

Prostate cancer, tumor immunity and a renewed sense of optimism in immunotherapy

Nicolò Rigamonti · Matteo Bellone

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Abstract The recent FDA approval of the first therapeutic vaccine against prostate cancer has revitalized the public interest in the fields of cancer immunology and immunotherapy. Yet, clinical results are modest. A reason for this limited success may reside in the capacity of the tumor to convert inflammation in a tumor-promoting condition and eventually escape immune surveillance. Here we present the main known interactions between the prostate tumor and the immune system, showing how the malignancy can dodge the immune system by also exerting several immunosuppressive mechanisms. We also discuss experimental and clinical strategies proposed to counteract cancer immune evasion and emphasize the importance of implementing appropriate murine models like the transgenic adenocarcinoma of the mouse prostate model for investigating the biology of prostate cancer and novel immunotherapy approaches against it.

Keywords Immunotherapy · Prostate cancer · Mouse model · Tumor immunity · Immunosuppression

Introduction

Prostate cancer (PC) is the most frequently diagnosed non-skin cancer among men in economically developed

countries and one of the leading causes of cancer-related death [1]. One in 6 men in the United States will be diagnosed with PC at some time during his lifetime, and 1 in 34 will die from it [2]. Although the mortality for PC has steadily declined in recent years, due in part to early diagnosis and better therapeutic approaches, more men are being diagnosed with early-stage, low-grade disease and younger than 50 years of age [3]. Differences in PC incidence among populations around the world underline the contribution of both genetic and environmental factors. As an example, the risk of PC is particularly high in people of sub-Saharan African ancestry, and African-American men are almost twice more likely to be diagnosed and 2.4 times more likely to die from PC [4]. On the other hand, the increased risk of PC in Asians who have migrated to the United States underlines the importance of environmental factors, notably the diet.

When the tumor is at an early stage, characterized by a slowly progressing disease that does not extend beyond the prostate gland itself [5], prostatectomy, even for low-risk patients [6], and radiotherapy are the therapeutic options, and more recent results suggest to combine radiotherapy and androgen-deprivation therapy (ADT) for those with intermediate- or high-risk localized PC [7]. Conversely, a valid therapeutic option for locally advanced or metastatic PC is still lacking. Therapies based on androgen deprivation are indeed the current most effective against metastatic disease, but often lead to androgen-independent progression and death of the patients within a few years. The standard of care for castration-resistant metastatic PC is docetaxel [8] with some new and very promising therapeutic options in the pipeline [9]. Therapies able to augment the natural immune response have lately become valuable alternatives to delay disease progression. Cancer vaccines have been demonstrated to be safe and induce

N. Rigamonti (✉) · M. Bellone (✉)
Cellular Immunology Unit, Program of Immunology,
Gene Therapy and Bio-Immunotherapy of Cancer (PIBIC),
San Raffaele Scientific Institute, via Olgettina 58,
20132 Milan, Italy
e-mail: rigamonti.nicolo@hsr.it

M. Bellone
e-mail: bellone.matteo@hsr.it

tumor-specific immune responses in cancer patients. Indeed, the FDA has recently approved the use of sipuleucel-T, a cell-based vaccine, for the treatment of asymptomatic or minimally symptomatic castration-resistant PC [10]. In general, however, the clinical benefits of immunotherapy are still small [11]. A reason for this limited success may reside in the capacity of the tumor to shift from an anti- to a pro-tumor inflammatory response and escape immune surveillance. Indeed, growth of PC and other neoplasms associates with mechanisms of immunosuppression that transform the tumor in a tissue of acquired immune privilege. We refer to a number of excellent reviews for a detailed description of all these mechanisms [12–15]. This review focuses instead on a large body of evidence, collected both in humans and mostly in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model of human PC, suggesting a link between chronic inflammation and PC. We also show how PC can dodge the immune system involved in the inflammatory reaction by converting inflammation in a tumor-promoting condition and exerting several immunosuppressive mechanisms. We finally report on strategies that thwart cancer immune evasion and increase the efficacy of immunotherapy.

Inflammation in cancer: with a little help from new friends

Inflammation is a complex and broad biological process that maintains tissue homeostasis in the presence of either infections or tissue damage by inducing tissue remodeling and angiogenesis. Inflammation may be acute or chronic. The latter appears to play a pivotal role in tumorigenesis and cancer, although the factors linking inflammation and cancer (e.g., inherited traits, immune deregulation and autoimmunity, virus and bacterial infections, tobacco smoking, inhaled pollutants and dietary factors) and the mechanisms by which inflammation drives cancer development and progression have not been fully elucidated yet [16]. Conversely, oncogenes may trigger proinflammatory pathways. As an example, while the release by inflammatory cells of reactive oxygen species (ROS), such as peroxynitrites, causes DNA damage leading to genetic instability and cancer progression [17], the activation of the oncogene RAS triggers the transcription of interleukin (IL)-8, leading to a host inflammatory response that promotes tumor growth [18]. In addition, inflammation also promotes the release of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) [19] and metalloproteinases that favor metastasis [20]. Finally, inflammation stimulates an aberrant myelopoiesis that leads to the accumulation within the tumor of myeloid-

derived suppressor cells (MDSC), alternatively activated M2-like macrophages, and dysfunctional dendritic cells (DC), cancer foes transformed in new friends that contribute to tumor growth and immune escape [21–24].

