Regulatory T cells in melanoma: the final hurdle towards effective immunotherapy?

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Immunotherapy studies in patients with melanoma have reported success in the expansion of tumour-specific effector T cells in vivo, but even in the presence of substantial numbers of functional T cells circulating in the blood, favourable clinical outcomes are scarce. This failure to induce robust clinical responses might be related to tumour-induced immune evasion, rendering the host tolerant to melanoma antigens. Immunosuppression in the tumour microenvironment mediated by regulatory T cells (Treg) is a dominant mechanism of tumour immune escape and is a major hurdle for tumour immunotherapy. Accumulation of Treg in melanoma is frequently recorded and the ratio of CD8-positive T cells versus Treg in the tumour microenvironment is predictive for survival of patients with melanoma. Hence, depletion of Treg seems to be a promising strategy for the enhancement of melanoma-specific immunity. Indeed, murine studies have shown that Treg depletion greatly increases the efficacy of immunotherapy. But despite the success of some strategies in depletion of Treg in patients, overall clinical efficacy has been disappointing. The lack of Treg specificity of the Treg depleting strategies applied so far imply that well-designed studies into dosage, timing, and administration regimens with more specific agents are urgently needed. Depletion of functional Treg from the tumour microenvironment as part of multifaceted immunotherapeutic treatments is a major challenge to induce clinically relevant immune responses against melanomas.

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Melanoma and immunity

Worldwide, the incidence of malignant melanoma is steadily increasing, with a growing proportion of patients showing advanced disease for which the outlook is very poor.[1] Melanomas are less sensitive to standard treatment options, such as radiotherapy and chemotherapy, than other cancers. As a result, no therapeutic regimens that greatly prolong survival of patients with melanoma are available.[2] Alternative treatment opportunities or combination cancer therapies that will improve effectiveness are much needed. New developments include targeted therapies reactive against oncogenes such as *BRAF*, which is frequently mutated in patients with melanoma, and immunotherapy-based approaches, which is the focus of our Review.[3,4]

Melanoma is deemed one of the most immunogenic types of cancer for several reasons. First, several melanoma-specific antigens have been identified, such as those expressed by the cancer germline genes *MAGE* and *NY-ESO-1*.[5] Second, large numbers of melanoma-specific antibodies and functional lymphocytes are present in patients with melanoma.[5] Third, metastatic melanoma responds to immune-stimulating agents, such as interferons and interleukin 2.[2] And lastly, spontaneous regression of melanoma with simultaneous onset of vitiligo has been reported.[6] Immunotherapy has therefore had a prominent position in experimental melanoma therapy throughout the past few decades.[7] Experiments in animals have provided compelling evidence that immunity to melanoma with tumour clearance can be induced effectively and can proceed through both antibody-mediated and T-cell-mediated pathways. Despite induction of tumour-specific immune responses in early-phase immunotherapeutic trials in patients with melanoma, favourable clinical outcomes are scarce.[8] The presence of progressive disease despite the occurrence of tumour-infiltrating melanoma-specific T cells is indicative of immune-regulatory mechanisms in the tumour microenvironment.[9]

Treg have an essential role in sustaining self-tolerance and immune homeostasis by suppressing many physiological and pathological immune responses.[10] In both human beings and rodents, these cells infiltrate into the tumour microenvironment and dampen immune responses to tumour cells. Naturally occurring Treg are produced in the thymus and form a functionally distinct T-cell population in the periphery that makes up 5–10% of the CD4-positive T cells in peripheral blood. Treg can be induced in the periphery from naive T cells under certain conditions; they are designated induced Treg. A cardinal feature of both of these subsets is their expression of the transcription factor forkhead box P3 (FOXP3).[11] This transcription factor controls the expression of proteins capable of mediating Treg suppressive function.[12] The naturally occurring Treg use a large unrestricted αβ repertoire specific for a broad range of self antigens, including tumour-associated antigens. Induced Treg display the T-cell receptor repertoire of naive conventional T cells, and their development seems to be stochastic and dependent on chronic antigen exposure.[10] Treg functions depend on activation of T-cell receptors, but the cells exert their effect in an antigen-non specific manner.[13] Although not all of the cellular and molecular mechanisms of Treg-induced immunosuppression are completely understood, various cell contact-dependent and contact-independent mechanisms are known.[14] The functional importance of Treg in a tumour-bearing host is shown in murine models of melanoma in which transient Treg depletion induces antitumour immunity and improves tumour clearance and survival.[15]

