Asian Journal of Andrology (2013) 15, 461–465

www.nature.com/aja

ORIGINAL ARTICLE

Analysis of circulating regulatory T cells ($CD4^+CD25^+CD127^-$) after cryosurgery in prostate cancer

Tong-Guo Si[1,*], Jun-Ping Wang[2,*] and Zhi Guo[1]

This study was performed to assess the response of regulatory T cells (Tregs) following cryosurgery in prostate cancer (PCa) patients by measuring their frequency and immune function. Blood was collected prior to and at 4 and 8 weeks after treatment in 30 patients with high-risk PCa who underwent cryosurgery and from 15 healthy volunteers. Circulating $CD4^+CD25^+CD127^-$ Tregs were isolated. Their frequency was detected by flow cytometry, and immune suppressive function was evaluated by measuring the proliferation of $CD4^+CD25^-$ T cells cocultured with Tregs. The results showed that the percentage of circulating $CD4^+CD25^+CD127^-$ Tregs was increased in PCa patients compared to healthy volunteers (7.6%±0.73% *vs.* 5.8%±0.54%, $P<0.001$). The frequency of circulating $CD4^+CD25^+CD127^-$ Tregs was reduced 4 weeks after cryosurgery compared to before surgery (6.3%±0.58% *vs.* 7.6%±0.73%, $P<0.001$), and the decrease persisted for 8 weeks. However, the suppressive function of Tregs was increased in eight of 12 patients, which might contribute to cancer recurrence. Then the response of circulating Tregs is complicated after cryosurgery for PCa, and further studies are warranted.

Asian Journal of Andrology (2013) **15,** 461–465; doi:10.1038/aja.2013.22; published online 3 June 2013

Keywords: cryosurgery; immune response; prostate cancer (PCa); regulatory T cell (Tregs)

INTRODUCTION

Cryosurgery is a minimally invasive technique that has proven to be effective in the treatment of patients with prostate cancer (PCa).[1] This treatment destroys tumour tissue *in situ* by freezing, leaving tumour proteins and tumour-associated antigens intact, which can potentially modulate the host's immune response.[2] In a mouse melanoma model, den Brok and collaborators[3] demonstrated that tumour debris generated by cryosurgery could be captured by dendritic cells (DCs) and reach the draining lymph nodes, leading to a mild tumour-specific immune response. Further studies showed that the immune response was enhanced by the coadministration of anticytotoxic T-lymphocyte-associated antigen 4 blocking antibodies. Blocking anticytotoxic T-lymphocyte-associated antigen 4 abolishes the function of naturally occurring $CD4^+CD25^+Foxp3^+$ T-regulatory cells.[4,5] To the best of our knowledge, no direct assessment of the effect of cryosurgery on the Treg population and Treg function has been reported in clinical studies.

$CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) play an important role in immune homeostasis because of their ability to suppress the activation of T cells, and an increase in the number or functionality of Tregs could thus favour tumour development. Increased levels of $CD4^+CD25^+FoxP3^+$ Tregs have been detected in peripheral blood mononuclear cells, the tumour microenvironment, and the draining lymph nodes in patients with PCa,[6–8] other solid tumours[9] and haematological malignancies.[10] Clinical studies have demonstrated that Tregs can inhibit both antigen-specific and nonspecific T-cell responses[11,12] and that an increase in $FoxP3^+$ Tregs is associated with an increased risk of recurrence.[13,14]

Currently, the isolation and expansion of human Treg subsets into functionally active, disease-specific T cells is difficult due to the paucity of Tregs in the peripheral blood and the lack of specific identity markers for Tregs. In humans, $CD4^+CD25^+$ T cells are a mixed population, including suppressor $CD4^+CD25^{high}$ T cells as well as $CD4^+CD25^{low}$ T cells, which are non-suppressive, activated $CD4^+$ T cells. Furthermore, the expression of Treg markers such as cytotoxic T–lymphocyte antigen 4 (CTLA-4) or glucocorticoid-induced tumour-necrosis factor receptor (GITR) can vary depending on cell activation, and these markers have not been useful for discriminating Tregs from effector T-cell populations. Similarly, Foxp3 expression, although more specific for Tregs, may also be upregulated on effector cells following activation.[15] Furthermore, due to its intracellular localisation, Foxp3 cannot be used for the isolation of live Tregs. Recently, two groups have independently shown that CD127 expression, which is the α chain of the interleukin-7 receptor, discriminates $CD127^{low}$ Tregs from $CD127^{high}$ conventional T cells within the $CD25^+CD45RO^+/RA^-$ effector/memory and the $CD45RA^+RO^-$ naive compartments in human peripheral blood and lymph nodes.[16,17]

