Anti-idiotype antibody induced cellular immunity in mice transgenic for human carcinoembryonic antigen

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Summary

In the present study, we have analysed the detailed cellular immune mechanisms involved in tumour rejection in carcinoembryonic antigen (CEA) transgenic mice after immunization with dendritic cells (DC) pulsed with an anti-idiotype (Id) antibody, 3H1, which mimics CEA. 3H1-pulsed DC vaccinations resulted in induction of CEA specific cytotoxic T lymphocyte (CTL) responses *in vitro* and the rejection of CEA-transfected MC-38 murine colon carcinoma cells, C15, *in vivo* (Saha *et al.*, *Cancer Res* 2004; 64: 4995–5003). These CTL mediated major histocompatibility complex (MHC) class I-restricted tumour cell lysis, production of interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α), and expression of Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) in response to C15 cells. CTL used perforin-, FasL-, and TRAIL-mediated death pathways to lyse C15 cells, although perforin-mediated killing was the predominant lytic mechanism *in vitro*. The cytokines IFN-γ and TNF-α synergistically enhanced surface expression of Fas, TRAIL receptor, MHC class I and class II on C15 cells that increased the sensitivity of tumour cells to CTL lysis. CTL activity generated in 3H1-pulsed DC immunized mice was directed against an epitope defined by the idiopeptide LCD-2, derived from 3H1. *In vivo* lymphocyte depletion experiments demonstrated that induction of CTL response and antitumour immunity was dependent on both $CD4^+$ and $CD8^+$ T cells. The analysis of splenocytes of immunized mice that had rejected C15 tumour growth revealed up-regulated surface expression of memory phenotype Ly-6C and CD44 on both $CD4^+$ and $CD8^+$ T cells. The adoptive transfer experiments also suggested the role of both $CD4^+$ and $CD8^+$ T cells in this model system. Furthermore, mice that had rejected C15 tumour growth, developed tumour-specific immunological memory.

Keywords: dendritic cells; vaccination; T cells; tumour immunity; transgenic mice

Introduction

Certain anti-idiotype (Id) antibodies that bind to the antigen-combining sites of antibodies can effectively mimic the three-dimensional structures and functions of the external antigens and can be used as surrogate antigens for active specific immunotherapy. Several monoclonal anti-Id antibodies, which mimic distinct human tumour-associated antigens (TAAs) have been developed and characterized by others and us (reviewed in 1). The greatest challenge in immunotherapy by means of anti-Id vaccines is to identify the optimal anti-Id antibody (Ab2β) that will function as a true surrogate antigen for a TAA system and ideally will generate both humoral and

Abbreviations: CEA, carcinoembryonic antigen; DC, dendritic cells; Id, idiotype; FasL, Fas ligand; TRAIL, TNF-related apoptosis-inducing ligand; TAA, tumour-associated antigen; CRC, colorectal carcinoma; CEA-Tg, CEA transgenic; CMA, concanamycin A; BLT, *N*-a-benzyloxycarbonyl-L-lysine-thiobenzyl ester hydrochloride.

cellular immune responses. In most preclinical and clinical studies, immunizations with anti-Id antibody, however, have elicited humoral immunity only and has not induced human leucocyte antigen (HLA) class I antigen restricted TAA-specific cytotoxic T lymphocyte (CTL) response. This is because of the requirement of association of heavy and light chains of anti-Id antibodies for the TAA mimicry in most of the antigenic system analysed. In general, the antigen mimicry by anti-Id antibodies reflects structural homology in majority of the cases. In few cases, the anti-Id antibody that mimics the TAA shows linear amino acid sequence homology with the nominal tumour antigen.[2–5] Idio-peptides, which are derived from the heavy and/or light chains of an anti-Id antibody, may be presented by HLA class I antigens to CTL. These particular anti-Id vaccines were capable of inducing both $CD4^+$ T helper activity as well as $CD8^+$ cytolytic activity.[2–10] However, little attention has been paid to the detailed mechanism involved in CTL responses after anti-Id vaccination either in animal model or in patients.

We have described a murine monoclonal anti-Id antibody, designated 3H1,[11] which mimics a distinct and specific epitope of carcinoembryonic antigen (CEA) over expressed on human colorectal carcinoma (CRC) and other adenocarcinomas. Anti-Id 3H1 can be used as a surrogate for CEA and induced anti-CEA immunity in different species of animals as well as humans and showed promise as a potential vaccine candidate in the phase I/II clinical trials of CRC patients. Anti-Id 3H1 could easily break immune tolerance to CEA and induced high-titre anti-CEA antibody as well as $CD4^+$ T helper 1 (Th1) responses in CRC patients.[12–14] In our clinical studies, 3H1 was injected after absorption onto alum, which we believe favours the humoral immune responses. A similar phenomenon was observed when the efficacy of 3H1 as a tumour vaccine was evaluated in a murine tumour model.[15] Recently we have reported that immunizations of CEA transgenic (CEA-Tg) mice with 3H1-pulsed DC could reverse CEA unresponsiveness and result in induction of CEA-specific humoral and cellular immune responses and the rejection of CEA cDNA-plasmid transfected MC-38 murine colon carcinoma cells, C15[16] in nearly 100% of experimental mice.[17]

In the present study, we have dissected the cellular mechanisms involved in these events and provided convincing evidence to support the notion that TAA-specific cytotoxic T-cell responses could be generated in CEA-Tg mice after vaccination with DC pulsed with anti-Id antibody, 3H1. The sensitivity of C15 cells to CTL lysis was mediated by, granule, FasL, and tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-dependent pathways. We have also identified a peptide from 3H1 (designated LCD-2) that induces CTLs recognizing CEA positive colon carcinoma cells in a major histocompatibility complex (MHC) class I-restricted fashion. 3H1-pulsed DC immunized mice were immune to tumour rechallenge and adoptive transfer experiments also suggested the induction of long-lived memory response. To the best of our knowledge, this is the first report documenting the details of the mechanisms of T-cell mediated immunity invoked by an anti-Id vaccine in a clinically relevant animal model.

Materials and methods

Mice and cell lines

CEA-Tg mice [C57BL/6J-TgN(CEAGe)18FJP], male and female, were obtained from Dr F. James Primus and have been described previously.[16,17] For *in vivo* studies all mice were used at 6–8 weeks of age. The mice were treated in accordance with Institutional Animal Care and Use Committee guidelines.

The murine chemically induced colon carcinoma cells, MC-38 (C57BL/6, H-2^b), and the human CEA-transfected MC-38 clone (C15-4.3) have been described previously.[16] EL4 (thymoma, H-2^b), B16-F10 (melanoma, H-2^b), RMA (lymphoma, H-2^b), RMA-S (lymphoma, H-$2b^-$ TAP_2 deficient), SCC VII (squamous cell carcinoma, H-2^k), AG104A (fibrosarcoma, H-2^k), TSA (mammary adenocarcinoma, H-2^d), and murine natural killer (NK)-sensitive YAC-1 (lymphoma, H-2^a) cells were maintained in tissue culture in Dulbecco's modified Eagle's minimal essential medium or RPMI-1640 medium.

Anti-Id vaccine and immunization

Generation, purification, and characterization of anti-Id monoclonal antibody (mAb) 3H1, designated as CeaVac have been described previously.[11] Isotype matched control anti-Id mAb 1A7[18] was also used in this study. Mature DC were generated from bone marrow and were pulsed with 3H1 or 1A7 as described previously.[17] After loading, DC were injected subcutaneously (s.c.) in the lower right flank of syngeneic mice and boosted twice on every other week as described.[17]

Cytotoxicity assays

Assays were performed according to the standard protocols.[19] Mice were killed 2 weeks after the third immunization and lymphocytes were isolated from harvested spleen of three mice per group. 3H1-specific CTL were generated as described previously,[17] and the CTL activity was determined by a 6–8 hr ^{51}Cr release assay using a variety of target cell lines. Cytotoxicity assays of 6–8 hr were used in preference to standard 4 hr assays which, although adequate for granule-mediated killing, tend to underestimate death receptor-mediated lysis. For inhibition experi-

ments, various monoclonal antibodies (mAbs) were added to the culture at a final concentration of 10 μg/ml. Isotype-matched mAbs were used as control. For the inhibition of perforin-dependent cytotoxicity, CTL were pretreated with concanamycin A (CMA, Sigma-Aldrich, St. Louis, MO) for 2 hr at 500 nM, and then cytotoxicity assays were performed in the continuous presence of CMA. All antibodies used in inhibition experiments were purchased from BD PharMingen (San Diego, CA). In select experiments, purified $CD4^+$ and $CD8^+$ T cells were obtained from antigen-specific CTL culture by using magnetic beads (Miltenyi Biotec, Auburn, CA), and were used in cytotoxicity assays. Spontaneous release was <25% of maximum release.