For more on **melanoma-specific antigens** see http://www.cancerimmunity.org

	Method (n)	Treg definition	Treg (%)*
Peripheral blood—healthy controls			
Viguier and colleagues (2004)[22]	FCM (10)	CD4+, CD25+	5·8
Gray and colleagues (2003)[19]	FCM (14)	CD4+, CD25+	9·1
Cesana and colleagues (2006)[17]	FCM (16)	CD4+, CD25+	2·4
McCarter and colleagues (2007)[21]	FCM (13)	CD4+, CD25+	6·0
Ahmadzadeh and colleagues (2008)[28]	FCM (12)	CD4+, FoxP3+	4·9
Jandus and colleagues (2008)[20]	FCM (53)	CD4+, FoxP3+	2·9
Correll and colleagues (2010)[18]	FCM (28)	CD4+, FoxP3+, CD25+	5·0
Jacobs JFM (unpublished)	FCM (15)	CD4+, FoxP3+, CD25+, CD127–	3·6
Peripheral blood—metastatic melanoma			
Viguier and colleagues (2004)[22]	FCM (8)	CD4+, CD25+	4·9
Gray and colleagues (2003)[19]	FCM (20)	CD4+, CD25+	16
Cesana and colleagues (2006)[17]	FCM (36)	CD4+, CD25+	7·5
McCarter and colleagues (2007)[21]	FCM (15)	CD4+, CD25+	12
Ahmadzadeh and colleagues (2006, 2008)[16,28]	FCM (24)	CD4+, FoxP3+	7·7
Jandus and colleagues (2008)[20]	FCM (23)	CD4+, FoxP3+	6·9
Correll and colleagues (2010)[18]	FCM (18)	CD4+, FoxP3+, CD25+	8·0
Jacobs JFM (unpublished)	FCM (15)	CD4+, FoxP3+, CD25+, CD127–	4·2
Primary melanoma—cutaneous			
Mourmouras and colleagues (2007)[27]	IHC (50)	CD4+, FoxP3+	8·4
Miracco and colleagues (2010)[25]	IHC (66)	CD25+, FoxP3+	6·7
Ladanyi and colleagues (2010)[23]	IHC (97)	FoxP3+	11·9
Primary melanoma—uveal			
Mougiakakos and colleagues (2010)[26]	IHC (90)	FoxP3+	<5%
Lagouros and colleagues (2009)[24]	IHC (42)	FoxP3+	<5%
Melanoma-draining lymph node			
Jandus and colleagues (2008)[20]	FCM (10)	CD4+, FoxP3+	7·3
Jacobs JFM (unpublished)	FCM (4)	CD4+, FoxP3+, CD25+CD127–	7·2
Melanoma-infiltrated lymph node			
Jandus and colleagues (2008)[20]	FCM (10)	CD4+, FoxP3+	17·4
Jacobs JFM (unpublished)	FCM (4)	CD4+, FoxP3+, CD25+, CD127–	25·8
Melanoma metastasis			
Mourmouras and colleagues (2007)[27]	IHC (26)	CD4+, FoxP3+	4·6
Ahmadzadeh and colleagues (2008)[28]	FCM (26)	CD4+, FoxP3+	25·8
Jandus and colleagues (2008)[20]	FCM (3)	CD4+ FoxP3+	18·3
Jacobs JFM (unpublished)	FCM (3)	CD4+, FoxP3+, CD25+, CD127–	15

n=number of patients. Treg=regulatory T cell. IHC=immunohistochemistry. FCM=flow cytometry. *Treg as % of total CD4+ cells.

Table: **Regulatory T cells in melanoma patients**

The translational relevance of this observation is supported by many reports on Treg recruitment in patients with melanoma.