[1]Department of Interventional Therapy, Tianjin Cancer Hospital, Tianjin Medical University, Tianjin 300060, China and [2]Department of Radiology, Tianjin Medical University General Hospital, Tianjin 300052, China
* These authors contributed equally to this work.
Correspondence: Dr Z Guo (drguozhi@163.com)
Received: 21 July 2012; Revised: 27 October 2012; Accepted: 2 February 2013; Published online: 3 June 2013

In this study, we used these methods to evaluate changes in qualitative and quantitative parameters of Treg populations in patients with PCa after cryosurgery.

PATIENTS AND METHODS

Patient characteristics

Thirty PCa patients, with a median age of 67 years, who underwent cryosurgery of the prostate for clinically localized, T1c–T2b PCa with high-risk features were identified. The inclusion criteria were as follows: patients with prehormone therapy high-risk features of PCa, which were defined as either a prostate-specific antigen (PSA) level $\geqslant 10$ ng ml^{-1}, a pathology report indicating a Gleason sum score $\geqslant 8$, or both; no previous hormonal, immunosuppressive, or radiation therapy before this treatment; and no evidence of metastatic disease by bone scan, computer-assisted tomography, or magnetic resonance imaging. The patient characteristics are summarized in **Table 1**.

Cryosurgery treatment

All patients received a rectal enema the night before the procedure. Under spinal anaesthesia, the patients were placed in a lithotomy position. Cystoscopy was performed, and a suprapubic bladder catheter was placed under direct vision. All procedures were performed according to the modified Onik technique using an ENDOCare unit with argon and helium gas for freezing and thawing, respectively, for a total of two freeze–thaw cycles. Under transrectal ultrasound guidance, 5–7 cryoprobes were introduced into the prostate, and four thermoprobes were located bilaterally in the neurovascular bundles, one in Denonvilliers' fascia and the other at the sphincter. The freezing process was monitored in real time by transrectal ultrasonography and using thermoprobes to enable the direct visualisation of the ice ball and avoid creating lesions on adjacent tissues. During the procedure, the urethra was protected with a warming device at 37 °C degrees that was maintained in place until the patient left the operating room. All patients were discharged within 24 h, and the suprapubic catheter was removed after 1 week.

Blood sample preparation

Blood samples were obtained from 30 PCa patients prior to and 1 month after the treatment and from 15 age-matched healthy volunteers as controls (NCs). All subjects signed an informed consent approved by the Institutional Review Board of Tianjin Medical University. Peripheral blood mononuclear cells were isolated by centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech). The cells were washed twice in phosphate-buffered saline without calcium and magnesium (pH 7.2) and resuspended in X-VIVO 15 medium (BioWhittaker and In Vitro) for further analysis.

Table 1 Patient characteristics and treatment outcome

Characteristic	*Value, mean (range)*
Total no.	30
Median age (year)	67 (55–74)
TNM	
T1c	11
T2a	12
T2b	7
Gleason score	
6	5
7	8
8	13
9	4
Mean PSA (ng ml^{-1})	
Before treatment	14.5 (9.2–31.4)
1 month after treatment	0.12 (0–0.3)
3 months after treatment	0.17 (0–0.8)
6 months after treatment	0.51 (0–6.7)[a]

Abbreviations: PSA, prostate-specific antigen.
[a] The values of PSA in patient 6 and patient 18 were 6.7 and 4.7 ng ml^{-1}, respectively; cancer recurrence was proven by biopsy.

Flow cytometry

Four-colour flow cytometry was performed on an FACSCalibur (BD Biosciences) with CellQuest Pro software. All monoclonal antibodies and isotype controls used were purchased from eBiosciences (San Diego, CA, USA): PE-Texas Red (ECD)-labelled anti-CD3, PC5-labelled anti-CD4, FITC-labelled anti-CD25 and PE labelled anti-CD127. After red blood cell lysis (Q-prep System; Beckman Coulter, Hialeah, FL, USA), naturally occurring Tregs were characterized by the expression of CD4 and CD25 and the lack of expression of CD127. The results are expressed as percentages of the $CD4^+$ lymphocyte population and as the number of cells per microlitre of whole blood. In addition, $CD56^+/CD16^+$ cells were identified as natural killer (NK) cells.

