Growth Stimulation of Tumor-Specific Cytotoxic T Lymphocytes on Concanavalin A-Immobilized Carrier Beads

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Abstract

Human tumor-specific CD4⁺ cytotoxic T lymphocytes (CTL) were generated against duodenum papilloma cell line TGBC18TKB from HLA type-matched peripheral blood mononuclear cells. Concanavalin A (Con A) immobilized on carrier beads stimulated growth of the CTL in a long-term culture without repeated antigen stimulation, while soluble Con A induced death of the CTL. The CTL exhibited the target-specific cytotoxicity in a more potent manner than those before the long-term culture in the presence of the immobilized Con A. Enhanced expression of the adhesion molecule, CD11b, was observed on the CTL. These results suggest that immobilized Con A will be useful for continuous growth stimulation and large scale expansion of CTL without tumor antigen.

Abbreviations: CTL – cytotoxic T lymphocytes; HLA – human leucocyte antigens; PBMC – peripheral blood mononuclear cells

Introduction

Tumor-specific cytotoxic T lymphocytes (CTL) are expected for adoptive immunotherapy of human tumors. They can be generated from the peripheral blood of tumor-bearing patients or from tumor-infiltrating lymphocytes (Ioannides and Whiteside, 1993). However, successful generation has been limited to cases where sufficient tumor cells (Abo and Balch, 1982; Crowley et al., 1991; Goedegebuure et al., 1995; Kawakami et al., 1994a; Wolfel et al., 1994) or tumor antigenic peptides (Slingluff et al., 1994; Tjoa et al., 1994) were available for repeated stimulation of CTL growth for a prolonged culture period. CTL keep growing on the stimulation of tumor-antigens, and most of them die if the stimulation is removed (Crowley et al., 1991; Liu et al., 1995b; Slingluff et al., 1994; Tjoa et al., 1994; Wolfel et al., 1993). This phenomenon can also be observed *in vivo* (Lau et al., 1994).

The supply of target tumor cells is a key problem due to difficulty of establishing the tumor cell line from every patient (Hay et al., 1994). The alternative method, tumor antigenic peptides, is a useful tool for CTL induction. However, diversity of tumor antigenic peptides exists in the same type of tumors from different individuals (Bakker et al., 1995; Fisk et al., 1995; Moudgil and Sercarz, 1994; Liu et al., 1995a). Therefore, the CTL induced with known antigenic peptides (Bakker et al., 1995; Kawakami et al., 1994b; Welfel et al., 1994) will be insufficient to kill the tumors with unknown antigen. Although we have successfully generated CTL on formalin-fixed paraffin-embedded sections with unknown antigen (Liu et al., 1995b), supply of sections will also be insufficient when the CTL have grown to a considerably large scale.

Anti-CD3 antibodies (Goedegebuure et al., 1995; Groux et al., 1993; Takayama et al., 1991) and plant-derived lectins (Abo and Balch, 1982; Mazumder et al., 1982 and 1983) have been used for the non-

specific stimulation of T lymphocyte growth. They are useful if CTL have been once induced at the early phase of the induction culture, although excess stimulation with anti-CD3 antibodies will induce the death of CTL (Groux et al., 1993). Short-term stimulation with anti-CD3 antibodies fixed on the solid surface is then developed to provide mild stimulation for lymphocytes (Takayama et al., 1991; Goedegebuure et al., 1995) and used for the lymphocyte culture in the clinical trials (Goedegebuure et al., 1995). However, the stimulative effect lasts only for 10-14 days after single stimulation by the fixed anti-CD3 antibodies (our unpublished data).

The plant-derived lectins, concanavalin A (Con A) (Baker et al., 1979; Quentmeier et al., 1992; Shinomura et al., 1986) and phytohemagglutinin (PHA) (Mazumder et al., 1983) are able to induce cytokine production from peripheral blood mononuclear cells. Considering the cost of long term culture for expansion of CTL, Con A is one of the candidate non-specific stimulators of CTL growth. However, since Con A itself shows a toxic effect on lymphocytes, direct addition of Con A should be avoided to the culture for CTL induction (Baker et al., 1979; Mukherji et al., 1989; Quentmeier et al., 1992; Shinomura et al., 1986). We here investigated the effect of Con A immobilized on carrier beads, expecting to reduce the toxicity and stimulate continuous growth of CTL without tumor-antigens.