Treg in patients with melanoma

Several investigations have shown that Treg are over-represented in peripheral blood of patients with metastatic melanoma, compared with age-matched healthy controls (table).[16–28] Treg in patients with melanoma are highly enriched in the tumour micro-environment including primary lesions,[23–27] affected lymph nodes,[20] and metastatic lesions (table).[20,27,28] The Treg in these patients are reported to be functionally immunosuppressive.[20,29,30]

Although sample sizes were small, Curiel and colleagues[31] reported that accumulation of Treg in the tumour microenvironment predicted reduced survival of patients with cancer. Several subsequent studies confirmed the correlation between the extent of Treg infiltration and prognosis in patients with melanoma. All these studies were retrospective and the results are somewhat contradictory. Some researchers reported that a high percentage of Treg in both primary cutaneous melanomas and lymph-node metastases predicted local recurrence and reduced overall survival.[25,32,33] By contrast, other investigators did not find such correlations.[23,34] The variable that best correlates with favourable clinical outcome and survival in patients is the ratio of CD8-positive T cells to Treg cells in the tumour microenvironment.[35] In our opinion, a truly accurate assessment will be possible only if the ratio between functional tumour-infiltrating effector lymphocytes and Treg can be calculated.

If Treg quantities are to be used as a prognostic marker, accurate identification of Treg is imperative. Treg in patients with melanoma have been analysed and quantified ex vivo by various techniques, each of which has advantages and disadvantages (figure 1). Immunohistochemistry can visualise only a small number of Treg markers; however, this technique has the advantage that Treg can be visualised within the tissue context. And the experiments can be done on fixed and stored pathology samples, which facilitates large retrospective studies. However, study of Treg in human beings with immunohistochemistry was complicated by the discovery that activated non-regulatory lymphocytes transiently express both CD25 and FOXP3.[36] Downregulation of the interleukin 7 receptor (CD127) on Treg might distinguish Treg from activated T cells.[37] This lack of a unique Treg-specific marker makes broad phenotypic characterisation of Treg (eg, by multicolour flow cytometry) essential. A specific advantage of flow cytometry is that labelled Treg can be sorted, after which the Treg can be further characterised with microarrays, mass spectrometry, or functional assays. However, the suppressive activity in vivo might be dictated by the tissue environment and might thus not be apparent in

the ex-vivo analyses. Well-designed crossover suppression assays with Treg and effector cells from blood and tumour tissue or with cells from patients versus those from healthy controls will be necessary to provide insight into the regulating capabilities of Treg and the susceptibility of effector T cells to Treg-mediated suppression. A promising novel Treg-specific molecular feature is the demethylation of a conserved region in the *FOXP3* intron 1,[38] which is needed for stable *FOXP3* expression in Treg.[39] This region is completely methylated in other human cells of haemopoietic origin, even after activation. This Treg-identifying element can be quantified by use of a methylation-specific quantitative PCR that enables activated T cells to be distinguished from naturally occurring Treg.

The exact definition of Treg and the technique of Treg analyses greatly affect the result of Treg measurements and thus should be taken into account in meta-analyses (table). In the context of clinical studies, multicolour flow cytometry, combined with molecular analysis of the methylation status of *FOXP3*, is recommended to characterise and quantify Treg. Ideally, cell suspensions should be isolated and analysed from peripheral blood, tumour, and tumour-draining lymph nodes. Additional immunohistochemical investigations can provide qualitative spatial information about Treg in the tissue context. Repeated tumour sampling to monitor the effect of Treg modulating therapies in patients is not feasible. All human studies reported have monitored Treg depletion in peripheral blood, which is a clear limitation because the intratumour ratio of effector CD8-positive T cells to Treg might be a better prognostic marker for treatment efficacy.[35,40,41] Further refinement in the methods to monitor spontaneous and vaccine-induced T-cell responses within functional and molecular studies is awaited to differentiate between tumour-specific T helper cells and Treg.

