Cancer Immunol Immunother (2001) 49:573–586
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ORIGINAL ARTICLE

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Tumour-induced suppression of immune response and its correction

Received: 22 June 2000 / Accepted: 6 September 2000

Abstract Immunosuppressive features of tumour cells are a major obstacle for immunotherapy of cancer. We recently noted that RENCA cells effectively interfere with the in vivo activation of RENCA-specific T cells. To unravel the underlying mechanism, we evaluated the influence of RENCA cells on a mixed-lymphocyte/tumour reaction as well as an allogeneic mixed-lymphocyte reaction. We observed that RENCA cells were not directly immunosuppressive. Instead, they initiated deviation of an immune response in at least two independent directions: (i) expansion of a population of $NK1.1^{+}/CD3^{+}$ cells, which was accompanied by elimination of mainly $CD4^{+}$ lymphocytes, and (ii) production of a leukocyte-derived inhibitory factor. Expression of the costimulatory molecule B7.1 by RENCA cells prevented induction of anergy, while expression of MHC class II molecules prevented expansion of $NK1.1^{+}$ cells, which was accompanied by a significant decrease in cell death. Hence, an unimpaired response was observed only when RENCA cells expressed B7.1 plus MHC class II molecules. Thus, even if a tumour itself is not immunosuppressive, it can induce a strong deviation of the immune response. It is concluded that the first contact between elements of the immune system and the tumour cell can confer a severe bias on immunoregulatory circuits.

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Key words Mouse · Renal cell carcinoma · T cell activation

Introduction

The cellular immune response against tumour cells is, in most instances, very weak and mostly inefficient (reviewed in [28]). Although cytotoxic T cells (CTL) could be established in vitro against human and animal tumours [21, 37, 69], tumour-specific CTL have been detected only in a minority of patients [48, 63]. In particular, it has proven notoriously difficult to establish tumour-specific CTL from tumour-infiltrating lymphocytes [73]. Several features of tumour cells might contribute to the inefficacy of the antitumour immune attack. Tumour cells frequently express low levels of MHC class I antigens [46] and rarely MHC class II antigens [8]. Tumour cells may have defects in antigen processing, e.g. a reduced activity of TAP-1 and TAP-2 molecules [36, 59]. Most importantly, tumour cells are often devoid of ligands for costimulatory molecules such as B7.1 and B7.2 [17]. Since T cell activation requires a costimulatory "second" signal provided by the interaction of, for example, CD28 on the T cell with molecules of the B7 family on the accessory cells [22], the lack of B7 expression on tumour cells may well contribute to their poor immunogenicity. In fact, transfection of genes encoding B7 and/or MHC class II molecules has been shown to enhance T-cell-mediated antitumour responses significantly [5, 18, 35, 44, 47, 52, 53, 57, 77].

Alternatively, though the two possibilities are not mutually exclusive, the weak stimulatory capacity of tumour cells might rely on their genuine immunosuppressive activities [13, 40, 55]. Certain types of tumour cell secrete transforming growth factor β (TGFβ), prostaglandins or interleukin-10 (IL-10) [15, 19, 31, 41, 74], i.e. cytokines and mediators with potent inhibitory effects on lymphocyte activation, proliferation and cytotoxic potency [9, 12]. Induction of apoptosis could be another reason for the inefficacy of an antitumour

immune response [3]. It is worthwhile noting that apoptosis of T cells, triggered through a CD95–CD95L interaction [25, 60], could be initiated by a tumour cell–T cell interaction [16, 64, 76], but also by an interaction between different subsets of activated lymphocytes [14, 16, 51, 76]. This, in fact, represents a third mechanism of tumour escape, where the tumour is not directly immunosuppressive, but re-directs an immune response.

We have described recently [68] that individual clones of the murine renal cell carcinoma line RENCA differ significantly in antigenicity and immunogenicity. To elucidate further the mechanisms underlying the differences in immunogenicity, we explored the regulatory effect of RENCA cells on the activation of alloantigen-reactive T lymphocytes in a mixed-lymphocyte reaction. We show that RENCA cells drastically inhibit T cell reactivity in a mixed-lymphocyte culture (MLC). Suppression is mediated by at least two elements: (i) prevention of cytokine expression, possibly by factor(s) produced upon coculture of lymphocytes with RENCA cells, and (ii) activation of $NK1.1^{+}/CD3^{+}$ lymphocytes, which support apoptosis.

Materials and methods

Mice and tumours

BALB/c (H-2^{d}) and C57BL6 (H-2^{b}) mice were obtained from Charles River, Sulzfeld, Germany. Mice were kept under specific-pathogen-free conditions and were fed dry food and water ad libitum. Mice were used for experiments at the age of 8–10 weeks. The RENCA tumour line [49], clones derived therefrom (P1 and P2) [68] and B16F1 cells [29] were maintained in RPMI-1640 medium, supplemented with antibiotics and 10% fetal calf serum. Confluent cells were trypsinized and split into 3.

Transfection of RENCA cells and RENCA-derived clones

RENCA cells as well as the RENCA clones P1 and P2 were transfected with MHC class II and/or B7.1 cDNA. The MHC class II (H-2^{d}) α and β chain cDNA was kindly provided by R. Lechler, Department of Immunology, Royal Postgraduate Medical School, London, UK. The B7.1 cDNA was a kind gift from F. Momburg, Department of Immunogenetics, German Cancer Research Centre, Heidelberg, Germany. The MHC class II α and β chain cDNA was cloned into the phβAPr-1 vector, which contains the neomycin-resistance gene. The B7.1 cDNA was inserted into the pcDNA3 vector, which also contains the neomycin-resistance gene. Uncloned RENCA cells and cells of clones P1 and P2 were transfected by electroporation (260 V, 1050 μF) with either MHC class II α and β chain cDNA or with B7.1 cDNA or consecutively with both MHC class II and B7.1 cDNA. After transfection, cells were seeded in 24-well plates at a density of 1×10^{4} cells/well in RPMI-1640 medium, containing 10% fetal calf serum and 700 mg/ml G418. In the case of double transfections, cells expressing B7.1 were enriched by repeated fluorescence-activated cell sorting starting 2 weeks after transfection.

Monoclonal antibodies and flow cytometry

The following hybridomas were obtained from the European or the American Type Culture collections: anti-CD4 (YTA.3.2.1, rat IgG2b, ECACC), anti-CD8 (YTS169.4.2.1, rat IgG2b, ECACC), anti-CD3 (145-2C11, hamster IgG, ATCC). Anti-H-2^{d} (K9-18, mouse IgG3) [6] and anti-I-A^{d} (K24-199, mouse IgG2a) [38] were kindly provided by G. Hämmerling, Department of Immunogenetics, German Cancer Research Centre, Heidelberg, Germany. Antibodies were purified by passing the culture supernatant over a ProteinG column. Where indicated, purified antibodies were labelled with fluorescein isothiocyanate (FITC) according to the manufacturer's suggestion (Molecular Probes, Eugene, Oregon, USA). Unlabelled, FITC-labelled or phycoerythrin(PE)-labelled anti-B7.1, anti-CD95, anti-CD95L, anti-CD28, anti-CTLA-4, FITC-labelled anti-NK (anti-Ly-49C, clone DX5), unlabelled and biotinylated anti-IL-2, anti-IL10, anti-(interferon γ) (anti-IFNγ) and anti-(tumour necrosis factor α) (anti-TNFα) as well as FITC-, PE- or biotin-labelled secondary antibodies, FITC-labelled mouse IgG and PE-labelled hamster Ig were obtained from Pharmingen, Hamburg, Germany. Cell death was determined by propidium iodine (Sigma, Munic, Germany) uptake (2 μg/ml phosphate-buffered saline, PBS). Cells were stained according to routine procedures using 5×10^{5} cells/well. For the evaluation of cytokine expression, monensin (5 μM) was added during the last 8 h of culture. Thereafter cells were fixed for 30 min in 4% paraformaldehyde (w/v in PBS) and were permeabilized by incubation for 15 min in 0.5% (v/v in PBS) Triton X-100. After washing and blocking of non-specific binding sites, cells were incubated with the anti-cytokine antibody. Fluorescence staining was evaluated either by an EPICS XL flow cytometer (Coulter Corp, Krefeld, Germany) or a FACS-Star (Becton-Dickinson, Heidelberg, Germany).