Materials and methods

Reagents and culture media

Con A was purchased from Seikagaku Kogyo, Inc. (Tokyo, Japan). Alginate, 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride), and glycine ethyl ester hydrochloride were obtained from Aldrich Co. (Milwaukee, WI, U.S.A.). Na¹²⁵I was from ICN Biomedicals (Irvine, CA, U.S.A.). Interleukin (IL) -1, -2, -4 and -6 were kindly provided by Otsuka Pharmaceuticals, Inc. (Tokushima, Japan), Takeda Chemical Industries, Inc. (Osaka, Japan), Ono Pharmaceuticals, Co. Ltd. (Tokyo, Japan) and Ajinomoto Co. Ltd. (Kawasaki, Japan), respectively. Tumor cell lines were established in RIKEN Cell Bank. They were routinely maintained in Dulbecco's modified MEM containing 10% fetal calf serum. RHAM α for generation and expansion of CTL was prepared as described (Liu et al., 1995b).

Preparation of radiolabelled Con A

¹²⁵I-labelled Con A was prepared by incubating the Con A solution (100 ml, 1.0 mg ml⁻¹) with Na¹²⁵I (1 mCi) in 0.5 M K₂PO₄ (pH 7.5) containing Chloramine-T (0.4 mg ml⁻¹) at room temperature for 10 min under stirring, then a sodium thiosulfate solution (2 mg ml⁻¹, PBS, pH 7.2) was added to stop the reaction. A Dowex resin-packed column in which the non-specific protein binding sites were previously blocked with the bovine serum albumin solution (3%; PBS, pH 7.2) was employed to purify the radiolabelled Con A (Liu et al., 1992).

Preparation and determination of immobilized Con A

Crosslinked alginate beads were prepared by incubating 0.5 g water-soluble sodium alginate beads (50–100 μ m in diameter) in 40 ml BDDE-containing basic dimethylformamide (DMF) aqueous solution (mixing 1 part of 0.1 N NaOH with 3 parts of DMF, adding 2% (w/v) of BDDE) under constant shaking for 12 h (Fig. 1). The beads were washed, dried, then activated with tresyl chloride in pre-dried acetone (Liu et al., 1996). Con A was coupled onto the activated beads by incubating overnight the beads (2 g) in 10 ml of phosphate-buffered solution (PBS, pH 8.0) of Con A (1.0 mg ml⁻¹) at room temperature. The unreacted sulfonyl groups on the beads were quenched by replacing the reaction solution with glycine ethyl ester hydrochloride-PBS solution (100 mM, in 0.5 M PBS, pH 8.0) and incubating at room temperature for another 1 h, then washed with a large volume of acetate buffer (0.02 M, pH 3.5), or phosphate buffer (0.01 M, pH 5.6 or pH 8.0) until free Con A became undetectable in the washing solution. The completion of the coupling reaction was confirmed by sulfur elemental analysis of the reacted beads. To determine the amount of Con A bound on the beads, radiolabelled [¹²⁵I]-Con A was added as a tracer. The immobilized amount of Con A was then calculated based on the measurement of radioactivity. After washing as above, the beads were sterilized with 70% ethanol, washed thoroughly with PBS, resuspended in cell culture medium and kept in a refrigerator until use. No leakage of the [¹²⁵I]-Con A from the beads was found so far we have observed the beads incubated in the culture medium at 37 °C in a CO₂ incubator for 5 weeks.

Figure 1. Diagram of preparation of Con A-immobilized alginate beads.

