagnostika GmbH, Strassberg, Germany).

RNA isolation and RT-PCR analysis of gp70 expression

Expression of gp70 was analysed by RT-PCR on WT Sp6 cells, their transfectants and spleen cells from naive animals and injected with WT Sp6 and transfectants. The presence of gp70 transcripts was also investigated in spleen cells from naive mice cultured for 48 hr in the presence of 100 ng/ml HMGB1, BSA, or supernatants from 5-day co-cultures of spleen cells from mice injected s.c. with Sp6/B7, Sp6/B7/L^d or WT Sp6 and γ-irradiated Sp6/B7, Sp6/B7/L^d or WT Sp6 cells.

Total RNA was extracted by using the TRizol® reagent (Invitrogen S.R.L., S. Giuliano Milanese, Milan, Italy), according to the manufacturer's instructions. The RNA pellet was resuspended in ethanol, precipitated overnight at −20° and finally re-dissolved in water. Contaminating DNA was removed from the RNA preparation using the DNA-*free*™ kit (Ambion-Applied Biosystems, Monza, Milan, Italy). RT-PCR was performed using the Gene Amp® Gold RNA PCR reagent kit (Applied Biosystems), as well as control PCR on RNA samples that were not retro-transcribed.

PCR amplifications were performed for 40 cycles, with the following conditions:

gp70: 60 seconds at 94°, 60 seconds at 62°, 60 seconds at 72°;
β-actin: 60 seconds at 94°, 60 seconds at 57°, 180 seconds at 72°.

The following primers were used:

gp70 sense: 5′ ACCTTGTCCGAAGTGACCG 3′
gp70 antisense: 5′ GTACCAATCCTGTGTGGTCG 3′
β-Actin sense: 5′ CACCCTGTGCTGCTCACCGAGG CC 3′
β-Actin antisense: 5′ CCACACAGATGACTTGCGCT-CAGG 3′[18,27]

Electron microscope analysis

Pellets of cell culture were fixed in 1% phosphate-buffered glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in acetone and embedded in Epon. Then, 80-nm-thick sections were stained with uranyl acetate and lead citrate and observed on a Zeiss EM 109 electron microscope.

ELISA analysis for HMGB-1 release monitoring

Supernatants derived from MLTCs were tested to determine the release of the DAMP molecule HMGB-1 by an ELISA kit, according to the manufacturer's instructions (IBL INTERNATIONAL GMBH, Hamburg, Germany).

Statistics

Data presented in Figs 5(b), 6, 7(b,c), 8 and 9(d,f) are expressed as mean value ± standard deviation (SD). Survival analysis has been carried out using the method developed by Kaplan–Meier, and the log-rank test was used to compare survival. Differences in tumour incidence were also assessed using χ^2 test with Yates correction. Differences were considered statistically significant for P values $\leq 0{\cdot}05$. All statistical calculations were performed using STATA™ IC v.10.0 (StataCorp, College Station, TX) for Microsoft Windows®.

Results

H-2 L^d expression influences tumour immunogenicity in the Sp6 tumour model

Wild-type Sp6 and Sp6/B7 cells showed expression levels of the MHC-I molecular subset H-2 L^d that were much lower than those of the other subsets H-2 K^d and H-2 D^d and compared to control B cells from spleens of naive BALB/c mice (Fig. 1). H-2 K^d and H-2 D^d were expressed at similar levels when comparing WT Sp6 and Sp6/B7 to B cells (Fig. 1). To assess the effect of increased H-2 L^d expression on the tumour-specific immune response, WT Sp6 and Sp6/B7 were transfected with the cDNA coding for H-2 L^d (Sp6/L^d and Sp6/B7/L^d). As previously found for the transfection of B7-1 cDNA in WT Sp6,[17] transfection raised H-2 L^d levels without altering either growth kinetics *in vitro* or the expression of other immunologically relevant molecules.

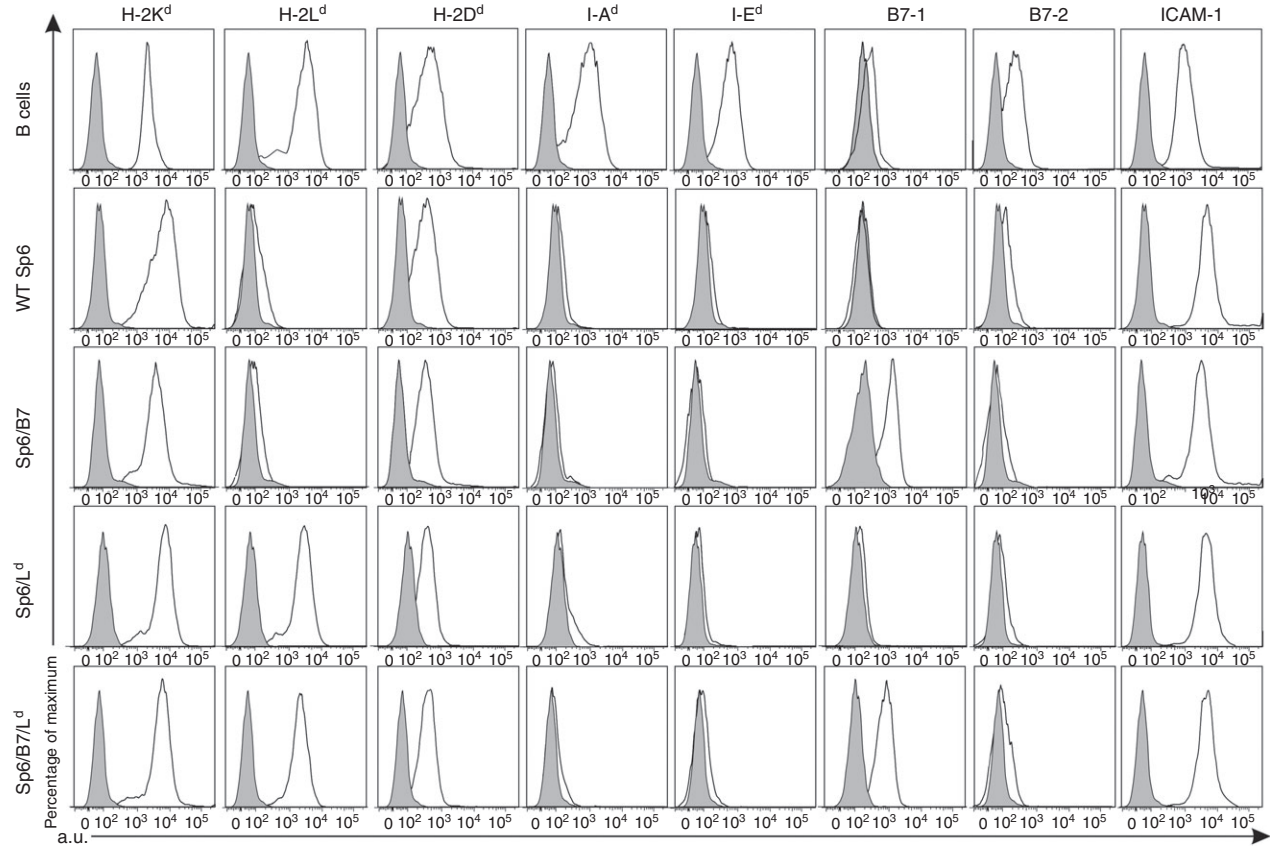


Figure 1. Cytofluorimetric analysis of B cells from a mix of the spleens of three naive mice, wild-type (WT) Sp6 mouse plasmacytoma-derived hybridoma and the three WT Sp6-derived transfectants Sp6/B7, Sp6/L^d, Sp6/B7/L^d after indirect immunofluorescence staining with monoclonal antibodies specific for the mouse MHC-I molecules H-2K^d, L^d and D^d, the MHC-II molecules H-2 I-A^d and I-E^d, the B7-1 and B7-2 co-stimulatory molecules and intercellular adhesion molecule 1 (ICAM-1), as indicated at the top of each lane. The different cells are indicated to the left of each horizontal series of histograms. Grey histograms indicate the fluorescence of each sample treated with the secondary monoclonal antibody only. Fluorescence values are expressed in absorbance units (a.u.).

As previously applied to Sp6 and Sp6/B7,[17] a sensitive, reproducible cytofluorimetric technique that compares cell counts in a cytofluorimeter every 8 hr was applied to Sp6/L^d and Sp6/B7/L^d transfectants as well.[33] Wild-type and transfected tumour cells grew following mono-exponential kinetics for approximately 80 hr. Thereafter, cells reached a stationary growth phase, which was monitored for a further 60 hr. No significant differences in the growth kinetics were observed for WT and transfected tumour cells during both growth phases. Previously, the growth rates and doubling times calculated for the exponential growth phase had been comparable for WT Sp6, Sp6/pSRαneo and Sp6/B7 (k_p = 0·0207, 0·0210, 0·0220 hr^{-1} and DT = 14·54, 14·33, 13·68 hr, respectively).[17] Similarly, the growth kinetics of Sp6/L^d and Sp6/B7/L^d transfectants were compared *in vitro* with those of WT Sp6 and no significant differences were observed (k_p = 0·0214, 0·0218, 0·0207 hr^{-1} and DT = 14·06, 13·80, 14·54 hr, respectively).