Immunosuppression assays

$CD4^+CD25^-$ T cells (1×10^4 cells per well) were cultured alone or with Tregs in three different ratios with 1 μg ml^{-1} of anti-CD3 antibody (OKT3; eBiosciences) in the presence of irradiated (3500 rad) T-cell-depleted peripheral blood mononuclear cells (1×10^5 cells per well) in a 96-well flat-bottomed plate at 37 °C and 5% CO_2. Cells were cultured in RPMI 1640 (Mediatech; Manassas, VA, USA) supplemented with 10% heat-inactivated human AB serum (Gemini BioProducts; West Sacramento, CA, USA), 100 units per ml of penicillin, 100 μg ml^{-1} of streptomycin (Mediatech) and 2 mmol l^{-1} of *L*-glutamine (Mediatech). Proliferation was measured by $[^3H]$ thymidine (PerkinElmer, Waltham, MA, USA) incorporation at 1 μCi (0.037 MBq) per well. Cells were pulsed on day 4 and quantified 18 h later using a liquid scintillation counter (PerkinElmer). All experiments were performed in triplicate. Proliferation of $CD4^+CD25^-$ T cells without coculturing with Tregs was considered 100% proliferation.

Statistical analysis

The data are presented as the mean±s.d. because all variables were normally distributed. The statistical significance of differences between two groups was assessed by a two-tailed *t*-test. When comparisons were performed at multiple time points, a one-way analysis of variance followed by Bonferroni's post-test was used. $P<0.05$ was considered statistically significant.

RESULTS

The PSA scores were all significantly decreased 4 weeks after treatment, and no cases of PSA failure were observed within 2 months. After 6 months, an elevated PSA score was found in two patients, and cancer recurrence was proven by biopsy (**Table 1**); hormone therapy was recommended. The observed complications were mostly minor, with 20% (4/20) reporting mild haematuria, and 15% (3/20) reporting perineal/scrotum haematoma with no need for further treatment. Rectal injury and urinary incontinence, which was defined as the need for one or more pads per day, were not observed.

We evaluated the levels of Tregs in the peripheral blood of PCa patients and healthy volunteers. CD4 cells were gated for $CD25^+$ and $CD127^-$ cells. There was a significantly increased percentage of

Table 2 The lymphocyte sub-populations on admission

	Patients	*Healthy volunteers*	t	P
$CD4^+$ lymphocytes (10^3 μl^{-1})	0.67±0.06	0.72±0.05	2.11	0.04
Tregs (cell μl^{-1})	51.5±6.81	41.7±5.43	4.85	<0.001
Tregs (% of CD4)	7.6±0.73	5.8±0.54	8.57	<0.001
NK cells (cell μl^{-1})	64.3±5.13	66.9±4.28	1.73	0.09
NK cells (% of lymphocytes)	6.3±0.42	6.6±0.52	1.83	0.07

Abbreviations: NK, natural killer; Tregs, regulatory T cells.

$CD4^+CD25^+CD127^-$ cells in PCa patients compared to healthy volunteers (7.6%±0.73% *vs.* 5.8%±0.54%, respectively, $P<0.001$) (**Figure 1**). Although the absolute count of $CD4^+$ cells in the patients was decreased compared to healthy volunteers, the absolute count of $CD4^+CD25^+CD127^-$ cells in patients was also increased significantly compared to healthy volunteers. However, there was no significant difference between the two groups in either the percentage or absolute number of NK cells found (**Table 2**).

The effects of cryosurgery on peripheral $CD4^+CD25^+CD127^-$ lymphocytes were evaluated, and the results showed that the percentage of $CD4^+CD25^+CD127^-$ cells in the patients was decreased significantly (6.3%±0.58% *vs.* 7.6%±0.73%, $P<0.001$) at 4 weeks after cryosurgery. Although the absolute count of $CD4^+$ lymphocytes did not change markedly, the absolute count of $CD4^+CD25^+CD127^-$ cells did decrease significantly (42.3±5.01 μl^{-1} *vs.* 51.5±6.81 μl^{-1}, $P<0.001$). Moreover, the decrease persisted up to 8 weeks after treatment (**Table 3**).