Inflammation and prostate cancer

Compelling data both in mice and humans support chronic inflammation (i.e., chronic prostatitis) as an important factor in prostate carcinogenesis, as excellently reviewed in [25]. While in most cases, the triggering inflammatory event is unknown, prostate infection, urine reflux, estrogens and consumption of red meat and animal fats, either alone or in combination, favor prostatic inflammation and are indicted for inflammatory-associated prostate carcinogenesis [25].

Relevant are also inherited traits that may render the subject more susceptible to chronic inflammation. As an example, a strong correlation exists between genetic polymorphisms of toll-like receptor (TLR)4 and susceptibility to PC in humans [26]. Although the biological mechanism of this observation remains to be elucidated, it is plausible that modifying the genetic sequence of TLR4 could result in high state of TLR4 activation, favoring chronic inflammation and carcinogenesis. Indeed, overexpression in PC cells of endogenous TLR4 ligands such as peroxiredoxin 1 (Prx1) lead to prostate tumor growth through TLR4-mediated regulation of angiogenesis [27]. In addition, TLR4 gene expression is induced by IL-6 via signal transducer and activator of transcription 3 (STAT3), an important proinflammatory transcription factor [28]. Thus, inactivation of STAT3 would result in impaired TLR4 expression, reduced inflammation and consequently reduced tumor growth. Indeed, pharmacologically inactivation of STAT3 turned out to have therapeutic effects in mice transplanted with syngenic TRAMP-C2 PC cells [29], a PC cell line established from a tumor spontaneously developed in a TRAMP mouse [30].

Interestingly, a direct correlation has been recently reported between TLR and the expression of alternatively spliced isoforms of the *TMPRSS2/ERG* fusion gene, present in the majority of PC lesions [31]. Wang et al. [32] reported that *TMPRSS2/ERG* isoforms differently increase the expression of a number of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)-associated genes including TLR3 and TLR4, therefore, linking gene instability, inflammation and carcinogenesis.

Major insights on the role of inflammation and TLR in PC have been obtained taking advantage of the TRAMP model, in which the transgene [i.e., the SV40 early genes (large and small T antigens: Tag)] is expressed under the control of the probasin regulatory element [33]. As a

consequence, starting at puberty, male mice invariably and progressively develop spontaneous mouse prostate intraepithelial neoplasia [mPIN; week (wk) 6–12], adenocarcinoma (wk 12–18), seminal vesicle invasion, and lymph node and lung metastases (wk 18–30; Ref. [34]). While the natural history of the disease in TRAMP mice is remarkably similar to the human counterpart, there are several diverging anatomical and biological characteristics between mice and humans that should be taken into account [35]. As few examples, the adult mouse prostate is divided in distinct lobes that are not found in the human prostate. The mouse prostate lacks the abundant dense fibromuscular stroma surrounding ductules and forming the prostate capsule in humans, making the concept of extracapsular extension of the disease hardly applicable to the mouse. Conversely, a relevant stromal hypercellularity, unusual in human PC, is associated with epithelial cell proliferation and increase extracellular matrix in the TRAMP model, and can be utilized as sign of past disease occurrence in treated animals [36]. In addition to the natural history of the disease, cancer development [35], androgen sensitivity [37], fine aspects of neo-angiogenesis [38] and metabolic activity [39] all resemble human PC and make the TRAMP mouse the most frequently utilized preclinical model in this research field.

It has been demonstrated that inactivation of signaling of NF- κ B, a master transcription factor for genes related to inflammation, has a relevant effect on PC growth and metastatogenesis in TRAMP mice [40]. Indeed, a mutation that prevents I κ B kinase α (IKK α) activation and nuclear translocation of NF- κ B also results in upregulation of maspin, a metastasis suppressor protein (Table 1), and reduced PC growth and metastasis occurrence. Conversely, activation of IKK α by RANK ligand (RANKL) inhibits maspin expression and promotes metastatogenesis. Since inflammation contributes to the recruitment of macrophage and T cells into the tumor, tumor-infiltrating leukocytes might be a source of RANKL, establishing a loop between cancer progression and inflammation.

Curiously, mice transplanted with syngenic TRAMP-C2 PC cells knocked out for TLR3, develop a more aggressive tumor as compared to their wild-type counterpart, whereas the opposite is true for TRAMP mice treated with the TLR3 agonist polyinosinic-polycytidylic acid (polyI:C) [41]. The therapeutic effect is mainly mediated by TLR3-induced IFN signaling that promotes natural killer (NK) cells activation and reduces expansion of CD4⁺CD25⁺ Foxp3⁺ regulatory T cells (Treg). Thus, this study implicates the involvement of TLR3 in cancer immune surveillance, suggesting the use of TLR3 agonists as a therapeutic approach in PC patients. Additional studies are warranted to define the role of TLR3 in human PC [42, 43].

TGF β and prostate cancer

TGF β is a relevant molecule in cancer biology, as it works both as tumor-suppressor and tumor-promoter [44, 45], and several studies have documented increased levels of TGF β both in serum and prostate of PC patients, which may negatively correlate with patients' survival [46–48].

Pu et al. [49] have reported that a dysfunctional TGF β receptor II (TGF β RII) in epithelial cells promotes inflammation and PC in TRAMP mice (Table 1), therefore, underlying the role of TGF β as a tumor-suppressor in the early stages of prostate tumorigenesis. In these mice, the inflammatory process leads to macrophage infiltration that correlates with elevated VEGF and monocyte chemoattractant protein-1 (MCP-1) protein levels in the prostate of TRAMP mice, and consequently, with increased angiogenesis that could contribute to the more aggressive form of the tumor [49]. In addition, TGF β signaling disruption favors the intratumor recruitment of CD11b⁺Gr1⁺ myeloid cells that promote metastasis [50].