The expression levels of MHC-I H-2 K^d and D^d, MHC-II H-2 I-A^d and I-E^d, B7-1/-2 and ICAM-1 were comparable in WT Sp6 and transfectants (Fig. 1). Tumorigenicity was also unchanged (Fig. 2, see plots of WT Sp6 and Sp6/L^d challenge of naive mice).

Naive animals were immunized with 0·5 × 10^6 of either Sp6/B7 or Sp6/B7/L^d cells given s.c., and 3 weeks later they received the tumorigenic dose[17] of 0·5 × 10^6 WT Sp6 or Sp6/L^d cells via the s.c. and i.p. routes (Figs 2a–d and 3a). According to previously published results,[17] challenge s.c. yielded different results depending upon the anatomical quarter of delivery relative to that of immunization: same anatomical quarter (ipsilateral) or contralateral relative to site of immunizing inoculum. The differences in tumour incidence comparing mice immunized with either Sp6/B7 or Sp6/B7/L^d and challenged with WT Sp6/Sp6/L^d s.c. or i.p. to naive mice injected with WT Sp6 or Sp6/L^d were statistically significant (P < 0·05–0·001), except when challenge with WT Sp6 was given either i.p. to Sp6/B7-immunized mice, or s.c. contralaterally (relative to immunizing inoculum) to Sp6/B7/L^d-immunized mice (Figs 2a–d and 3a).

The highest levels of protection were found against challenge with Sp6/L^d, with both vaccines (tumour incidences

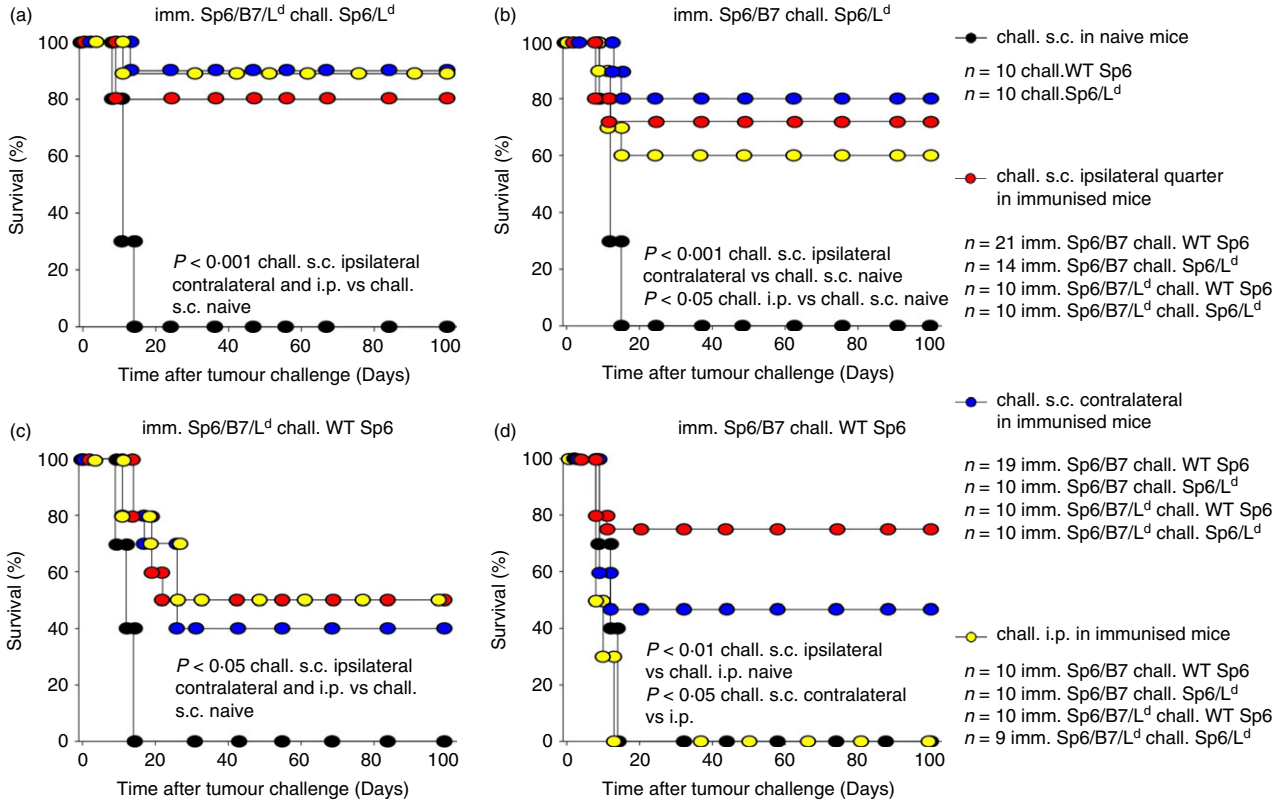


Figure 2. Overall survival of mice immunized with 0·5 × 10^6 Sp6/B7/L^d (a and c) or Sp6/B7 (b and d) delivered subcutaneously (s.c.), challenged s.c. by an injection given locally relative to the immunizing injection, within a radius of 0·5 cm around the immunizing injection (in the same anatomical quarter or ipsilateral) or contralateral or intraperitoneally (i.p.), with either 0·5 × 10^6 wild-type (WT) Sp6 (c and d) or Sp6/L^d (a and b), and of control naive mice inoculated s.c. with 0·5 × 10^6 Sp6/L^d (a and b) or WT Sp6 (c and d), as indicated in the legend above each plot.

of 10–20% when immunizing with Sp6/B7/L^d, 20–40% when immunizing with Sp6/B7), as shown in Figs 2(a,b) and 3(a). Conversely, the lowest levels of protection were observed when challenge was performed with WT Sp6, with both vaccines as well (tumour incidence of 50–60% when immunizing with Sp6/B7/L^d; tumour incidence of 24–100% when immunizing with Sp6/B7), as shown in Figs 2(c,d) and 3(a). Hence, both B7-1-expressing vaccines (Sp6/B7 and Sp6/B7/L^d) showed improved efficacy against challenging tumour cells with increased cell surface amounts of H-2 L^d (Sp6/L^d).

Immunization with Sp6/B7/L^d cells protected at 90–80% against challenge s.c. and i.p. with Sp6/L^d cells compared with the not-previously-immunized controls (tumour incidence 10–20%) ($P < 0·001$, Figs 2a and 3b), but only at 40–50% against WT Sp6 (tumour incidence 50–60%) ($P < 0·05$ for challenge i.p. or s.c either ipsilateral or contralateral versus not-immunized, Figs 2c and 3b).

Immunization with 0·5 × 10^6 Sp6/B7 cells was more protective against Sp6/L^d than WT Sp6 when challenge was given both s.c. contralateral or i.p. (tumour incidence of WT Sp6: 53–100%, tumour incidence of Sp6/L^d: 20–40%) (compare Fig. 2b,d and see Fig. 3a); protection was similar against both WT Sp6 and Sp6/L^d, or even slightly more protective against WT Sp6 only when challenging cells were given s.c. in the same anatomical quarter (tumour incidence of WT Sp6 = 24%; tumour incidence of Sp6/L^d = 28%, Fig. 3a).

An enhanced H2-L^d expression on immunizing cells (Sp6/B7/L^d) increased protection against challenge i.p. not only against Sp6/L^d, but also against WT Sp6 ($P < 0·05$ for challenge i.p. of WT Sp6 comparing immunization with Sp6/B7/L^d versus Sp6/B7). This was not the case for contralateral challenge s.c., where tumour incidence of WT Sp6 in Sp6/B7-immunized mice was lower than in mice immunized with Sp6/B7/L^d (tumour incidence = 53% versus 60%, Fig. 3a).