Mixed-lymphocyte and mixed-lymphocyte/tumour cell cultures (MLC/MLTC)

Spleens and lymph nodes were excised, pushed through fine gauze and freed from debris and dead cells by Ficoll Hypaque centrifugation. Antigen-presenting cells (APC) were prepared from spleen cells. Cells were seeded on petri dishes and incubated for 2 h at 37 °C. Thereafter, non-adherent cells were discarded and the adherent cells were cultured for 7 days in RPMI-1640 medium, supplemented with 10% fetal calf serum, 100 U/ml IFNγ, 150 U/ml granulocyte/macrophage-colony-stimulating factor (GM-CSF) and 100 U/ml IL-4. APC were loaded with tumour cell extracts, which had been prepared by three rounds of freezing and thawing. Loading was performed by overnight incubation at 37 °C at an estimated ratio of 1 APC to 10 lysed tumour cells. After loading, APC were washed and used as stimulator cells.

In MLC, 1×10^{5} BALB/c lymph node cells were cultured with 5×10^{4} irradiated (3 Gy) C57BL6 spleen cells; in MLTC, BALB/c or C57BL6 lymph node cells (1×10^{5}) were cultured either with 1×10^{4} irradiated (300 Gy) tumour cells (RENCA, P1, P2, B16F1) or with tumour-lysate-pulsed APC (5×10^{3}/well). In some experiments, proliferative activity in response to the mitogen concanavalin A (7.5 μg/ml) was evaluated. Cultures were set up in triplicate and incubated for 1–3 days. For the determination of proliferative activity, 1 μCi [^{3}H]thymidine was added during the last 6 h of culture. Thereafter cells were harvested onto filter-papers by an automatic harvester and emission was determined in a β-counter. Proliferative activity is presented either as cpm/number of cells or as the proliferation index, where the radioactivity (cpm) in test wells was divided by that in control wells containing only responder cells.

To investigate the influence of soluble mediators on lymphocyte proliferation in MLC and MLTC, double-chamber experiments were performed. Distinct cell populations or mixtures of cell populations were cultured for 3–6 days in two compartments of 24-well culture plates, which were separated by a transwell (Nunc, Nürtlingen, Germany). These cultures contained 2×10^{6} responder cells and either 1×10^{6} irradiated allogeneic lymphocytes or 2×10^{5} irradiated syngeneic tumour cells as stimulators in a total volume of 2 ml. After the indicated time, 100 μl responder cell suspension was transferred in triplicate to 96-well round-bottom plates,

[^{3}H]thymidine was added and proliferative activity was determined as described above.

Supernatants were collected from uncloned RENCA cells and from clones P1 and P2 as well as from the B16F1 line. Where indicated, tumour cells were stimulated by IFNγ (100 U/ml). Tumour cells were seeded at a density of 1×10^5/ml. For collecting supernatants of MLC or MLTC, the responder population was seeded at a density of 1×10^6/ml. Supernatants were collected after 1–3 days; they were filter-sterilised and stored at −20 °C until use.

ELISA

Standard enzyme-linked immunosorbent assay (ELISA) procedures were used. Plates were coated with anti-cytokine antibodies in bicarbonate buffer, pH 8.9. Unspecific binding of antibodies was blocked by incubation of the coated plates with 100 μg/ml bovine serum albumin for 1 h at 37 °C, and plates were incubated with 50 μl culture supernatant overnight. After the plates had been washed, 50 μl biotinylated antibody at the appropriate dilution was added and the plates were incubated for 3 h at room temperature. After washing, alkaline-phosphatase-coupled streptavidin and finally substrate solution (50 μl) in diethanolamine buffer, pH 8.9, were added. The absorbance at 405 nm was read after 60–120 min. Assays were run in triplicate.

Statistics

Significance of differences was calculated by the two-tailed Student's *t*-test.

Results

Immunosuppression by RENCA-derived clones

We recently described how standard preparations of RENCA cells are composed of different clones, some of which are highly immunogenic (e.g. P2), while others apparently are non-immunogenic (e.g. P1) [68]. Nonetheless, LNC from untreated mice proliferated in response to dendritic cells pulsed with extracts not only of the immunogenic clone P2 but also of the "non-immunogenic clone". However, LNC did not proliferate in response to irradiated P1 cells and only marginally in response to uncloned RENCA cells (Fig. 1A). The same observation accounted for LNC from tumour-immune mice (Fig. 1B). This finding indicated that RENCA cells, irrespective of their "immunogenicity", may exert some kind of suppression. The interpretation was supported by the observation that LNC from RENCA-bearing mice hardly responded to dendritic cells pulsed with tumour extracts (Fig. 1C). Furthermore, they showed reduced responsiveness towards an alloantigenic stimulus (Fig. 1D).

The reduced proliferation was accompanied by a very low percentage of CD8$^+$ and CD4$^+$ blasts in a syngeneic MLTC of BALB/c LNC and RENCA cells (Fig. 2). The percentage of small (resting) CD4$^+$ and CD8$^+$ cells appeared largely unaltered. The phenomenon was RENCA-related inasmuch as, in a syngeneic MLTC using C57BL6 LNC and the B16F1 melanoma, no such decrease in CD4$^+$ and CD8$^+$ blasts was observed. In an allogeneic MLC the percentage of CD4$^+$ blasts was particularly high. Concomitantly with the low percentage of CD4$^+$ and CD8$^+$ blasts we observed an increase in NK1.1$^+$ blasts (Fig. 2). The increase in NK1.1$^+$ cells was predominantly due to an increase in NK1.1$^+$/CD3$^+$ cells, whereas the percentage of NK1.1$^+$/CD3$^-$ cells increased only slightly.

Finally we noted that the cultures contained a significantly increased number of dead cells (Fig. 3A). The phenomenon, too, was RENCA-related, i.e. it has not been observed in an allogeneic MLC nor in a syngeneic MLTC using C57BL6 LNC and irradiated B16F1 cells. Concomitantly with DNA fragmentation (data not shown), the percentage of cells expressing CD95 increased significantly in a MLTC with RENCA cells. The increase in CD95$^+$ cells was even noted in the population of small cells, i.e. before blast transformation. The percentage of CD95L$^+$ blasts was also higher in RENCA-MLTC than in B16-MLTC or an allogeneic MLC (Fig. 3B).

The findings confirmed that RENCA cells disturb the process of mounting an immune response. This can be due to immunosuppression by the tumour itself or the tumour can initiate a deviation of immune response. Because of the high percentage of dead cells in RENCA-MLTC, we first asked whether RENCA cells express the CD95L or produce factors supporting the lysis of lymphocytes.

RENCA cells do not produce soluble factors that initiate apoptosis and do not express CD95L

RENCA cells and clones derived therefrom express CD95, and expression was slightly increased upon stimulation with IFNγ (Table 1). Yet neither unstimulated nor IFNγ-stimulated RENCA cells express CD95L. These findings exclude the possibility that RENCA cells lyse lymphocytes via CD95 binding.

Apoptosis could also be induced by binding of TNFα to its receptor. Furthermore, activation of lymphocytes could be hampered by IL-10 or a deviation of response could be induced by IL-4. Therefore, we next evaluated whether these cytokines are produced by RENCA cells (Tables 2, 3). Less than 20% of uncloned and cloned RENCA cells expressed TNFα. RENCA cells did not express IL-4. Roughly one third of the RENCA cells expressed IL-10 (Table 2). Although stimulation of RENCA cells by IFNγ treatment led to a strong expression of MHC class II molecules, cytokine expression was not markedly altered by this treatment.