To determine whether cryosurgery could affect the levels of Treg suppressive function, *in vitro* functional studies were performed using $CD4^+CD25^+CD127^-$ cells collected from patients before and 4 weeks after cryosurgery (**Figure 2**). Because large volumes of peripheral blood were required to purify Tregs and evaluate Treg function, only 12 patients were included in this part of the study. As shown in **Table 4**, the suppressive activity of $CD4^+CD25^+CD127^-$ cells increased in seven patients, and cancer recurred in two of the patients 6 months after treatment (**Table 4**).

DISCUSSION

PCa is the most common noncutaneous malignancy diagnosed in males, and it has become a large threat for ageing patients in Asia.[18] Cryosurgery is an effective treatment strategy for PCa that destroys tumour and prostate gland tissue through freezing. The development of cryotherapy for localized PCa provides a potentially curative option for patients with primary or recurrent disease, with less morbidity than radical surgery.[19] However, the American Urological Association has only recently accepted cryosurgery as a primary modality in the treatment of localized PCa, as published in the 'Best practice statement on cryosurgery for the treatment of localized PCa'.[20] Nonetheless, cryotherapy is currently indicated as a primary therapy for low-risk patients as an alternative to prostatectomy or radiotherapy in higher surgical risk patients and as a salvage procedure for patients who have not responded to radiation therapy. The Cryotherapy On-Line Data Registry (COLD Registry) used the Phoenix definition (nadir+2 ng ml^{-1} definition) to report 5-year biochemical disease-free survival rates in 1198 patients: 91% in low-risk patients, 78% in intermediate-risk patients and 62% in high-risk patients. These biochemical results compare quite favourably to the results of radiation therapy as a monotherapy.[21] A recent randomized trial comparing cryotherapy with radiation therapy reported similar biochemical outcomes and a significantly lower rate of positive post-treatment biopsies in the cryotherapy-treated patients.[22]

Cryosurgery leaves tumour proteins and tumour-associated antigens intact. The presence of residual tumour antigens in an inflammatory microenvironment can stimulate anti-tumour immune responses.[23] The immunological effects of cryosurgery were first documented by demonstrating the production of antibodies against rabbit male reproductive tissues after cryosurgery in the 1960s.[24] Unfortunately, immunological assays at that time were limited. Therefore, the existence of a cryoimmunological response remained controversial, and the mechanisms by which this may occur were unknown. However, increased interest in the clinical potential of cryosurgery and a more detailed understanding of the mechanisms by which the immune system recognizes and targets tumour antigens have generated a renewed interest in the field of cryoimmunology. In experiments in several animal models of cancer including melanoma,[3] colon cancer[25] and breast cancer,[26] the tumour debris generated by tumour cryosurgery was captured by DCs and transported to the draining lymph nodes, resulting in a weak but tumour-specific immune response. In a PCa study, Lubaroff *et al.*[27] demonstrated that cryosurgery alone was not effective in producing an immune response that was protective against rechallenge in the Dunning R3327 adenocarcinoma model, but a recent clinical study showed that cryosurgery for PCa could induce limited tumour-specific cytotoxic T-cell stimulation.[28] Several studies have demonstrated that the cryoimmunological response could be enhanced by other adjunct treatments such as the intratumour administration of DCs,[25,29] Toll-like receptor stimulation[30] and Treg depletion.[3]

Tregs (5%–10% of peripheral $CD4^+$ T cells) are primarily generated in the thymus and represent an essential mechanism of peripheral tolerance to self-antigens.[31] These cells may suppress or kill $CD8^+$ or $CD4^+$ T cells, resulting in suppressed antitumour immunity. Increased Treg numbers have been observed in patients with malignant tumours, including PCa, gastric cancer, lung cancer, pancreatic cancer and breast cancer.[32] Additionally, some evidence suggests that increased Treg numbers are correlated with a poor prognosis for patients with malignant tumours.[33,34] Furthermore, the elimination of these cells or the inhibition of their function may increase the antitumour immune response.[35,36]