Flow cytometric analysis

Cell surface antigens on C15 cells were analysed after overnight treatment with rm interferon-γ (rmIFN-γ; 10^3 U/ml) and/or rmTNF-α (250 U/ml; BD PharMingen) or media alone by flow cytometry using fluoroscein isothiocyanate (FITC)- or phycoerythrin (PE)-labelled mAbs reactive to H-$2K^b$ MHC class I alloantigen, I-A^d and I-E^d MHC class II alloantigens, Fas, TRAIL-R2, or isotype-matched controls. Cells were also stained for surface expression of CEA using an anti-CEA mAb (8019), followed by incubation with a secondary antibody, FITC-labelled goat anti-mouse immunoglobulin (IgG; Southern Biotechnology, Birmingham, AL). After staining cells were washed twice and analysed in a flow cytometer as described previously.[17]

For the detection of FasL or TRAIL on CTL, CTL (2×10^6/ml) generated from immunized mice splenocytes were first incubated for 6 hr at 37° with media alone, untreated or IFN-γ and TNF-α treated C15 cells (2×10^5/ml), or phorbol 12-myristate 13-acetate (PMA; 20 ng/ml) plus ionomycin (1 μg/ml). Cells were then stained simultaneously with FITC-labelled anti-CD4 or anti-CD8 mAb, and PE-labelled anti-FasL or anti-TRAIL mAb, or isotype-matched controls. After washing, the cells were subjected to flow cytometric analysis. CTL were stained for perforin expression using an intracellular staining technique as described previously.[17] Briefly, cells were stained with PE-labelled anti-CD4 or anti-CD8 mAb, washed, then fixed and permeabilized, followed by staining with anti-perforin antibodies (clone KM585; Kamiya Biomedical, Seattle, WA), washed, and then additionally incubated with a secondary antibodies, FITC-labelled goat anti-rat IgG (Southern Biotechnology). The cells were then washed twice and analysed. Cells from CTL culture were also stained and analysed for the surface expression of Ly-6C, CD44, CD122, and CD127 on CD4 and CD8 T cells. Anti-TRAIL-PE and anti-TRAIL-R2-PE mAbs were purchased from eBioscience (San Diego, CA) and all other mAbs were purchased from BD PharMingen.

N-a-benzyloxycarbonyl-L-lysine-thiobenzyl ester hydrochloride (BLT)-esterase release assay

CTL (2×10^5/well) generated from immunized mice splenocytes were incubated with untreated or IFN-γ and TNF-α treated C15 cells (2×10^4/well) at 37° for 6 hr. Cell-free supernatant was harvested and the BLT esterase activity of the supernatant was measured as described previously.[20]

Detection of Fas-induced cell death

Cell death was measured by ^{51}Cr release assays. Briefly, C15 cells were treated overnight with IFN-γ and/or TNF-α or left untreated. Cells were then labelled with ^{51}Cr and were analysed for Fas-mediated killing by incubation for 8–18 hr with anti-mouse Fas mAb (10 μg/ml, Jo2; BD PharMingen) and protein G (10 μg/ml; Amersham Pharmacia, Piscataway, NJ) to maximize cross-linking of anti-Fas mAb.

Proliferation assays

In vitro T-cell proliferation was measured by [^{3}H]thymidine incorporation as described previously.[17] Based on the amino acid sequence homology of 3H1 and CEA, several peptides were designed and synthesized.[3] Peptide LCD-2 (TLIYRANR*LIDG*VP) and HFW-1 (GPELVKP GASL) were obtained from 3H1. Peptide CEA-B (PPA QYSW*LIDG*N) and CAP-1 (YLSGANLNL) were obtained from CEA. CAP-1 is a class I, HLA-A2 restricted CEA epitope that can induce human T cells for cytolytic activity against CEA-positive tumour cells. In select experiments, these peptides were used as stimulants at a concentration of 5 μg/well. Assays were performed in triplicate wells.

Peptide pulsing of RMA-S cells for CTL-mediated lysis

RMA-S cells were cultured with different peptides (100 μg/ml) for 18–20 hr at 37° and 5% CO_2 in RPMI 1640 medium. Ovalbumin (OVA) peptide$_{257-264}$ (SIINFEKL), which encodes an H-$2K^b$-restricted epitope,[21] was used as control. Following incubation, cells were harvested, washed, and labelled with ^{51}Cr for use as targets in CTL assay. Peptide pulsed RMA-S cells were also stained with FITC-labelled anti-mouse H-$2K^b$ mAb and analysed for MHC class I antigen expression by flow cytometry.

In vivo *depletion of immune cell subsets*

Depletion studies were performed by intraperitoneal administration of anti-CD4 (GK1·5), anti-CD8 (2·43), anti-NK (PK136; ATCC, Manassas, VA), or isotype

control antibody (rat IgG, Sigma). Mice were injected with 200 μg of antibody on days −3 and +3 of each immunization and 2 days before tumour cell challenge. Specific depletion was >85% as determined by flow cytometry (data not shown).

Prophylactic treatment in s.c. tumour model and rechallenge experiments

Groups of mice ($n = 8$) were immunized as described.[17] Two weeks after the third immunization, mice were challenged with 1×10^6 of CEA-transfected murine colon carcinoma cells, C15[16] or 5×10^5 of non-transfected parental MC-38 cells s.c. in the lower left flank. Mice were monitored on a regular basis for tumour growth and survival. 3H1-pulsed DC immunized mice that had rejected C15 tumour growth and survived longer than 90 days were also rechallenged with either C15, MC-38, or EL4 (1×10^5) tumour cells s.c. In all rechallenge experiments naïve CEA-Tg mice were included as controls and were challenged with the same tumour dose.

Adoptive transfer experiments

3H1-pulsed DC immunized mice that had rejected C15 tumour growth and survived longer than 90 days served subsequently as donors of lymphocytes for adoptive transfer experiments. Splenocytes were cultured for 5 days in presence of 3H1 and rhIL-2 as described and at the end of culture $CD4^+$ and $CD8^+$ T cells were isolated at >90% purity. Purified T cells or total lymphocytes were then transferred (4×10^6 cells/mouse) intravenously (i.v.) into naïve CEA-Tg mice. Adoptive transfer of *in vitro* stimulated naïve mice splenocytes served as control. After 1 day, each group of mice were challenged with 1×10^6 of C15 tumour cells s.c.

Statistical analysis

Statistical analyses were performed by Student's two-tailed *t*-test or non-parametric Mann–Whitney rank-sum test using SigmaStat software (Jandel, San Rafael, CA). The data are presented as mean ± SE. $P < 0{\cdot}05$ was considered significant.