Two methods were used to determine whether expression of IL-10 or additional undefined factors, which may be secreted by RENCA cells, initiated lymphocyte death or influenced initiation of an immune response: (i) addition of RENCA cell supernatants to an allogeneic MLC and (ii) the Boyden chamber system, where the upper chamber contained

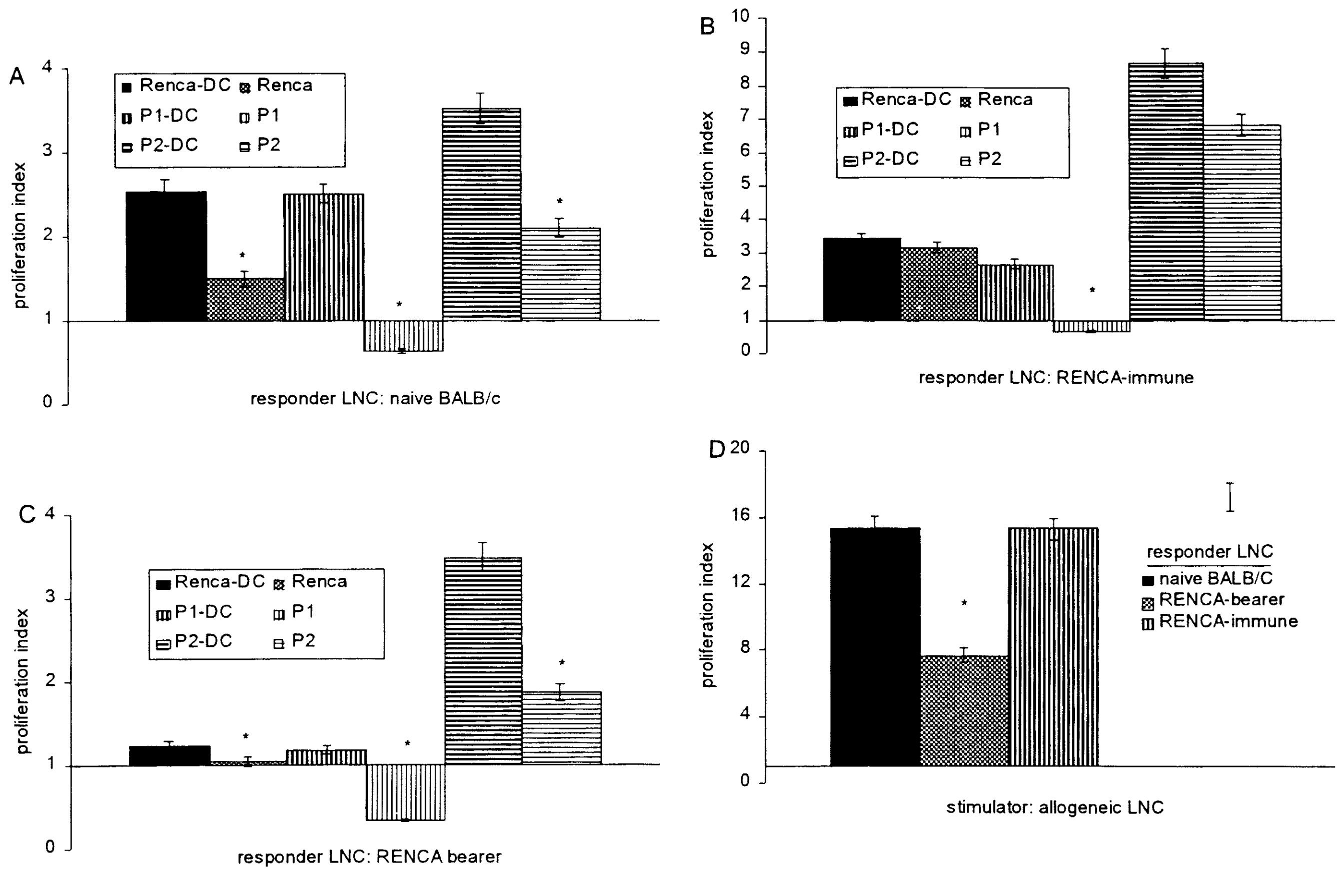


Fig. 1A–D Proliferative activity of lymph node cells in response to RENCA cells. (Draining) lymph node cells (*LNC*) were collected from untreated BALB/c mice, from BALB/c mice bearing a RENCA tumour and from mice immunized with irradiated RENCA cells. LNC were either cultured in the absence of a stimulus or with irradiated tumour cells (uncloned RENCA cells, clone P1, clone P2) or with dendritic cells (*DC*) after loading with extracts of the tumour cells (**A**, **B**, **C**) or with irradiated allogeneic (C57BL6) lymphocytes (**D**). Proliferative activity was determined by [^{3}H]thymidine incorporation after 3 days of culture. Mean values ± SD of the proliferation index (cpm of test well/cpm of control) of triplicate cultures are shown. *Significance of differences ($P < 0.01$) (**A**, **B**, **C** RENCA-cell-extract-loaded DC versus irradiated RENCA cells; **D** LNC from naive versus LNC from RENCA-bearing or RENCA-immune BALB/c mice). The experiment was repeated three times providing similar results

an allogeneic MLC and the lower chamber non-irradiated RENCA cells (Table 3). Supernatants of neither unstimulated nor stimulated RENCA cells, nor RENCA cells when separated by a membrane from the allogeneic MLC, exerted any effect on an allogeneic MLC. Thus, supernatants of RENCA cells, although expressing and secreting (data not shown) IL-10 and– at a low level – TNFα, did not suppress an allogeneic MLC.

Because supernatants of RENCA cells did not induce lymphocyte death and because RENCA cells were CD95L-negative and expression of TNFα was low, it appeared unlikely that lymphocyte death was mediated by RENCA cells. Instead, RENCA cells apparently impacted on lymphoid cells and their regulatory circuits.

RENCA cells initiate a suppression of immune response

When irradiated RENCA cells (uncloned, clones P1 or P2) were added to an allogeneic MLC, they clearly suppressed proliferative activity (Fig. 4). The strongest effect was seen with clone P1, but uncloned RENCA cells and even clone P2 also exerted a significant suppressive effect. Furthermore, supernatants of MLTC, when added to an allogeneic MLC, were also strongly immunosuppressive.

Because of the latter finding, we hypothesised that RENCA cells may produce cytokines that are immunosuppressive. However, the levels of IL-4 and IL-10, two cytokines known to suppress or deviate an immune response, were not or only slightly increased in supernatants of MLTC and a strong increase in TNFα was only seen in MLTC containing clone P2. Instead, cultures contained only very low levels of IL-2 and IFNγ (Table 4). This also accounted for an allogeneic MLC containing RENCA cells and for an allogeneic MLC fed with supernatants of a MLTC (data not shown). The cytokine expression profile (data not shown) corresponded to the secretion profile, i.e. the only consistently observed strong deviation, as compared to an allogeneic MLC not containing RENCA cells (nor