Table 3 The effect of cryoablation on the frequency of Tregs

	Before treatment	*4 weeks after treatment*	*8 weeks after treatment*
$CD4^+$ lymphocytes (10^3 μl^{-1})	0.67±0.07	0.70±0.06*	0.68±0.05*
Tregs (cell μl^{-1})	51.5±6.81	42.3±5.01*	44.1±5.06*
Tregs (% of CD4)	7.6±0.73	6.3±0.58*	6.4±0.46*
$CD4^+CD25^-$ (% of CD4)	73.2±7.2	74.6±6.3	75.2±8.1

Abbreviation: Tregs, regulatory T cells.
*Significant differences were observed between before and 4 weeks after treatment or 8 weeks after treatment ($P<0.001$), but no difference was observed between 4 weeks after treatment and 8 weeks after treatment.

Table 4 The effect of cryoablation on the suppressive function of Tregs

Patients	*% Treg suppression*		*Δ Suppression (post–pre)*
	Pre–treatment	*Post-treatment*	
#2	54.6	63.2	8.6
#3	38.4	28.9	−9.5
#5	46.5	31.4	−15.1
#6[a]	35.5	52.4	16.9
#9	50.6	40.2	−10.4
#11	47.6	43.4	−4.2
#12	45.2	67.3	22.1
#15	66.2	42.5	−23.7
#18[a]	35.4	51.3	15.9
#21	24.6	33.5	8.9
#24	43.2	52.6	9.4
#27	35.6	49.0	13.4

Abbreviation: Tregs, regulatory T cells.
[a] Cancer recurrence was proven by biopsy 6 months after treatment.

Previous studies have suggested that cryosurgery can reduce the release of immunosuppressive factors and that tumour antigens are released into the circulation by necrotic tumour cells, leading to increased antitumour immunity.[37] The present study indicated that the frequency and absolute number of peripheral Tregs were increased in patients with PCa, which is consistent with a previous study.[6] More importantly, this study provided clinical evidence that cryosurgery for PCa can affect the peripheral Treg pool. Indeed, 4 weeks after treatment, the number of these cells was reduced significantly, and this effect lasted for at least 8 weeks after treatment. Surprisingly, the results showed that the suppressive effects of Tregs were increased in most patients (8/12) after cryosurgery. Moreover, cancer recurrence was observed in two of these patients. These results appear to be controversial because no direct evidence of an influence of cryosurgery on the frequency or function of Tregs in PCa patients was found.

Several studies have found that the carcinoma microenvironment can induce, recruit, and/or activate and expand Tregs.[38] It is possible that the tumour cells or other cells inside the tumour secrete chemokines that attract Tregs to migrate into the tumour. Tregs have been shown to express a variety of chemokine receptors, including CCR4, CCR7, CCR8, CXCR4 and CXCR5, depending on their activation status and tissue location.[38] A previous study on PCa demonstrated that some PCa cell lines, malignant ascites fluid and prostate tumour biopsies in culture contain or secrete CCL22 and chemoattract Tregs in an *in vitro* migration assay.[6] Cryosurgery of tumour tissue leads to cellular coagulative necrosis and causes cellular breakdown and the release of intracellular contents and proinflammatory cytokines that initiate the innate immune response and attract granulocytes and/or macrophages.[40] We therefore hypothesized that some cytokines released after cryosurgery attract Tregs into the tumour microenvironment and trigger the suppressive function simultaneously. The frequency of Tregs in the circulation would therefore be reduced, but increased suppressive function would be detected. To prove this hypothesis, further studies of the molecular mechanisms involved in the origin, function and interactions of Tregs after cryosurgery are urgently required. As animal studies have shown that cryosurgery combined with Treg depletion can enhance the antitumour immunological response to reduce cancer recurrence,[3] our findings suggest that monitoring Treg frequency and function in addition to administering anti-Treg treatment, such as cyclophosphamide [41] or ipilimumab,[42] might be crucial to allow the development of effective

