Depletion of T_{reg} cells inhibits minimal residual disease after surgery of HPV16-associated tumours

JANA SÍMOVÁ, JAN BUBENÍK, JANA BIEBLOVÁ, RODNEY ALEXANDER ROSALIA, JAN FRIC and MILAN REINIS

Institute of Molecular Genetics Academy of Sciences of the Czech Republic, Flemingovo nám. 2, CZ-166 37 Prague 6, Czech Republic

Received August 11, 2006; Accepted September 18, 2006

Abstract. It is generally accepted that T regulatory cells (T_{reg} $CD4^+CD25^+Foxp3^+$) play an important role in the suppression of tumour immunity. We examined the impact of T_{reg} cell depletion with anti-CD25 antibody as adjuvant therapy in the treatment of minimal residual disease after excision of murine HPV16-associated tumours. We found that the depletion of T_{reg} cells inhibited growth of the recurrences after surgery of HPV16-associated MHC class I^+ as well as MHC class I-deficient tumours transplanted in syngeneic mice. These results demonstrate that depletion of $CD25^+CD4^+$ T_{reg} cells can be used as an efficient adjuvant treatment improving the results of surgery in the experimental systems mimicking human MHC class I^+ and MHC class I-deficient, HPV16-associated neoplasms. Therefore, this therapeutic modality is worth being examined in patients with minimal residual HPV16-associated tumour disease after surgery.

Introduction

Local recurrence of tumours at the site of tumour resection represent a major problem in oncology. It has been found in experimental systems that cytokine-based immunotherapy and gene therapy performed after tumour resection can substantially improve the therapeutic results (1,2) (reviewed in ref. 3).

Regulatory $CD4^+CD25^+$ T cells (T_{reg} cells) play an important role in maintaining immunological homeostasis to both self and foreign antigens and control a wide spectrum of immune responses. The activity of T_{reg} cells is not always beneficial for the hosts; the T_{reg} cells may be important for the suppression of T cell-mediated tumour immunity and thus, effective immunotherapeutic strategies should consider elimination of their effects (reviewed in ref. 4).

In various murine models, it has indeed been demonstrated that the depletion of $CD25^+$ T_{reg} cells may result in the inhibition of tumour growth (5-10). *In vitro*, the regulatory T cells could inhibit the antitumour activity of tumour-specific $CD8^+$ cytotoxic T lymphocytes as well as the activity of $CD4^+$ T cells and NK cells (reviewed in ref. 11). Thus, these data suggest that the T_{reg} cells can inhibit both the MHC class I-restricted and -unrestricted immune responses. This is of particular importance, since downregulation of the MHC class I expression is a frequent mechanism of tumour escape from the immune response (12) in the course of tumour development and progression. In preclinical models, experimental immunotherapy protocols of the MHC class I-deficient HPV16-associated tumours have been investigated and inhibition of the tumour growth was achieved with tumour vaccines, cytokine therapy and CpG oligodeoxynucleotide treatment (reviewed in refs. 13-15). However, so far the information on the efficacy of the *in vivo* treatment of MHC class I-deficient tumours by depletion of $CD25^+$ cells with specific monoclonal antibodies (mAb) in this experimental system has been lacking. To study this problem, we used a model mimicking human tumours associated with human papilloma virus type 16 (HPV16) (16), murine tumours expressing HPV16 E6/E7 oncoproteins and differing in the expression of MHC class I molecules, the MHC class I-positive tumour cell line TC-1 (17) and its MHC class I-deficient subline TC-1/A9 (18) and examined the possibility to enhance the anti-tumour immune responses by $CD25^+$ cell depletion performed at the time of tumour excision.

Materials and methods

Mice. C57BL/6 males, 2-4 months old, were obtained from AnLab Co., Prague, Czech Republic. The mice were housed in the animal facility of the Institute of Molecular Genetics. Experimental protocols were approved by Institutional Animal Care Committee of the Institute of Molecular Genetics, Prague.