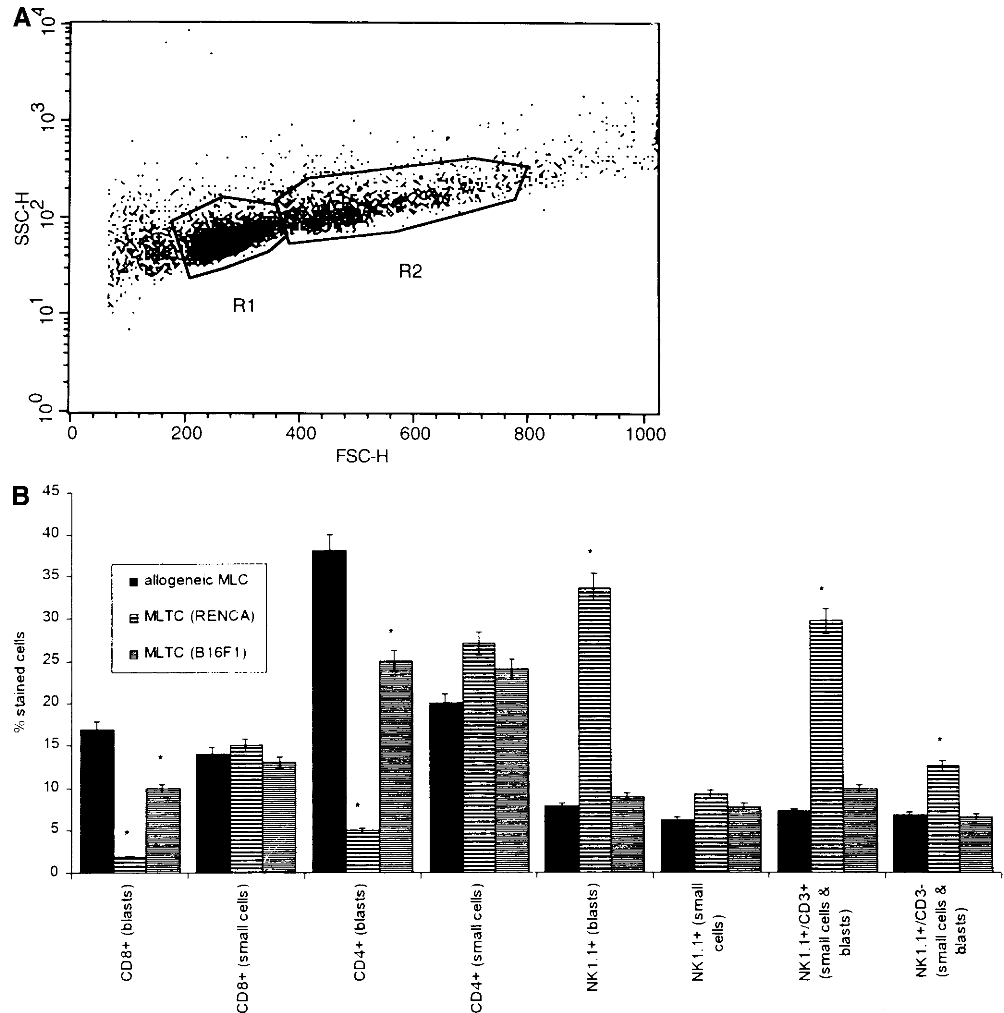


Fig. 2A, B Blast transformation and expansion of NK1.1$^+$ cells in (mixed-lymphocyte culture (*MLC*) and mixed-lymphocyte/tumour cell culture (*MLTC*). LNC from untreated mice were stimulated for 3 days with irradiated allogeneic LNC or with irradiated syngeneic tumour cells. Expression of CD4, CD8 and the percentage of NK1.1$^+$ and NK1.1$^+$/CD3$^+$ were determined by flow cytometry. Resting cells (*R1*) were differentiated from blast cells (*R2*) by gating as exemplified in **A**. **B** Mean values ± SD of three independently performed experiments are shown. *Significance of differences ($P < 0.01$) between the allogeneic MLC and the syngeneic MLTC

supernatants from a MLTC), was the low level of IL-2 and IFNγ expression.

The findings support the interpretation that lymphocytes, upon contact with RENCA cells, produce a factor that interferes with activation of the IL-2 and the IFNγ genes. Because CD28 is known to be involved in the activation of the IL-2 gene, which – on the other hand – is hampered by CTLA-4, it was of interest to evaluate the expression profile of these two molecules on lymphocytes recovered from an MLTC or an allogeneic MLC containing irradiated RENCA cells. Expression of CD28 was unaltered (data not shown). However, CTLA-4 was expressed in a significantly higher percentage of lymphocytes than in an allogeneic MLC. As could have been expected by the low level of IL-2, expression of the IL-2 receptor (CD25) was significantly reduced in an allogeneic MLC (Table 5).

Alternatively, though the two possibilities are not mutually exclusive, lymphocytes may receive a stimulus to express or secrete ligands for death-domain-bearing receptors. TNFα has not been found to be strongly upregulated (see Table 4), but, expression of CD95 and CD95L was increased (Table 5). The increased percentage of CD95$^+$ and CD95L$^+$ lymphocytes in RENCA-containing cultures indicated that lymphocyte suicide could well have been initiated. In fact, when an allogeneic MLC contained irradiated RENCA cells, we observed the same distribution of lymphocytes as described above (Fig. 2) for the syngeneic MLTC. As revealed by double staining with anti-CD3 and propidium iodide, over 50% of cells had died by day 3 of culture (Table 6) and over 90% of cells had died by day 5 (data not shown). In the allogeneic MLC fewer than 20% of cells were dead. Furthermore, the percentage of NK1.1$^+$/CD3$^+$ blasts was high and, as could have been expected, cultures contained very few CD4$^+$ blasts.

From these observations, RENCA cells do not directly kill lymphocytes, but they hamper the induction of an allogeneic immune response. Since supernatants of MLC containing RENCA cells were suppressive too, a contribution by soluble factors, which have not yet been identified, appears likely. Furthermore, the RENCA-cell-induced immunosuppression apparently has two components: (i) the absence of IL-2 and IFNγ production, as well as the reduced expression of IL-2 receptor and the missing blast transformation of CD4$^+$ and CD8$^+$ cells, suggest the induction of a state of anergy; (ii) a mechanism of lymphocyte suicide is supported by

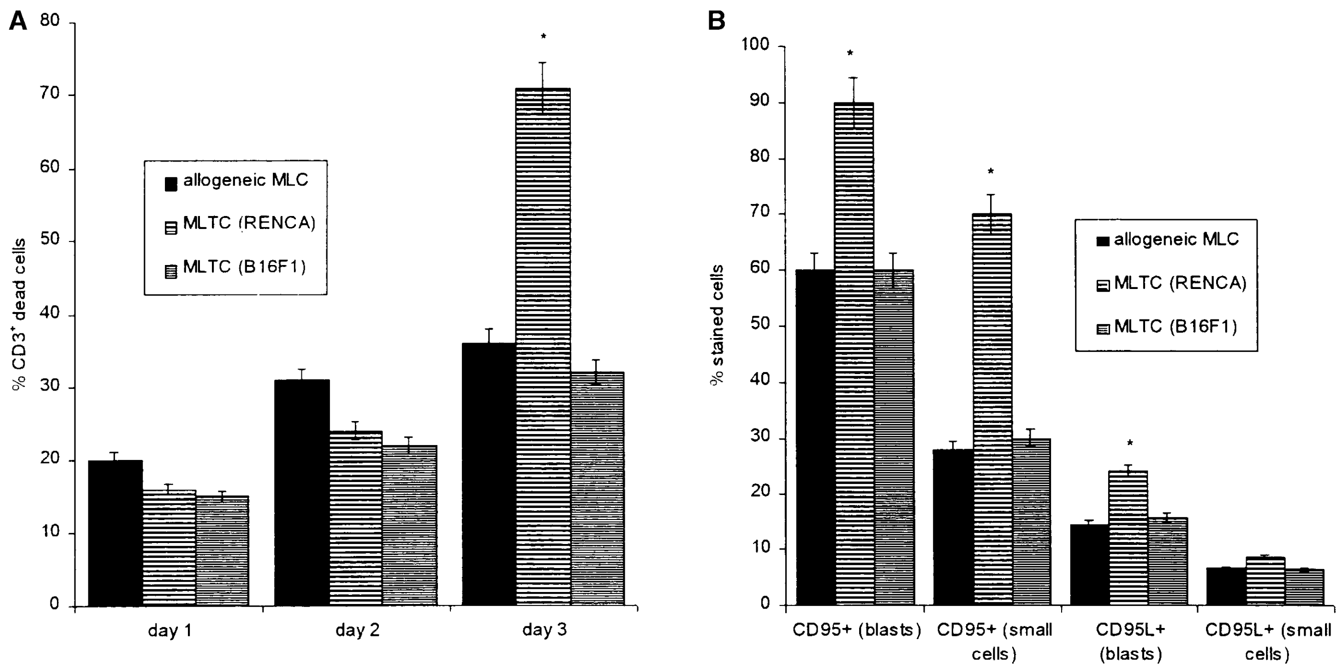


Fig. 3A, B Cell death in allogeneic MLC and syngeneic MLTC and expression of CD95 and CD95L. LNC from untreated mice were stimulated for 1–3 days with irradiated allogeneic LNC or with irradiated syngeneic tumour cells. The percentage of dead (propidium iodide uptake) CD3$^+$ cells (**A**) and (only day 3) the percentage of CD95$^+$ and CD95L$^+$ cells (**B**) were determined by flow cytometry. Resting cells were differentiated from blast cells by gating (see Fig. 2A). Mean values ± SD of three independently performed experiments are shown. *Significance of differences ($P < 0.01$) between the allogeneic MLC and the syngeneic MLTC

Table 1 CD95 and CD95L expression by RENCA cells and RENCA-derived clones. CD95 and CD95L expression was evaluated by flow cytometry; for interferon γ (*IFNγ*) stimulation tumour cells were cultured for 3 days in the presence of 100 U/ml IFNγ

Tumour cell	Cells (%) expressing	
	CD95	CD95L
RENCA	34.1 ± 0.7	3.1 ± 1.0
Clone P1	36.9 ± 1.5	5.4 ± 0.9
Clone P2	34.7 ± 1.3	3.7 ± 1.2
RENCA, IFNγ-stimulated	45.3 ± 1.1	4.2 ± 1.2
Clone P1, IFNγ-stimulated	56.4 ± 1.6	6.2 ± 1.5
Clone P2, IFNγ-stimulated	54.3 ± 1.4	3.9 ± 1.1

the strong expansion of NK1.1$^+$/CD3$^+$ cells, the high percentage of dead cells and the up-regulation of CD95.