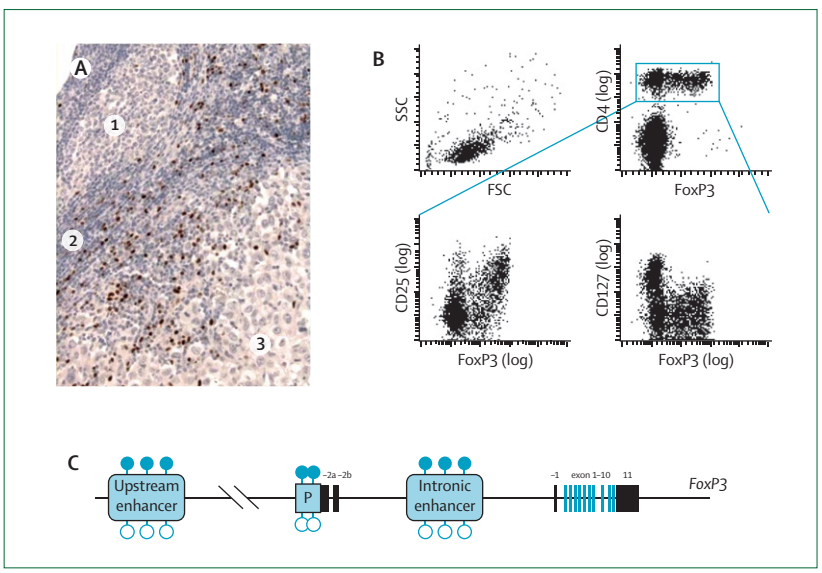

Figure 1: **Regulatory T cell analysis**
A=Immunohistochemistry with FOXP3 antibodies. A lymph node infiltrated by melanoma cells is shown. FOXP3-positive cells do not reside in the B-cell follicles (1) but are exclusively detected in the T-cell areas (2) or deeply infiltrated into the melanoma metastasis (3); B=Multicolour flow cytometric analysis of Treg; C=Gene structure of *FOXP3*. Black bars represent non-coding exons, blue bars represent coding exons. DNA stretches in the *FOXP3* promoter region (P), and in the upstream-enhancers and intronic-enhancers can either have hypermethylated CpG and are transcriptionally inactive (closed circles) or unmethylated CpG (open circles).

Melanoma-specific Treg

The generation and maintenance of Treg to regulate autoimmunity requires the presence of target antigens.[42] Wang and colleagues[43] were the first to isolate Treg that recognised epitopes from the tumour-associated antigen LAGE-1 from patients with melanoma, which provided evidence that this mechanism is relevant in the melanoma setting. Tumour-specific Treg that can recognise a broad range of melanoma-associated antigens circulate in patients with melanoma.[30] Fourcade and colleagues[13] showed that the same melanoma-associated antigens can stimulate both T helper cells and Treg. As a consequence, immunotherapeutic vaccinations with melanoma-associated antigens in patients with melanoma can result in expansion of both induced and naturally occurring melanoma-associated Treg.[44] Naturally occurring Treg and induced Treg contribute independently to tumour-specific tolerance. In mice, induction of antigen-specific Treg from naive cells in the tumour microenvironment does not seem to be intrinsically affected by naturally occurring Treg.[45] At tumour sites, these tumour-specific Treg could have a profound effect on the inhibition of T-cell responses against cancer. Once activated by a specific antigen, Treg suppress responder T cells in an antigen non-specific (so-called bystander) manner.[10] This finding implies that tumour-infiltrating Treg that are activated by one tumour-associated antigen can suppress both vaccine-induced and naturally occurring antitumour immune responses against a broad range of tumour antigens. The relative potency of in-vivo naturally occurring Treg and induced Treg is unclear and could depend on antigen density, T-cell receptor affinity, and the cell type expressing the antigen (eg, tumour cells, tumour stroma, or endothelial cells).

Mechanism of Treg accumulation

The selective accumulation of Treg in the tumour microenvironment suggests that this process is tumour-driven. In theory, four non-mutually exclusive mechanisms could account for Treg accumulation in the tumour microenvironment (figure 2). The dynamic expression of various chemokine receptors and integrins on the cell surface of Treg suggests that selective migration and retention of Treg in vivo is controlled by local chemokine secretion and integrin-ligand expression.[46] Curiel and colleagues[31] showed that CCL22, secreted by ovarian cancer cells and macrophages in the tumour microenvironment,