TGF β also exerts a pivotal role in maintaining immune homeostasis [51] and can impair CD8⁺ T-cell function by repressing the production of cytolytic factors including granzyme A and B, perforin and the pro-apoptotic cytokines IFN γ and Fas-ligand [52]. By crossing TRAMP mice with a series of transgenic mice deficient in TGF β signaling by the expression of a dominant-negative form of TGF β RII (DNTGF β RII) or lacking TGF β production by either all T cells or selectively by Foxp3⁺ Treg, Li and colleagues [53] have demonstrated that TGF β produced by CD8⁺ T cells is crucial to impair the CD8⁺ cell-mediated tumor immunity and, consequently, for tumor progression (Table 1). Thus, either blocking TGF β signaling [54, 55] or production [53] in CD8⁺ T cells results in augmenting anti-tumor immunity, supporting the hypothesis of targeting TGF β for cancer immunotherapy (Table 1). However, systemic blocking of TGF β may be dangerous. Indeed, loss of functionality of TGF β induces malignant transformation of non-tumorigenic rat prostate epithelial cells [56], and restoration of TGF β signaling, by overexpression of TGF β RII, results in suppression of PC growth [57]. Disruption of TGF β signaling also correlates with a pathologic manifestation of human PIN and PC [58, 59]. Collectively, the observations obtained in human and murine PC argue for a more selected targeting of self-directed TGF β signaling in T cells.

It has also been described that TGF β decreases the expression of the activating receptor NKG2D in NK cells and CD8⁺ T cells and reduces the expression of NKG2D ligands in tumor cells [60], therefore, reducing the NKG2D-mediated immune surveillance in PC [61]. A relevant role for NKG2D⁺ cells in PC has also been

Table 1 List of transgene-modified TRAMP mice with their phenotype and the potential therapeutic application

Targeted gene	Gene function	Knockout strategy ^a	Phenotype	Therapeutic application	References
IKK α	Activation of NF- κ B	Mutation that prevents IKK α activation	Prolonged tumor onset, decreased metastasis occurrence and delayed mortality	Pharmacologic regulation of either expression of maspin or inactivation of NF- κ B	[40]
TGF β /RII	Initiates the downstream TGF β signaling cascade	DNTGFR/RII expressed in epithelial cells	Appearance of early malignant changes	Administration of TGF β . However, TGF β is also an immunosuppressive cytokine. Hence, administration of TGF β might exert opposing effects	[49]
TGF β /RII	Initiates the downstream TGF β signaling cascade	DNTGFR/RII expressed in CD8 ⁺ and CD4 ⁺ T	Reduced tumor growth associated with enhanced tumor antigen-specific T cell responses	This strategy could be used in adoptive immunotherapy to render tumor-specific T cells less susceptible to TGF β -mediated immunosuppression	[53]
TGF β	Immunoregulatory cytokine	Crossing mice with floxed and null alleles of TGF β 1 (Tgf β 1 ^{fln}) with CD4-Cre mice	As above	Inhibition of TGF β by specific monoclonal antibodies. However, systemic inhibition of TGF β might favor tumor cell growth	[53]
TGF β	Immunoregulatory cytokine	Crossing Tgf β 1 ^{fln} with Foxp3-Cre mice	No effects on tumor growth	As above	[53]
Klrk1	Codifies for NKG2D	Deletion of the exons 1b to 6 of NKG2D	Appearance of more aggressive tumors	Treatment with IFN α and IL-2 may result in in vivo expansion and activation of NK cells	[62]
TCR δ	Allows thymic selection of $\gamma\delta$ T cells and antigen recognition	B6.129P2 Tcr δ ^{mi1Mom/J} mice are deficient for $\gamma\delta$ T cells	Appearance of more aggressive tumors	Zoledronate and IL-2 activate in vivo $\gamma\delta$ T cells	[91]
J α 18	Allows thymic selection of iNKT cells and antigen recognition	B6.129-Tcra-J α 18 mice are deficient for iNKT cells	Appearance of more precocious and aggressive tumors	Treatment with α GalCer or α GalCer-modulated DC	[98]
IDO	Promotes the catabolism of tryptophan	Deletion of the exons 3–5 of the IDO gene	Delayed appearance of palpable tumor in TRAMP IDO ^{-/-} mice	Pharmacological inhibition of IDO by 1-methyl-tryptophan	[151]

^a TRAMP mice were crossed with mice carrying the indicated genetic alteration for several generations to generate TRAMP mice of nearly identical background and carrying the additional genetic alteration

suggested by the finding that NKG2D-deficient TRAMP mice have increased and more precocious incidence of large, highly malignant PC when compared with wild-type TRAMP mice (Table 1) [62]. Since NKG2D is also expressed in T, $\gamma\delta$ and iNKT cells, further studies are needed to clarify the role of the different components of the immune system in the NKG2D-mediated tumor immunity. These data also suggest therapeutic interventions aimed at increasing the natural NKG2D-mediated immune surveillance against PC.

Discoveries in the near future will shed more light on the mechanistic interplay between chronic inflammation and prostate carcinogenesis, and especially on factors that dynamically influence the shift from tumor-suppressive to tumor-promoting inflammation.