Tumour cell lines. The TC-1 tumour cell line was established by transformation of primary C57BL/6 mouse lung cells with

Correspondence to: Professor Jan Bubeník, Institute of Molecular Genetics AS CR, Flemingovo nám. 2, CZ-166 37 Prague 6, Czech Republic
E-mail: bubenik@img.cas.cz

Key words: HPV16, residual tumour disease, T_{reg} cells, MHC class I-deficient tumours

HPV16 E6/E7 and activated Ha-ras DNA (17). The MHC class I-deficient variant of the TC-1 cells, TC-1/A9 (a generous gift from Dr M. Smahel) was derived from TC-1 tumours formed in mice preimmunized repeatedly with HPV16 E7-containing plasmid DNA (18).

Antibodies. In vivo depletion of CD25+ cells was performed using mAb PC61 (19) produced by Exbio, Prague, Czech Republic. Depletion of the CD4+CD25+ cells was monitored in lymph nodes by flow cytometry using monoclonal antibodies, the FITC-labelled anti-mouse CD4 (clone RM4-4) and PE-labelled anti-mouse CD4 (clone GK1.5), the PE-labelled anti-mouse CD25 (PC61) and FITC-labelled anti-mouse CD25 (7D4) recognizing distinct epitopes on the CD25 molecule than PC61 mAb, purchased from Pharmingen, San Diego, CA.

CD25 and Foxp3 mRNA analysis. Total RNA was extracted from 15 to 20x10^6 lymph node cells using Tri reagent (Sigma-Aldrich, St. Louis, MO) and mRNA was transcribed into cDNA using the GeneAmp® RNA PCR kit (Applied Biosystems) and random hexamers as the primers. PCR primers were purchased from Generi Biotech, Czech Republic. Real-time PCR was performed using the IQ™ SYBR Green Supermix (170-8880) as described by the manufacturer and the BioRad iCycler version 2.039. The sequences of specific primers were: *FoxP3* 5'-CCCAGGAAAGACAGCAACCTT-3' and 5'-TCT CACAACCAGGCCACTTG-3' (20); *CD25* 5'-CCAGCAAC TGGGATGACAAA-3' and 5'-GCTCTTTCTGGTGTTCAA GTTGAG-3' (21); *ß-actin* 5'-GATGAGATTGGCATGGC TTT-3' and 5'-CACCTTCACCGTTCCAGTTT-3'. Amplification was performed in a total volume of 25 μl for 35 cycles of 15 sec at 95˚C, 1 min at 60˚C and 2 min at 72˚C. Samples were ran in triplicate, and their relative gene expression were determined by normalizing of the expression of each target to *ß-actin* expression.

In vivo experiments. The TC-1 or TC-1/A9 tumour cells were transplanted subcutaneously into syngeneic mice. CD25+ cell depletion was performed by the administration of 0.3 mg of the PC61 mAb per mouse two days before transplantation of tumour cells, or at the time of the appearance of palpable tumours, or during the surgical removal of the growing tumours. To investigate the effects of CD25+ T cell depletion at the time of tumour excision, mice with growing tumours (8-12 mm in diameter) were randomly distributed to experimental and control groups. In the experimental mice the PC61 antibody was injected i.p. two days before excision and s.c. one day after the excision, in the vicinity of the excised tumours. The experimental and control mice were then observed twice a week, and the number of tumour-bearing mice and size of tumours were recorded.

Statistical analyses. For statistical analyses of differences between the growth of tumours in experimental and control groups, the analysis of variance from NCSS, Number Cruncher Statistical System (Kaysville, UT), statistical package was used. For comparison of tumour takes and recurrences in experimental and control groups, the χ^2 comparison test was utilized.