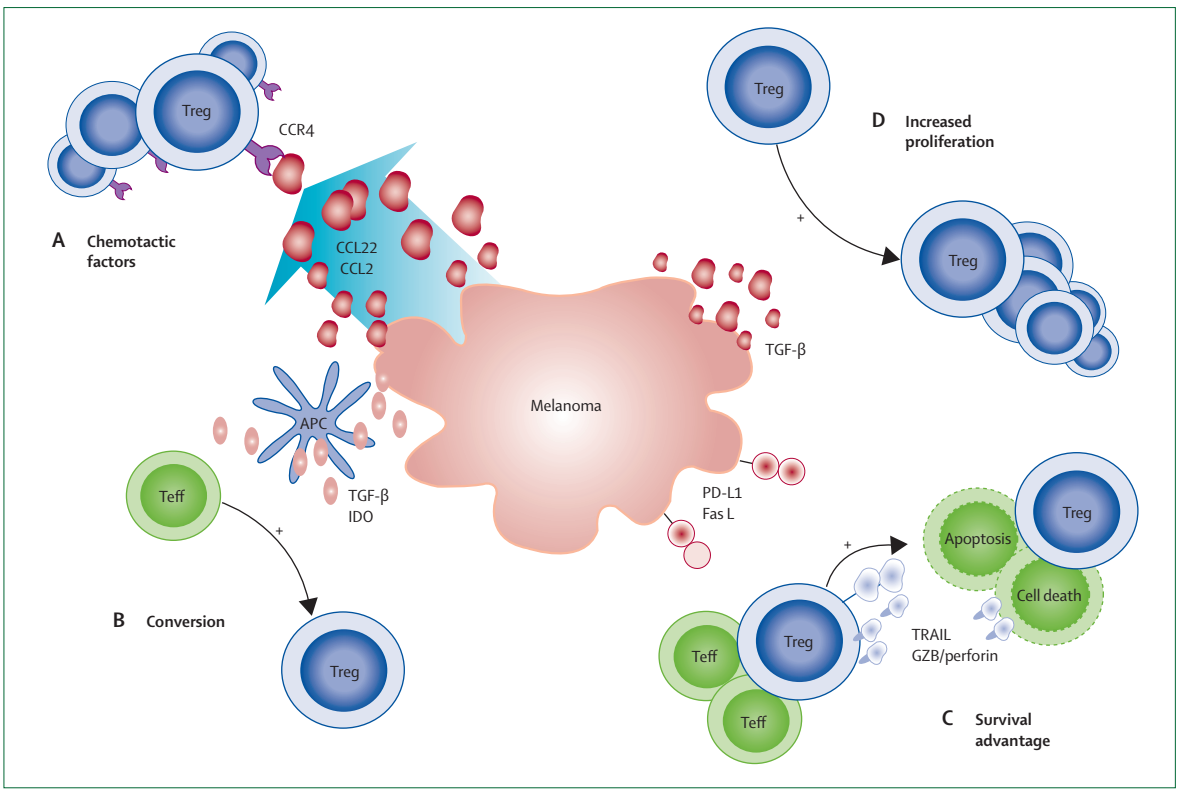

Figure 2: **Mechanisms of intratumoral regulatory T cell accumulation**
A) Chemokine secretion to induce selective migration and retention of Treg that constitutively express high quantities of CCR4 (CCL22, CCL2). B) Secretion of anti-inflammatory mediators that induce conversion from conventional T cells (Teff) to Treg either directly or via antigen-presenting cells (transforming growth factor β, IDO). C) Selective survival advantage of Treg over other tumour-infiltrating lymphocytes occurs when negative costimulatory signals selectively affect the effector T cells (PD-L1, FasL). Additionally, Treg can induce direct receptor-mediated or cytotoxin-mediated Teff depletion. D) Immunosuppressive factors locally secreted by melanoma can cause Treg proliferation (transforming growth factor β). APC=antigen-presenting cell. CCL=chemokine ligand. CCR=chemokine receptor. IDO=indoleamine 2,3-dioxygenase. GRZ-B=granzyme B. PD-L1=programmed death-ligand 1. Teff=effector T cell. TGF=transforming growth factor. TRAIL=TNF-related apoptosis inducing ligand. Treg=regulatory T cell.

induces selective migration of Treg that constitutively express large quantities of CCR4, which is the chemokine receptor that recognises CCL22. Several researchers have shown that the CCL22 signal mediates CCR4^{high} Treg trafficking in other tumours.[46] The CCR4-mediated Treg migration towards melanomas seems to be caused by secretion of the alternative CCR4 ligand CCL2, not CCL22.[47] Because CCR4 is broadly expressed on other immune cells, CCL2 and CCL22 could attract other cells towards the tumour microenvironment and the overall suppressive effect would depend on the balance between Treg and effector immune cells. The possibility that chemokine-mediated Treg migration to tumour tissue could be blocked, by targeting of either the chemokines or their specific receptors, provides opportunities to prevent Treg accumulation in the tumour microenvironment.