RENCA-initiated immunosuppression can be prevented by ligation of costimulatory molecules and/or targeting of CD4$^+$ cells

The immunosuppression described was not observed when RENCA-associated antigens were presented by conventional APC (see Fig. 1). Hence it was tempting to speculate that suppression could be circumvented by triggering costimulatory molecules during the initial contact with the tumour cell. To support the hypothesis, we evaluated whether RENCA cells expressing B7.1 and/or MHC class II are immunosuppressive. RENCA cells expressing the transfected cDNAs at a high level were selected by cloning or by FACS as detailed in Materials and methods (Fig. 5).

MHC class II$^+$, B7.1$^+$ and MHC class II$^+$ plus B7.1$^+$ clones were tested for their stimulatory capacity in a syngeneic MLTC (Fig. 6A). BALB/c lymphocytes responded strongly to RENCA cells expressing B7.1 and weakly to RENCA cells expressing MHC class II molecules. When P1 cells were used as stimulators, expression of both MHC class II and B7.1 led to a further increase in the proliferative response. Furthermore, RENCA cells expressing B7.1 or B7.1 plus MHC class II molecules also did not (clone P2) or only weakly (clone P1) suppress an allogeneic immune response (Fig. 6B).

Evaluation of cytokine production in an MLTC with B7.1- and/or MHC-class-II-expressing P1 cells as well as in an allogeneic MLC containing transfected P1 cells (Table 7) revealed a strong increase in IL-2 and IFNγ production when the tumour cells expressed B7.1. Expression of B7.1 as well as of MHC class II molecules also sufficed for expansion of CD4$^+$ cells, which was accompanied by up-regulation of CD25 and down-regulation of CTLA-4 expression (Table 8). Furthermore, expression of CD95 and of CD95L was reduced, particularly when RENCA cells expressed MHC class II molecules. The percentage of NK1.1$^+$/CD3$^+$ decreased and concomitantly a reduction in the percentage of dead cells (Table 9) was noted. The reduction in NK1.1$^+$/CD3$^+$ as well as in dead cells was strongest when RENCA cells expressed MHC class II

Table 2 RENCA cells do not produce immunosuppressive factors: cytokine expression by RENCA cells and RENCA-derived clones. MHC class II and cytokine expression was evaluated by flow cytometry (see Materials and methods for details); for IFNγ-stimulation tumour cells were cultured for 3 days in the presence of 100 U/ml IFNγ; BALB/c LNC were cultured with irradiated C57BL6 lymphocytes. Cultures contained either supernatants of unstimulated or IFNγ-stimulated RENCA cells or RENCA cells separated by a membrane; *IL* interleukin, *TNFα* tumour necrosis factor α

Tumour cell	Cells (%) expressing:			
	MHC class II	IL-4	IL-10	TNFα
RENCA	1.4 ± 0.2	3.7 ± 1.1	26.8 ± 2.8	10.8 ± 1.8
Clone P1	1.3 ± 0.5	2.4 ± 0.7	31.5 ± 2.6	14.1 ± 1.7
Clone P2	2.5 ± 1.3	3.6 ± 1.5	33.9 ± 3.3	12.9 ± 2.3
RENCA, IFNγ-stimulated	43.6 ± 5.1	7.2 ± 3.2	29.2 ± 3.9	14.2 ± 2.5
Clone P1, IFNγ-stimulated	54.5 ± 3.6	8.2 ± 2.7	34.7 ± 3.1	13.5 ± 1.9
Clone P2, IFNγ-stimulated	61.3 ± 6.4	6.9 ± 1.3	32.2 ± 3.9	12.2 ± 2.3

Table 3 RENCA cells do not produce immunosuppressive factors: influence of RENCA-derived soluble factors on an allogeneic response. Experimental details as for Table 2. *MLC* mixed-lymphocyte culture, *LNC* lymph node cells, *NS* not significant, *NT* not tested

Source of soluble factor	Allogeneic MLC (cpm/10^5 LNC) in the presence of	
	RENCA supernatants	Membrane-separated RENCA cells
Medium	16,341 ± 622	9,431 ± 451
RENCA	17,632 ± 711 (NS)	9,122 ± 433 (NS)
Clone P1	16,409 ± 581 (NS)	8,730 ± 427 (NS)
Clone P2	16,880 ± 623 (NS)	9,521 ± 481 (NS)
Medium, IFNγ	15,639 ± 476	NT
RENCA, IFNγ-stimulated	17,565 ± 479 (NS)	NT
Clone P1, IFNγ-stimulated	19,833 ± 518 (NS)	NT
Clone P2, IFNγ-stimulated	15,872 ± 526 (NS)	NT

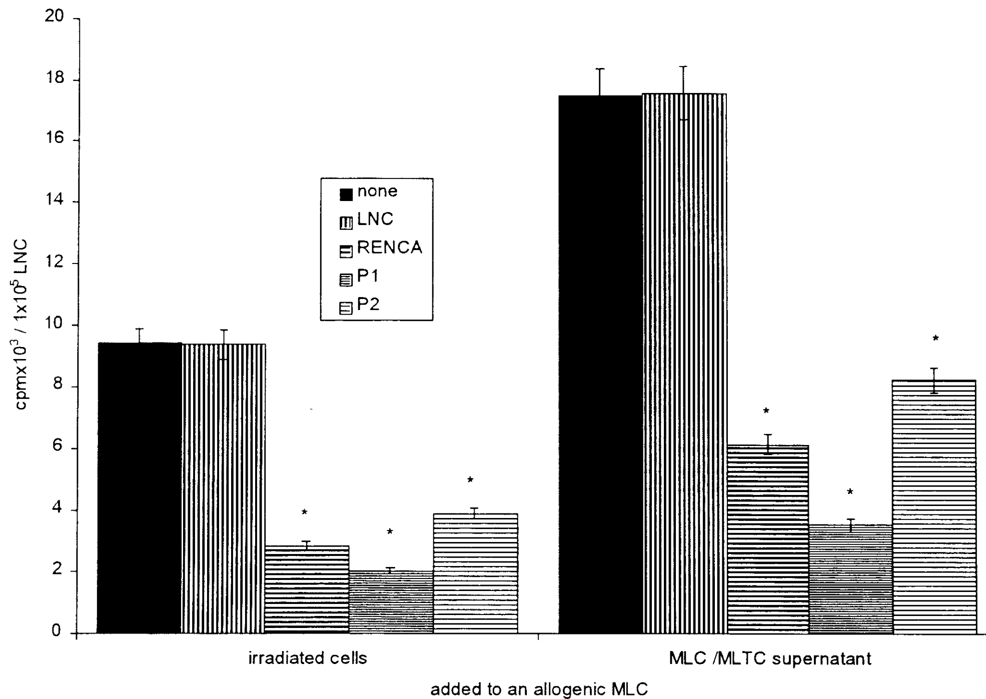


Fig. 4 Suppression of an allogeneic mixed-lymphocyte reaction by RENCA cells and supernatants of MLTC. BALB/c LNC were cocultured with irradiated C57BL6 lymphocytes. Cultures contained in addition irradiated LNC or RENCA cells (BALB/c LNC:RENCA cells = 50:1) or supernatants of a MLC or a MLTC (50% of culture medium). Proliferative activity was determined by [^{3}H]thymidine incorporation after 3 days of culture. Mean values ± SD of triplicate cultures are shown. *Significance of differences ($P < 0.01$) from the allogeneic MLC containing neither irradiated RENCA cells nor supernatants of a MLTC

or MHC class II plus B7.1. Similar findings were observed with transfectants of clone P2 (data not shown).