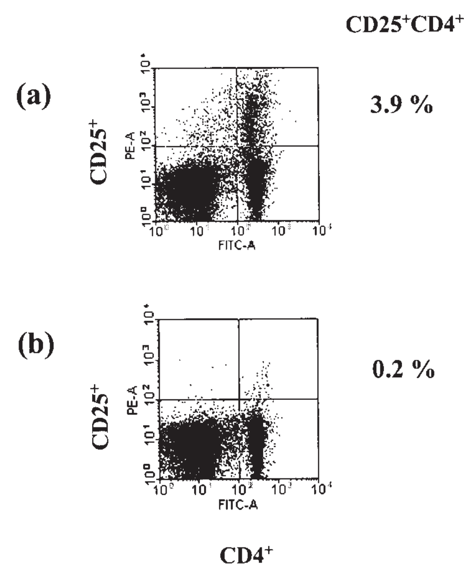


Figure 1. Efficacy of CD4+CD25+ depletion after administration of PC61 mAb: (a) surgically-treated mice, (b) surgically-treated and PC61 mAb-treated mice. Data are representative of all experiments.

Results

Efficacy of in vivo anti-CD25 mAb administration. To determine the efficacy of T_{reg} cell depletion we examined the presence of CD25+ and Foxp3+ cells in regional lymph nodes of experimental mice. The representative data obtained by flow cytometry, confirming the efficacy of CD25+ cell depletion in the experimental groups, are shown in Fig. 1. The percentage of T_{reg} cells in regional lymph nodes decreased after treatment of mice with the PC61 mAb from 3.9 to 0.2%. Similar results were obtained by using anti-CD25 mAB 7D4 (data not shown). Further, we determined the presence of CD25 and Foxp3 mRNA in lymph node cells. The representative data of mRNA expression (lymph node cells pooled from six mice in each group) are shown in Fig. 2. The administration of the anti-CD25 mAb resulted only in the decrease of the expression, but not in the absence of the Foxp3 and CD25 mRNA in both control and tumour-bearing mice.

Effects of anti-CD25 mAb administration on the growth of MHC class I+ (TC-1) and MHC class I-deficient (TC-1/A9) tumours. PC61 mAb was injected i.p. two days before transplantation of the tumour cells. Administration of the anti-CD25 mAb substantially inhibited the growth of the MHC class I+ TC-1 cell line (Fig. 3a) as well as growth of its MHC class I-deficient subline TC-1/A9 (Fig. 3b). We further examined the effect of the PC61 mAb administration on the growth of tumours that were already palpable. When the tumours reached ~1-2 mm in diameter, the mice were randomly distributed to the experimental and control groups. Experimental groups of mice were repeatedly treated i.p. with PC61 antibody. In this scheme of anti-CD25 antibody administration, the growth of neither MHC class I positive nor MHC class I-deficient tumours was inhibited (Fig. 4).

Effects of anti-CD25 mAb administration on the recurrences after surgical removal of the established tumours. TC-1 or