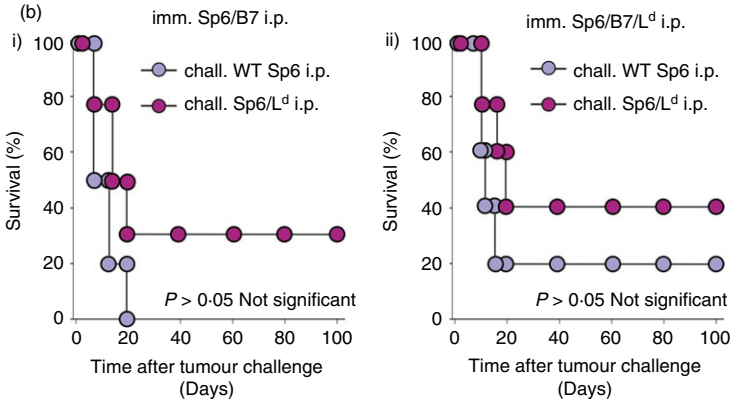
Immunization i.p. with 0·5 × 10^6 Sp6/B7/L^d cells did not impart statistically significant levels of protection against challenge with both WT Sp6 and Sp6/L^d cells (Fig. 3b,c), confirming the subcutaneous route as the most efficient for immunization with Sp6/B7/L^d cells, similarly to previously published results obtained with Sp6/B7.[17]

Naive animals were also immunized s.c. with the dose of 5 × 10^6 Sp6/B7/L^d or Sp6/B7 cells and then challenged with 0·5 × 10^6 WT Sp6 or Sp6/L^d cells i.p. (Fig. 3a):

(a)

Immun. ($0{\cdot}5\times10^6$ cells) s.c.	Challenge ($0{\cdot}5\times10^6$ cells) WT Sp6	Challenge ($0{\cdot}5\times10^6$ cells) Sp6/L^d	Immun. ($0{\cdot}5\times10^6$ cells) s.c.	Challenge ($0{\cdot}5\times10^6$ cells) WT Sp6	Challenge ($0{\cdot}5\times10^6$ cells) Sp6/L^d	Immun.	Challenge ($0{\cdot}5\times10^6$ cells) WT Sp6	Challenge ($0{\cdot}5\times10^6$ cells) Sp6/L^d
Sp6/B7 s.c. 0/84 (0%)[a]	s.c. 5/21 (24%)[b,e] 10/21 (53%)[c,f] i.p. 10/10 (100%)[f]	s.c. 4/14 (28%)[b,e] 2/10 (20%)[c,e] i.p. 4/10 (40%)[f]	Sp6/B7 s.c. 0/29 (0%)	i.p. 4/19 (20%)[i,m]	i.p. 0/10 (0%)[i,m]	none	10/10 (100%)	
Sp6/B7/L^d s.c. 0/59 (0%)	s.c. 5/10 (50%)[b,h] 6/10 (60%)[c] i.p. 5/10 (50%)[h,m]	s.c. 2/10 (20%)[b,g] 1/10 (10%)[c,g] i.p. 1/19 (11%)[g]	Sp6/B7/L^d s.c. 0/20 (0%)	i.p. 0/10 (0%)[i,m]	i.p. 0/10 (0%)[i,m]	none		10/10 (100%)

(b)



(c)

Immun. ($0{\cdot}5\times10^6$ cells) i.p.	Challenge ($0{\cdot}5\times10^6$ cells) i.p. WT Sp6	Challenge ($0{\cdot}5\times10^6$ cells) i.p. Sp6/L^d
Sp6/B7 0/10 (0%)	10/10 (100%)	7/10 (70%)
Sp6/B7/L^d 0/10 (0%)	8/10 (80%)	6/10 (60%)

Figure 3. (a) Tumour incidence indicated as the number of animals developing tumours/total number of animals injected and as percentage of animals developing tumours (in parenthesis), of mice immunized with $0{\cdot}5 \times 10^6$ Sp6/B7 or Sp6/B7/L^d and challenged subcutaneously (s.c.) [b]ipsilaterally, s.c. [c]contralaterally or intraperitoneally. The differences in tumour incidence comparing mice immunized with Sp6/B7, challenged with wild-type (WT) Sp6 s.c. ipsilateral or Sp6/L^d s.c. either ipsilateral or contralateral to naive mice injected with WT Sp6 or Sp6/L^d are statistically significant ($P < 0{\cdot}001$)[e]. The differences in tumour incidence comparing mice immunized with Sp6/B7 and challenged with WT Sp6 s.c. contralateral or Sp6/L^d i.p. to mice that received the same immunization but were challenged with WT Sp6 i.p. are statistically significant ($P < 0{\cdot}05$)[f]. The differences in tumour incidence between mice immunized with Sp6/B7/L^d and challenged with Sp6/L^d s.c. or i.p. are statistically significant compared with naive mice injected with Sp6/L^d ($P < 0{\cdot}001$)[g]. The differences in tumour incidence between mice immunized with Sp6/B7/L^d and challenged with WT Sp6 s.c. ipsilateral or i.p. are statistically significant compared with naive mice injected with WT Sp6 or Sp6/L^d ($P < 0{\cdot}05$)[h]. The differences in tumour incidence between mice immunized s.c. with 5×10^6 Sp6/B7 or Sp6/B7/L^d and challenged i.p. with WT Sp6 are statistically significant compared with mice immunized s.c. with $0{\cdot}5 \times 10^6$ Sp6/B7 challenged i.p. with WT Sp6 ($P < 0{\cdot}001$)[i], or with Sp6/L^d ($P < 0{\cdot}05$), or compared with mice immunized s.c. with Sp6/B7/L^d challenged i.p. with WT Sp6 ($P < 0{\cdot}05$)[m]. (b) Overall survival of mice immunized with $0{\cdot}5 \times 10^6$ Sp6/B7 (i) or Sp6/B7/L^d (ii) by i.p. delivery and challenged i.p. with WT Sp6 or Sp6/L^d, as indicated above each plot. (c) Tumour incidence indicated as the number of animals developing tumours/total number of animals injected and as percentage of animals developing tumours (in parenthesis), of mice immunized i.p. with $0{\cdot}5 \times 10^6$ Sp6/B7 or Sp6/B7/L^d and challenged i.p. with WT Sp6 or Sp6/L^d. The differences in tumour incidence between mice immunized with Sp6/B7 or Sp6/B7/L^d and challenged with WT Sp6 or Sp6/L^d are not statistically significant compared either among differently immunized mice or to naive mice injected with WT Sp6 or Sp6/L^d.

80% of animals immunized with Sp6/B7 were protected against i.p. challenge with WT Sp6 and 100% against Sp6/L^d tumour cells ($P < 0{\cdot}001$); 100% of animals immunized with Sp6/B7/L^d were protected against both WT Sp6 and Sp6/L^d tumour cells ($P < 0{\cdot}001$). These data indicate that a 10-fold higher dose of either Sp6/B7 or Sp6/B7/L^d cells appeared to bypass the effect of quantitative differences in H-2 L^d expression.

Wild-type Sp6- and Sp6/L^d-specific cytotoxic responses increased dramatically when mice were immunized with the 10-fold higher dose of 5×10^6 Sp6/B7-Sp6/B7/L^d cells (Fig. 6a,b), according to our previously published data concerning Sp6/B7 only.[17]

In vivo depletion of CD4$^+$ or CD8$^+$ T-cell subsets indicates that both are necessary for a memory tumour-protective response

To investigate the role of the different T-cell subsets in the elimination of Sp6/B7 cells *in vivo*, 5×10^6 of Sp6/B7 cells were injected s.c. in four groups, each of 12 animals, that were depleted of the CD4$^+$ and/or CD8$^+$ T-cell subsets by *in vivo* treatment with anti-CD8 and/or anti-CD4 antibodies as described in Materials and methods. Figure 4 shows the cytofluorimetric analysis performed to verify depletion occurrence. As shown in Fig. 5(a), depletion of CD8$^+$ T cells rendered Sp6/B7 cells tumorigenic in 100% of cases,