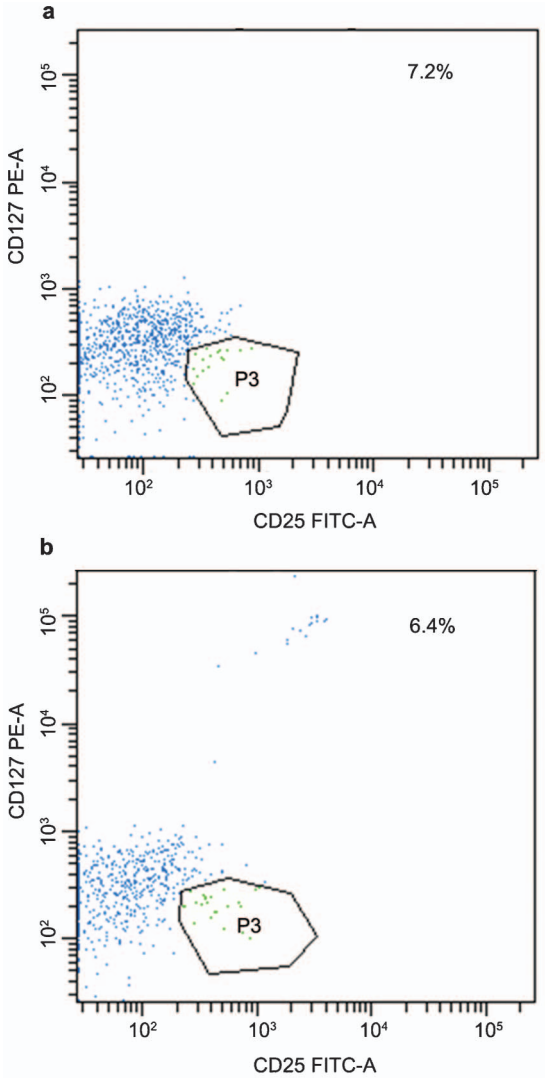

Figure 1 Tregs ($CD4^+CD25^+CD127^-$) were isolated before cryosurgery (**a**, 7.2%) and 4 weeks after cryosurgery (**b**, 6.4%). Tregs, regulatory T cells.

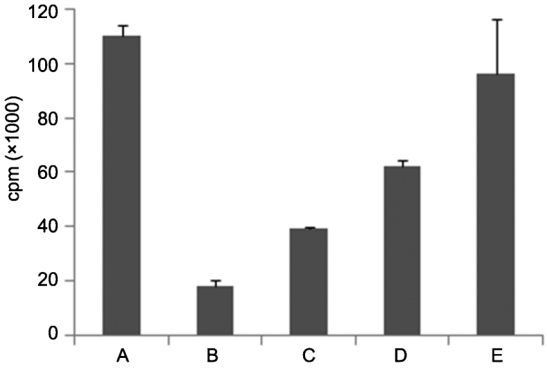

Figure 2 Suppression of $CD4^+CD25^-$ T-cell proliferation by $CD4^+CD25^+CD127^-$ Tregs in a patient with PCa. Isolated effectors ($CD4^+CD25^-$) and Tregs ($CD4^+CD25^+CD127^-$) were cultured alone or at three different ratios: column C 62.3% at 1 : 1, column D 42.6% at 1 : 0.5 and column E 15.2% at 1 : 0.1. PCa, prostate cancer; Tregs, regulatory T cells.

antitumour immunity after cryosurgery. Further studies are warranted.

In this study, we attempted to measure the function of Tregs in the peripheral blood of patients by evaluating the ability of peripheral blood cells to facilitate $CD4^+CD25^-$ T-cell proliferation. There was the potential concern of artefacts induced by peripheral blood handling, and the results do not represent the same sub-population present at the site of tumour ablation.

AUTHOR CONTRIBUTIONS

TGS and JPW performed the blood collection and flow cytometry analysis and drafted the manuscript. ZG designed the study and collected the clinical features. All authors read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

ACKNOWLEDGMENTS

This work was supported in part by the National Natural Science Foundation of China (No. 81001002 and No. 30973438) and Tianjin Health Bureau Foundation of China (No. 2010KZ7).