Cancer immune surveillance and immunoediting, two sides of the same coin

Extensive experimental and clinical data support the notion that the immune system plays an active and crucial role in hampering tumor growth. This process, referred to as immune surveillance [63, 64], has been clearly documented in rodents. Indeed, totally or selectively immunodeficient mice are more susceptible to carcinogen-induced and spontaneous tumors than immunocompetent mice, and tumors arisen in immunodeficient mice are more immunogenic than those from immunocompetent mice [65]. This because interferon (IFN) γ and lymphocytes participate in the selection of tumor variants with reduced immunogenicity that have better chances of surviving in immunocompetent hosts [66].

Similarly, in humans, the frequency of both hematopoietic and solid malignancies is higher in patients rendered immunodeficient by HIV infection [67] or in transplanted patients treated with immunosuppressive drugs to facilitate organ engraftment [68–70]. Clinical evidence that further supports the concept of cancer immune surveillance is the prognostic value of tumor-infiltrating lymphocytes (TIL). Indeed, a better prognosis correlates with a higher number of effector cytotoxic T lymphocytes (CTL) infiltrating the tumor [71, 72]. Conversely, tumor infiltration by Treg, a cell population with immunoregulatory function, fosters immune privilege and predicts reduced survival in patients affected by PC or other tumors [73]. Consistent with this notion, the CD8⁺/Treg ratio has a more reliable prognostic value than only TIL [74].

The dark side of the immune system is in its capacity to promote tumor development. This dual host-protective and tumor-promoting action is at the basis of the cancer immunoediting hypothesis [64].

Immune surveillance and immune escape in prostate cancer

PC is among the tumors at increased risk in transplanted patients [75–77], and several cell-intrinsic and cell-extrinsic mechanisms of immunosuppression have been reported in PC patients [12]. As few examples, impairment in tumor antigen expression, processing and presentation by both tumor cells and antigen presenting cells (APC) and altered expression of costimulatory molecules by APC may likely decrease immune surveillance in PC. Pro-apoptotic mechanisms, including FasL expressed on tumor cells, may favor T-cell apoptosis at the tumor site. In addition, cytokines such as TGF β , IL-10, IL-6 and prostaglandins and even prostate-specific antigen (PSA) are all factors that have negative effects on T-cell function while favoring tumor growth. Finally, the PC microenvironment might promote development and recruitment of cells endowed with suppressive function like Treg [78, 79] and myeloid-derived cells [80].

While these immunosuppressive processes have been clearly documented in PC patients, transgenic models of spontaneous cancer development have shown to be indispensable tools to investigate the timing and dynamics of the immunosuppressive mechanisms. We [81, 82] and others [83–85] have shown that disease progression gradually renders TRAMP mice tolerant to tumor-associated antigens (TAA). As an example, whereas marginal expression of Tag, here considered as a *bona fide* TAA, in the thymus causes deletion of high avidity T-cell clones [86], in the periphery Tag is selectively expressed in prostate epithelial cells under the influence of sex hormones. Hence, young TRAMP mice (up to 10 wk of age) are able to mount a Tag-specific T-cell response upon vaccination with bone marrow-derived DC pulsed with the immunodominant CTL epitope Tag-IV (sequence 404–411), whereas older mice fail to do so, and develop Tag T-cell tolerance [81], therefore, well recapitulating the situation found for non-mutated TAA in patients with advanced PC [87]. All these characteristics, together with the recent identification of an integrated SV40 Tag cancer signature in aggressive human PC [88], make the TRAMP a unique model to investigate interactions between PC and the immune system.

A role for $\gamma\delta$, iNKT and NK cells, innate effectors, in prostate cancer

$\gamma\delta$ T cells are unconventional T-cell subsets, bearing invariant TCR, exhibiting several characteristics that place them at the border between the innate and the adaptive immune system. As opposed to $\alpha\beta$ T cells, which mount an immune response against specific antigens, $\gamma\delta$ T cells

recognize generic antigens expressed by stressed cells including neoplastic cells. A role for $\gamma\delta$ T cells in PC has been suggested by the evidence that $\gamma\delta$ T cells can kill PC cells in vitro [89]. Also, the frequency of $\gamma\delta$ T cells infiltrating the tumor positively correlates with the tumor expression of NKG2D ligands [90]. Interestingly, $\gamma\delta$ T-cell-deficient TRAMP mice show a more aggressive disease when compared with the wild-type counterpart [91] (Table 1). Conversely, adoptive transfer of syngenic $\gamma\delta$ T cells into mice bearing a subcutaneous TRAMP-C2 tumor delays tumor growth [91]. All together, these data provide a biological rationale for an active role of $\gamma\delta$ T cells in PC immune surveillance and the basis for developing $\gamma\delta$ T-cell-based immunotherapies against PC. To this purpose, zoledronate and IL-2 have shown to allow large-scale ex vivo expansion [92], and in vivo activation and expansion of $\gamma\delta$ T cell in cancer patients [93]. Interestingly, the number of peripheral $\gamma\delta$ T cells with an activated effector memory-like phenotype showed a statistically significant correlation with the decline of serum PSA levels and objective clinical outcomes [94].