Generation of CTL

CTL were generated as described (Liu et al., 1995b) by the co-culture of a duodenum papilloma cell line TGBC18TKB, of which human leukocyte antigen (HLA)-type was A2, A11, and HLA-type matched peripheral blood mononuclear cells (PBMC) derived from a healthy donor. Briefly, TGBC18TKB cells in an appropriate amount of culture medium was placed at 1×10^5 cells/cm² in the 6-well culture plate and incubated overnight. They were irradiated with 30 Gy of X-ray and washed twice with PBS before the addition of PBMC. Heparinized blood was collected from the healthy donor under informed consents. PBMC was prepared by the common method with Ficoll-Paque. PBMC were suspended in RHAM α medium supplemented with 5% autologous plasma, IL-1, -2, -4 and -6 (all 67 U ml⁻¹) at a concentration of 1×10^6 cells ml⁻¹ (Liu et al., 1995b). Five ml of the PBMC suspension was added to the well with X-ray-irradiated TGBC18TKB cells. After the initial induction culture (14 days) and followed weekly stimulation with TGBC18TKB for 3 weeks, tumor-specific CTL were generated. They were then routinely maintained in the RHAM α medium without autologous plasma and the four interleukins but with 0.5% human serum albumin and IL-2 (67 U ml⁻¹).

Comparison of soluble and immobilized Con A on CTL growth

A definite amount of soluble (50 μ g) and immobilized Con A (25 μ g) in 100 μ l CTL culture medium was

added in the 1st row of the 96-well plate. Fresh CTL culture medium was also dispensed in 2nd-12th row. Another 100 μ l medium was added with a 8-channel pipetteman into the 1st row, mixed by repeatedly pipetting and removed 100 μ l into the 2nd row. Repeatedly mixing and removing were also made on the 2nd-11th row in the order. The 12th row was used as control.

CTL were resuspended at a concentration of 5×10^5 cells ml⁻¹ in fresh RHAM α medium containing IL-2 (67 U ml⁻¹), and placed 100 μ l into the 96-well that contained the diluted Con A solution. They were cultured for 48 h. CTL numbers were then determined with WST-1 assay (Liu et al., 1995b). The cell number in the presence of IL-2 without any addition of Con A was used as a control and defined as 100% to calculate the relative cell growth in the presence of soluble or immobilized Con A by dividing the sample cell number with the control cell number. The data were represented as the average and standard error (n=8).

Long-term culture of CTL

CTL were placed in the 6-well culture plate at a concentration of 5×10^5 cells ml⁻¹ in RHAM α supplemented with 0.5% human serum albumin. Con A-immobilized beads (Con A concentration, 2 μ g ml⁻¹) or naked control beads and IL-2 (67 U ml⁻¹) were added to the medium. CTL were then incubated at 37 °C in a 5% CO₂ humidified incubator. Half of the culture medium was changed every other day. Number of cells was counted and adjusted to 5×10^5 cells ml⁻¹ if cell population density became higher than 1×10^6 cells ml⁻¹.

Table 1. Amount of Con A bound on the alginate beads

Washing buffer	Amount of Con A (mg/g)
Acetate buffer (0.02 m, pH 3.5)	12.1
Phosphate buffer (0.01 m, pH 5.6)	23.9
Phosphate buffer (0.01 m, pH 8.0)	24.4

Killing assay of CTL

Killing activity of CTL was determined by the crystal violet-staining method after the coculture with the target tumor cells for 24 or 48 h as previously described (Liu et al., 1995b) except the definition of the percentage of surviving target tumor cells, since the control target cells cultured without CTL grew considerably for the coculture period. To exclude the possibility of simple growth inhibition of tumor cells by CTL, we separately measured OD560 of the well of target cells at the start of the killing assay and taken this value as the denominator in the calculation of the percentage of surviving target cells after the coculture of CTL and the target cells. Thus the percentage of surviving target cells of the control tumor cells exceeds 100%. We defined that, when the surviving target cells cocultured with CTL was less than 100%, killing, but not growth inhibition, of the target tumor cells have happened.