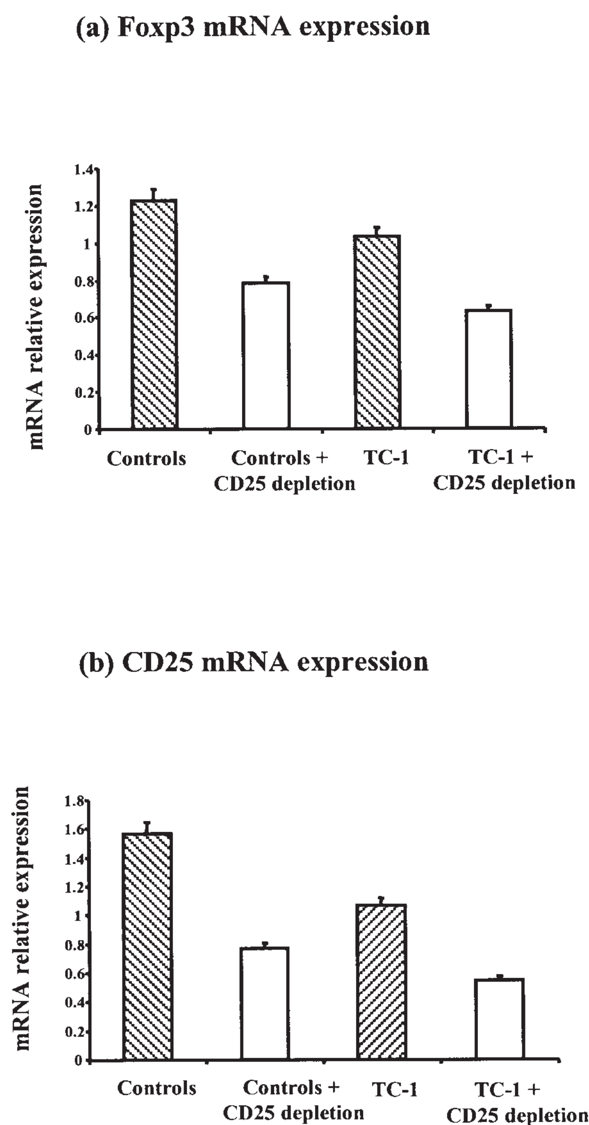


Figure 2. Foxp3 and CD25 mRNA expression in lymph node cells of mice. Control and TC-1 tumour-bearing mice were i.p. injected with PC61 mAb and relative mRNA expression was determined by RT PCR; (a) relative FoxP3 mRNA expression, (b) relative CD25 mRNA expression. Data are representative of all experiments.

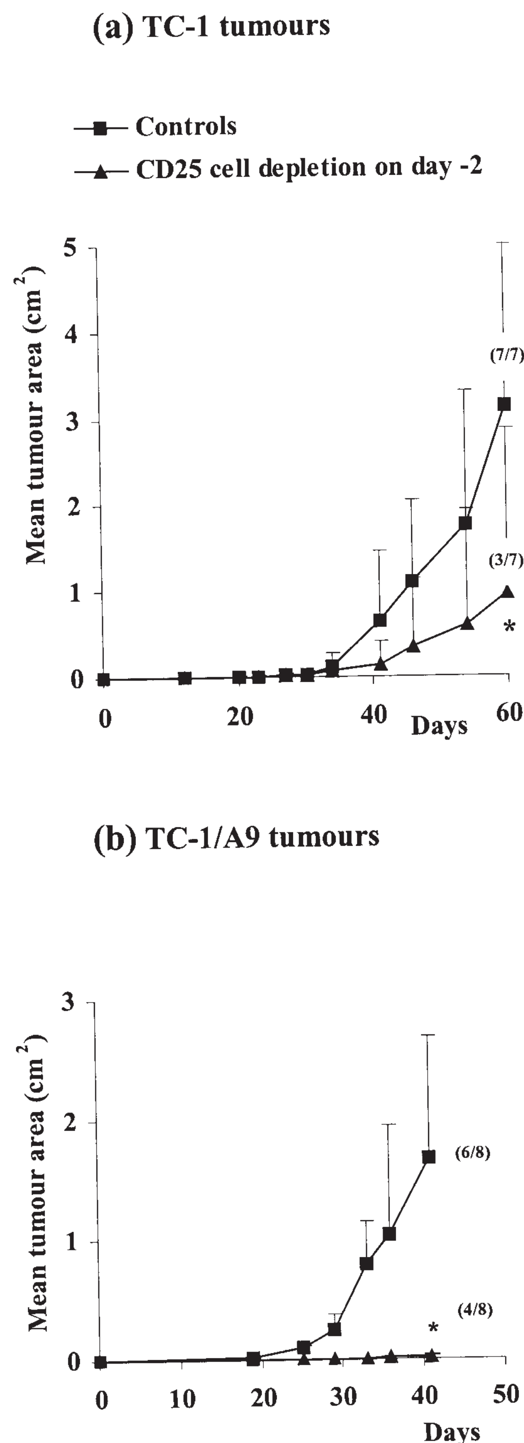
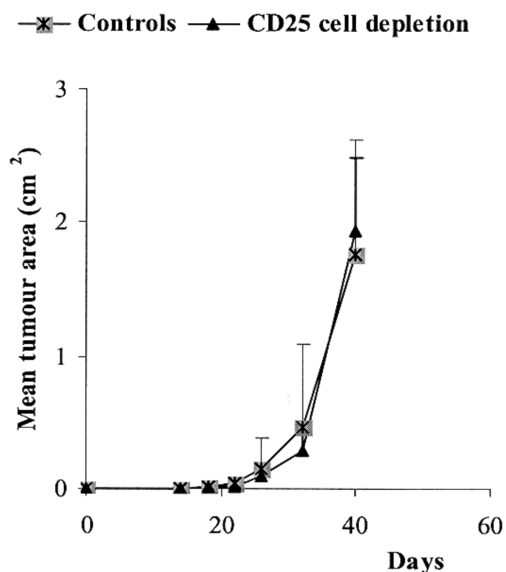