Immunosuppressive factors that are locally secreted by melanomas, such as transforming growth factor β and interleukin 10, could promote both expansion of naturally occurring Treg and de-novo generation of induced Treg.[15] Likewise, molecular mechanisms of tumour immune suppression mediated by indoleamine 2,3-dioxygenase (IDO) have a direct anergic effect on effector T cells and enhance local Treg-mediated immunosuppression. Moreover, expression of IDO on tumour-infiltrating antigen-presenting cells stimulates the conversion of conventional T cells to Treg.[48] Upregulation of IDO expression in melanoma lymph-node metastases is associated with an increased number of tumour-infiltrating Treg and consequently shorter survival.[32]

Finally, selective survival of Treg occurs when interactions between cancer cells and effector T cells activate receptor-mediated negative regulatory pathways. Expression of Fas ligand (FasL) and programmed death-ligand 1 (PD-L1) in melanomas causes apoptosis of effector T cells via Fas receptor and PD-1, respectively.[9] A further selective survival advantage occurs when activated Treg induce receptor-mediated and cytotoxin-mediated lysis of effector T cells.[14] Importantly, the dominant mechanism of Treg recruitment might be distinct in different cancer types, or even in different subsets of patients.[46] In this context, blocking of CCL1

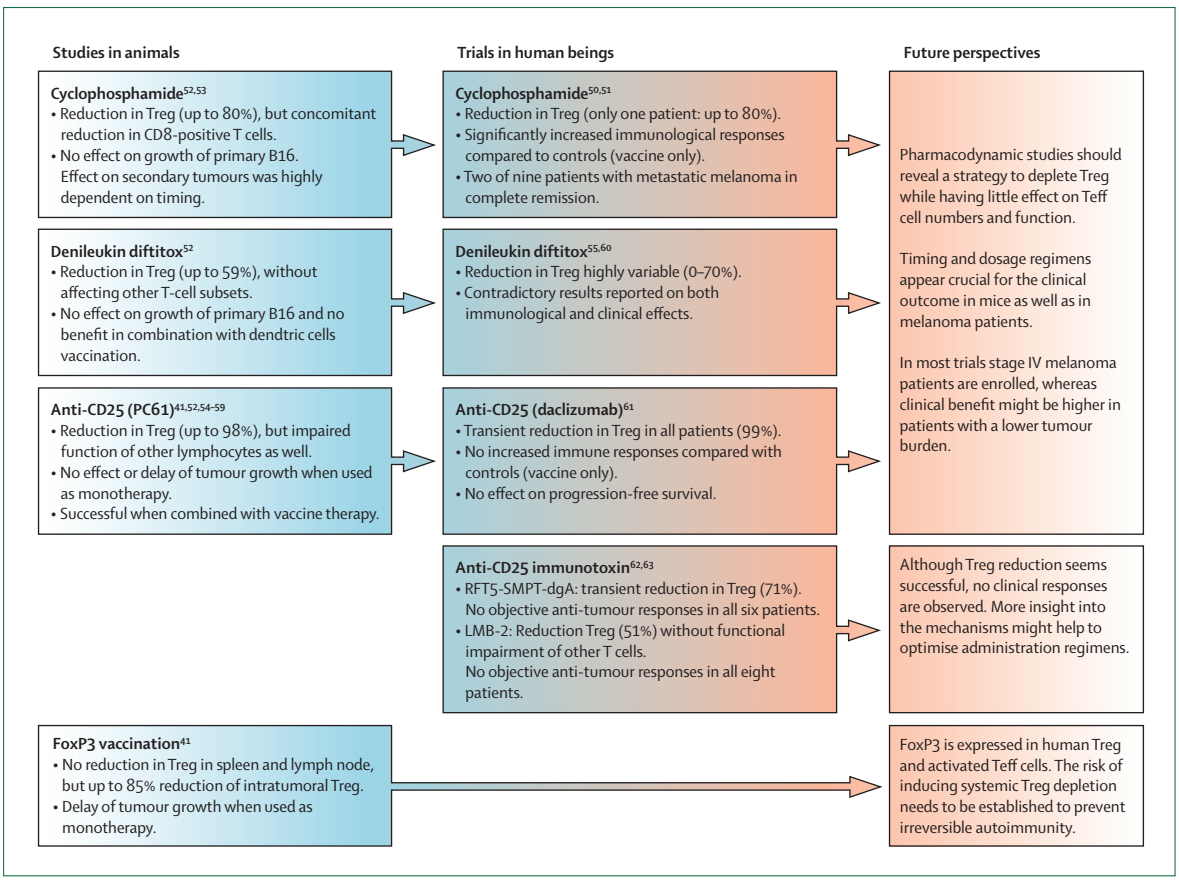

Figure 3: **Regulatory T-cell depleting strategies in melanoma immunotherapy**
Teff=effector T cell. Treg=regulatory T cell.