FACSscan analyses of adhesion molecules

CTL were stained with mouse anti-human leukocyte surface protein (CD2, CD4, CD8, CD44, CD45, CD45RO, CD45RA and LFA-1) antibodies (IgG family) and further stained with FITC-labeled goat anti-mouse IgG antibody (Becton Dickinson, Co.) by incubating at 4 °C for 30 min in PBS. The concentration of cells and antibodies was adjusted as described in the manufacturer's manual. After the staining, CTL were analyzed by a FACSscan (Becton Dickinson, Co.). The results were represented as the relative fluorescent intensity.

Results

Immobilization of Con A

After the immobilization reaction of Con A (Fig. 1) and subsequent wash, no sulfur was detected in the Con A preparation by elemental analysis, indicating that no

Figure 2a.

Figure 2b.

Figure 2. Characterization of the tumor-specific CD4⁺ CTL. (a) Specific cytotoxicity in a 24 h assay before the culture in the presence of immobilized Con A. Note that, to exclude possibility of simple growth inhibition of tumor cells by CTL, we have separately measured OD560 of the well of target cells at the start of the killing assay and taken this value as the denominator in the calculation of the percentage of surviving target cells after killing by the target cells. (b) Effects of the supernatant of CD4⁺ CTL, exogenously added IFN- γ and TNF- α on growth of TGBC18TKB cells. Cells (1×10^4 /well) were placed in a 96-well plate with 100 μ l of medium and incubated for 48 h.

active tresyl groups remained on the beads. As shown in Table 1, the amount of bound Con A was dependent on pH. Con A bound on the microsphere after washing with the acetate buffer (pH 3.5) was approximately half

Figure 3a.

Figure 3. Effects of immobilized and soluble Con A on growth of the CTL. (a) Dose-dependence of CTL growth on immobilized and soluble Con A in the 48 h incubation. (b) Photograph of CTL on the Con A-immobilized beads (see next page). CTL culture was performed as described in **Materials and methods**.

of that washed with phosphate buffers at pH 5.6 or 8.0. Therefore, in the present study, we adopted washing at pH 8.0 for the preparation of the immobilized Con A.

Characterization of the CTL

Fig. 2a shows specific cytotoxicity of the CTL in the killing assay for 24 h. In the present experiments, the generated CTL were CD4⁺ CD8⁻ and their HLA-type was A2 as revealed by FACSscan analysis (data not shown). The CTL killed half of seeded TGBC18TKB cells (HLA-type is A2,11) at an effector/target (E/T) ratio of 10. However, they did not affect the other 3 cell lines at this E/T ratio, i.e., HLA-type matched brain tumor TKB-1 (HLA A2), lung squamous carcinoma SQ-5 (HLA A2), and HLA-type mismatched gastric adenocarcinoma GT3TKB (HLA A2402).

To examine whether the target cell killing was caused by direct action of IFN- γ or TNF- α possibly secreted by the CD4⁺ CTL, we added exogenous IFN- γ and TNF- α to the culture supernatant of the CTL. However, none of these killed TGBC18TKB cells after 48 h incubation, although IFN- γ at more than 20 U ml⁻¹ reduced the relative growth of the target tumor cells (Fig. 2b).

Effects of immobilized- and soluble-Con A on CTL growth

Fig. 3a shows growth of the CD4⁺ CTL in the presence of soluble and immobilized Con A. Soluble Con A inhibited CTL growth at 0.1 $\mu\text{g ml}^{-1}$ or more, while immobilized Con A increased CTL growth slightly in the range of 0.025–2 $\mu\text{g ml}^{-1}$. Although maximum cell growth were observed at around 0.05 $\mu\text{g ml}^{-1}$, no enhancement of cell growth could be obtained at the lower concentration as compared with the control. The total amount of Con A in the beads was used to calculate the concentration of immobilized Con A. So, the effective Con A to live cells was limited only to those on the surface of beads. This must be one of the reasons why CTL kept their growth potential at the higher concentration of immobilized Con A.

Microscopic observation showed the previously documented effect (Reeke et al., 1974) of soluble Con A on cell aggregation (data not shown) and attachment of the CTL on Con A-immobilized beads (Fig. 3b). This attachment on the immobilized-Con A beads was observed during the whole culture period of CTL. This is different from the general situation of CTL growth in the absence of antigen stimulation where the CTL grew as a single cell suspension after several subcultures. The results showed that CTL bound by immobilized Con A could maintain their growth.