Results

Role of perforin, FasL, and TRAIL pathways in CTL-mediated cytotoxicity of C15 targets *in vitro*

Previously we have shown that 3H1-pulsed DC immunizations in CEA-Tg mice resulted in induction of CEA-specific effector $CD8^+$ T cells capable of displaying cytotoxic activity against C15 tumour cells in an MHC class I-restricted manner.[17] To better understand the potential contribution of antigen-specific CTL in tumour rejection *in vivo*, we first examined the CTL-mediated cytotoxicity and cytokine production *in vitro* in response to various cell lines (Table 1). CTL generated from 3H1-pulsed DC immunized mouse splenocytes showed

Table 1. Cytotoxicity and cytokine production by CTL on coincubation with various cell lines[1]

Tumour cells	Histology	MHC class I isotype control	mAb	Lysis (%)	IFN-γ (pg/ml)	TNF-α (pg/ml)	IL-4 (pg/ml)
C15	Colon carcinoma	+		48.7 ± 4.1	2000 ± 40*	450 ± 30*	<20
			Anti-CD4	40.0 ± 3.9	1260 ± 30	285 ± 15	<20
			Anti-CD8	9.8 ± 1.8	360 ± 10	180 ± 15	<20
			Anti-I-A^b	38.9 ± 4.2	1190 ± 25	270 ± 15	<0
			Anti-H-$2K^b$/H-$2D^b$	10.9 ± 2.1	425 ± 15	165 ± 15	<0
MC-38	Colon carcinoma	+		10.8 ± 2.4	1105 ± 25	180 ± 15	<0
EL4	Thymoma	+		4.3 ± 2.0	1075 ± 20	135 ± 15	<0
RMA	Lymphoma	+		10.9 ± 2.6	1030 ± 25	150 ± 15	<0
B16-F10	Melanoma	–		7.5 ± 2.1	990 ± 20	135 ± 15	<0
SCC VII	Squamous cell carcinoma	+		10.6 ± 3.0	885 ± 20	135 ± 15	<0
AG104A	Fibrosarcoma	+		2.3 ± 1.6	955 ± 20	150 ± 15	<0
TSA	Mammary carcinoma	+		5.2 ± 2.0	825 ± 15	165 ± 15	<0
YAC-1	Lymphoma	ND		8.8 ± 2.8	1010 ± 20	150 ± 15	<0
		–		1090 ± 25	165 ± 15		<0

[1]Cytotoxicity against various cell lines was measured in a 6 hr ^{51}Cr release assay at an E : T ratio of 25 : 1. CTL were generated from 3H1-pulsed DC immunized mice as described. CTL (2×10^5/well) were incubated with tumour cells (2×10^4/well) or media alone in 96-well tissue culture plates at 37° for 6 hr. Cell-free supernatants were harvested and analysed for the production of IFN-γ, TNF-α, and IL-4 by standard ELISA kits.[17] For inhibition experiments, blocking mAb was added to the culture at a concentration of 10 μg/ml. Surface MHC class I antigen expression was analysed by flow cytometry. The representative data of three independent experiments is shown.

*$P < 0.002$ with respect to other culture conditions for IFN-γ and TNF-α production.

ND, not determined.

specific lysis of C15 cells but not other syngeneic or allogeneic cell lines. In addition, increased amounts of IFN-γ and TNF-α were produced in response to C15 cells. These specific responses against C15 cells were mostly inhibited ($P < 0{\cdot}001$) by anti-CD8 (53-6.7) or anti-H-2K^b/H-2D^b (28-8-6) mAb, but not by either anti-CD4 (GK1.5) or anti-I-A^b (KH74) mAb ($P > 0{\cdot}06$). The data clearly demonstrate the specificity for C15 cells and H-2K^b restriction of these CTL. Immunization of mice with DC alone generated some non-specific lytic activity (data not shown), which is typically seen with bone-marrow-derived DC as evidenced by previous studies.[22]

CTL showed cytotoxic activity against C15 cells in relatively short-term assay (6–8 hr) and the overall lytic activity did not increase significantly in long-term (18 hr) assay (e.g. at 25 : 1 ratio, 48·0 versus 52·9). However, if C15 cells were pretreated for overnight with IFN-γ and TNF-α before the cytotoxicity assay, these cells became more susceptible to CTL lysis in short-term assay (Fig. 1b, closed bars). Most of the CTL lysis was blocked (80% inhibition) in the presence of CMA, a potent inhibitor of perforin-mediated lysis,[23] and the inhibition was significant ($P < 0{\cdot}005$), suggesting a major effector role for granule-mediated lysis *in vitro* (Fig. 1b). To delineate the nature of the perforin-independent pathways, CTL activity was examined in the presence of neutralizing anti-FasL (MFL3) and anti-TRAIL (N2B2) mAbs. The inclusion of anti-FasL and/or anti-TRAIL mAb had a partial inhibitory effect (29·8–43·6%), suggesting that the perforin pathway was still the dominant effector mechanism under these *in vitro* assay conditions (Fig. 1b). However, the combination of CMA, anti-FasL mAb, and anti-TRAIL mAb exerted maximal inhibition of lysis compared with isotype-matched control mAb ($P < 0{\cdot}0004$). Thus under conditions in which the perforin pathway was blocked, the contribution of the CTL-directed FasL and TRAIL pathways were more easily revealed. Taken together, these data suggest that maximal CTL-mediated lysis of C15 targets predominantly involved perforin-, FasL-, and TRAIL-based effector mechanisms.

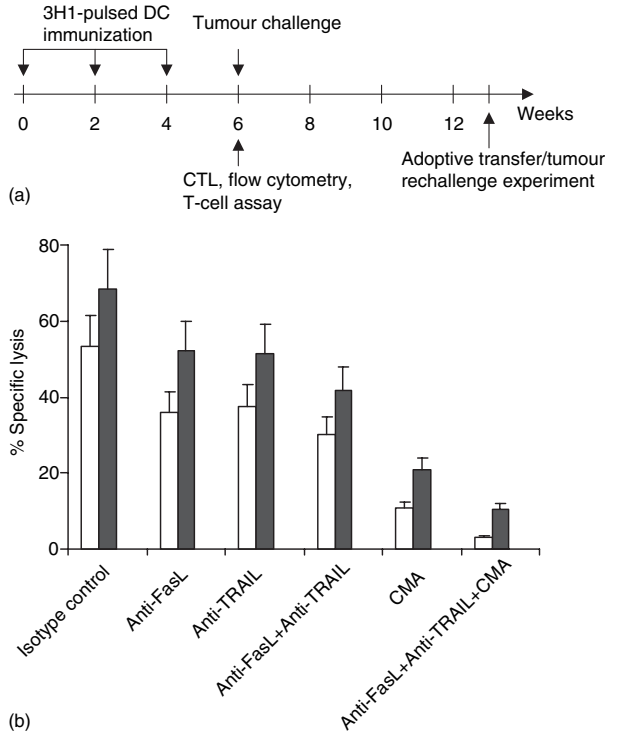


Figure 1. (a) Schematic diagram depicting immunization protocol and experiments. (b) Role of perforin, FasL, and TRAIL on CTL-mediated lysis of C15 tumour cells. Mice were immunized with 3H1-pulsed DC and CTL were generated from splenocytes as described in Materials and methods. Lytic activity of CTL was tested against untreated (open bars) or IFN-γ- and TNF-α-treated (closed bars) C15 cells by ^{51}Cr release assay. The 8 hr cytotoxicity assays (E : T, 50 : 1) were performed in the presence of CMA (500 nM), neutralizing mAb (10 μg/ml), isotype-matched control, or combinations of these agents as indicated. A representative experiment of three is shown.