Also iNKT cells belong to the innate arm of the immune system. At difference with $\gamma\delta$ T cells, iNKT cells express an invariant TCR V α 14J α 18 chain in mice and V α 24J α 18 in humans, combined with variable TCR β -chains. This semi-invariant TCR is restricted for CD1d, a member of the non-MHC encoded family of CD1 antigen presenting molecules, and recognizes lipid antigens of both cell endogenous or exogenous origin, whose prototype is the glycosphingolipid α galactosylceramide (α GalCer). iNKT cells produce a broad range of cytokines within short time of antigenic stimulation, therefore, alerting both innate and adaptive immunity [95]. A role for iNKT in PC has been suggested by the evidence that in advanced PC patients and TRAMP mice, the numbers of circulating iNKT cells and their production of IFN γ are reduced [96, 97]. In addition, we have recently reported that lack of iNKT cells in TRAMP mice results in the appearance of more precocious and aggressive tumors that significantly reduce animal survival (Table 1) [98]. TRAMP mice bearing or lacking iNKT cells respond similarly to a Tag-specific vaccination and develop tolerance to Tag at comparable rate [98]. Thus, these data argue for a critical role of iNKT cells in the immune surveillance of carcinoma that appears to be independent of tumor-specific CTL. Although the mechanism by which iNKT exerts immune surveillance in PC remains to be determined, promising results have been already reported in cancer patients treated with α GalCer or α GalCer-modulated DC [99], and PC will likely be the target of similar clinical trials.

Less is known about NK cells in PC. Early studies documented a reduced activity of NK cells in patients with advanced disease [100, 101], which is likely due to a

reduced surface NKG2D expression in NK cells [61]. Indirect evidence of a role for NK in PC is provided by the fact that in TRAMP mice dietary intake of sulforaphane, a synthetic analog of cruciferous vegetable-derived L isomer reduces carcinogenesis and pulmonary metastases. This correlates with enhanced cytotoxicity of NK cells against TRAMP-C1 target cells, and increased infiltration of the prostate by T cells [102]. In addition, as reported above, effective treatment of TRAMP mice with polyI:C associates with TLR-mediated NK cells activation and reduced expansion of Treg [41].

Two-faced CD8⁺ and CD4⁺ T cells in prostate cancer

Studies both in humans and mice have clearly documented the determinant role of the adaptive immunity, and especially of CD8⁺ T cells in PC immune surveillance [11]. Indeed, PC lesions are mainly infiltrated by CD8⁺ T cells characterized by an effector memory (CCR7⁻CD45RA⁻CD62L⁻) or terminally differentiated phenotype (CCR7⁻CD45RA⁺CD62L⁻) [103]. CD4⁺ T cells are also found within PC lesions and include CD4⁺CD25⁺ effectors, Treg and Th17 [78, 79, 104–106], although the role of Th17 in PC is still debated [105, 106]. In addition, the success obtained with active immunotherapy in PC is an indirect evidence of the potential effects of the immune system against PC [11, 13]. Among the most promising vaccines for PC, the Sipuleucel-T vaccine recently approved by the FDA involves administration of autologous DC pulsed with a chimeric protein containing the granulocyte-macrophage colony-stimulating factor (GM-CSF) and prostatic acid phosphatase (PAP) as a TAA. The clinical trial has shown a relative reduction of 22% in the risk of death and a 4.1-month improvement in median survival as compared with the placebo group [10]. While a correlation was found between the titer of anti-PAP antibodies and patients' survival, no correlation was found with T-cell proliferation to the same antigen.

At difference, GVAX is a vaccine composed by two allogenic cancer cell lines secreting GM-CSF. While results in phase I/II clinical trials were promising [107, 108], phase III clinical trials were terminated for lack of success [109]. Also in this case, only antibody-mediated responses to the vaccine were detected.

A promising vaccine is PROSTVAC-VF that is constituted by two recombinant viral vectors that express PSA and three immune costimulatory molecules. In a phase II study, PROSTVAC-VF improved the median survival of 8.5 months [110]. Like sipuleucel-T, PROSTVAC-VF improved the overall survival, without improving progression-free survival. Since PC is a slowly progressing disease, and the therapeutic effect of vaccination may reside in its long-lived memory response, an objective

response is more likely demonstrated in a long-term clinical study. One might also argue that the clinical results obtained by these immunological approaches are still under expectation. However, the treatments have been administered in advanced patients affected by castration-resistant disease, in which immunosuppressive mechanisms engaged by the tumor are already present. Active immunotherapy is expected to be far more effective in adjuvant settings after surgery and in patients with minimal residual disease.

Despite the consistent number of vaccine trials for PC patients, a few studies have investigated the characteristics of prostate TIL. Unexpectedly, TIL, comprising CD4, CD8 and CD20 lymphocytes, resulted as an independent predictor of short PSA recurrence-free survival in PC patients affected by localized disease [111]. As demonstrated for other tumors [112], this is likely due to a partial characterization of the infiltrate. Indeed, PC lesions may harbor both CD4⁺ and CD8⁺ T cells that either are inactive or may exert immunosuppressive functions [78].

CD4⁺CD25⁺Foxp3⁺ Treg in prostate cancer

There are several subset of Treg that are classified according to their surface markers, and Treg manifest their function through a myriad of mechanisms that include the secretion of immunosuppressive soluble factors such as IL-9, IL-10 and TGFβ cell contact-mediated regulation via the high affinity TCR and other proteins such as cytotoxic T-lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor family related gene (GITR) and cytolytic activity [113, 114]. While in cancer patients, different populations of Treg have been identified that either express or not the master gene regulator Foxp3 [113], the best characterized Treg express CD4, CD25 and Foxp3 [115]. In several animal models, most of which were based on transplantable tumors, CD4⁺Foxp3⁺ Treg cells showed to be essential for tumor immune escape, and their depletion favored tumor rejection or increases overall survival [116]. While early studies in several human tumors showed that the frequency of CD4⁺ Treg negatively correlates with disease aggressiveness and/or prognosis [73], more recent results challenge this conclusion [117].