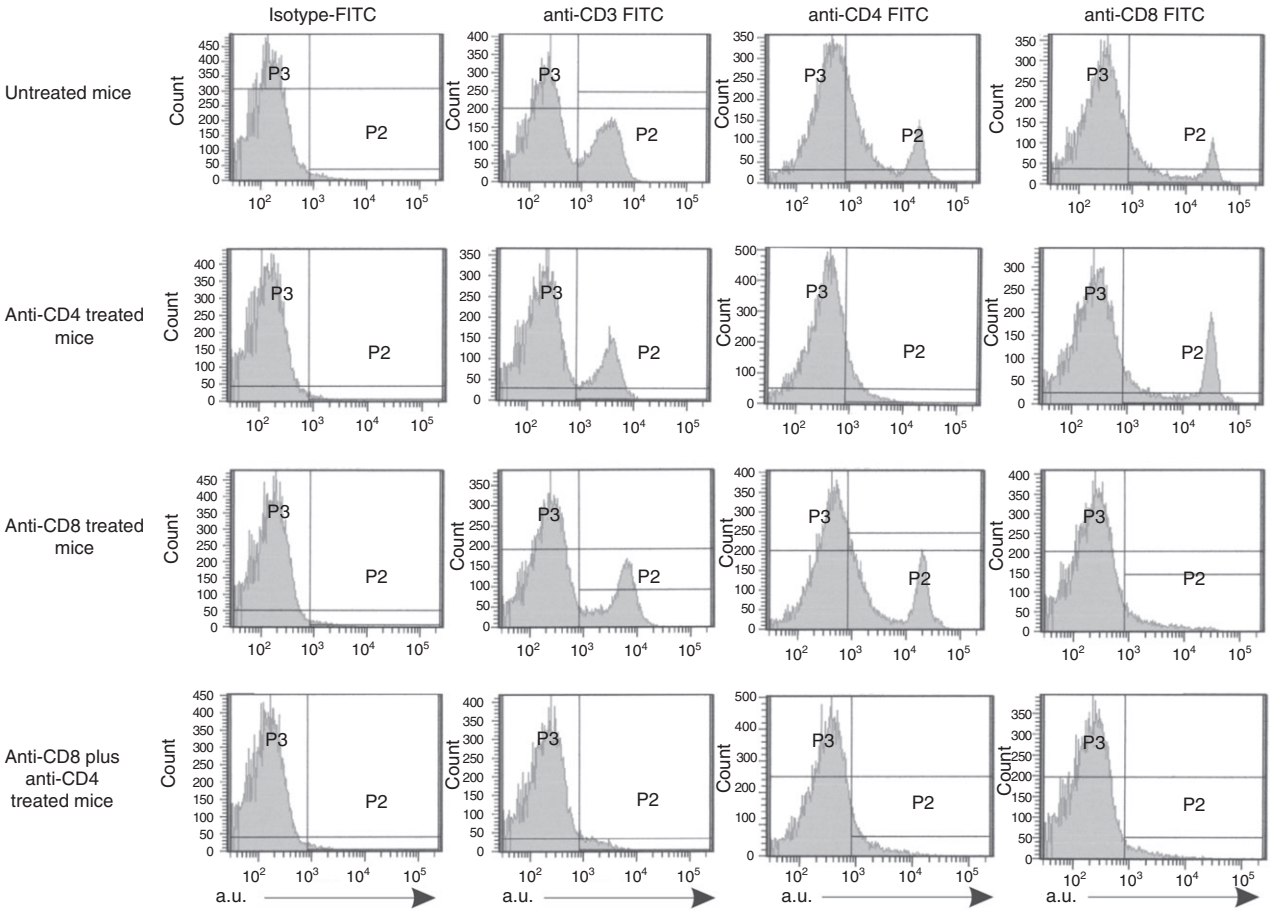


Figure 4. Cytofluorimetric analysis of spleen cells of mice depleted of the $CD4^+$ and $CD8^+$ T-cell subsets by *in vivo* treatment with anti-CD8 and/or anti-CD4 antibodies as described in Materials and methods. Untreated mice are included as control. Spleen cells were analysed after direct immunofluorescence staining with anti-mouse CD3, CD4 and CD8 monoclonal antibodies as indicated at the top of each lane. The treatment of mice is indicated on the left of each horizontal series of histograms. Fluorescence values are expressed in absorbance units (a.u.).

whereas this was not the case when the $CD4^+$ T-cell subset was depleted. These results demonstrate that the $CD8^+$ T-cell population, not the $CD4^+$, is required to eliminate Sp6/B7 tumour cells. Then six animals among the mice depleted of the T $CD4^+$ cells together with six animals of the control group (treated with complete medium only and not T-$CD4^+$-depleted), tumour-free following s.c. injection of 5×10^6 Sp6/B7, were challenged s.c. with $0{\cdot}5 \times 10^6$ WT Sp6 cells in the same anatomical quarter as the immunizing injection, without performing any further treatment with GK1.5 antibody. The s.c. inoculum of WT Sp6 cells grew in all T $CD4^+$-depleted mice, but it did not grow in control un-depleted animals, indicating that the $CD4^+$ T-cell subset is necessary for the induction of the Sp6-specific T $CD8^+$ memory anti-tumour response (Fig. 5a). The cells derived from the spleens of the remaining six animals among the T $CD4^+$-depletion-treated mice and of the control group (treated with complete medium only and not T $CD4^+$-depleted), tumour-free following s.c. injection of 5×10^6 Sp6/B7, were used to analyse the WT Sp6- and Sp6/L^d-specific CTL response in *in vitro* cytotoxicity assays, performed as described in the Materials and methods section. Spleen cells from mice depleted of T $CD4^+$ cells and immunized with Sp6/B7 showed almost undetectable levels of specific lysis of WT Sp6 and Sp6/L^d cell targets, compared with un-depleted, control mice immunized with Sp6/B7 (Fig. 5b). Wild-type Sp6- and Sp6/L^d-specific CTL effector activity, found in un-depleted control mice, was absent in the spleens of T $CD4^+$-depleted mice. These results indicate that the $CD4^+$ T-cell population, although not involved in the direct initial elimination of the B7-1-expressing Sp6 tumour cells, is required to induce the memory immune response against WT Sp6.

H-2 subset-specific blocking antibodies reveal the different contributions to the Sp6-specific tumour immune response given by each single restriction element

Inhibition of WT Sp6 and Sp6/L^d-specific *in vitro* cytotoxicity by H-2 subset-specific blocking antibodies showed that both immunizations with 5×10^6 Sp6/B7

(a)

T cell depletion	Tumour incidence after immunization s.c with 5×10^6 Sp6/B7 cells	Tumour incidence after challenge s.c with 5×10^6 WT Sp6 cells
None	0/12 (0%)	0/6 (100%)
T CD8$^+$	12/12 (100%)	-
T CD4$^+$	0/12 (0%)	6/6 (100%)
T CD8$^+$ and T CD4$^+$	12/12 (100%)	-

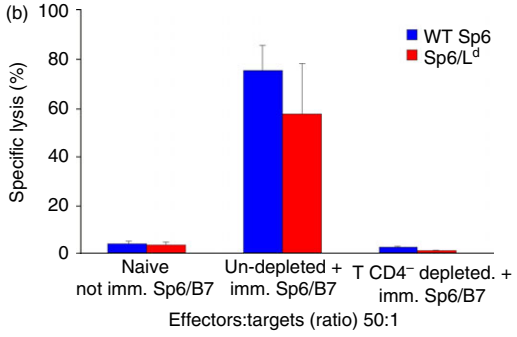


Figure 5. (a) Tumour incidence indicated as the number of animals developing tumours/total number of animals injected and as percentage of animals developing tumours (in parenthesis), of mice depleted of the CD4$^+$ and/or CD8$^+$ T-cell subsets, after injection of 5×10^6 Sp6/B7 cells. Tumour-free mice were challenged with $0{\cdot}5 \times 10^6$ WT Sp6 cells. (b) Cytotoxic response against WT Sp6 and Sp6/L^d targets of spleen cells from three groups of mice: (i) untreated naive mice as control, (ii) mice immunized with 5×10^6 Sp6/B7 cells, and (iii) mice depleted of T CD4$^+$ cells and immunized with 5×10^6 Sp6/B7 cells, as indicated below each histogram. The percentage of specific lysis measured as ^{51}Cr release is on the ordinate, at the 50 : 1 effector : target ratio, as indicated at the bottom of the figure. Each histogram indicates the mean values with standard deviation of the results from six immunized animals.

and Sp6/B7/L^d cells were able to elicit a multi-component immune response against antigenic epitopes restricted by all three MHC-I molecules H-2 L^d, K^d and D^d (Fig. 6c,d). The multi-component feature appeared more consistent when immunizing with Sp6/B7. In fact, in mice immunized with Sp6/B7, the response to WT Sp6, although primarily K^d-restricted (40–60% inhibition induced by anti-K^d blocking antibody), displayed a relevant component restricted by H-2 L^d and D^d, as a 25–30% inhibition was induced by anti-L^d and -D^d blocking antibodies (Fig. 6c, histogram on the left). In these same mice immunized with Sp6/B7, the response against Sp6/L^d target showed a main L^d-restricted component (30–45% inhibition mediated by anti-L^d blocking antibody), with K^d- and D^d-restricted responses of lower intensity (Fig. 6d, histogram on the left). Similarly, the WT Sp6-specific immune response of Sp6/B7/L^d immunized mice showed a multi-component restriction with a prevalent K^d-restricted component (Fig. 6c, histogram on the right). Only the response of Sp6/B7/L^d immunized mice to Sp6/L^d was almost exclusively restricted by L^d (60–80% lysis inhibition by anti-L^d blocking antibody) (Fig. 6d, histogram on the right).

P1A and gp70 tumour antigens are not involved in the rejection of WT Sp6 and Sp6/L^d

Wild-type Sp6 and transfectants were monitored for the capacity to present gp70 and P1A to specific CTL cells and be lysed, restricted by the H-2 L^d molecule. In spite of their down-regulated cell surface expression of H-2 L^d, WT Sp6 and Sp6/B7 could still be lysed *in vitro* by CTL cell lines specific for the gp70-AH1 immunodominant epitope (gp70$_{423–431}$ peptide)[18,19] (Fig. 7a, histogram on the left), although the transfection-mediated increase of H-2 L^d expression in Sp6/L^d and Sp6/B7/L^d raised gp70-AH1-specific cytotoxicity (Fig. 7a, histogram on the left). In contrast, the CTL clone specific for the H-2 L^d-restricted P1A-immunodominant epitope (P1A$_{35–43}$ peptide) did not show specific cytotoxic activity, indicating that the P1A antigen was not presented by WT Sp6 and its transfectants (Fig. 7a, histogram on the right).