1 Mouraviev V, Johansen TE, Polascik TJ. Contemporary results of focal therapy for prostate cancer using cryoablation. *J Endourol* 2010; **24**: 827–34.
2 Sabel MS. Cryo-immunology: a review of the literature and proposed mechanisms for stimulatory versus suppressive immune responses. *Cryobiology* 2009; **58**: 1–11.
3 den Brok MH, Sutmuller RP, Nierkens S, Bennink EJ, Frielink C *et al*. Efficient loading of dendritic cells following cryo and radiofrequency ablation in combination with immune modulation induces anti-tumour immunity. *Br J Cancer* 2006; **95**: 896–905.
4 Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J *et al*. Immunologic self-tolerance maintained by $CD25^+CD4^+$ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* 2000; **192**: 303–10.
5 Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of $CD25^+CD4^+$ regulatory cells that control intestinal inflammation. *J Exp Med* 2000; **192**: 295–302.
6 Miller AM, Lundberg K, Ozenci V, Banham AH, Hellstrom M *et al*. $CD4^+CD25^{high}$ T cells are enriched in the tumor and peripheral blood of prostate cancer patients. *J Immunol* 2006; **177**: 7398–405.
7 Yokokawa J, Cereda V, Remondo C, Gulley JL, Arlen PM *et al*. Enhanced functionality of $CD4^+CD25^{high}FoxP3^+$ regulatory T cells in the peripheral blood of patients with prostate cancer. *Clin Cancer Res* 2008; **14**: 1032–40.
8 Sfanos KS, Bruno TC, Maris CH, Xu L, Thoburn CJ *et al*. Phenotypic analysis of prostate-infiltrating lymphocytes reveals TH17 and Treg skewing. *Clin Cancer Res* 2008; **14**: 3254–61.
9 Woo EY, Chu CS, Goletz TJ, Schlienger K, Yeh H *et al*. Regulatory $CD4^+CD25^+$ T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res* 2001; **61**: 4766–72.
10 Beyer M, Kochanek M, Darabi K, Popov A, Jensen M *et al*. Reduced frequencies and suppressive function of $CD4^+CD25^{high}$ regulatory T cells in patients with chronic lymphocytic leukemia after therapy with fludarabine. *Blood* 2005; **106**: 2018–25.
11 Mukherji B, Guha A, Chakraborty NG, Sivanandham M, Nashed AL *et al*. Clonal analysis of cytotoxic and regulatory T cell responses against human melanoma. *J Exp Med* 1989; **169**: 1961–76.
12 Chakraborty NG, Twardzik DR, Sivanandham M, Ergin MT, Hellstrom KE *et al*. Autologous melanoma-induced activation of regulatory T cells that suppress cytotoxic response. *J Immunol* 1990; **145**: 2359–64.
13 Bates GJ, Fox SB, Han C, Leek RD, Garcia JF *et al*. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol* 2006; **24**: 5373–80.
14 Wolf AM, Rumpold H, Wolf D, Gastl G, Reimer D *et al*. Role of forkhead box protein 3 expression in invasive breast cancer. *J Clin Oncol* 2007; **25**: 4499–500.
15 Godfrey WR, Spoden DJ, Ge YG, Baker SR, Liu B *et al*. Cord blood $CD4^+CD25^+$-derived T regulatory cell lines express FoxP3 protein and manifest potent suppressor function. *Blood* 2005; **105**: 750–8.
16 Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR *et al*. CD127 expression inversely correlates with FoxP3 and suppressive function of human $CD4^+$ T reg cells. *J Exp Med* 2006; **203**: 1701–11.
17 Hartigan-O'Connor DJ, Poon C, Sinclair E, McCune JM. Human $CD4^+$ regulatory T cells express lower levels of the IL-7 receptor alpha chain (CD127), allowing consistent identification and sorting of live cells. *J Immunol Methods* 2007; **319**: 41–52.
18 Zhang L, Yang BX, Zhang HT, Wang JG, Wang HL *et al*. Prostate cancer: an emerging threat to the health of aging men in Asia. *Asian J Androl* 2011; **13**: 574–8.
19 Cohen JK, Miller RJ Jr, Ahmed S, Lotz MJ, Baust J. Ten-year biochemical disease control for patients with prostate cancer treated with cryosurgery as primary therapy. *Urology* 2008; **71**: 515–8.
20 Babaian RJ, Donnelly B, Bahn D, Baust JG, Dineen M *et al*. Best practice statement on cryosurgery for the treatment of localized prostate. *J Urol* 2008; **180**: 1993–2004.