Fas and TRAIL-R2 expression by C15 tumour cells

The cytokines IFN-γ and TNF-α enhanced susceptibility of C15 cells to CTL lysis (Fig. 1b). Therefore, the effects of these cytokines on target cells were examined in more detail. C15 cells express low levels of Fas; however, treatment with IFN-γ or TNF-α up-regulated Fas expression, and the combination of these cytokines synergistically enhanced surface Fas expression on C15 cells (Table 2). Several studies have suggested that cell surface Fas expression on both mouse and human tumour cell lines can be enhanced by treatment with proinflammatory cytokines, namely, IFN-γ and/or TNF-α.[24,25] The expression of TRAIL receptor 2 (TRAIL-R2[26]); on C15 cells was also increased by treatment with TNF-α and the combination

Table 2. IFN-γ and TNF-α treatment of targets resulted increased expression of surface antigens[1]

Surface antigen expression	Cytokine treatment of C15 cells			
	None	IFN-γ	TNF-α	IFN-γ + TNF-α
CEA	97.4	97.7	97.8	98.6
MHC class I	87.0	99.2	87.3	99.5
MHC class II	6.2	11.6	16.4	20.3
Fas	19.1	33.5	31.4	56.0
TRAIL-R2	36.1	36.3	49.1	54.5

[1]C15 cells were cultured overnight in the presence or absence of IFN-γ and/or TNF-α. These cells were then stained with FITC- or PE-labelled mAb or isotype-matched control, washed, and analysed in a flow cytometer. Results are presented as percentage of positive cells after subtraction of isotype control values. The representative data of three independent experiments is shown.

of IFN-γ with TNF-α. C15 cells express high endogenous levels of MHC class I. IFN-γ and the combination of IFN-γ with TNF-α further enhanced expression of this molecule on C15 cells. The surface expression of MHC class II was also enhanced to some extent with IFN-γ and/or TNF-α (Table 2). Functionally, treatment of C15 cells with either IFN-γ or TNF-α led to a moderate increase in sensitivity to Fas-mediated death. However, the combination of IFN-γ-? with TNF-α-sensitized C15 cells to Fas-mediated death more efficiently (data not shown). Similar results were observed with soluble TRAIL as a TRAIL-R2 stimulus (data not shown). Soluble TRAIL-induced apoptosis of tumour cell lines has also been reported in other studies.[27]

Interactions between CTL and cytokine-treated tumour cells resulted in enhanced granule exocytosis and up-regulated expression of FasL and TRAIL

In vitro tumour cell lysis by CTL was mostly granule-mediated and cytokine treatment of tumour targets increased the extent of lysis (Fig. 1b). Therefore, we wanted to examine whether there was any direct correlation between granule exocytosis and tumour killing. Degranulation of CTL was estimated by the release of BLT esterase activity (Fig. 2a). BLT esterase is stored in the granules together with perforin/granzymes, and its secretion correlates with the exocytosis of lytic granules. Supernatants from the coculture of CTL and cytokines treated C15 cells showed higher levels of BLT esterase activity (closed bars) than those from CTL cocultured with untreated C15 cells (open bars). The BLT esterase activity was largely inhibited in presence of anti-CD8 or anti-MHC class I mAb and the inhibition was significant ($P < 0{\cdot}005$) compared with isotype-matched control antibody. The perforin expression by $CD8^+$ T cells from CTL culture indicated that IFN-γ- and TNF-α-treated C15 cells induced higher accumulation of intracellular perforin compared with untreated C15 cells (Fig. 2b).

Next, we investigated the expression of FasL and TRAIL on CTL at different culture conditions. FasL expression was up-regulated on CTL after 6 hr specific interaction with C15 cells (Fig. 2c). The expression of FasL was increased further when CTL were cocultured with IFN-γ- and TNF-α-treated C15 cells. FasL expression by CTL was also increased after stimulation with PMA and ionomycin. A similar phenomenon was observed when TRAIL expression on CTL was analysed (Fig. 2c).

Peptide-specific proliferative responses in immunized mice

Immunization of mice with 3H1 can induce CEA-specific T-cell responses because of linear homology of amino acid sequence between 3H1 and CEA.[3,5] We have found several regions of homology in 3H1 heavy and light chain variable domains, as well as in the framework regions. To search for potential cross-reactive T-cell epitopes, a number of peptides were synthesized based on 3H1 and CEA homology and two partially homologous peptides, designated as LCD-2 (from 3H1) and CEA-B (from CEA) containing the common sequence LIDG were identified.[3] Therefore, we tested whether mice immunized with 3H1 had T cells that can recognize peptides from anti-Id mAb as well as nominal antigen CEA. As shown in Fig. 3(a), when incubated with LCD-2 or CEA-B peptide, bulk splenocyte populations and purified $CD4^+$ cells proliferated significantly ($P < 0{\cdot}005$), whereas $CD8^+$ T cells were relatively unresponsive. Control peptides HFW-1 (from 3H1) and CAP-1 (from CEA) did not stimulate the proliferation. Furthermore, antigen-induced T-cell proliferation was inhibited significantly in the presence of anti-CD4 ($P < 0{\cdot}002$) but not against anti-CD8 mAb ($P > 0{\cdot}06$) (Fig. 3b).

RMA-S cells pulsed with 3H1-derived peptide is target for antigen-specific MHC class I-restricted lysis

HLA motif analysis of cross-reacting peptides (LCD-2 and CEA-B) shows low but positive scores with H-2Kb molecules.[28] To assess whether any of these peptide contain CTL epitope, peptide-pulsed RMA-S cells[29] were used as targets for lysis by antigen-specific CTL. As shown in Fig. 3(c) 99·4% of RMA-S cells were positive for MHC class I expression when pulsed with OVA peptide (amino acids 257–264), whereas 68·3% and 38·5% cells expressed MHC class I molecules when pulsed with LCD-2 and CEA-B peptides, respectively. $CD8^+$ T cells obtained from 3H1-pulsed DC immunized mice splenocytes could lyse target cells only when RMA-S cells were pulsed with LCD-2 peptide (Fig. 3d). The presence of CEA-B peptide on RMA-S cells was probably suboptimal for lysis by CTL. HFW-1 peptide, derived from 3H1 heavy chain framework region was used as a control in this assay system. These results confirm that CTL activity generated in 3H1-pulsed DC immunized mice was directed against the epitope defined by the LCD-2 peptide.

$CD8^+$ as well as $CD4^+$ T cells are required for DC-mediated antitumour immune response *in vivo*

We next implemented *in vivo* depletion studies with anti-NK, anti-CD4, and/or anti-CD8 antibodies in order to assess the relative contribution of immune cell subsets in antitumour immunity. As shown in Fig. 4(a), in non-depleted immunized mice, *in vitro* lysis against C15 target cells was mostly mediated by purified $CD8^+$ T cells ($P < 0{\cdot}007$), whereas, $CD4^+$ T cells did not display any significant cytolytic activity. However, *in vivo* depletion of $CD4^+$ and/or $CD8^+$ T cells led to significant reduction of