Figure 3. Administration of anti-CD25 mAb inhibits growth of MHC class I positive as well as deficient tumours in syngeneic mice. Mice were challenged s.c. with the respective tumours cells. The experimental mice were treated two days prior to challenge with the PC61 mAbs. (a) TC-1 (MHC I$^+$) tumours, *P<0.05; number of tumour-bearing mice/total number of mice; 7/7 control group vs. 3/7 experimantal group - P<0.025; (b) TC-1/A9 (MHC I$^-$) tumours, *P<0.05.

TC-1/A9 tumour cells were transplanted into syngeneic mice. The mice with growing tumours were randomly distributed into experimental and control groups and the tumours were surgically removed. The CD25$^+$ cell depletion was performed in the experimental groups by injecting PC61 antibody i.p. two days before excision of tumours and by s.c. injection one day after the excision in the vicinity of the excised tumours. As seen in Fig. 5, the recurrence rate of the TC-1 tumours decreased after treatment of mice with the anti-CD25 mAb from 74 to 43% (Fig. 5a) and the growth of both, MHC class I$^+$ (TC-1) and MHC class I deficient (TC-1/A9) tumour recurrences was substantially inhibited (Fig. 5a and b).

Discussion

In the past several years there has been substantial advances in the development of prophylactic HPV16 vaccines. Vaccines based on the HPV virus-like particles and inducing genotype-specific virus-neutralizing antibodies were found to prevent infection from HPV16 and, in controlled clinical trials, to significantly reduce HPV16-associated cervical cancer (22) (reviewed in ref. 23).



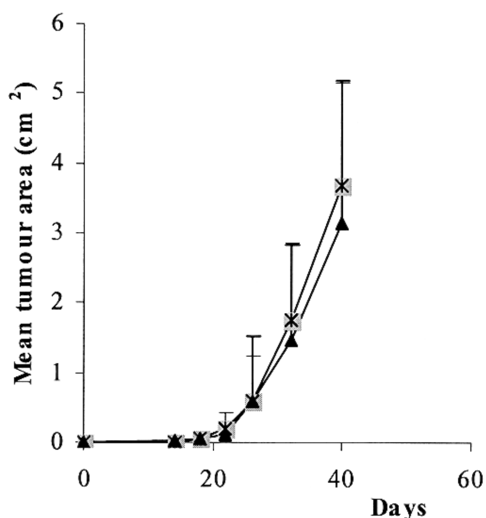
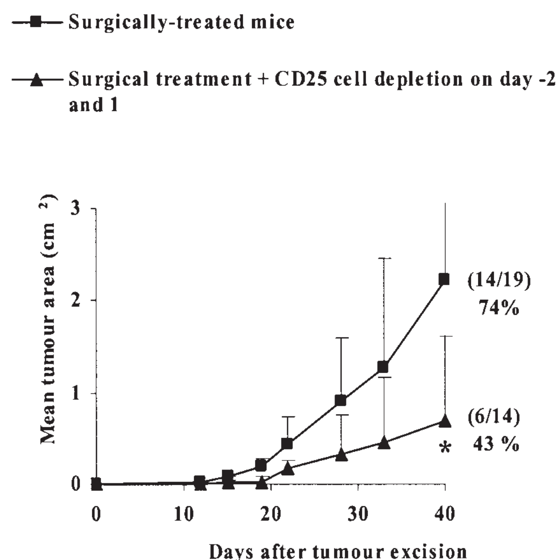