Long-term culture of CTL

To confirm whether CTL can keep growing without antigen stimulation in the presence of immobilized Con A, long-term culture of CTL was applied. From Fig. 3, the concentration (2 $\mu\text{g ml}^{-1}$) of immobilized Con A was chosen for the observation of long-term effect on CTL growth. A typical result is shown in Fig. 4. When the CD4⁺ CTL were cultured without tumor antigen stimulation in the presence of immobilized Con A or naked beads as the control under the serum-free condition (RHAM α supplemented with 0.5% human serum albumin) for 45 days, the CTL grew continuously in the former, but, in the latter, the growth rate decreased gradually after 3 weeks and growth ceased after 34 days (Fig. 4).

Specific cytotoxicity of the CTL was retained against the target TGBC18TKB cells after long-term culture with the immobilized Con A (Fig. 5). They did not kill the other 3 malignant tumor cell lines, TKB-1, SQ-5, and GT3TKB, and additionally tested renal carcinoma cell lines, OS-RC-2 (HLA-type was A9),

Figure 3b.

Figure 4. Growth of the tumor-specific CD4⁺ CTL without antigen stimulation in the presence or absence of immobilized Con A. CTL were incubated in the serum-free RHAM α medium at 37 °C in a 5% CO₂ humidified incubator in the presence (2 μ g ml⁻¹) or absence of immobilized Con A. The medium was changed every other day.

Hpt.10 (A2,2402), and TUHR4TKB (A2402,25). As the CTL killed almost all the target TGBC18TKB cells at the E/T of 5, the cytotoxic activity against the target

Figure 5. Specific cytotoxicity of the CTL cultured long-term with the Con A-immobilized beads.

cells became more potent than those before the long term culture (Figs. 2a and 5).

Adhesion molecules of CTL

Expression of adhesion molecules on the surface of the CTL were analysed by flow cytometry after long term culture (Table 2). So far as observed on CD2, CD11b, CD44 and CD45 which have been considered essential for the initial recognition and lytic process of target cells (Norris, 1990), CD11b on the CTL cul-

Table 2. Expression of adhesion molecules on CTL

Surface markers	Expression of adhesion molecules on CTL		
	With Con A-beads	Without Con A-beads	Ratio of expression (+/- Con A beads)
CD2	125.0	109.4	114.2
CD45	325.4	285.4	114.0
CD44	587.2	503.5	116.6
CD11b	57.1	34.8	163.9
CD45RO	107.3	115.2	93.1
CD45RA	93.7	103.3	90.7

* Data represent the average of fluorescent intensity of cells measured by flow cytometry. Cells were labeled with FITC-labelled monoclonal antibodies at 4 °C for 30 min, washed twice with the phosphate-buffered saline (PBS) containing 4% fetal bovine serum (FBS), then resuspended in 0.5 ml of PBS containing 4% FBS for the measurement.

tured on Con A-immobilized beads was up-regulated to 164% of the control CTL that have been cultured on naked beads. Levels of CD2, CD44 and CD45 increased slightly, while a decrease in CD45RA and CD45RO was observed.

Discussion

In the present study, the immobilized Con A was used to substitute the unknown tumor antigenic molecules for the stimulation of CTL growth. Although tumor specific CTL died gradually without antigen stimulation (Crowley et al., 1991; Liu et al., 1995b; Slingluff et al., 1994; Tjoa et al., 1994; Wolfel et al., 1993; our Fig. 4 after 34 days), they grew continuously without specific antigen stimulation in the presence of the immobilized Con A. In Fig. 4, due to the lasting effect of the tumor antigen stimulation that was done during the daily maintenance, CTL kept growing in the initial culture for 34 days in the control. In the separate culture, recovery of rapid growth was observed once the CTL on the 45th day or later were stimulated with the tumor antigen (data not shown). Therefore, although immobilized Con A promoted CTL growth to some extent even in the absence of antigens, Con A was the less potent stimulator for CTL growth compared to tumor antigens.