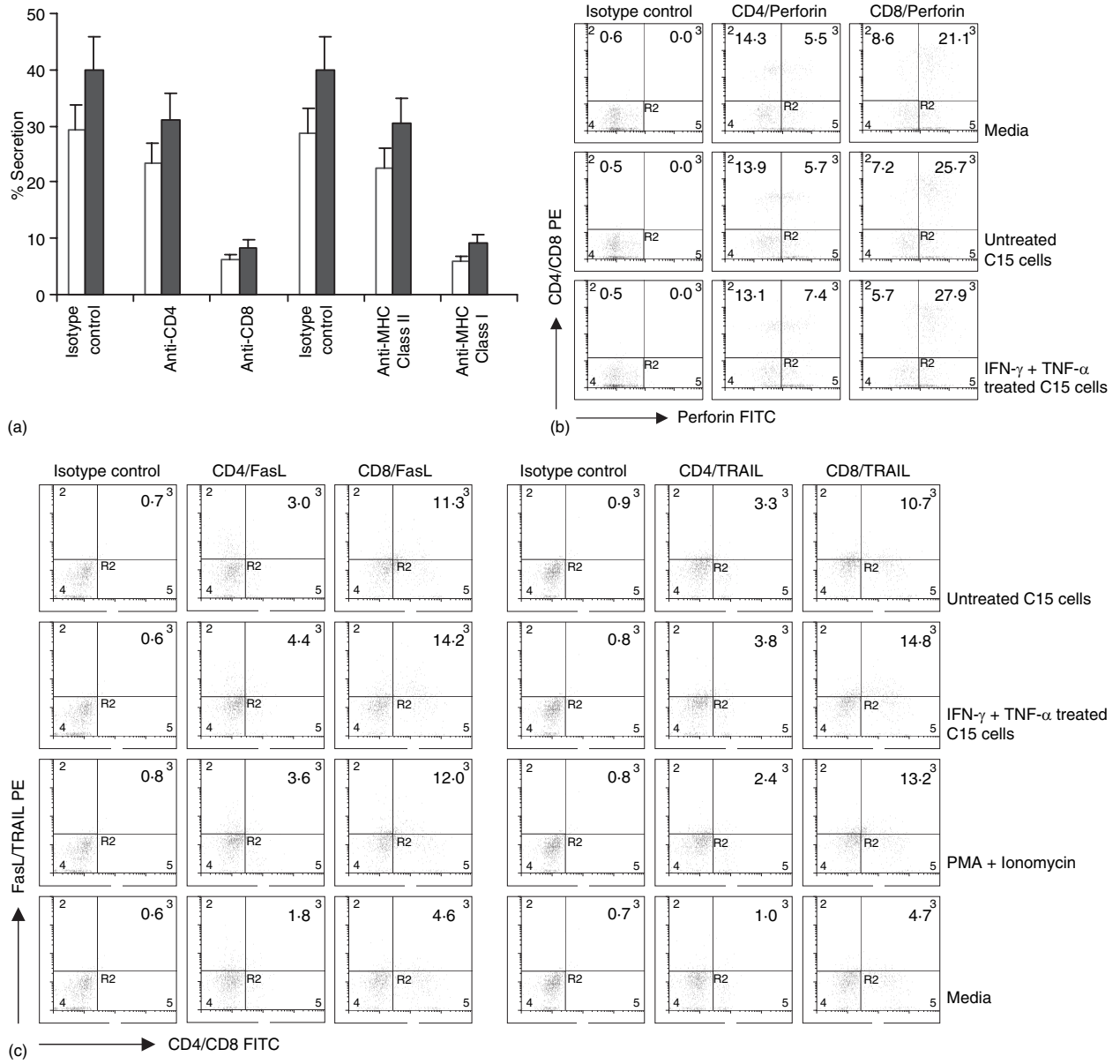


Figure 2. BLT esterase release activity and expression of perforin, FasL, and TRAIL by CTL in presence of various stimuli. Mice were immunized with 3H1-pulsed DC and CTL were generated from splenocytes as described. (a) CTL were incubated with untreated (open bars) or IFN-γ- and TNF-α-treated (closed bars) C15 cells for 6 hr in 96-well tissue culture plates in presence of neutralizing mAb or isotype-matched control (10 μg/ml). Next, the BLT activity in the supernatant was measured. (b and c) CTL were cocultured with untreated or IFN-γ- and TNF-α-treated C15 cells, media alone, or combination of PMA and ionomycin for 6 hr. These CTL were then stained and analyzed in a flow cytometer for the detection of intracellular accumulation of perforin (b) and surface expression of FasL or TRAIL (c) by $CD4^+$ and $CD8^+$ T cells. Results are presented as percentage of positive cells. (a–c) A representative experiment of three is shown.

antigen-specific *in vitro* cytolytic activity ($P < 0{\cdot}006$), indicating that both T-cell subsets were required for antigen specific lytic activity, whereas *in vivo* depletion of NK cells had no effect in cytolysis (Fig. 4b, c). Interestingly, *in vitro* proliferation of splenocytes from depleted mice indicated that antigen-specific proliferation was mostly mediated by $CD4^+$ T cells (Fig. 4d). Of note, all of the non-depleted or NK-depleted mice were protected from C15 tumour development, whereas control mice or mice depleted of both $CD4^+$ and $CD8^+$ T cells succumbed to death at the same time (Fig. 4e, f). Mice depleted of $CD8^+$ T cells alone or mice depleted of $CD4^+$ T cells alone had partial survival benefit. Therefore, these data suggest that neither T-cell subset alone was sufficient to confer complete antitumour immunity after immunization with 3H1-pulsed DC. This protection was specific to

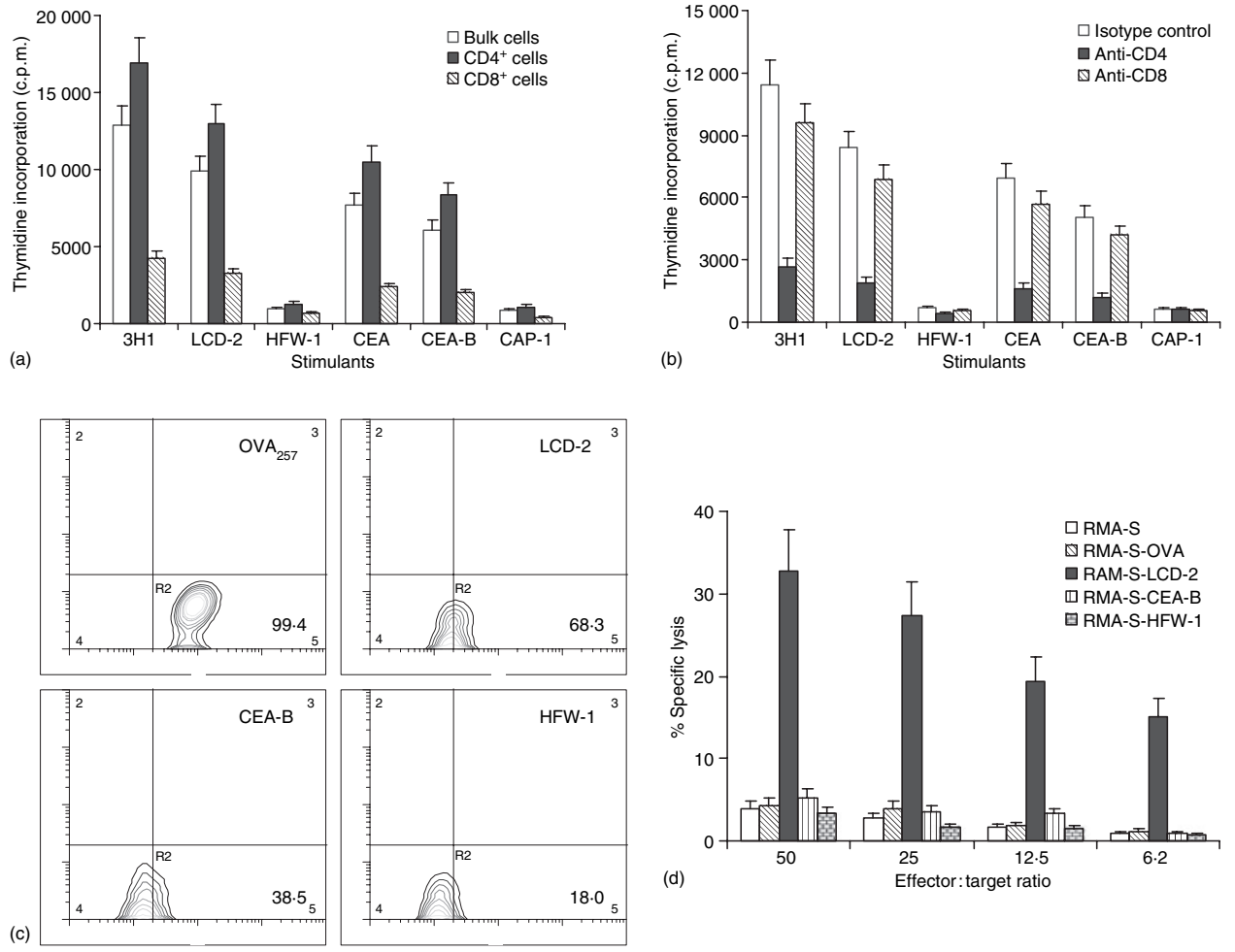
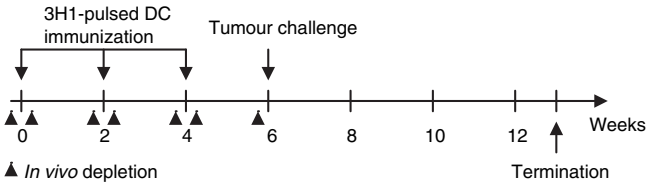


Figure 3. Peptide-specific proliferation and $CD8^+$ T cell-mediated lysis of peptide pulsed RMA-S cells. (a and b) 3H1-pulsed DC immunized mice splenocytes were cultured *in vitro* in presence of peptides (LCD-2, HFW-1, CEA-B, and CAP-1), antigen (CEA), or anti-Id mAb (3H1) for 5 days and T-cell proliferation was measured by determining $[^3H]$thymidine incorporation. Proliferation in the presence of media had been subtracted from values obtained in the presence of different stimulants. (a) $CD4^+$ and $CD8^+$ T cells were separated by magnetic-activated cell sorting micro beads as described. Macrophages isolated from splenocytes were added as antigen presenting cells in this assay. (b) Antibodies (10 μg/ml) were added at the beginning of culture. (c) RMA-S cells were cultured overnight in the presence or absence of different peptides. These cells were then stained with FITC-labelled anti-MHC class I mAb and analysed by flow cytometry. Results are presented as percentage of positive cells. (d) CTL were generated from 3H1-pulsed DC immunized mice splenocytes and lytic activity of purified $CD8^+$ T cells was determined by 6 hr ^{51}Cr release assay using peptide-pulsed RMA-S cells as targets. (a–d) A representative experiment of three is shown.