Figure 4. Administration of anti-CD25 mAb does not inhibit growth of palpable tumours. Mice were challenged s.c. with tumour cells. When the tumours reached ~1-2 mm in diameter, mice were randomly distributed to the experimental and control groups. The experimental groups of mice were treated with PC61 antibody i.p. on day 14 and s.c. on day 20. (a) TC-1 (MHC I^+) tumours; (b) TC-1/A9 (MHC I^-) tumours.



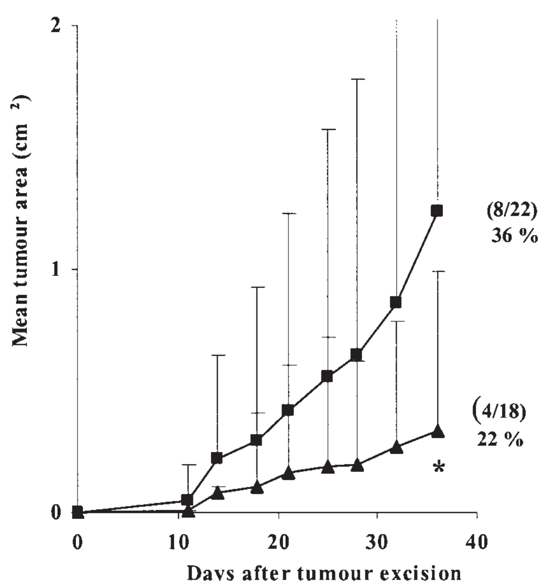


Figure 5. Administration of anti-CD25 mAb inhibits growth of recurrences after surgical removal of tumours. PC61 mAb was injected i.p. two days before excision of the respective tumours and s.c. one day after the excision of tumours. (a) TC-1 (MHC I^+) tumours; (b) TC-1/A9 (MHC I^-) tumours; $^*P<0.05$

Also, therapeutic vaccines were examined both in the animal models (reviewed in ref. 16) and in clinical trials. In the experimental models, substantial therapeutic and anti-metastatic effects of cytokine and dendritic cell-based vaccines were observed (2,24-26) (reviewed in ref. 3). However, the clinical results of therapeutic vaccination have been disappointing, particularly due to the fact that the immunotherapy was carried out in patients with late-stage disease, who were already partially immune compromised by radiotherapy and/or chemotherapy. Therefore, the development of efficient immunotherapy strategies for clinical purposes is needed.

It is generally accepted that the immunotherapy trials in patients with minimal residual disease after surgery are more likely to be successful than the immunotherapy of the late-stage disease patients. Therefore, we focused our attention on preventing the cancer-associated immunosuppression accompanying the progression of tumour recurrence after