Miller et al. originally reported an enrichment of CD4⁺CD25⁺ Treg both in peripheral blood and prostate tissue of early-stage PC patients [78]. More recently, Derhovanessian and colleagues showed that the frequency of CD4⁺CD25⁺Foxp3⁺ Treg was significantly higher in hormone-resistant non-bone metastatic PC patients than in age-matched healthy control subjects [105]. Interestingly, the frequency of Th17 cells and not of Treg inversely correlated with time to disease progression, suggesting a tumor-promoting role for the Th17 subpopulation of CD4⁺ T cells [118].

As in PC patients, also in TRAMP mice, Treg accumulate in the prostate and tumor draining lymph nodes during disease progression, and the effects of Treg depletion on PC progression are varied. We have found that neither the transient antibody-mediated depletion of Treg nor inhibition of their functions by cyclophosphamide is able to break tumor T-cell tolerance and impact on tumor progression [82]. Conversely, Drake and colleagues [119] reported that cyclophosphamide administration in TRAMP mice cause a transient depletion of Treg and augmented antitumor immunity. While these conflicting results may depend on the different vaccine and cyclophosphamide treatment schedule used in the two studies, a better definition of the contribution of CD4 Treg in PC would be important for the design of more effective immunotherapeutic treatments.

CD8⁺ regulatory T cells

An intriguing concept that is emerging is the existence both in humans [120] and mice [121] of CD8⁺CD25⁺Foxp3⁺ Treg. These CD8⁺ Treg require IL-10 for expansion, express also CTLA-4, TGFβ, GITR and inhibit mainly through a cell–cell contact mechanism. Similar CD8⁺CD25⁺Foxp3⁺ Treg are present in human PC TIL [104]. Importantly, TLR8 agonists reverse their suppressive function [104]. CD8⁺CD25⁺Foxp3⁺ Treg may express the lymphocyte activation gene-3 (LAG-3), a marker for CD4⁺ Treg in mice, and suppress T cells partly through the secretion of chemokine (C-C motif) ligand 4 (CCL4) [122]. Curiously, LAG-3 is upregulated in antigen-specific CD8⁺ T cells accumulating in the prostate of ProHAXTRAMP mice, and antibody blocking of LAG-3 favors antigen-specific CD8⁺ T-cell expansion in the prostate gland [123]. Hence, it would be interesting to verify whether LAG-3⁺CD8⁺ T cells are Treg.

We have obtained in vitro and in vivo evidence for the existence of immunoregulating CD8⁺ T cells in tumor-bearing TRAMP mice. Indeed, CD8⁺ T splenocytes from tumor-bearing TRAMP mice, but neither those from healthy TRAMP nor wild-type mice, upon adoptive transfer in CD45.1 recipients, impaired the Tag-specific CTL response induced by DC pulsed with Tag-IV (Rigamonti N. et al., manuscript in preparation). Others have recently reported that TCR transgenic CD8⁺ T cells specific for Tag-IV, upon adoptive transfer in tumor-bearing TRAMP mice, become tolerant and acquire an immunoregulatory phenotype. These CD8⁺ Treg are found only in the prostate of TRAMP mice and require a passage within the prostate to acquire their immunosuppressive function [124]. Interestingly and at odds with our findings, they did not find a population of CD8⁺ Treg in the spleen of TRAMP mice. Our hypothesis is that endogenous low-avidity (Rigamonti N et al., manuscript in preparation) and adoptively

transferred high-avidity T cells [124] may behave differently in TRAMP mice. Alternatively, CD8⁺ Treg reside primarily in the prostate and are stochastically found in the spleen. Further studies are needed to better define the role of Treg in PC.

Other cells of the immune system
with immunosuppressive functions in prostate cancer

DC are critical for the generation and maintenance of antitumor immune responses. However, the tumor environment can promote DC differentiation toward an immunosuppressive phenotype. Plasmacytoid dendritic cells (pDC) are a relatively rare DC subpopulation that may also exert immunosuppressive activities. Human pDC express the IL-3 receptor (CD123) and CD304, whereas the murine counterpart expresses B220 and CD11c [125]. It has been recently reported that murine tumor-associated DC (TADC), mainly pDC induce infectious tolerance [126], an *in vivo* process by which immune tolerance is passed on from one cell population to another. More precisely, CD11c⁺B220⁺CD137⁺CD11b⁻ pDC, purified from the prostate of tumor-bearing TRAMP mice, induced tolerance in tumor-specific CTL by activating immunomodulatory pathways mediated by arginase (Arg), indoleamine 2,3-dioxygenase (IDO), TGF β and programmed cell death (PD)-ligand 1 (PD-L1). In turn these tolerant CD8⁺ T cells inhibited the nearby lymphocytes. Watkins et al. [126] identified Foxo3 both in mouse and human TADC as the master regulator responsible for TADC tolerogenic activity. Thus, Foxo3 inactivation could be a promising therapeutic approach to prevent tumor tolerance and enhance cancer immunity by reducing the immunosuppressive activity of TADC.