These observations suggested that, in the Sp6 tumour model, gp70 could behave as a tumour-associated antigen and stimulate the generation of gp70-specific CTLs. To test this hypothesis, spleen cells of mice immunized with either 5×10^6 Sp6/B7 or Sp6/B7/L^d cells were re-stimulated *in vitro* in MLPC with gp70$_{423–431}$ and P1A$_{35–43}$ peptides (P1A as negative control), but specific lysis of 293L^d control target cells pulsed with gp70 peptides was not observed, even if the same spleen cells did show cytotoxic activity specific against WT Sp6 and Sp6/L^d (Fig. 7b,c).

The activation of gp70-AH1-specific CTL clones in mice immunized with Sp6/B7-Sp6/B7/L^d was investigated by means of more sensitive techniques. A measure of the IFN-γ production by CTL clones in response to the gp70$_{423–431}$-AH1 H-2 L^d-restricted peptide compared with the unrelated β-gal$_{876–884}$ negative control was performed by ELISPOT test on spleen cells. The results shown in Fig. 8(a) indicate that immunization with either Sp6/B7 or Sp6/B7/L^d was able to activate gp70-AH1-specific T-cell clones, in numbers higher than those found in naive (not-immunized) mice, but quite limited, compared with control immunization with γ-irradiated CT26 cells, correlating with a strong tumour-rejection gp70-driven CTL response.[34] The presence of gp70-specific T CD8$^+$ cells in Sp6/B7- and Sp6/B7/L^d-immunized mice was also examined by MHC-I tetramer-based cytofluorimetric analysis, as described in the Materials and methods section. In agreement with the results of the ELISPOT test on spleen cells, the MHC-I tetramer-based cytofluorimetric analysis revealed dramatically reduced numbers of gp70-AH1-specific CD8$^+$ T cells in draining lymph nodes of mice immunized with Sp6/B7-Sp6/B7/L^d, compared with mice immunized with γ-irradiated CT26 cells (Fig. 8b). These

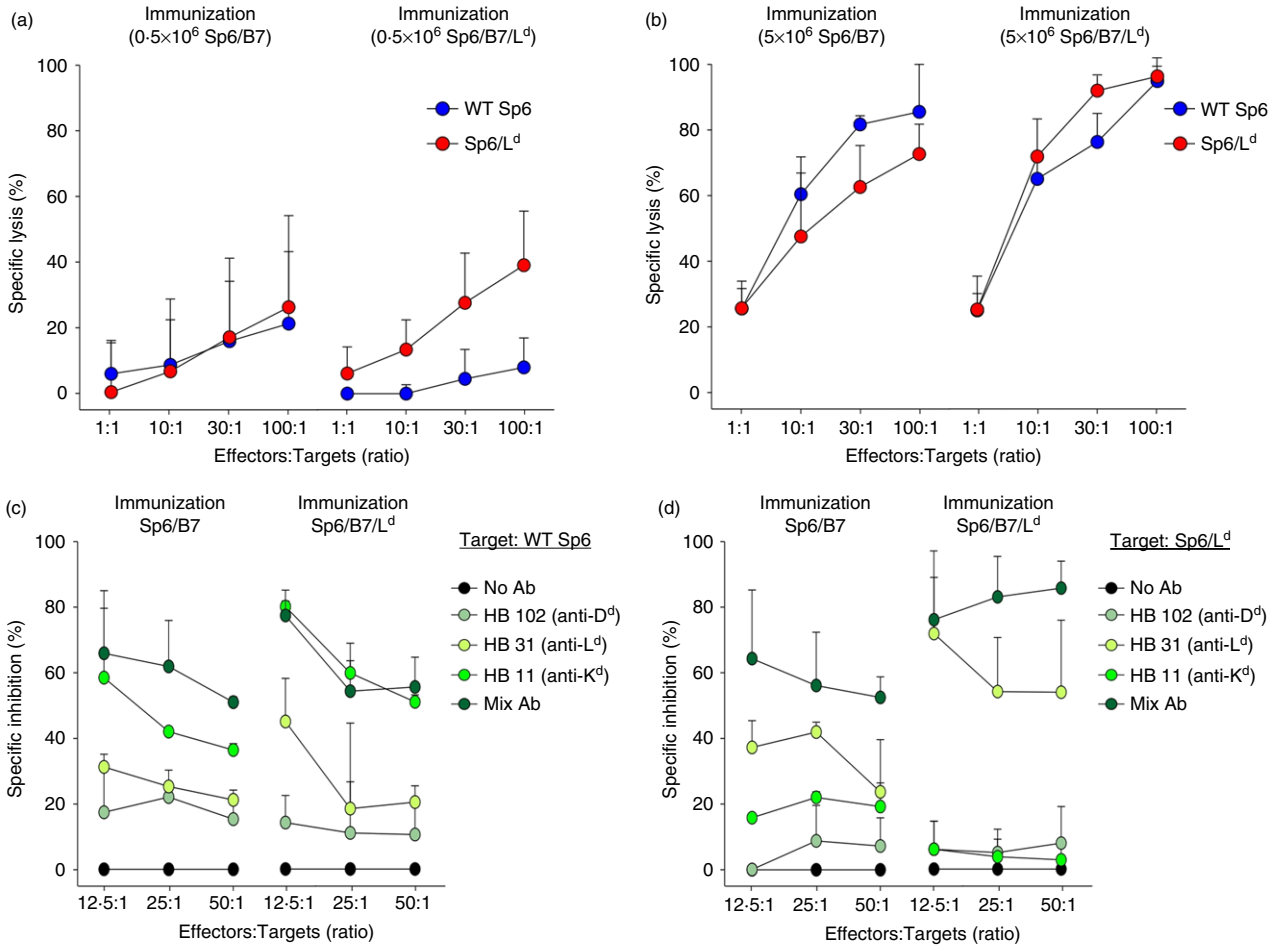


Figure 6. (a) Splenic cytotoxic response against wild-type (WT) Sp6 and Sp6/L^d targets, of mice immunized by one subcutaneous (s.c.) injection with 0·5 × 10^6 Sp6/B7 or Sp6/B7/L^d cells, as indicated above each plot. Spleen cells were re-stimulated in mixed leucocyte tumour culture (MLTC) for 5 days with Sp6/B7 or Sp6/B7/L^d irradiated cells, according to immunization. The percentage of specific lysis measured as ^{51}Cr release is on the ordinate. Each histogram indicates the mean values with standard deviation of the results from three immunized animals. (b) Splenic cytotoxic response against WT Sp6 and Sp6/L^d targets, of mice immunized by one s.c. injection with 5 × 10^6 Sp6/B7 or Sp6/B7/L^d cells, as indicated above each plot. The experiments were performed as described in (a). (c) Percentage of specific inhibition of splenic cytotoxic response against WT Sp6 target, of mice immunized by one s.c. injection with 5 × 10^6 Sp6/B7 or Sp6/B7/L^d cells, as indicated above each plot. Spleen cells were re-stimulated in MLTC for 5 days with 5 × 10^5 Sp6/B7 or Sp6/B7/L^d irradiated cells, according to immunization. For MHC-I subset-restricted blocking of cytotoxic cell function, before being added to effectors, labelled target cells were incubated for 30 min at 4° with anti-H-2 L^d (HB-31), anti-H-2 K^d (HB-11), anti-H-2 D^d (HB-102), or a mix of them, at the final concentration of 10 μg/ml. Data are means of triplicate of percentages of specific lysis measured as ^{51}Cr release at the indicated effectors:targets ratios. The percentage of specific inhibition of lysis measured as ^{51}Cr release is on the ordinate, at the indicated effector : target ratios. Each histogram indicates the mean values with standard deviation of the results from three immunized animals. (d) Percentage of specific inhibition of splenic cytotoxic response against Sp6/L^d target, of mice immunized by one s.c. injection with 5 × 10^6 Sp6/B7 or Sp6/B7/L^d cells, as indicated above each plot. The experiments were performed as described in (c).

data indicate that, although WT Sp6 and transfectants do express and present the gp70-AH1 immunodominant epitope even when H-2 L^d is poorly expressed, as in WT Sp6 and Sp6/B7 (Fig. 7a), most of the Sp6/L^d and WT Sp6-tumour protective CTL-dependent response elicited by the immunization with Sp6/B7-Sp6/B7/L^d cells is not gp70-specific, despite few $CD8^+$ T cells reactive to gp70-AH1 being detected by either tetramer staining or ELISPOT analysis.