Thus, RENCA-cell-induced immunosuppression was mitigated by expression of MHC class II molecules as well as of B7.1. By triggering the costimulatory molecule CD28, an efficient proliferative response and cytokine secretion has been initiated. Ligation of CD4$^+$ cells was

Table 4 Cytokine production in mixed-lymphocyte/tumor cell culture (MLTC) and in MLC in the presence and absence of RENCA cells. Cytokine production was determined after 3 days of culture by enzyme-linked immunosorbent assay (ELISA). Quantification was performed by comparison with a calibration curve using recombinant cytokines. *P* values are shown in parentheses

Culture	Cytokine production (ng-ml)				
	IL-2	IL-4	IL-10	IFNγ	TNFα
LNC	0.47	0.15	0.71	0.51	0.37
LNC + RENCA	0.14 (< 0.01)	0.12 (NS)	0.87 (0.02)	0.09 (< 0.01)	0.24 (< 0.01)
LNC + P1	0.13 (< 0.01)	0.17 (NS)	1.01 (< 0.01)	0.18 (< 0.01)	0.58 (< 0.01)
LNC + P2	0.22 (< 0.01)	0.14 (NS)	0.69 (NS)	0.17 (< 0.01)	0.76 (< 0.01)
Allogeneic MLC	0.97	0.22	1.26	0.88	0.66
Allogeneic MLC + RENCA	0.55 (< 0.01)	0.13 (< 0.01)	1.38 (NS)	0.19 (< 0.01)	0.42 (< 0.01)
Allogeneic MLC + P1	0.53 (< 0.01)	0.10 (< 0.01)	0.59 (< 0.01)	0.31 (< 0.01)	1.00 (< 0.01)
Allogeneic MLC + P2	0.56 (< 0.01)	0.03 (< 0.01)	0.84 (< 0.01)	0.54 (< 0.01)	1.62 (< 0.01)

Table 5 Leucocyte subset distribution and survival in allogeneic MLC in the presence and absence of RENCA cells: expression of CD95, CD95L and activation markers. Marker expression was determined after 3 days of culture, by flow cytometry. Mean values of three independently performed experiments are shown, with *P* values in parentheses

Culture	Stained cells (%)			
	CD95	CD95L	CD25	CTLA-4
Allogeneic MLC	38.3	5.2	36.9	4.7
Allogeneic MLC + RENCA	55.8 (< 0.01)	14.3 (< 0.01)	19.9 (< 0.01)	11.6 (< 0.01)
Allogeneic MLC + P1	61.1 (< 0.01)	15.0 (< 0.01)	17.4 (< 0.01)	11.9 (0.01)
Allogeneic MLC + P2	51.5 (0.02)	19.6 (< 0.01)	20.4 (< 0.01)	11.7 (< 0.01)

Table 6 Leucocyte subset distribution and leucocyte survival in the presence and absence of RENCA cells. The population of blasts (as shown in Fig. 2) has been gated; marker expression was determined by flow cytometry after 3 days of culture. Mean values of three independently performed experiments are shown, with *P* values in parentheses. *PI* propidium iodide

Culture	Stained blasts (%)			
	CD4	CD8	NK1.1 + CD3	PI + CD3
Allogeneic MLC	46.2	25.5	6.3	19.7
Allogeneic MLC + RENCA	24.8 (< 0.01)	20.8 (NS)	23.3 (< 0.01)	52.8 (< 0.01)
Allogeneic MLC + P1	18.7 (< 0.01)	17.5 (0.02)	36.2 (< 0.01)	50.9 (< 0.01)
Allogeneic MLC + P2	29.0 (< 0.01)	23.8 (NS)	21.5 (< 0.01)	54.7 (< 0.01)

less efficient in these respects, but prevented deviation towards suicide.

Discussion

Despite the presence of tumour-associated antigens, which can be recognised by cytotoxic T cells [21, 69, 70], induction of an efficient immune response in tumour patients is a very rare event and the in vitro generation of tumour-directed CTL has been proven to be particularly difficult with tumour-infiltrating lymphocytes, as compared to peripheral blood T cells or T cells from draining lymph nodes, for example [63]. These observations suggested that, besides the known phenomena of tumour escape [1, 17, 20, 36, 42, 46, 59, 61, 62], tumour cells may actively suppress antitumour immune responses [5, 47]. Discussions of immunosuppression by tumour cells mainly focus on the production of suppressive factors, like IL-10 or TGFβ [15, 19, 26, 31, 34, 43, 74] and expression of ligands for receptors of the death-signalling cascade, like CD95L or TNF [24, 30, 32, 67]. Recently the possibility has been described that tumour cells may escape an immune attack by modulating an immune response such that activation of T helper (Th1) cells and CTL is redirected in favour of Th2 cells [33, 43, 65], possibly with the consequence of activation-induced death of the Th1 population [75]. However, the mechanisms of immunosuppression by tumour cells are not well defined and there are indications that individual tumours may interfere differently with the mounting of an immune response.

We have described recently that the RENCA line is composed of clones that differ widely in antigenicity [68].

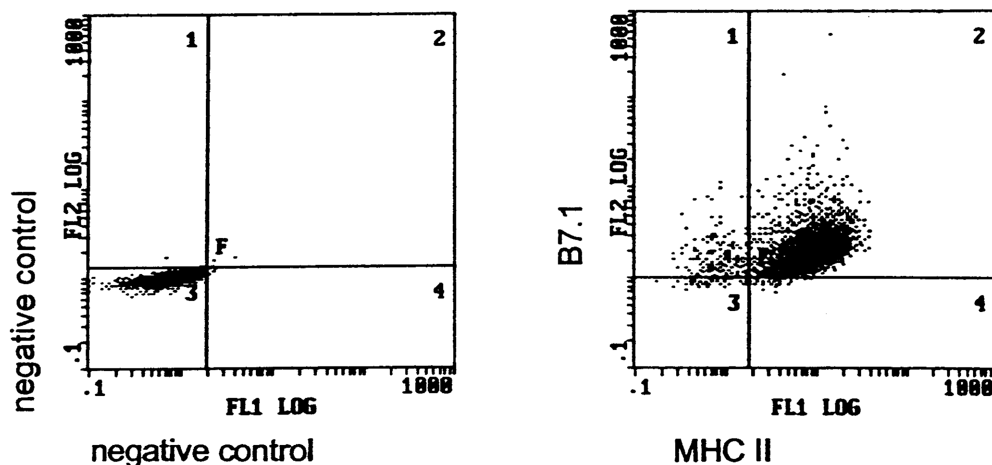
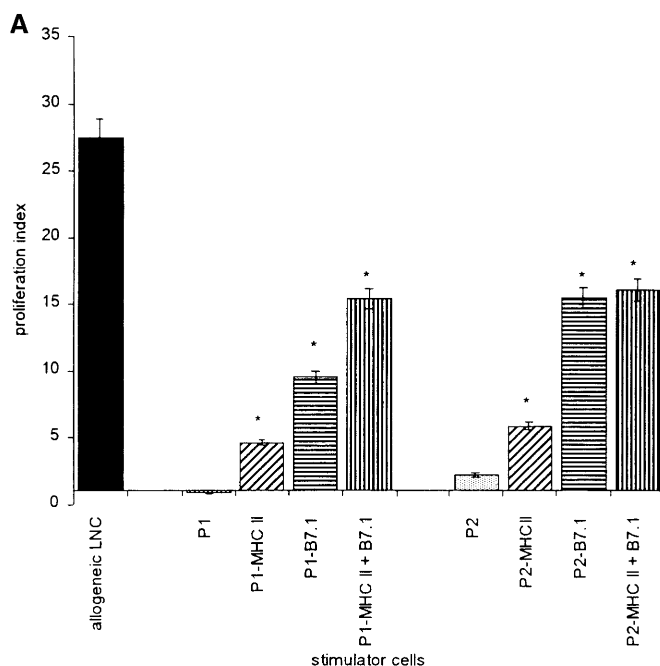


Fig. 5 Expression of MHC class II and/or of B7.1 on transfected RENCA cells. RENCA cells were transfected with either the B7.1 cDNA or the MHC class II α and β chain cDNA of the H-2^d haplotype or both. Cells expressing MHC class II and/or B7.1 were selected as described in Materials and methods. Surface expression was evaluated by flow cytometry and is demonstrated for the doubly transfected clone P1. *Left* negative control (FITC-labelled mouse IgG, PE-labelled hamster Ig); *right* staining with FITC-labelled anti-I-Ad and PE-labelled anti-B7.1

In addition, the discrepancy between the ready induction of CTL in vitro and the inefficacy of therapeutic vaccination specifically points towards RENCA-induced immunosuppression. Since the question of immunosuppression by tumour cells is of utmost importance in the immunotherapy of cancer, we strove to elucidate the underlying mechanisms.