CEA-expressing tumours, as 3H1-pulsed DC immunized mice did not mount an antitumour response when inoculated with the parental MC-38 cells.[17]

Long-lived memory T cells are maintained in mice that have rejected C15 tumour

The goal of vaccination strategies is to elicit effective long-term immunity. Our findings show that immunization of CEA-Tg mice with 3H1-pulsed DC was effective at inducing CEA-specific antitumour immunity, which helped those mice to reject a lethal challenge of CEA-transfected tumour cells. We sought to investigate at different time points the expression of Ly-6C, CD44, and CD122, which are associated with memory T-cell populations.[30–32] The data presented in Fig. 5 show that expression of Ly-6C and CD44 increased in tumour-rejected mice. The expression on $CD4^+$ T cells was maximal at 12 weeks post tumour challenge, whereas the expression on $CD8^+$ T cells was maximal at 6 weeks post tumour challenge. However, there was no difference of the CD122 (the β-subunit of interleukin (IL)-2 and IL-15 receptor) expression levels following immunization and tumour cells challenge. IL-7 has been implicated in the regulation of homeostasis and survival of $CD8^+$ memory T cells *in vivo*[33] and surface expression of CD127 (the α-subunit of IL-7 receptor) has been described as a marker for long-lived memory T cells.[34] The analysis of CD127



Schematic diagram depicting immunization protocol and *in vivo* depletion

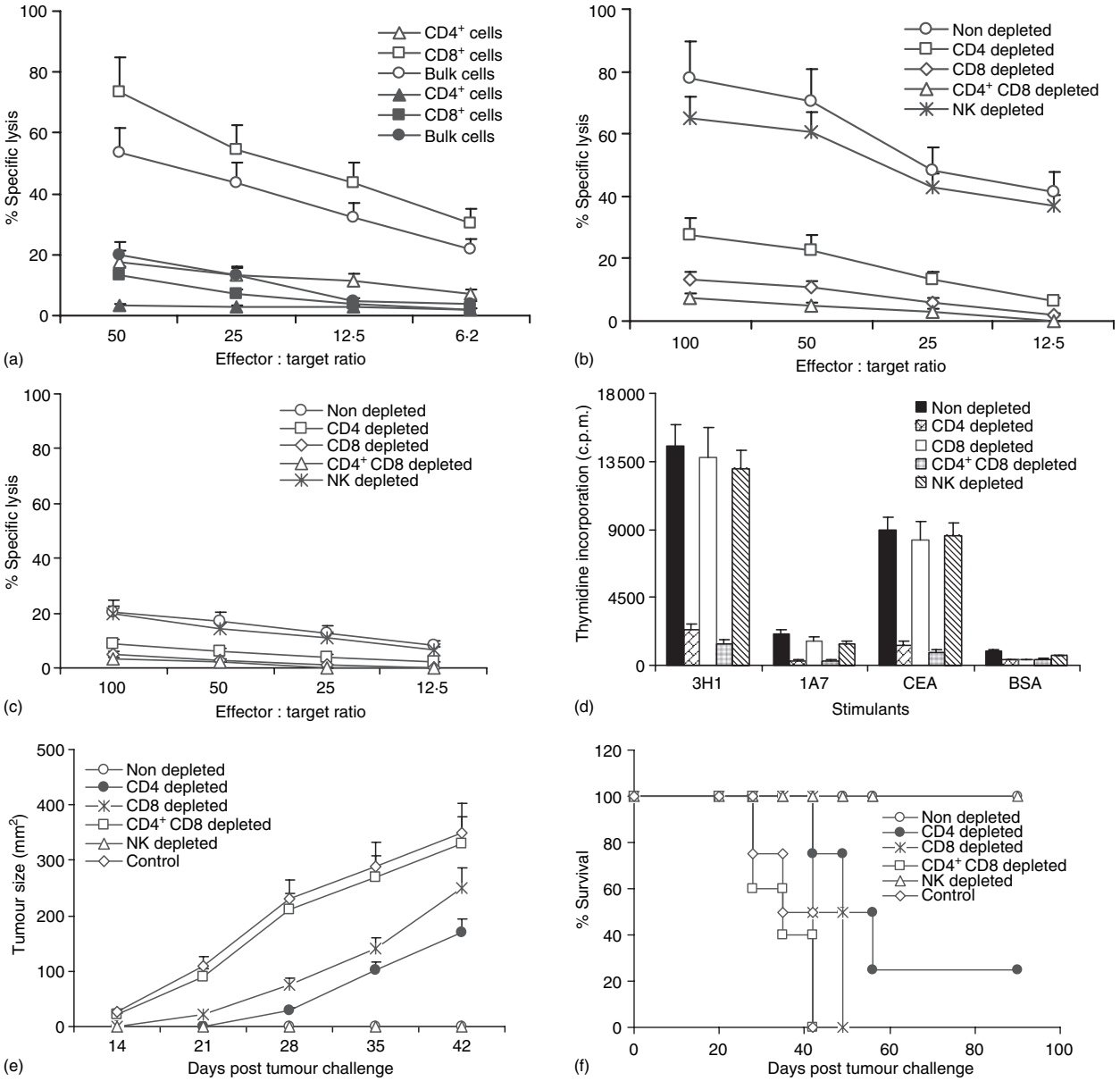


Figure 4. Involvement of T-cell subsets in the induction of immune response and rejection of C15 tumour cells *in vivo*. (a) CTL were generated from 3H1-pulsed DC immunized mice and cytotoxicity was measured by 6 hr ^{51}Cr release assay using C15 (open symbol) and MC-38 (closed symbols) as target cells. (b–f) mice were depleted from $CD4^+$ and/or $CD8^+$ T cells, or NK cells during immunizations with 3H1-pulsed DC and before challenge with C15 tumour cells by repeated injections of specific mAbs as described in Materials and methods. CTL were generated from splenocytes and cytotoxicity was measured against C15 (b) and MC-38 (c) target cells. (d) Splenocytes were cultured *in vitro* in presence of anti-Id mAbs (3H1, 1A7), antigen (CEA), or BSA for 5 days and T-cell proliferation was measured by determining $[^3H]$thymidine incorporation. Proliferation in the presence of media had been subtracted from values obtained in the presence of different stimulants. (e and f) Two weeks after the third immunization, mice were challenged with 1×10^6 of CEA-transfected C15 tumour cells s.c. Mice immunized with 1A7-pulsed DC and challenged with C15 tumour cells served as control. Tumour growth (e) and survival (f) were recorded over time. Mice were killed when tumours became ulcerated or when they reached a size >250 mm^2 and survival was recorded accordingly. (a–f) Similar results were obtained in three independent experiments.