However, by stimulating with the immobilized Con A, the CTL were accompanied with higher expression of the adhesion molecule, CD11b, (Table 2) which has been known to be expressed on the activated T lymphocytes (van Nessel et al., 1988). Con A resulted in

a stronger stimulation of RNA synthesis than PHA in the serum-free culture medium although PHA stimulated RNA synthesis more potently in the plasma-supplemented medium (Powell and Leon, 1970). In the study, immobilized Con A up-regulated the CD11b expression – probably the results of the CD11b RNA synthesis.

Since the present culture medium, RHAM α , was developed for serum-free culture of LAK cells (Kawai et al., 1992), we could maintain the CTL under the serum-free condition. This enabled us to avoid the complicated quality control of human plasma for possible clinical application of the CTL.

Soluble Con A inhibited CTL growth (Fig. 3a), probably due to the formation of tight aggregation of the cells through Con A-tetramer that crosslinks CTL surface mannoside residues and restricts membrane mobility of cells (Yahara et al., 1972). Binding of excess amount of Con A on CTL ($>1 \times 10^6$ sites per cell) (Novogrodsky et al., 1971) induces the dense cluster formation, hence may have induced the growth inhibition by the lack of nutrients. The phenomenon was also observed in conventional static culture of CTL in which CTL formed large clusters. When CTL have not been dispersed by pipetting for 2 days, the CTL in the center of the cluster died as revealed by trypan blue staining (Liu, unpublished data).

On the other hand, dimeric Con A is able to maintain its function with reduced toxic side effects (Gunter et al., 1973). Modification of Con A by chemical reaction to inhibit the tetramer formation showed an enhanced thymidine incorporation into the cells at a significantly higher concentration (upto $200 \mu\text{g ml}^{-1}$) than the toxic concentration of the soluble Con A.

The immobilized Con A did not induce death of the CTL so far examined at a high concentration where the growth inhibition was observed (Fig. 3a). This will be ascribed to the ability of the immobilized Con A not to crosslink CTL each other but on the surface of the carrier beads (Fig. 3b). Since the immobilized Con A will not be internalized into the cells, it will continuously stimulate the CTL growth without antigen, although the stimulation will be localized on the carrier surface.

Since gentle pipetting of the CTL-carrier beads suspension causes detachment of the CTL from the carrier, recycled use of the immobilized Con A will be possible. This system makes CTL culture easier in the scale-up process because CTL detached from the beads will attach again on the newly added beads and will keep growing. Harvesting of the cells is possible by filtration (pore size of approximately $30 \mu\text{m}$).

The present results, shown in Figs. 2a and 2b, suggest that CTL were specific to the target and did not kill by excreting IFN- γ and/or TNF- α . Unexpectedly, their killing activity was enhanced after the long-term serum-free culture with the immobilized Con A beads (Fig. 5 compared to Fig. 2a). We consider this enhancement should at least partly be ascribed to the enhanced expression of the surface molecule CD11b, an adhesion molecule. The higher expression should keep the contact of CTL and tumor cells stronger. The contact has been suggested as the essential step for the specific lysis (Norris, 1990), although further investigation should be made for the precise mechanistic explanation of Con A – stimulated enhancement of lasting specific CTL cytotoxicity. This phenomenon will yet be helpful in the expected adoptive immunotherapy for human tumors with CTL where only a tiny tumor antigen is available for the generation and maintenance of CTL specificity and cytotoxicity.