surgery of HPV16-associated tumours. We performed *in vivo* depletion of immunosuppressive T_{reg} cells by the administration of anti-CD25 mAb PC61 that resulted in the depletion of cells expressing membrane $CD4^+CD25^+$ markers but only the decrease of Foxp3 and CD25 mRNA expression. This is in accordance with recent findings of Kohm *et al* (27) that the administration of anti-CD25 monoclonal antibody results in only the functional inactivation, but not physical depletion of T_{reg} cells. Nevertheless, our *in vivo* experiments demonstrated that the administration of anti-CD25 mAb was sufficient for efficient adjuvant antitumour therapy. Since the $CD25^+$ T_{reg} cells can inhibit not only the effects of specific $CD8^+$ cytotoxic T lymphocytes but also the tumour cell-directed nonspecific NK cell functions (28), we examined the anti-suppressoric treatment in parallel in a doublet of HPV16-associated tumour model systems, the MHC class I^+ conventional TC-1 (17) and in its MHC class I-deficient TC-1/A9 counterpart (18). We found that the depletion of the T_{reg} cells reduced the percentage of tumour recurrence after surgery and inhibited the growth of the recurrences in the T_{reg} cell-depleted mice carrying both, the MHC class I^+ and MHC class-deficient tumours. Since the anti-immunosuppressive treatment was found to be an efficient adjuvant therapy improving the results of surgery in our preclinical models mimicking human HPV16-associated tumours, it is worthwhile applying T_{reg} cell depletion also in patients with HPV16-associated neoplasms.

Acknowledgements

This work was supported by grant No. 301/04/0492 from the Grant Agency of the Czech Republic; No. AV0Z50520514 from the Grant Agency of the Academy of Sciences of the Czech Republic; by the League Against Cancer, Prague; and by the EU-FP6-NoE Clinigene Project No. 018933.