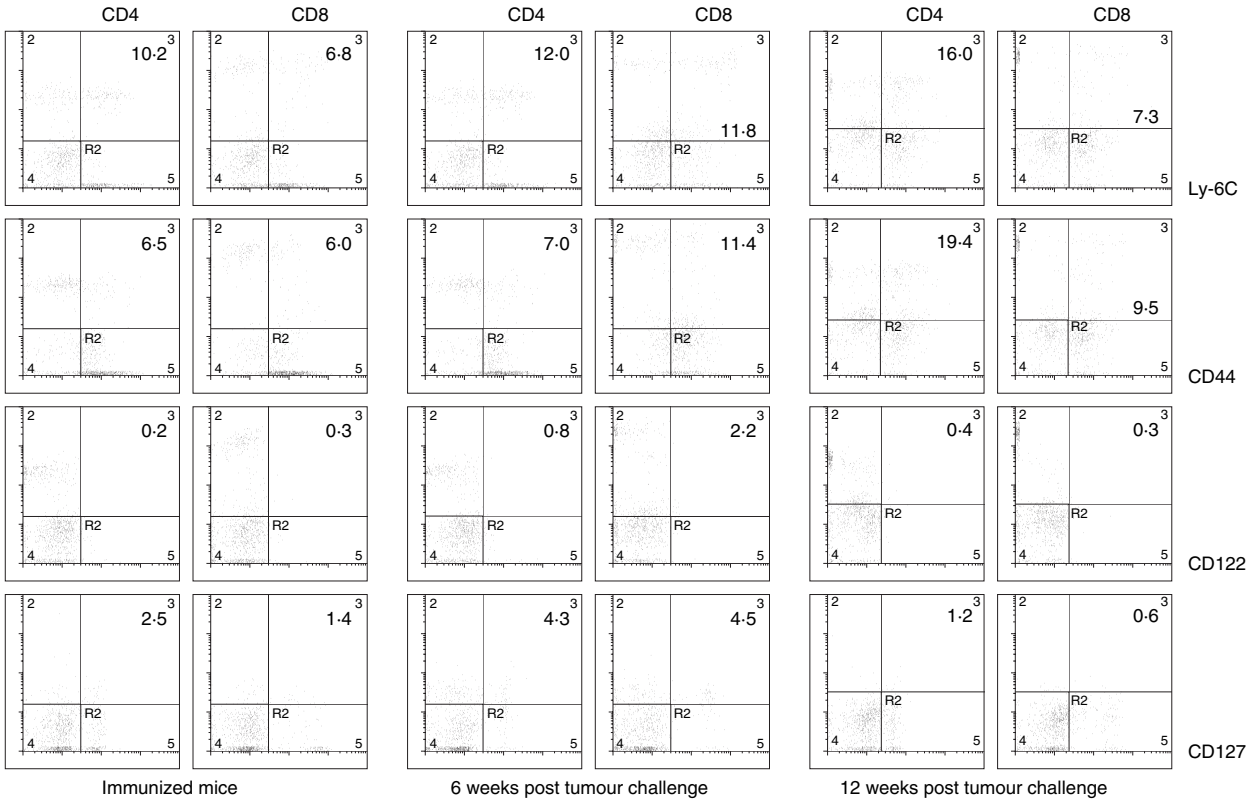


Figure 5. $CD4^+$ and $CD8^+$ T cells up-regulated surface expression of memory phenotype Ly-6C, CD44, and CD127 following tumour challenge. Splenocytes harvested 2 weeks after the third immunization or 6 weeks and 12 weeks post tumour challenge were stimulated *in vitro* in the presence of 3H1 for 5 days. For the detection of surface expression of Ly-6C, CD44, or CD122 cells were stained simultaneously with FITC-labelled anti-Ly-6C, anti-CD44, or anti-CD122 mAb, and PE-labelled anti-CD4 or anti-CD8 mAb. For the detection of surface expression of CD127 cells were stained simultaneously with PE-labelled anti-CD127 mAb and FITC-labelled anti-CD4 or anti-CD8 mAb. Cells were also stained with respective isotype-matched controls. After staining cells were washed and analysed by flow cytometry. Results are presented as the percentage of positive cells. A representative experiment of three is shown.

expression on $CD4^+$ and $CD8^+$ T cells indicated two- to threefold increases at 6 weeks post tumour challenge (Fig. 5). However, the expression was diminished at later time points. Together, these results suggest that tumour rejected mice developed antigen-specific memory T cells as determined by higher expression of Ly-6C and CD44.

Development of tumour-protective immunological memory

3H1-pulsed DC immunized mice that had rejected CEA-transfected C15 tumour remained tumour free for more than 90 days after challenge (Fig. 4f). To determine whether these mice developed tumour-protective long-term immunity *in vivo*, they were rechallenged with the same tumour cells used for the first challenge or with other syngeneic tumour cells. Mice cured from C15 tumour rejected subsequent challenges with the same tumour or non-transfected parental MC-38 and remained tumour free for more than 90 days when the experiment was terminated, while mice could not reject syngeneic unrelated tumour, EL4 (Fig. 6a). The results indicate that the tumour protection was mediated by persistent antitumour immunity specific for antigens relevant to C15 tumour. It is of interest that the tumour-free mice also had protective immunity against subsequent challenge with the parental MC-38 cells, suggesting that mice rejecting CEA-transfected carcinoma developed immunity to other antigens expressed on C15 and shared with the non-transfected parental MC-38 cell line, resulting in long-lasting memory against these tumours. Induction of immunity to shared tumour antigens has also been observed in other tumour models during tumour rejection after particular antigen immunization.[35,36] All control CEA-Tg mice were previously untreated and died of progressive tumour growth, irrespective of challenge with C15, MC-38, or EL4 cells (Fig. 6b).

We finally investigated whether this immunological memory could be transferred into naïve (non tumour-bearing and untreated) adult CEA-Tg mice. Recipient mice were challenged with C15 tumour cells 1 day after the adoptive transfer of *in vitro* stimulated splenocytes. The