Immunization with Sp6/B7-Sp6/B7/L^d induces expression of gp70 transcripts in secondary lymphoid organs

It has been extensively described that the genome of all mouse strains currently employed in medical research contains Mu-MLV DNA sequences, including those coding for the gp70 antigen.[21,43] Virus-like particles were detected in WT Sp6 cells and transfectants by electron

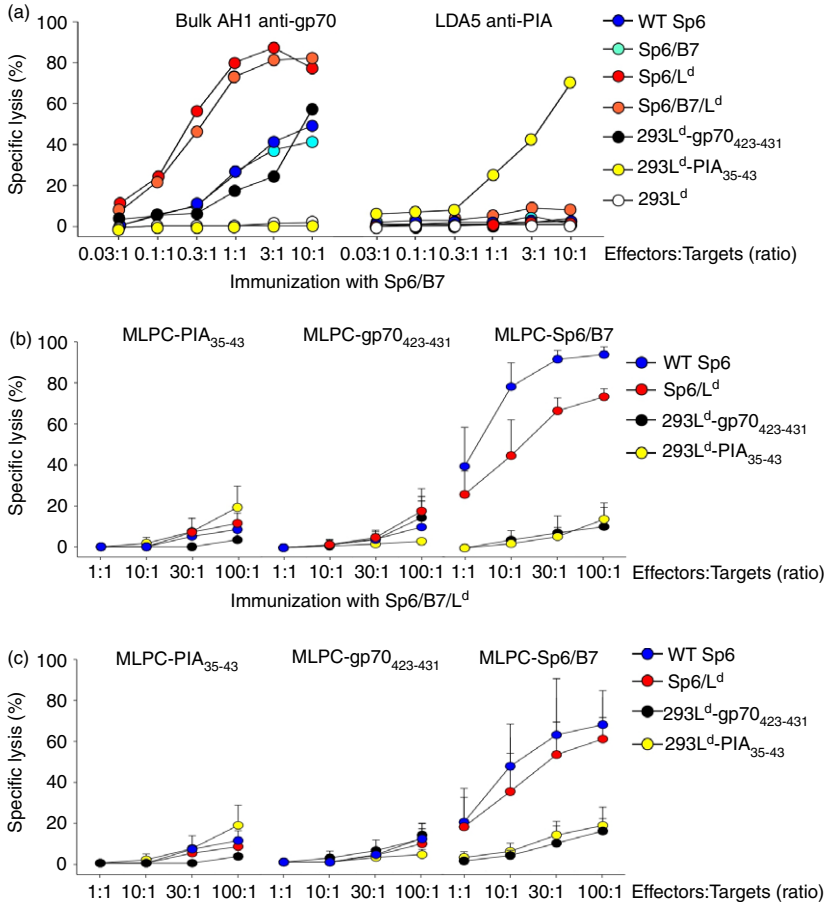


Figure 7. (a) Cytotoxic response of the gp70-specific cytotoxic T lymphocyte (CTL) line Bulk AH1 and the P1A-specific CTL clone LDA5 (as indicated above each plot) against wild-type (WT) Sp6, Sp6/L^d, Sp6/B7/L^d and 293L^d unloaded and loaded with $gp70_{423–431}$ or $P1A_{35–43}$ peptide, as indicated in the legend on the right of the plot. Data are means of triplicates of percentages of specific lysis measured as ^{51}Cr release at the indicated effector : target ratios. The data shown are from one representative experiment out of three with similar results. (b) Cytotoxic response of spleen cells from mice immunized with 5×10^6 Sp6/B7 cells against WT Sp6, Sp6/L^d, Sp6/B7/L^d and 293L^d unloaded and loaded with $gp70_{423–431}$ or $P1A_{35–43}$ peptide, as indicated on the right of the plots. Spleen cells were re-stimulated in mixed leucocyte–peptide culture (MLPC) for 5 days with gp70- or P1A-derived peptides or in mixed leucocyte tumour culture (MLTC) with Sp6/B7 irradiated cells, as indicated above each plot. The percentage of specific lysis measured as ^{51}Cr release is on the ordinate, at the indicated effector : targets ratios. Each histogram indicates the mean values with standard deviation of the results from three immunized animals. (c) Cytotoxic response of spleen cells from mice immunized with 5×10^6 Sp6/B7/L^d cells against WT Sp6, Sp6/L^d, Sp6/B7/L^d and 293L^d unloaded and loaded with $gp70_{423–431}$ or $P1A_{35–43}$ peptide, as indicated on the right of the plot. Spleen cells were re-stimulated in MLPC for 5 days with gp70- or P1A-derived peptides or in MLTC with Sp6/B7/L^d irradiated cells, as indicated above each plot. The percentage of specific lysis measured as ^{51}Cr release is on the ordinate, at the indicated effector : target ratios. Each histogram indicates the mean values with standard deviation of the results from three immunized animals.

microscope analysis (Fig. 9a), which was very similar the description by Watson *et al.*[44] in mouse plasmacytoma cells. As expected, WT Sp6 cells and transfectants expressed gp70-specific transcripts (Fig. 9b, lanes 1–4), at levels comparable to those of the CT 26 colon carcinoma (Fig. 9b, lane 5), positive control cell line for gp70 expression.[34]

Transcripts specific for gp70 were not present in secondary lymphoid organs (lymph nodes and spleen) of mice injected with WT Sp6 or Sp6/L^d cells and developing tumours or naive (Fig. 9b lanes 9, 10, 13, 14 and Fig. 9c lane 20). However, they became detectable after immunization with Sp6/B7 or Sp6/B7/L^d cells (Fig. 9b lanes 7, 8, 11 and 12). These results suggested that expression of gp70 in peripheral lymphoid organs could be a consequence of vaccination.

It has been described that triggering of TLR2, TLR4 and TLR7 by DAMPs can induce gp70 expression.[22,23] Apoptotic target cells lysed by CTLs do release DAMPs.[45] Hence, we hypothesized that CTL-mediated lysis of immunizing B7-1^+ cells that reached secondary lymphoid organs may induce TLR-triggered *de novo* expression of gp70 in resident APCs, by the release of DAMPs. Therefore, spleen cells from naive mice were incubated for

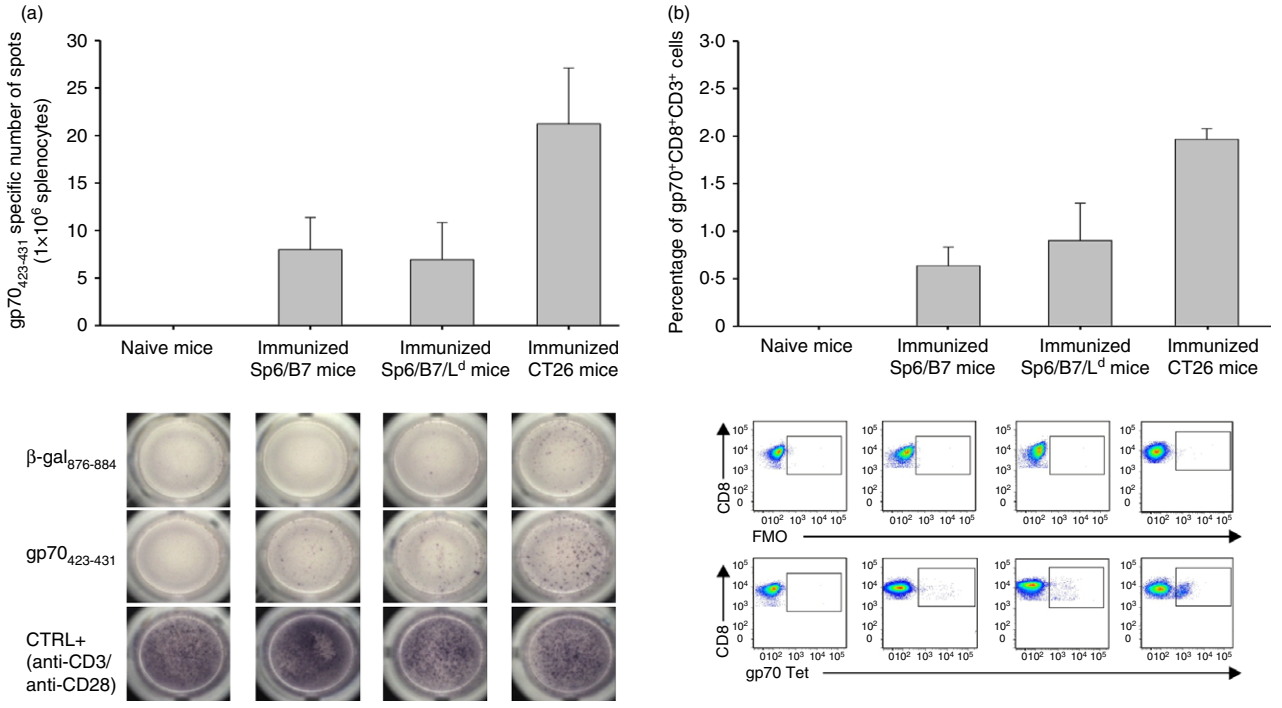


Figure 8. (a) Evaluation of gp70-specific immune response by mouse interferon-γ (IFN-γ) ELISPOT analysis. Spleen cells (1×10^6) from naive mice and mice immunized with Sp6/B7, Sp6/B7/L^d and γ-irradiated CT26 cells were re-stimulated for 20 hr with β-gal$_{876–884}$, gp70$_{423–431}$, or anti-CD3/anti-CD28. The histogram shows the number of gp70-specific spots normalized by subtracting the background spots relative to unrelated antigen (β-gal spots). Below the histogram is a representative image of the ELISPOT wells of the experiment, showing the positive control reaction with anti-CD3/anti-CD28, indicating the presence of seeded CD3$^+$ T cells. (b) MHC-I Pentamer labelling of gp70 T-cell receptor-specific CD8$^+$ T cells: 1×10^6 lymph node cells from mice immunized with Sp6/B7, Sp6/B7/L^d, γ-irradiated CT26 cells were stained with Pro5 MHC class I Pentamer loaded with H-2Ld gp70$_{423-431}$ peptide and then with CD3, CD8 surface antibodies. The chart shows the percentage of gp70 T-cell receptor-specific cells gated on CD8$^+$ T lymphocytes.