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References

- Abo T and Balch CM (1982) Characterization of HNK-1+ (Leu-7) human lymphocytes. II. Distinguishing phenotypic and functional properties of natural killer cells from activated NK-like cells. *J Immunol* 129: 1758–1761.
- Baker PE, Gillis S and Smith KA (1979) Monoclonal cytolytic T-cell lines. *J Exp Med* 149: 273–278.
- Bakker ABH, Schreurs MWJ, Tafazzul G, De Boer AJ, Kawakami Y, Adema GJ and Figdor CG (1995) Identification of a novel peptide derived from the melanocyte-specific gp100 antigen as the dominant epitope recognized by an HLA-2.1 restricted anti-melanoma CTL line. *Int J Cancer* 62: 97–102.
- Crowley NJ, Darrow TL, Quinn-Allen MA and Seigler HF (1991) MHC-restricted recognition of autologous melanoma by tumor-specific cytotoxic T cells – evidence for restriction by a dominant HLA-A allele. *J Immunol* 146: 1692–1699.
- Fisk B, Blevins TL, Wharton JT and Ioannides CG (1995) Identification of an immunodominant peptide of HER2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. *J Exp Med* 181: 2109–2117.
- Freedman RS, Tomasovic B, Templin S, Atkinson EN, Kudelka A, Edwards CL and Platsoucas CD (1994) Large-scale expansion in interleukin-2 of tumor-infiltrating lymphocytes from patients with ovarian carcinoma for adoptive immunotherapy. *J Immunol Methods* 167: 145–160.
- Goedgebuure PS, Douville LM, Li H, Richmond GC, Schoof DD, Scavane M and Eberlein TJ (1995) Adoptive immunotherapy with tumor-infiltrating lymphocytes and interleukin-2 in patients with metastatic malignant melanoma and renal cell carcinoma: a pilot study. *J Clin Oncol* 13: 1939–1949.
- Groux H, Monte D, Plouvier B, Capron A and Ameisen JC (1993) CD3-mediated apoptosis of human medullary thymocytes and activated peripheral T cells: respective roles of interleukin-1, interleukin-2, interferon- γ and accessory cells. *Eur J Immunol* 23: 1623–1629.
- Gunther GR, Wang JL, Yahara I, Cunningham BA and Edelman GM (1973) Concanavalin A derivatives with altered biological activities. *Proc Natl Acad Sci U.S.A.* 70: 1012–1016.
- Hay RJ, Park JG and Gazdar A (eds.) (1994) Atlas of human tumor cell lines, Academic Press, Inc. (C.A. U.S.A.).
- Ioannides CG and Whiteside T (1993) T cell recognition of human tumors: implications for molecular immunotherapy of cancer. *Clin Immunol Immunopath* 66: 91–106.
- Kawai K, Sasaki T, Saijo-Kurita K, Akaza H, Koiso K and Ohno T (1992) Additive effects of antitumor drugs and lymphokine-activated killer cell cytotoxic activity in tumor cell killing determined by lactate-dehydrogenase-release assay. *Cancer Immunol Immunother* 35: 225–229.
- Kawakami Y, Eliyahu S, Delgado CH, Robbins PF, Sakaguchi K, Appella E, Yannelli JR, Adema GJ, Miki T and Rosenberg SA (1994a) Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with *in vivo* tumor rejection. *Proc Natl Acad Sci USA* 91: 6458–6462.
- Kawakami Y, Eliyahu S, Sakaguchi K, Robbins PF, Rivoltini L, Yannelli JR, Appella E and Rosenberg SA (1994b) Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. *J Exp Med* 180: 347–352.
- Lau LL, Jamieson BD, Somasundaram T and Ahmed R (1994) Cytotoxic T-cell memory without antigen. *Nature* 369: 648–652.
- Liu SQ, Ito Y and Imanishi Y (1992) Cell growth on immobilized cell-growth factors; 4. Interactions of fibroblast cells with insulin immobilized on poly(methyl methacrylate) membrane. *J Biochem Biophys Methods* 25: 139–148.
- Liu B, Farrington SM, Petersen GM, Hamilton SR, Parsons R, Papadopoulos N, Fujiwara T, Jen J, Kinzler KW, Wyllie AH, Vogelstein B and Dunlop MG (1995a) Genetic instability occurs in the majority of young patients with colorectal cancer. *Nature medicine* 1: 348–352.
- Liu SQ, Saijo K, Todoroki K and Ohno T (1995b) Induction of human autologous cytotoxic T lymphocytes on formalin-fixed and paraffin-embedded tumour sections. *Nature medicine* 1: 267–271.
- Liu LS, Liu SQ, Ng SY, Froix M, Ohno T and Heller J (1997) Controlled release of interleukin-2 for tumour immunotherapy using alginate/chitosan porous microspheres. *J Control Release* 43: 65–74.
- Mazumder A, Grimm EA and Rosenberg SA (1983) Characterization of the lysis of fresh human solid tumors by autologous lymphocytes activated *in vitro* with phytohemagglutinin. *J Immunol* 130: 958–964.
- Mazumder A, Grimm EA, Zhang HZ and Rosenberg SA (1982) Lysis of fresh human solid tumors by autologous lymphocytes activated *in vitro* with lectins. *Cancer Res* 42: 913–918.
- Moudgil KD and Sercarz EE (1994) Can antitumor responses discriminate between self and nonself. *Immunol Today* 15: 353–355.
- Mukherji B, Guha A, Chakraborty NG, Sivanandham M, Nashed AL, Sporn JR and Ergin MT (1989) Clonal analysis of cytotoxic and regulatory T cell responses against human melanoma. *J Exp Med* 169: 1961–1976.