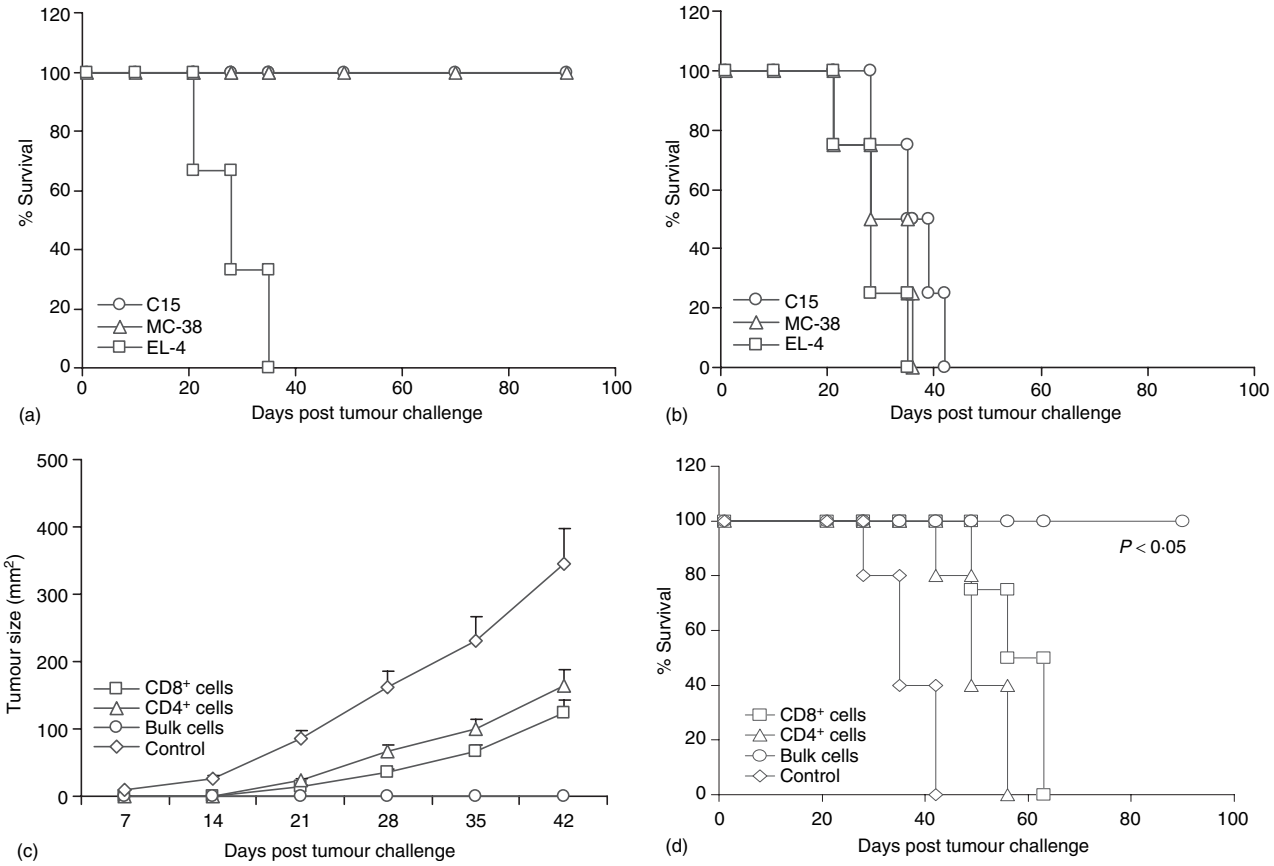


Figure 6. 3H1-pulsed DC immunized mice that had rejected C15 tumour cells developed tumour-specific long-lived memory response. (a) Surviving mice that had been immunized and challenged as described (Fig. 4) were rechallenged either with 1×10^6 of CEA-transfected C15, 5×10^5 of non-transfected parental MC-38, or 1×10^5 of syngeneic unrelated EL4 tumour cells s.c. 13 weeks after first tumour inoculation. (b) A set of age-matched naive CEA-Tg mice were also challenged either with C15, MC-38, or EL4 tumour cells and used as controls. Survival was recorded as described. (c and d) Adoptive transfer of immune splenocytes to prevent the growth of subsequent tumour challenge. 3H1-specific lymphocytes were generated from C15 tumour-free mice at 13 weeks of post tumour challenge as described in Materials and methods. Stimulated cells (4×10^6) were infused i.v. into naïve mice and on the next day mice were challenged with 1×10^6 of C15 tumour cells s.c. Tumour growth (c) and survival (d) were recorded over time. (a–d) Similar results were obtained in two independent experiments.

data depicted in Fig. 6(c, d) indicate that adoptive transfer of total splenocytes from tumour-free mice resulted tumour protection in 100% mice and these mice remained tumour free until day 90, when the experiment was terminated. Whereas C15 tumour developed rapidly in control group of mice adoptively transferred with total splenocytes from naïve CEA-Tg mice, and all of these mice died within 42 days. Tumour growth was relatively slower in groups of mice where adoptive transfer was performed with either $CD4^+$ or $CD8^+$ T cells which resulted in partial protection. Thus, both tumour antigen-specific $CD4^+$ and $CD8^+$ T cells played an important role in the control of tumour growth in this murine model of colon carcinoma.

Discussion

Previous studies have shown that anti-idiotypes are powerful stimulators of CD4 responses, while the evidence for stimulation of CD8 responses has been less convincing. This was thought to be caused by the inability of exogenous antigens to enter MHC class I pathways. However, Sigal *et al.*[37] have suggested that exogenous cross priming may indeed be the preferred route for CTL priming to viral antigens. This mechanism may also account for the generation of TAA specific CTL following immunizations with anti-Id antibodies. It is of interest that DC pulsed with soluble proteins can present peptide epitopes derived from these exogenous antigens to MHC class I molecules and induce an antigen-specific CTL response.[38] This is probably related to Fc receptor-mediated endocytosis or macro-pinocytosis of soluble antigens by DC for presentation on MHC class I molecules.[39] Therefore, it is expected that anti-Id 3H1 would be internalized and degraded to peptides by DC and those peptides bound to the MHC molecules would be presented to T cells. T cells with appropriate receptors will be

expanded and expected to constitute the anti3H1 cytotoxic, helper, and memory cells. The presence of candidate CTL epitopes in 3H1-peptides has been indicated by us[3] and others.[5]

Our findings clearly demonstrated that after 3H1-pulsed DC immunizations $CD8^+$ T cells could lyse C15 tumour cells *in vitro* by granule-, FasL-, and TRAIL-mediated pathways, although the antigen-specific CTL response was primarily mediated through the perforin/granzyme pathway. Interestingly, proinflammatory cytokines, such as IFN-γ and TNF-α, sensitized the tumour cells to be lysed by these pathways, suggesting that these cytokines could play an important role not only in the induction but also in the effector phase of the immune response. The interaction between cytokine-treated tumour cells and CTL produced enhanced expression of FasL, TRAIL, and intracellular accumulation of perforin by $CD8^+$ T cells, which implicated the importance of these cytokines on lysis of target cells. Physiologically, IFN-γ and TNF-α may have been secreted by activated CTL following TCR engagement and our *in vitro* studies demonstrated that CTL produced these cytokines in response to tumour-specific recognition. Several studies in humans suggest that host Fas/FasL system may be important for regulation of tumour growth[25,40,41] and TRAIL induced apoptosis in a variety of tumour cells makes it an excellent therapeutic candidate for treating cancer patients.[42,43] *In vivo* antitumour activity of TRAIL has also been demonstrated in athymic nude or SCID mice bearing human tumour xenografts derived from several carcinomas including colon carcinoma.[44–46]

Human CEA consists of three homologous repetitive domains and one 3H1 peptide (LCD-2) in particular showed high homology to this CEA region. The homology of the 3H1 peptide is maximum with repeat II domain showing 4 of 10 identity and results of the amino acid sequence alignment and reverse hydropathy analysis suggested that CDR2 of the 3H1 V_L and the region of CEA repeat domain may be the common epitope recognized by the anti-CEA Ab1, 8019.[3] Based on 3H1 and CEA sequences, eight different peptides were synthesized and used for *in vitro* proliferation of peripheral blood mononuclear cells (PBMCs) from colon cancer patients immunized with anti-Id mAb 3H1. Among these, only LCD-2 and CEA-B peptides containing the common sequence LIDG showed consistent stimulation in proliferation assays.[3] In our present study, we found that 3H1-pulsed DC immunized mice splenocytes could proliferate significantly in presence of LCD-2 and CEA-B peptides and the peptide LCD-2 also contains the epitope recognized by $CD8^+$ T cells for *in vitro* tumour cell lysis. Our results are in agreement with the recent findings of Murray *et al.*[10] who have reported that the peptide V_H3-11 derived from the anti-Id mAb MF11-30 induced proliferation and cytokine production by T cells, and induced HMW-MAA-specific CTL from PBMCs obtained from melanoma patients immunized with MELIMMUNE, a combination of murine anti-Id mAb MEL-2 and MF11-30. Recently it has also been reported that, PBMCs obtained from normal individuals could generate helper and cytotoxic T-cell responses that lysed CEA expressing human tumour cells after *in vitro* stimulation with a deimmunized anti-Id antibody that mimics CEA.[47] It is obvious that, $CD8^+$ T cells are important effector cells in our model because antigen-specific CTL activity could be detected *in vitro* and *in vivo* depletion of $CD8^+$ T cells abrogated the tumour protective immunity.

The development of long-lived immunological memory, as well as the ability to transfer immunity by the infusion of splenocytes suggests the presence of antigen-specific T cells in tumour-protected mice. The adoptive transfer experiment suggests that both $CD4^+$ and $CD8^+$ T cells are required to achieve tumour protection. It is possible that the cytokines produced by the transferred $CD4^+$ T cells are required for the maintenance of transferred $CD8^+$ T cells and/or activation of host $CD4^+$ or $CD8^+$ T cells. Several recent studies have documented that $CD4^+$ T-cell help is critical to the maintenance of $CD8^+$ T-cell population.[48–50]

Taken together, our findings strongly suggest that 3H1-pulsed DC vaccination induced antigen-specific $CD8^+$ T-cell responses in CEA-Tg mice and the results revealed involvement of perforin-, FasL-, and TRAIL-mediated pathways in tumour cell lysis *in vitro*. The cytokines IFN-γ and TNF-α had an important role for optimal target cell lysis by antigen-specific CTL. The immunity developed in immunized mice resulted in complete rejection of CEA-expressing murine colon carcinoma cells and the generation of long-lived memory response. These data suggest that 3H1-pulsed DC vaccination could be of future benefit for the treatment of colon cancer patients with minimal residual disease after surgery.

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