21 Jones JS, Rewcastle JC, Donnelly BJ, Lugnani FM, Pisters LL *et al*. Whole gland primary prostate cryoablation: initial results from the Cryo-On-Line Data registry. *J Urol* 2008; **180**: 554–8.
22 Donnelly BJ, Saliken JC, Brasher PM, Ernst SD, Rewcastle JC *et al*. A randomized trial of external beam radiotherapy versus cryoablation in patients with localized prostate cancer. *Cancer* 2010; **116**: 323–30.
23 Johnson JP. Immunologic aspects of cryosurgery: potential modulation of immune recognition and effector cell maturation. *Clin Dermatol* 1990; **8**: 39–47.
24 Yantorno C, Soanes WA, Gonder MJ, Shulman S. Studies in cryo-immunology. I. The production of antibodies to urogenital tissue in consequence of freezing treatment. *Immunology* 1967; **12**: 395–410.
25 Udagawa M, Kudo-Saito C, Hasegawa G, Yano K, Yamamoto A *et al*. Enhancement of immunologic tumor regression by intratumoral administration of dendritic cells in combinationwith cryoablative tumor pretreatment and bacillus calmetteguerin cell wall skeleton stimulation. *Clin Cancer Res* 2006; **12**: 7465–75.
26 Sabel MS, Nehs MA, Su G, Lowler KP, Ferrara JL *et al*. Immunologic response to cryoablation of breast cancer. *Breast Cancer Res Treat* 2005; **90**: 97–104.
27 Lubaroff DM, Reynolds CW, Canfield L, McElligott D, Feldbush T. Immunologic aspects of the prostate. *Prostate* 1981; **2**: 233–48.
28 Si T, Guo Z, Hao X. Immunologic response to primary cryoablation of high-risk prostate cancer. *Cryobiology* 2008; **57**: 66–71.
29 Machlenkin A, Goldberger O, Tirosh B, Paz A, Volovitz I *et al*. Combined dendritic cell cryotherapy of tumor induces systemic antimetastatic immunity. *Clin Cancer Res* 2005; **11**: 4955–61.
30 den Brok MH, Sutmuller RP, Nierkens S, Bennink EJ, Toonen LW *et al*. Synergy between in situ cryoablation and TLR9 stimulation results in a highly effective in vivo dendritic cell vaccine. *Cancer Res* 2006; **66**: 7285–92.
31 Rudensky AY, Gavin M, Zheng Y. FOXP3 and NFAT: partners in tolerance. *Cell* 2006; **126**: 253–6.
32 Baecher-Allan C, Anderson DE. Regulatory cells and human cancer. *Semin Cancer Biol* 2006; **16**: 98–105.
33 Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P *et al*. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004; **10**: 942–9.
34 Sasada T, Kimura M, Yoshida Y, Kanai M, Takabayashi A. $CD4^+CD25^+$ regulatory T cells in patients with gastrointestinal malignancies: possible involvement of regulatory T cells in disease progression. *Cancer* 2003; **98**: 1089–99.
35 Yamaguchi T, Sakaguchi S. Regulatory T cells in immune surveillance and treatment of cancer. *Semin Cancer Biol* 2006; **16**: 115–23.
36 Khazaie K, von Boehmer H. The impact of $CD4^+CD25^+$ Treg on tumor specific $CD8^+$ T cell cytotoxicity and cancer. *Semin Cancer Biol* 2006; **16**: 124–36.
37 Ravindranath MH, Wood TF, Soh D, Gonzales A, Muthugounder S *et al*. Cryosurgical ablation of liver tumors in colon cancer patients increases the serum total ganglioside level and then selectively augments antiganglioside IgM. *Cryobiology* 2002; **45**: 10–21.
38 Ha TY. The role of regulatory T cells in cancer. *Immune Network* 2009; **9**: 209–35.
39 Zou L, Barnett B, Safah H, Larussa VF, Evdemon-Hogan M *et al*. Bone marrow is a reservoir for $CD4^+CD25^+$ regulatory T cells that traffic through CXCL12/CXCR4 signals. *Cancer Res* 2004; **64**: 8451–5.
40 Sidana A, Chowdhury WH, Fuchs EJ, Rodriguez R. Cryoimmunotherapy in urologic oncology. *Urology* 2010; **75**: 1009–14.
41 Le DT, Jaffee EM. Regulatory T-cell modulation using cyclophosphamide in vaccine approaches: a current perspective. *Cancer Res* 2012; **72**: 3439–44.
42 O'Mahony D, Morris JC, Quinn C, Gao W, Wilson WH *et al*. A pilot study of CTLA-4 blockade after cancer vaccine failure in patients with advanced malignancy. *Clin Cancer Res* 2007; **13**: 958–64.