prevents de-novo conversion and suppressive function of Treg without affecting the function of conventional T cells.[49] Because several of the mechanisms of Treg accumulation discussed here are amenable to pharmacological inhibition, elucidation of the regulation of Treg trafficking in patients with melanoma can provide strategies to modulate intratumoral Treg accumulation in the future.

Treg in melanoma and immunotherapy

Treg-mediated immunosuppression is generally deemed one of the main hurdles for cancer immunotherapy. The ability to modulate selectively Treg in vivo is therefore a potential treatment option to augment antitumour immune responses (figure 3).[15,41,50–63]

Cyclophosphamide

In 1986, cyclophosphamide (a nitrogen mustard alkylating chemotherapeutic agent) was shown to increase delayed-type hypersensitivity responses; and it elicited clinical responses in (two of eight) patients with melanoma vaccinated with autologous melanoma cells.[50] The initial conclusion that cyclophosphamide inhibits so-called T-suppressor activity was confirmed by Ghiringhelli and colleagues[51] who showed that the agent reduces Treg numbers, leading to a restoration of peripheral T-cell proliferation and innate killing activities. The specificity of cyclophosphamide treatment seems to be highly controlled by the dose and timing of administration. Metronomic cyclophosphamide treatment selectively deleted Treg while preserving other lymphocytes in number and function. This selectivity was lost in patients receiving a high-dose regimen (200 mg/day).[51]

In preclinical models, cyclophosphamide did not affect the primary growth of B16 melanoma but greatly improved antitumour immunity after presensitisation.[52,53] The elicitation of concomitant immunity against a secondary tumour challenge strongly depended on the timing of the administration of the drug in relation to the inoculation of the primary tumour.[53] Hence, administration of cyclophosphamide as a monotherapy seems rather inefficient but it could improve antitumour immunity more effectively in combination therapy. Dudley and colleagues[64] reported 50–70% response rates in patients with metastatic melanoma when cyclophosphamide was used in combination with fludarabine phosphate and total-body irradiation for lympho-depletion before adoptive T-cell transfer. The success of

CD8-positive T-cell transfer might partly be related to the modulating effects of cyclophosphamide, but also of fludarabine[65] and total-body irradiation treatments.[66] In-depth studies in patients receiving this type of combination treatment are needed to show the extent to which Treg modulation can contribute to the reported effects.

Interleukin-2-based therapies

Interleukin 2 is an essential cytokine for the homeostasis, activation, proliferation, and survival of effector cells, particularly T cells. Studies in mice deficient for interleukin 2 or interleukin-2 receptor showed that interleukin-2 signalling is crucial for the generation, survival, and suppressive ability of Treg.[67] Moreover, immunotherapeutic administration of interleukin 2 significantly increased the number of Treg in patients with melanoma.[16,17] The interleukin-2 receptor α-chain, CD25, is constitutively expressed by Treg. Targeting of CD25 therefore seemed a promising strategy to modulate Treg numbers and function in vivo. Three major therapeutic options have been developed to target CD25: anti-CD25 depleting monoclonal antibodies, a fusion product of interleukin-2 protein and a toxin (denileukin diftitox), and CD25-directed immunotoxins.