MDSC are a heterogeneous population of cells of myeloid origin that include immature macrophages, granulocytes, DC and other myeloid cells [21, 127, 128]. In mice, MDSC are characteristically CD11b⁺, express the Gr-1 antigen at different levels, and may also express CD31, IL-4 receptor α -chain, CD115 and CD80 [127, 129]. The phenotype of human MDSC is ill defined, and a population of CD14⁺HLA-DR^{low/-} MDSC cells has been recently observed in the peripheral blood of PC patients that significantly correlate with circulating PSA levels [80]. MDSC may overexpress both inducible nitric oxide synthase (iNOS) and Arg1, enzymes involved in the metabolism of arginine. As reviewed in [21], depletion of arginine from the microenvironment inhibits T-cell activation and proliferation and favors T-cell apoptosis. Furthermore, iNOS produces nitric oxide (NO), which interferes with IL-2 receptor signaling, leading to cell cycle arrest. ROS and peroxynitrites, bioproducts of arginine metabolism, contribute to T-cell inhibition. Of relevance, T cells

infiltrating either human PC or the prostate of TRAMP are functionally impaired and contain high level of nitrotyrosines, suggesting *in loco* peroxynitrites production [103]. We have studied the therapeutic potential of modulators of the arginine metabolism, such as L-NAME and Sildenafil, reported to impair the immunosuppressive activity of MDSC [130], both in the transplantable TRAMP-C1 model and in TRAMP mice [131]. Flow cytometry analysis of the tumor showed a dramatic accumulation of CD11b⁺Gr1^{high} cells in mice bearing TRAMP-C1 tumors when compared with naïve age- and sex-matched littermates. Differently, TRAMP mice affected by PC were characterized by a recruitment of CD11b⁺Gr1⁻ cells rather than double positive CD11b⁺Gr1^{high} MDSC. We have also found that the drugs impaired the immunosuppressive activity of MDSC in both models, but exerted therapeutic effects only in the TRAMP-C1 model. Indeed, both treatments neither broke tumor-specific immune tolerance nor restrained tumor progression in the TRAMP model [131]. Besides the remarkable discrepancy between a transplantable and a spontaneous model of PC that underlines once more the need for an accurate choice of the pre-clinical model, at least in our experimental conditions, arginine depletion seems to cause negligible effect on spontaneous PC progression and the associated T-cell tolerance. It will be important to investigate the effects of novel and more powerful Arg and iNOS inhibitors, such as 3-(aminocarbonyl)furoxan-4-yl]methyl Salicylate [132] in TRAMP mice.

Getting down at the molecular level: PD-1 and CTLA-4 signaling pathways in prostate cancer

The T-cell-mediated response is powerful and wide being able to target a huge variety of antigens, but it must be efficiently regulated in order to avoid autoimmunity. Indeed, T-cell activation requires two signals: engagement of the TCR with specific peptide/MHC complexes, and a second co-stimulatory signal that is mediated by the interaction between the CD28 receptor on the surface of T cells and its counter ligands (CD80 and CD86) provided by professional APC. Co-inhibitory signals, mainly provided by PD-1/PD-L1 and CTLA-4/CD80-CD86 signaling pathways, are present as well, generating a regulatory feedback loop, which avoids excessive immune responses. Similar co-inhibitory signals can be exploited by the tumor to dodge the immune surveillance.

The interaction between PD-1 and its ligands PD-L1 [133] and PD-L2 (B7-DC) [134] is required to establish self-peripheral tolerance since PD-1 knockout mice spontaneously develop autoimmune diseases [135]. Human PC lesions are surrounded by clusters of immune cells

overexpressing PD-1 [136], and PC cell lines and fresh PC specimens express PD-L1 [137]. PD-L1 may also be provided by tumor-infiltrating immune cells [136]. Hence, tumor tolerance might be induced locally by the interaction of PD-1 expressed in T cell, and PD-L1 expressed in TADC presenting the TAA, rather than by direct co-inhibitory signaling provided by tumor cells. In support of this hypothesis, TADC, isolated from the prostate of TRAMP mice, express PD-L1 [126]. In addition, Sfanos et al. [106] have reported that the CD8⁺ T cells infiltrating human PC undergo clonal expansion in response to an unidentified tumor antigen and exhibit an exhausted phenotype also characterized by the expression of PD-1.

PD-L1 seems to be important also for the development and function of CD4⁺Foxp3⁺ Treg [138]. Hence, the PD-L1/PD-1 signaling pathway, in addition to being a cell-intrinsic immunosuppressive mechanism, could favor the local induction/expansion of Treg, and, therefore, act as a cell-extrinsic mechanism in the immunosuppressive tumor microenvironment. Additionally, IFN γ , one of the hallmarks of tumor immunity, can promote the expression of PD-L1 by triggering the production of IFN regulatory factor-1 (IRF-1), a transcription factor that activates the PD-L1 promoter [138]. Thus, PD-L1 and IFN γ signaling are connected by a regulatory feedback loop that may be involved in establishing a mechanism of immune escape in PC. Finally, it has been clearly documented that blocking the PD-1 signaling pathway by specific antibodies increases the tumor-specific immune response and favor tumor rejection in mouse models [139]. In a recent clinical trial designed to assess safety and tolerability of the single agent anti-PD-1 in 39 patients with treatment-refractory solid tumor, among which 8 were PC patients, the treatment was well tolerated, with only one adverse event likely due to autoimmunity [140]. More clinical studies are needed to evaluate the impact of this treatment on tumor-specific immunity and patients' overall survival.

Also CTLA-4 supplies an inhibitory signal in the T-cell compartment by increasing the threshold of signal necessary for full activation [141]. CTLA-4 blockade significantly impacts on the growth of well-established tumors in several transplantable tumor models [142], but it fails in the contest of the less immunogenic TRAMP mice [143].

Of relevance, the FDA has recently approved the use of ipilimumab (anti-CTLA-4 monoclonal antibody) to treat patients with late-stage (metastatic) melanoma [144]. A characteristic of CTLA-4 blockade is that the clinical response often associates with a strong autoimmune reaction, defined as immune breakthrough [144]. The new treatment has been already tested also in 14 hormone-refractory PC patients, in which, however, no immune breakthrough was registered [145], therefore, suggesting

that CTLA-4 alone, as in the TRAMP model [143], might not be effective in advanced PC patients.