48 hr with supernatants of 5-day *in vitro* MLTCs of spleen cells from Sp6/B7- or Sp6/B7/L^d-immunized mice. Following the above-mentioned treatment, spleen cells acquired *de novo* expression of gp70 transcripts (Fig. 9c, lanes 16–19), suggesting that the induction of gp70 expression could be the result of TLR triggering by DAMPs released by the apoptotic Sp6 cells lysed by activated CTLs.[23] A statistically significant increase ($P < 0{\cdot}05$) of the high mobility group box 1 (HMGB1) DAMP molecule was found in supernatants of WT Sp6 cell cultures after incubation with MLTC-induced spleen cells derived from mice injected with Sp6/B7 compared with supernatants with spleen cells from mice injected with WT Sp6 (Fig. 9d).

To confirm the hypothesis that HMGB1 could be involved in activation of gp70 expression, spleen cells from naive mice were incubated for 48 hr with medium containing 100 ng/ml of purified HMGB1 or BSA as a control. The incubation with HMGB1 was able to trigger gp70 expression in naive splenocytes at levels higher than BSA (where gp70 transcripts were barely detectable), confirming that HMGB1 is contributing to activate gp70 expression in naive spleen and lymph node cells (Fig. 9e). In addition, spleen cells from naive mice, following a 48-hr incubation with purified HMGB1, BSA or pulsation with β-gal$_{876–884}$/gp70$_{423–431}$ peptides, were co-cultured with Bulk AH1 anti-gp70 CTL line and activation was monitored in a functional IFN-γ ELISPOT assay, where mouse IFN-γ spots are induced by specific gp70$_{423–431}$ peptide recognition by the Bulk AH1 anti-gp70 CTL line. As can be seen in Fig. 9(f), naive splenocytes incubated with HMGB1 triggered the Bulk AH1 anti-gp70 CTL line to produce IFN-γ spots in numbers comparable to those obtained after proper pulse with the gp70$_{423–431}$ peptide, as the differences were not statistically significant (indicated above the histograms). This implies that HMGB1-TLR binding took place and activated antigen expression and presentation of the gp70 AH1 antigenic epitope to the specific CTL line.

Discussion

The present work highlights the capacity of whole-tumour–cell vaccines to overcome tumour-immunoescape mechanisms such as down-regulation of MHC-I subsets and inactivation of CTLs specific for immunodominant antigenic epitopes.

Immunization with one dose of $0{\cdot}5 \times 10^6$ cells of both Sp6/B7/L^d and Sp6/B7 induced a protection more

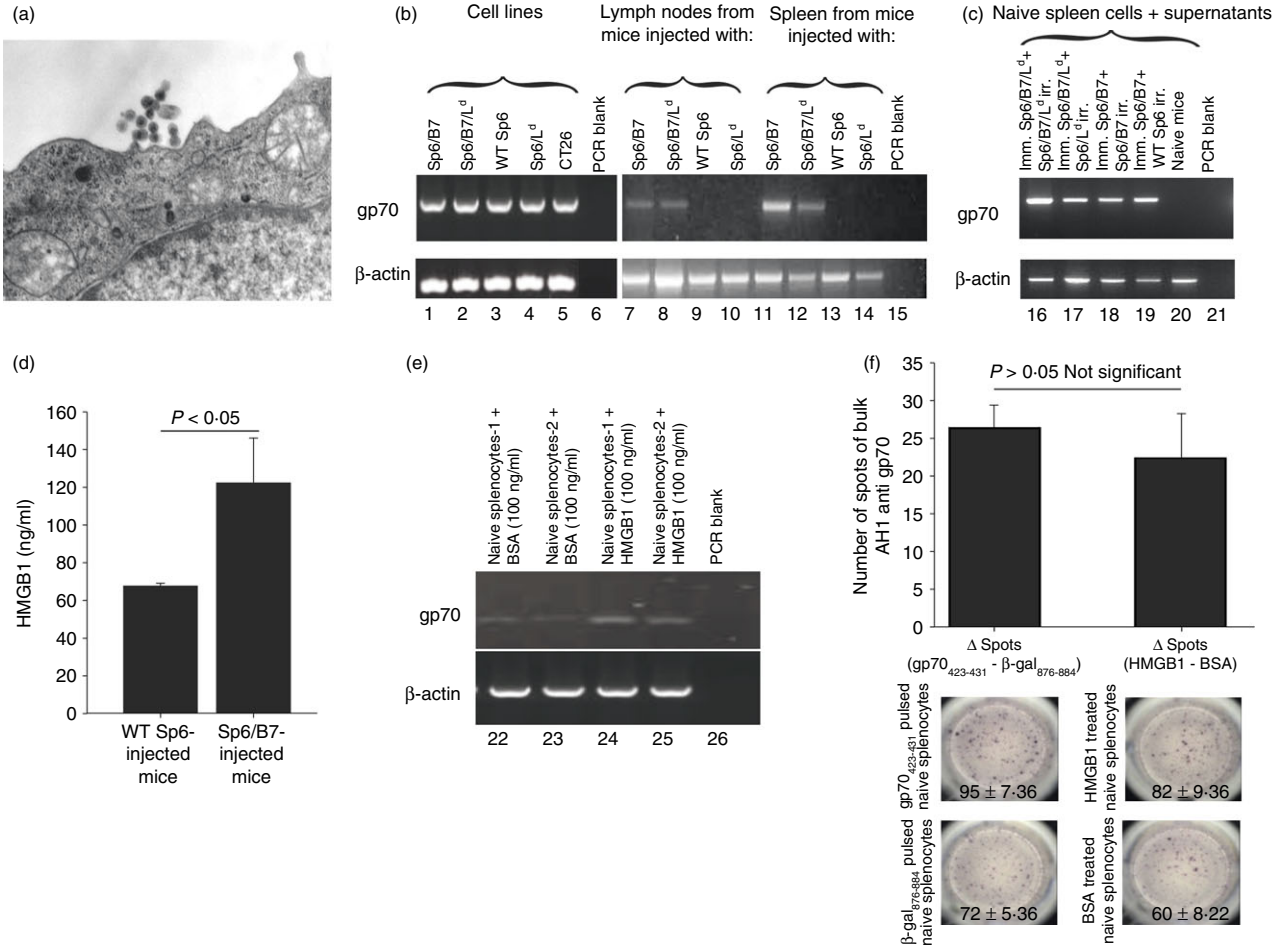


Figure 9. (a) A representative case of wild-type (WT) Sp6 cells, observed on a Zeiss EM 109 electron microscope. The presence of virus-like particles in both cytoplasm and extracellular spaces is detectable. (b) RT-PCR analysis for the expression of gp70 and β-actin genes in: Sp6/B7, Sp6/B7/L^d, WT Sp6, Sp6/L^d, CT26 tumour cells (lanes 1–5); lymph nodes and spleens from a mouse injected with the same tumour cells as indicated above (lanes 7–14). (c) RT-PCR analysis for the expression of gp70 and β-actin genes in spleen cells from a naive mouse incubated with supernatants of 5-day cultures of spleen cells from mice differently immunized and co-cultured, as reported above the lanes (lanes 16–20). 'PCR blank' indicates the result of a control PCR without any RNA addition (lanes 6, 15, 21, 26). (d) HMGB1 release by WT Sp6 cells after exposure to mixed tumour leucocyte culture (MLTC)-induced spleen cells derived from either WT Sp6- or Sp6/B7-injected mice, as indicated below each histogram. Spleen cells were cultured in MLTC for 5 days with irradiated Sp6/B7 cells. The MLTC was next incubated with WT Sp6 target cells or with medium alone, and the supernatant was collected after 48 hr. Histograms show HMGB1 concentrations normalized for the values of the MLTC incubated with medium alone. (e) RT-PCR analysis as described in (b) and (c) was performed in spleen cells from a naive mouse incubated for 48 hr with BSA or HMGB1 (100 ng/ml). (f) Spleen cells from a naive mouse previously incubated with BSA or HMGB1 (100 ng/ml) or pulsed with β-gal$_{876–884}$/gp70$_{423–431}$ peptides for 48 hr were co-cultured for 20 hr with Bulk AH1 anti gp70 CTL line in an IFN-γ-ELISPOT functional assay. The histogram shows the numbers of mouse interferon-γ (IFN-γ) spots induced by specific gp70$_{423–431}$ peptide recognition by the Bulk AH1 anti-gp70 CTL line triggered by naive splenocytes either pulsed with the gp70$_{423–431}$ peptide or incubated with HMGB1, subtracted from the spots from control reaction (e.g. β-gal$_{876–884}$ or BSA, respectively).