References

1. Vlk V, Rossner P, Indrova M, Bubenik J and Sobota V: Interleukin-2 gene therapy of surgical minimal residual tumour disease. Int J Cancer 76: 115-119, 1998.
2. Mikyskova R, Indrova M, Simova J, Jandlova T, Bieblova J, Jinoch P, Bubenik J and Vonka V: Treatment of minimal residual disease after surgery or chemotherapy in mice carrying HPV16-associated tumours: Cytokine and gene therapy with IL-2 and GM-CSF. Int J Oncol 24: 161-167, 2004.
3. Bubenik J and Simova J: Minimal residual disease as the target for immunotherapy and gene therapy of cancer (Review). Oncol Rep 14: 1377-1380, 2005.
4. Klein G and Klein E: Surveillance against tumors - is it mainly immunological? Immunol Lett 100: 29-33, 2005.
5. Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T and Nakayama E: Tumor rejection by *in vivo* administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. Cancer Res 59: 3128-3133, 1999.
6. Shimizu J, Yamazaki S and Sakaguchi S: Induction of tumor immunity by removing $CD25^+CD4^+$ T cells: a common basis between tumor immunity and autoimmunity. J Immunol 163: 5211-5218, 1999.
7. Tawara I, Take Y, Uenaka A, Noguchi Y and Nakayama E: Sequential involvement of two distinct $CD4^+$ regulatory T cells during the course of transplantable tumor growth and protection from 3-methylcholanthrene-induced tumorigenesis by CD25-depletion. Jpn J Cancer Res 93: 911-916, 2002.
8. Li J, Hu P, Khawli LA and Epstein AL: Complete regression of experimental solid tumors by combination LEC/chTNT-3 immunotherapy and CD25(+) T-cell depletion. Cancer Res 63: 8384-8392, 2003.
9. Kudo-Saito C, Schlom J, Camphausen K, Coleman CN and Hodge JW: The requirement of multimodal therapy (vaccine, local tumor radiation, and reduction of suppressor cells) to eliminate established tumors. Clin Cancer Res 11: 4533-4544, 2005.
10. Yu P, Lee Y, Liu W, Krausz T, Chong A, Schreiber H and Fu YX: Intratumor depletion of $CD4^+$ cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors. J Exp Med 201: 779-791, 2005.
11. Terabe M and Berzofsky JA: Immunoregulatory T cells in tumor immunity. Curr Opin Immunol 16: 157-162, 2004.
12. Levitsky HI, Lazenby A, Hayashi RJ and Pardoll DM: *In vivo* priming of two distinct antitumor effector populations: the role of MHC class I expression. J Exp Med 179: 1215-1224, 1994.
13. Bubenik J and Vonka V: MHC class I status of tumours and design of immunotherapeutic strategies. Immunol Lett 90: 177-178, 2003.
14. Bubenik J: MHC class I down-regulation: tumour escape from immune surveillance? (Review). Int J Oncol 25: 487-491, 2004.
15. Reinis M, Simova J and Bubenik J: Inhibitory effects of unmethylated CpG oligodeoxynucleotides on MHC class I-deficient and -proficient HPV16-associated tumours. Int J Cancer 118: 1836-1842, 2006.
16. Bubenik J: Animal models for development of therapeutic HPV16 vaccines (Review). Int J Oncol 20: 207-212, 2002.
17. Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM and Wu TC: Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. Cancer Res 56: 21-26, 1996.
18. Smahel M, Síma P, Ludvíková V, Marinov I, Pokorná D and Vonka V: Immunisation with modified HPV16 E7 genes against mouse oncogenic TC-1 cell sublines with downregulated expression of MHC class I molecules. Vaccine 21: 1125-1136, 2003.
19. Lowenthal JW, Corthesy P, Tougne C, Lees R, MacDonald HR and Nabholz M: High and low affinity IL 2 receptors: analysis by IL 2 dissociation rate and reactivity with monoclonal anti-receptor antibody PC61. J Immunol 135: 3988-3994, 1985.
20. Baratelli F, Lin Y, Zhu L, Yang SC, Heuze-Vourc'h N, Zeng G, Reckamp K, Dohadwala M, Sharma S and Dubinett SM: Prostaglandin E2 induces FOXP3 gene expression and T regulatory cell function in human $CD4^+$ T cells. J Immunol 175: 1483-1490, 2005.
21. Cai XP, Zhang H, Zhang YC, Wang Y, Su C, Ji MJ, Wu HW, Zhu X, Zhang ZS and Wu GL: Dynamics of $CD4^+CD25^+$ T cells in spleens and mesenteric lymph nodes of mice infected with Schistosoma japonicum. Acta Biochim Biophys Sin 38: 299-304, 2006.
22. Koutsky LA, Ault KA, Wheeler CM, Brown DR, Barr E, Alvarez FB, Chiacchierini LM and Jansen KU: A controlled trial of a human papillomavirus type 16 vaccine. N Engl J Med 347: 1645-1651, 2002.
23. Frazer IH: Prevention of cervical cancer through papillomavirus vaccination. Nat Rev Immunol 4: 46-54, 2004.
24. Mikyskova R, Bubenik J, Mendoza L, Vonka V, Smahel M, Simova J and Jandlova T: Local cytokine treatment of HPV16-associated tumours results in inhibition of their lung metastases. Clin Exp Metastasis 18: 581-587, 2001.
25. Bubenik J, Mikyskova R, Vonka V, Mendoza L, Simova J, Smahel M and Indrova M: Interleukin-2 and dendritic cells as adjuvants for surgical therapy of tumours associated with human papillomavirus type 16. Vaccine 21: 891-896, 2003.
26. Reinis M, Indrova M, Mendoza L, Mikyskova R, Bieblova J, Bubenik J and Simova J: HPV16-associated tumours: therapy of surgical minimal residual disease with dendritic cell-based vaccines. Int J Oncol 25: 1165-1170, 2004.
27. Kohm AP, McMahon JS, Podojil JR, Begolka WS, DeGutes M, Kasprowicz DJ, Ziegler SF and Miller SD: Cutting Edge: anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of $CD4^+CD25^+$ T regulatory cells. J Immunol 176: 3301-3305, 2006.
28. Ghiringhelli F, Menard C, Terme M, Flament C, Taieb J, Chaput N, Puig PE, Novault S, Escudier B, Vivier E, Lecesne A, Robert C, Blay JY, Bernard J, Caillat-Zucman S, Freitas A, Tursz T, Wagner-Ballon O, Capron C, Vainchencker W, Martin F and Zitvogel L: $CD4^+CD25^+$ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-{beta}-dependent manner. J Exp Med 202: 1075-1085, 2005.