Before a discussion of potential mechanisms of immunosuppression it should be mentioned that RENCA cells do not display common mechanisms of tumour escape, i.e. the majority of RENCA cells express MHC class I antigens, and the fact that CTL could be readily induced in vitro argues against major defects in antigen processing, though the latter has not been directly evaluated [68]. Thus, it is most likely that RENCA cells counterregulate the immune response.

Fig. 6A, B Abrogation of RENCA-induced immunosuppression by expression of MHC class II and/or B7.1. **A** BALB/c LNC were cultured with irradiated allogeneic C57BL6 lymphocytes or with irradiated RENCA cells expressing either MHC class II or B7.1 or both molecules. Proliferative activity was determined after 3 days of culture by [^{3}H]thymidine incorporation. Mean values ± SD of the proliferation index (cpm of test well/cpm of control) of triplicate cultures are shown. **B** BALB/c LNC were cultured with irradiated allogeneic C57BL6 lymphocytes. Where indicated, the cultures contained, in addition, irradiated untransfected RENCA cells or RENCA cells transfected with B7.1, MHC class II, or B7.1 plus MHC class II cDNA. Proliferative activity was determined by [^{3}H]thymidine incorporation after 3 days of culture. Mean values ± SD of triplicate cultures are shown. **A, B** *Significant differences ($P < 0.01$) between untransfected and transfected RENCA-derived clones. The experiments were repeated three times providing similar results



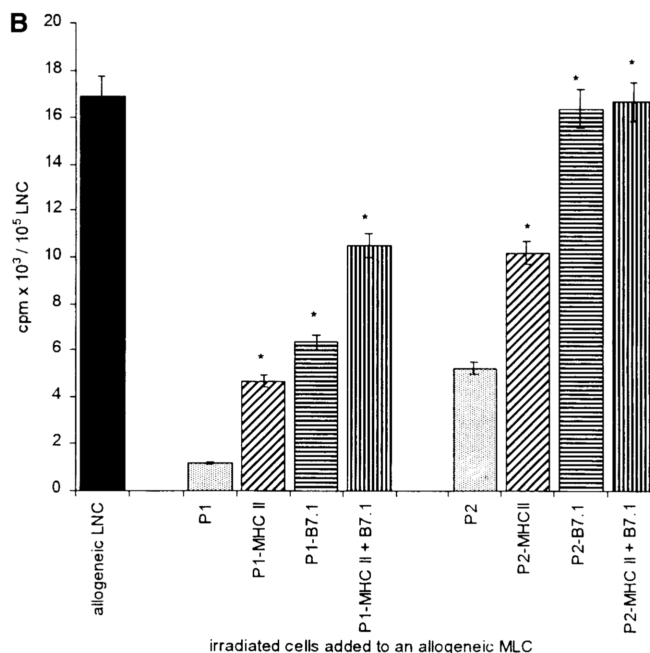


Table 7 Immunosuppression by RENCA cells (clone P1) expressing MHC class II and/or B7.1: cytokine production in MLTC and allogeneic MLC in the presence and absence of P1 cells transfected with of MHC class I and/or B7.1 cDNA. BALB/c LNC were cultured for 3 days with irradiated untransfected P1 cells or P1 cells transfected with B7.1, MHC class II or B7.1 plus MHC class II cDNA, or with irradiated allogeneic C57BL6 lymphocytes plus irradiated untransfected or transfected P1 cells (BALB/c LNC: P1 = 50:1). Cytokine production was determined after 3 days of culture by ELISA. Quantification was performed by comparison with a calibration curve using recombinant cytokines. *P* values are in parentheses

Culture	Cytokine production (ng/ml)			
	IL-2	IL-10	IFNγ	TNFα
LNC	0.30	0.60	0.41	0.37
LNC + P1	0.08	0.96	0.19	0.51
LNC + P1-MHC II	0.09 (NS)	0.74 (0.03)	0.25 (0.03)	0.46 (NS)
LNC + P1-B7.1	0.18 (< 0.01)	0.84 (NS)	0.38 (< 0.01)	0.42 (0.07)
LNC + P1-MHC II/B7.1	0.26 (< 0.01)	0.84 (NS)	0.38 (< 0.01)	0.46 (NS)
Allogeneic MLC	0.97	1.64	0.71	0.60
Allogeneic MLC + P1	0.24	1.06	0.24	1.06
Allogeneic MLC + P1-MHC II	0.31 (0.07)	1.08 (NS)	0.25 (NS)	0.58 (< 0.01)
Allogeneic MLC + P1-B7.1	0.72 (< 0.01)	0.88 (0.06)	0.60 (< 0.01)	0.60 (< 0.01)
Allogeneic MLC + P1-MHC II/B7.1	0.85 (< 0.01)	0.84 (0.04)	0.69 (< 0.01)	0.54 (< 0.01)

Table 8 Immunosuppression by RENCA cells (clone P1) expressing MHC class II and/or B7.1: leucocyte subset distribution in MLTC and allogeneic MLC in the presence and absence of P1 cells transfected with MHC II and/or B7.1 cDNA. Expression of CD4, CD8, CD25, CTLA-4, CD95L and of NK1.1 on $CD3^+$ cells was determined by flow cytometry. The culture was carried out as described in Table 7. *P* values are in parentheses

Culture	Stained cells (%)					
	CD4 (blasts)	CD8 (blasts)	CD25	CTLA-4	CD95L	$NK1.1^+/CD3^+$
LNC + P1	10.2	23.7	10.2	23.2	19.1	35.1
LNC + P1-MHC II	34.3 (< 0.01)	30.3 (0.05)	16.2 (< 0.01)	17.5 (0.07)	10.7 (< 0.01)	11.3 (< 0.01)
LNC + P1-B7.1	41.8 (< 0.01)	27.7 (NS)	21.7 (< 0.01)	15.6 (0.03)	11.6 (< 0.01)	15.0 (< 0.01)
LNC + P1-MHC II/B7.1	45.9 (< 0.01)	31.7 (0.02)	23.7 (< 0.01)	13.8 (0.01)	7.6 (< 0.01)	9.6 (< 0.01)
Allogeneic MLC	43.7	25.5	36.9	3.2	7.6	6.3
Allogeneic MLC + P1	31.3	14.7	17.4	15.6	21.6	24.6
Allogeneic MLC + P1-MHC II	33.1 (NS)	20.8 (0.02)	28.5 (< 0.01)	10.3 (< 0.01)	8.1 (< 0.01)	10.5 (< 0.01)
Allogeneic MLC + P1-B7.1	38.7 (0.05)	22.8 (< 0.01)	29.9 (< 0.01)	8.0 (< 0.01)	15.7 (0.02)	20.7 (NS)
Allogeneic MLC + P1-MHC II/B7.1	41.8 (0.02)	23.8 (< 0.01)	30.4 (< 0.01)	4.6 (< 0.01)	7.8 (< 0.01)	9.4

Table 9 Immunosuppression by RENCA cells (clone P1) expressing MHC class II and/or B7.1: cell death in MLTC and allogeneic MLC in the presence and absence of P1 cells transfected with MHC II and/or B7.1 cDNA. Culture conditions as in Table 7. Dead $CD3^+$ cells were determined by uptake of propidium iodide (flow cytometry). Mean values of three independently performed experiments are shown, with *P* values in parentheses

Added cells (irradiated)	Dead cells (%)	
	MLTC	Allogeneic MLC
None		15.3
P1	65.1	48.9
P1-MHC II	43.2 (< 0.01)	23.4 (< 0.01)
P1-B7.1	53.3 (0.07)	36.3 (0.03)
P1-MHC II/B7.1	39.8 (< 0.01)	19.5 (< 0.01)

RENCA cells are also not immunosuppressive by themselves. Tumour-mediated immunosuppression has been described to be most frequently mediated by soluble factors produced by the tumour cell. Those factors could be directly anti-proliferative, like TGFβ [7], they could favour the mounting of a Th2-mediated response and conversely down-regulate a Th1 response, like IL-4 [45, 56, 58], or they could interfere directly with the activation of Th1 cells and CTL, like IL-10 [33]. Although RENCA cells produce IL-10 and, at a low level, TNFα, these modes of action could be excluded. Supernatants of RENCA cells exerted no suppressive effect on an allogeneic MLC.