CTLA-4 blockade has been proposed also in association with GM-CSF-based immunotherapies [146]. Interestingly, the combination of anti-CTLA-4 with the GVAX platform exerts an effective anti-tumor action in TRAMP mice, resulting in a lower PC incidence and burden [143]. A note of caution has been posed by the finding that in PC patients treated with anti-CTLA-4 and GM-CSF both T effectors and Treg expand, due to proliferation, in a dose-dependent fashion [147]. Two phase III clinical trials are ongoing both in chemotherapy-naïve and chemotherapy-experienced castration-resistant PC patients that are expected to clarify all these issues.

Catabolism of tryptophan: indoleamine 2,3-dioxygenase

Since T lymphocytes undergo proliferation arrest in deficiency of tryptophan, an essential amino acid that cannot be synthesized *de novo*, modulation of the metabolism of this amino acid is an important way to regulate the immune system [148]. The enzyme IDO, also produced by DC [149], controls the immune response by promoting the catabolism of tryptophan and inducing tolerance. An IDO-mediated suppression of T-cell responses is documented in the physiologic control of events such as inhibition of maternal T-cell immunity to fetal tissues during pregnancy, and to avoid autoimmune disorders [148]. IDO can also contribute to the creation of a site of immune privilege in which the tumor is able to grow [15]. Most of the human tumors, among which PC, express IDO, and in transplantable tumor models, expression of IDO prevents tumor rejection since it impairs the accumulation of tumor-specific T cells in the tumor mass [150]. It has been reported an increased expression and activity of IDO in tumor-bearing TRAMP mice when compared with tumor-free TRAMP mice [151], supporting a specific enhancement of IDO activity led by tumor progression. Genetically modified TRAMP mice deficient in IDO production showed a delayed appearance of palpable tumors (Table 1) [151]. We have investigated the effects of 1-methyl-L-tryptophan (1-MT), an IDO inhibitor [150], alone or in combination with Treg depletion by anti-CD25 antibody, in preventing the tumor-associated T-cell tolerance and restraining tumor growth in both transplantable models and in TRAMP mice. While in transplantable models both treatments were alone sufficient to delay tumor growth, in the TRAMP model, neither 1-MT nor anti-CD25 antibodies or their combination impacted on disease progression and T-cell tolerance associated with tumor growth [82]. All together, our data [82] and those reported by Kallberg et al. [151] suggest that IDO has a more relevant direct effect on tumor cells [152],

which is not fully inhibited by 1-MT. IDO induces expression of a novel tryptophan transporter in mouse and human tumor cells that is responsible for 50% of the tryptophan uptake [153]. In a condition of tryptophan deficiency, IDO⁺ cells significantly increase the expression of this novel transporter and increase the tryptophan uptake. If the tumor cells do not express IDO, as in the case of TRAMP IDO^{-/-} mice, it will be less capable of overcoming the detrimental effects of local tryptophan shortage induced by IDO2, a recently discovered IDO isoform that is expressed mainly in sex organs [154], and tryptophan dioxygenase [155]. Thus, the effects of IDO on tumor development and progression might be more complex than what expected by the results reported above, and it will be important to clarify these issues before testing IDO inhibitors in the PC patients.

Conclusions

The tumor is a dynamic tissue that adapts to its microenvironment by selecting more resistant and aggressive cellular clones. Within this context, and especially in a host with a well-established cancer, immunotherapy has an unfair and uphill task. Thus, to successfully translate immunotherapy from the bench to the bedside active and/or adoptive immunotherapies should be combined to the removal of the immune-inhibitory brakes.

The difficulty of trafficking in the tumor mass, and eventually extravasate to get in direct contact with the tumor cells, is an additional hurdle that tumor-specific T cells must overcome. Indeed, tumor vessels are disorganized, tortuous, more branched and leaker than the normal ones [156]. In addition, tumor endothelial cells undergo a phenomenon of anergy, characterized by loss of adhesion molecules [157]. Several strategies have been designed to selectively modify the tumor-associated vessels and, therefore, favor vessel normalization and/or overcome endothelial cell anergy [158]. As an example, minute amounts of TNF, far below the maximal tolerated dose, can be efficiently targeted to tumor vessels by fusing TNF with a peptide sequence that selectively target CD13 on tumor endothelial cells [159]. We have evidence that targeted TNF, besides increasing the penetration and the efficacy of drugs in TRAMP-C1 tumors [160], activates endothelial cells and favors tumor infiltration by CTL with more efficient anti-tumor effects [161].

When the tumor has abundantly spread locally and/or to distant sites, the chances of cure with immunotherapy are dismal. In these cases, immunotherapy should be combined, either in neoadjuvant [162, 163] or adjuvant settings, to powerful tumor debulking approaches [164–166]. As a proof of principle, we have recently reported that in

TRAMP mice affected by advanced PC, non-myeloablative minor histocompatibility mismatched hematopoietic cell transplantation and donor lymphocyte infusion of unmanipulated lymphocytes combined with tumor-specific vaccination support tumor remission and long-term disease-free survival [167]. Interestingly, combined allotransplantation and vaccination, while largely ineffective if provided separately, overcomes functional peripheral T-cell tolerance and associates with a long-lasting tumor-specific memory response [167].

Collectively, these novel combined therapeutic approaches contribute to the Renaissance of cancer immunotherapy.

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Conflict of interest The authors declare that they have no conflict of interest.

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