extended anatomically versus Sp6/L^d than WT Sp6 (Fig. 2, compare a and c with b and d). Lysis of Sp6/L^d appeared mainly mediated by H-2L^d-restricted CTLs (Fig. 6d), whereas in the lysis of WT Sp6 the contribution of all three MHC-I subsets was more homogeneous, although with a slight prevalence of H-2 K^d-restricted CTL responses (Fig. 6c). These results suggest that the Sp6/B7 vaccine can still stimulate H-2 L^d-restricted responses in spite of the low surface amounts of the molecule, in a scenario where Sp6/B7 cells can be killed by differently MHC-restricted CTLs, previously activated by direct tumour antigen presentation. Apoptotic bodies are taken up by professional APCs, able to cross-prime H-2 L^d-restricted CTLs specific for Sp6-tumour-associated antigens, as described by Pavelic *et al.*[6]

Hence, as suggested by the results obtained with H-2 subset-specific blocking antibodies (Fig. 6c,d) as well as by the different tumour-protective effects observed

(Fig. 2), immunization with either Sp6/B7 or Sp6/B7/L^d induced multi-MHC-I-restricted immune responses. Nevertheless, the immunization with Sp6/B7/L^d is likely to be more prone to induce H-2 L^d-restricted responses than the immunization with Sp6/B7.

Wild-type Sp6 and H-2 L^d transfectants were able to present the immunodominant AH1 epitope of the gp70 tumour-associated antigen and be killed *in vitro* by CTL lines specific for the H-2 L^d-restricted-gp70-AH1 immunodominant epitope, in proportion to their H-2 L^d surface expression amounts (Fig. 7a). It was not the case for the P1A equivalent (Fig. 7a). On the contrary, *in vivo*, the tumour-protective CTL response elicited by immunization with B7-1-expressing Sp6 cells did not show specificity for gp70 (nor for P1A, as expected) (Fig. 7b,c). BALB/c mice, as well as almost all mouse laboratory strains, show endogenous murine leukaemia viruses (Mu-MLV) and virus-related sequences widely interspersed throughout the genome.[21,43] Gp70 is a viral antigen and evidence of infection by a Mu-MLV-1 was found in myeloma cells derived from BALB/c such as MOPC21 and derivatives like the P3/X63-Ag8 clone[46] and the P3/X63-Ag8-derived Sp6 hybridoma.[29,44] In fact, it has been shown that B cells represent permissive targets for virus replication, whereas T cells are resistant to infection.[47] As shown in Fig. 9(a), virus-like particles are detectable in WT Sp6 and derivatives, as described previously.[44,46] In addition, spleen cells from naive mice, after incubation with supernatants from Sp6 cells lysed by CTLs express *de novo* gp70 transcripts (Fig. 9c). Hence, it cannot be excluded that virus particles released by CTL-lysed cells may superinfect spleen cells in the *in vitro* experiments performed, as well as *in vivo*, during immunization, and lead to gp70 expression. Supernatants have been found to contain HMGB1 (and possibly other DAMPs), released by dying cells (Fig. 9d). HMGB1 is a ligand of TLR2 and TLR4.[45] In lupus-prone mice, injection of TLR agonists, as well as triggering of TLR4, TLR7 and TLR9 by apoptotic cells or nucleic acid-containing IgG immuno-complexes, induced high serum levels of gp70.[22,23] Hence, it is conceivable that the DAMPs (e.g. HMGB1) released during MLTCs by CTL-lysed Sp6 cells may trigger TLR-dependent gp70 expression on cells from naive spleens (Fig. 9c). WT Sp6 cells and transfectants, which are CXCR4 positive, are responsive to stromal cell-derived factor-1 (SDF-1 or CXCL12) *in vitro* (Sartoris S. *et al.*, manuscript in preparation), so they should be able to migrate from subcutaneous inoculation sites to SDF-1-expressing sites such as the secondary lymphoid organs, as seen for many other tumour-derived cell lines.[8] Hence we can hypothesize that during the immunization phase, the B7-expressing Sp6 cells migrate to draining lymph nodes and later to the spleen, where they encounter and directly prime the specific $CD8^+$ naive T cells. The activated CTLs kill Sp6/B7 cells, with the following release of HMGB1 and/or other DAMPs (e.g. single- and double-stranded RNA, DNA, etc.) that, through binding to TLRs on resident APCs, induce them to express gp70. A synergic stimulus for gp70 expression may be elicited by virus particles discharged by the CTL-lysed cells that may superinfect resident APCs. The APCs will process and present this *de novo* expressed gp70 via the endogenous route, and, in this context, immunodominant viral antigen epitopes will be presented by a large number of APCs resulting in the induction of anergy or clonal deletion of most gp70-specific $CD8^+$ T cells, probably through activation-induced exhaustion of the majority of high affinity TCR T cells.[48] The residual surviving gp70-AH1-specific T cells, detected by ELISPOT and tetramer staining (Fig. 8a,b), are probably low-affinity clones, likely to be overcome during immune response amplification by different and more Sp6-specific tumour antigens.

A similar link between TLR triggering and stimulation of viral product expression has been described in the human setting of Epstein–Barr virus infection, between TLR7 and Epstein–Barr virus latent membrane protein 1.[49] Hence, in Sp6/B7-immunized mice, a strong reduction of gp70-specific response associated with down-regulation of the H-2 L^d restriction element, probably shaped the immune response against different tumour antigens, preferentially H-2 K^d-restricted and, to a lesser extension, D^d-restricted and subdominant, as indicated by the fact that the immune response elicited by Sp6/B7 and Sp6/B7/L^d required recall stimulations or high vaccine doses to elicit a systemic protection against WT Sp6-Sp6/L^d.[17] These data are in agreement with results shown by other groups in different systems.[50,51]

As discussed elsewhere,[17] the mechanism of elimination of B7-1-expressing Sp6 cells is mainly mediated by the specific T-cell response, as revealed by T $CD4^+$ and $CD8^+$ depletion experiments (Fig. 5). Depletion of $CD8^+$ T cells rendered Sp6/B7 cells tumorigenic in 100% of cases (Fig. 5a). In contrast, depletion of the $CD4^+$ T-cell subset resulted in 0% tumour incidence (Fig. 5a). These data demonstrate that the $CD8^+$ T-cell population, not the $CD4^+$, was required to eliminate Sp6/B7 tumour cells. Indeed, according to the literature,[52,53] $CD4^+$ T cells were needed to induce the memory immune response against WT Sp6, as mice treated with anti-CD4 antibody and able to eliminate Sp6/B7, when subsequently challenged with WT Sp6 ($0{\cdot}5 \times 10^6$ Sp6 cells), developed tumours (Fig. 5a) and did not show cytotoxic activity specific for WT Sp6-Sp6/L^d (Fig. 5b). These results are in agreement with some authors, showing the action of $CD8^+$ T effector cells predominant in the elimination of tumour cells, and the need of $CD4^+$ T helper activity for the induction of a memory CTL response.[12,13,16] Nevertheless, $CD8^+$ T cells are not to be considered as universal anti-tumour T-cell effectors, as $CD4^+$ T-cell-mediated elimination of tumour cells has been extensively described (ref. 54, and

references therein). Based on MHC-II expression, two killing mechanisms have been identified: direct and indirect killing. Direct killing is mediated by intrinsic cytotoxic activity of the $CD4^+$ T cells and targets MHC-II-positive tumour cells.[55,56] Indirect killing of MHC-II-negative tumour cells relies on tumour antigens taken up by MHC-II-positive macrophages that are processed and presented to $CD4^+$ T cells in a bi-directional interaction leading to tumour cell killing by activated macrophages. In this context, the studies on the MOPC315 plasmacytoma tumour model produced relevant contributions.[57–59]

In conclusion, despite the identification of tumour antigens and their use as cancer vaccines, autologous tumour cell vaccines still remain a potent tool for generating anti-tumour immunity, especially when tumours avoid the immune response by down-regulating MHC haplotypes or by induction of peripheral tolerance to immunodominant tumour-associated antigens. In fact they bypass these immune escape strategies by amplifying protective immune responses against subdominant tumour antigens that show different MHC restriction from dominant ones, which otherwise would be predominant and ineffective if the tumour immune response relied on the cross-priming performed by the host's dendritic cells alone.[6]

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Author contributions

MM and MM performed most of the experiments and wrote the article. AR designed the study and contributed to the writing of the article. ES, AM and TC performed the initial experiments. SDS performed the *in vitro* experiments. FDS, SS and GF performed the final experiments. SF performed the electron microscope analysis. SU, PZ and VB contributed to the design of the study. SS designed the study and wrote the article.

Disclosures

There is no conflict of interest to disclose.

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