- Norris DA (1990) Cytokine modulation of adhesion molecules in the regulation of immunologic cytotoxicity of epidermal targets. *J Invest Derm* 95: 111S–120S.
- Novogrodsky A and Katchalski (1971) Lymphocyte transformation induced by concanavalin A and its reversion by methyl- α -D-mannopyranoside. *Biochim Biophys Acta* 228: 579–583.
- Powell AE and Leon MA (1970) Reverse interaction of human lymphocytes with the mitogen concanavalin A. *Exp Cell Res* 62: 315–325.
- Quentmeier H, Klaucke J, Muhlradt PF and Drexler HG (1992) Role of IL-6, IL-2 and IL-4 in the in vitro induction of cytotoxic T cells. *J Immunol* 149: 3316–3320.
- Reeke GN Jr, Becker JW, Cunningham BA, Gunther GR, Wang JL and Edelman GM (1974) Relationshs between the structure and activities of concanavalin A. *Ann NY Acad Sci* 234: 369–382.
- Shinomura N, Bluestone JA and Sachs DH (1986) Cloned cytotoxic T lymphocytes that recognized an I-A region product in the context of a class I antigen. *J Exp Med* 163: 972–980.
- Slingluff CL, Cox AL, Stover JMJ, Moore MM, Hunt DF and Engelhard AH (1994) Cytotoxic T-lymphocyte response to autologous human squamous cell cancer of the lung: epitope reconstitution with peptides extracted from HLA-Aw68. *Cancer Res* 54: 2731–2737.
- Takayama T, Makuuchi M, Sekine T, Terui S, Shiraiwa H, Kosuge T, Yamazaki S, Hasegawa H, Suzuki K, Tamagata M, Fujii M, Tanaka T and Kakizoe T (1991) Distribution and therapeutic effect of intraarterially transferred tumor-infiltrating lymphocytes in hepatic malignancies. *Cancer* 68: 2391–2396.
- Tjoa BA and Kranz DM (1994) Generation of cytotoxic T-lymphocytes to a self-peptide/class I complex: a model for peptide-mediated tumor rejection. *Cancer Res* 54: 204–208.
- ven Nossel C, Miedema F, Brouwer M, de Rie MA, Aarden LA and van Lier RAW (1988) Regulatory properties of LFA-1a and b chain in human T-lymphocyte activation. *Nature* 333: 850–852.
- Wolfel T, Herr W, Coulie P, Schmitt U, Buschenfelde KHM and Knuth A (1993) Lysis of human pancreatic adenocarcinoma cells by autologous HLA-class I-restricted cytolytic T-lymphocyte (CTL) clones. *Int J Cancer* 54: 636–644.
- Wolfel T, Van Pel A, Brichard V, Schneider J, Seliger B, Meyer zum Buschenfelde KH and Boon T (1994) Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes. *Eur J Immunol* 24: 759–764.
- Yahara I and Edelman GM (1972) Restriction of the mobility of lymphocyte immunoglobulin receptors by concanavalin A. *Proc Natl Acad Sci USA* 69: 608–612.

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