Tumour cells expressing CD95L could initiate activation-induced cell death in $CD95^+$ lymphocytes [16, 64, 76]. This, however, has not been the case. First, RENCA cells express CD95L very sparsely. Second, addition of RENCA cells to phytohaemagglutinin-stimulated lymphocytes was ineffective (data not shown). This excludes a RENCA-cell-induced death of lymphocytes by ligation of CD95, since mitogen-stimulated lymphocytes express CD95 at a level comparable to alloantigen-activated lymphocytes. Instead, the finding that a mitogen-induced immune response could not be suppressed by RENCA cells points towards an inappropriate initiation of response at the level of the T cell receptor and/or costimulatory molecules.

There remains the possibility that RENCA cells initiate a deviation of response. It should be mentioned at

this point that such a RENCA-induced immunosuppression was independent of the differing antigenicity of individual RENCA-derived clones, i.e. it was observed with clone P1, which apparently has lost antigenicity and, albeit more weakly, with clone P2, which expresses a new antigenic determinant [68]. Mechanisms underlying a tumour-cell-initiated deviation of response have not been fully elucidated. Thus, in a MLTR with $CD95^-$ tumour cells, autocrine production of CD95L by CTL has been observed, which initiated auto-lysis of CTL [23]. It also has been reported that activation of macrophages in the presence of apoptotic cells (irradiated tumour cells) leads to up-regulation of IL-10 [26, 27, 72] with the consequences of impaired Th1 and CTL activation. Finally, $NK1.1^+$ T cells [10] have been shown to suppress the generation of CTL and to lyse B7-expressing APC [4, 65, 66, 71]. Although our finding that irradiated RENCA cells suppress lymphocyte proliferation in an allogeneic MLC could theoretically be explained by all three observations, we consider two mechanisms, which may be interdependent or additive, as being most likely: initiation of anergy and autolysis.

RENCA-induced immunosuppression is at least partly mediated by a soluble-lymphocyte-derived factor. This assumption is supported by the observation that an allogeneic MLC was suppressed in the presence of supernatants of a mixed RENCA-cell/lymphocyte culture. Therefore, we primarily expected that, by initiation of IL-10 production, activation of Th1 and CTL would have been hampered. However, IL-10 production was not significantly up-regulated. Furthermore, cultures contained very low amounts of IL-4 (data not shown), which also argues against a RENCA-induced deviation towards a Th2 response. The low level of IL-10 and IL-4 and the insignificant increase in TNFα make it unlikely that the suppressive factor(s) will be a cytokine. So far, unfortunately, we have not succeeded in its identification.

Whereas IL-10 and IL-4 production by "responder" lymphocytes in a MLTC as well as in an allogeneic MLC containing RENCA cells appeared unaffected, lymphocytes hardly produced any IL-2 and IFNγ. It should be noted that the same phenomenon has been reported for lymphocytes stimulated with human renal cell carcinoma [39, 50]. Lymphocytes also did not express CD25 and only a minority of $CD4^+$ and $CD8^+$ cells underwent blast transformation. All these features indicate that, in the presence of RENCA cells or supernatants of a RENCA-containing MLTC, lymphocytes do not respond even to an allogeneic stimulus, i.e. they become anergic.

Finally, autolysis of lymphocytes, apparent by the high percentage of dead $CD3^+$ cells, may be partly due to apoptosis initiated by ligands of death-domain-bearing receptors, i.e. expression of CD95L and TNFα secretion were slightly up-regulated. Furthermore, high expression of CD95 was observed in the MLTC as well as in the allogeneic MLC containing irradiated RENCA cells. Most importantly, there was a significant expansion of $NK1.1^+/CD3^+$ lymphocytes, which are known to possess strong lytic activity [65].

There remained the question whether immunosuppression, which was initiated, but not mediated by RENCA cells, could be corrected by modulating RENCA cells. We did this by providing a ligand either for a costimulatory molecule or for CD4 by transfection of RENCA cells with MHC class II and/or B7.1 cDNA. We also expected that these modes of modulating RENCA cells would add further support to our interpretation of overlapping mechanisms of suicide and induction of anergy. RENCA-induced immunosuppression could be partly prevented by expression of MHC class II and could very efficiently be corrected by expression of B7.1. RENCA cells expressing both molecules, B7.1 and MHC class II, were no longer immunosuppressive, neither in the MLTC nor the allogeneic MLC containing irradiated RENCA cells. The findings are in line with the observation that immunosuppression was not observed when dendritic cells were pulsed with tumour cell lysates. It should be mentioned that cultures containing $B7.1^+$ RENCA cells, although displaying a significantly increased proliferative response, contained more dead cells than cultures containing MHC class II^+ RENCA cells, whereas the latter were less efficient in initiating proliferation. We interpreted these features and the finding that MHC class II plus B7.1 expression exerted an additive effect on correcting RENCA-induced immunosuppression, in the sense that RENCA cells may, indeed, stimulate two "suppressive" pathways, anergy and apoptosis.

How can expression of MHC class II molecules prevent T cell death? One possible explanation could be that irradiated RENCA cells provide an activation/expansion signal for $NK1.1^+/CD3^+$ cells, which lyse conventional APC [4, 65, 71]. In the absence of MHC class II molecules, $CD4^+$ cells are known to become anergic [35, 47, 57, 77] and susceptible to apoptosis [2, 11, 54]. In the presence of tumour cells expressing MHC class II molecules, expansion of $NK1.1^+/CD3^+$ cells was impaired. Consequently APC may not be lysed and $CD4^+$ cells binding to allogeneic APC (MLC plus RENCA cells) or MHC-class-II-expressing RENCA cells (MLTC) will be protected from apoptotic signals.

The fact that signal 2, initiated by binding of CD28 to B7.1/B7.2, triggers T cell activation is well known [35, 47, 57, 77]. Besides actively interfering with induction of a state of anergy, B7.1 expression on RENCA cells may also have bearing on the reduced lymphocyte suicide. It has been described that B7.1 prevents susceptibility towards activation-induced cell death [23] possibly by transferring signals, that lead to up-regulation of apoptosis-preventing members (bcl-xL) of the bcl-2 gene family [11]. Taking these observations together, we propose that expression of B7.1 prevented induction of a state of anergy and initiated cytokine production. Expression of MHC class II apparently interfered with activation/expansion of $NK1.1^+/CD3^+$ cells, which mitigated indirectly, i.e. by the survival of APC, lymphocyte suicide.

In summary, we have shown that RENCA cells/clones, whatever their antigenicity, initiate immunosup-

pression without actively attacking lymphocytes or secreting immunosuppressive substances. Instead, (i) a state of anergy is created and (ii) cytolytic cells of the non-adaptive immune system expand, their lytic activity directly or, more likely indirectly supporting apoptosis of $CD4^+$ cells. The fact that RENCA-induced immunosuppression could be efficiently prevented by provision of a ligand for costimulatory molecules and by targeting $CD4^+$ cells underlines the utmost importance of the first encounter of the immune system with a tumour cell for directing immunoregulatory circuits.

Acknowledgements This investigation was supported by the Mildred Scheel-Stiftung für Krebshilfe (M.Z.), and by a fellowship (G.A.B.) from the Deutsche Forschungsgemeinschaft. We thank Dr. S. Matzku, Merck AG, Darmstadt, for helpful suggestions and discussion during preparation of the manuscript.

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