Anti-CD25 antibody

In an experiment with cell-depleting anti-CD25 monoclonal antibodies in mice, prophylactic administration of the antibodies eradicated syngeneic tumours (leukaemia, sarcoma, myeloma) thus showing the role of Treg in tumour immunity.[54] Anti-CD25 monoclonal antibodies efficiently depleted CD4-positive, CD25^{high} Treg in preclinical models for poor immunogenic and malignant melanoma (B16), but did not affect or only delayed the growth of primary (parental) melanomas unless very high doses were used.[55] However, tumour rejection was much improved when anti-CD25 treatment was combined with dendritic-cell vaccinations,[41,52,55] cytotoxic T-lymphocyte antigen 4 (CTLA-4) blockade,[56] stimulation of glucocorticoid-induced tumour necrosis factor receptor family related protein (GITR),[57] or exposure to interferon γ,[58] or interleukin-12-transfected B16.[59]

CD25 is expressed not only by Treg cells but also by antigen-experienced CD4-positive and CD8-positive T cells, which implicates the simultaneous deletion of effector T cells. Although some researchers report no reduction in CD8-positive T cell numbers,[52] others conclude that anti-CD25 treatment resulted in the elimination of protective T cells that were elicited by the additional immunotherapeutic treatment of B16-bearing mice.[41,56] In fact, these studies showed that anti-CD25 monoclonal antibody treatment before a rechallenge in pre-vaccinated mice diminished the protective effect of vaccination.

We have confirmed the efficient depletion of CD4-positive, CD25^{high} Treg (up to 99%) in patients with metastatic melanoma who were pretreated with humanised antibodies against CD25 (daclizumab) before being vaccinated with peptide-pulsed dendritic cells.[61] Patients received a single low dose (0·5 mg/kg) of daclizumab to specifically target Treg and to minimise modulation of effector T cells. This regimen was chosen instead of high-dose treatment (1·0 mg/kg) every 2 weeks, which was particularly designed to target effector lymphocytes to prevent acute graft rejection after transplantation and additionally proved effective for the treatment of patients with autoimmune disease or leukaemia. Although we did not record an effect on the presence of antigen-specific T cells, daclizumab prevented CD25-positive T cells from acquiring effector functions and inhibited the generation of specific antibodies after peptide-pulsed dendritic-cell vaccinations. Most importantly, daclizumab had no significant effect on progression-free survival, which is by sharp contrast with the animal studies we have discussed. One other clinical study has reported the use of daclizumab (at a lower dose) in patients with cancer to deplete Treg before peptide vaccination.[68] An important point will be whether the reported hTERT and survivin-specific T cells that are elicited have effector functions. The timing of Treg deletion relative to the kinetics and the strength of the induced effector responses in itself seems crucial for the efficacy of anti-CD25 administrations.

Denileukin diftitox

In 1999, the US Food and Drug Administration approved the anti-neoplastic agent denileukin diftitox for the treatment of patients with cutaneous T-cell lymphoma whose malignant cells express CD25. This agent is a fusion protein consisting of a fragment of diphtheria toxin genetically fused to interleukin 2. It became an interesting compound to directly target CD25 on Treg. Intravenous infusion of the drug significantly reduced Treg in peripheral blood (26–76%) of patients with renal-cell carcinoma and increased the numbers of CD4-positive and CD8-positive T cells producing interferon γ after vaccination with RNA-transfected dendritic cells.[69] However, clinical trials in patients with melanoma report variable success in Treg-depletion and clinical efficacy. The first report showed a minimal decrease in Treg numbers after denileukin diftitox administration and no objective responses were recorded.[60] Other investigators reported a significant reduction in Treg and showed the presence of peptide-specific CD8-positive T cells in five of six patients after peptide immunisations and even regression of melanoma metastases in five of 16 patients.[15] These researchers did not agree about the effects of denileukin diftitox administration on other lymphocytes because one reported a transient reduction whereas the other claimed that other cell populations were

unaffected.[55] The latter findings are in agreement with results obtained with the murine B16 melanoma model in which prophylactic Treg depletion failed to enhance immunity against B16 tumours.[52]

Cells expressing the high-affinity interleukin-2 receptor, consisting of a trimer of the α-subunit (CD25), β-subunit (CD122), and γ-subunit (CD132) are most susceptible to the effects of denileukin diftitox.[70] This drug can bind to each subunit, but binding only to the intermediate and high-affinity receptor complex results in internalisation, which is required for its toxic effects to be exerted. This requirement, combined with the very short half-life of denileukin diftitox (70–80 min), implies that the timing of administration relative to the dynamics and kinetics of CD25 expression in Treg versus effector lymphocytes strongly dictates its efficacy in cancer immunotherapy.

CD25-directed immunotoxin

Powell and colleagues[62,63] reported two trials exploring the administration of immunotoxins linked to CD25 antibodies: RFT5-SMPT-dgIgA, CD25-specific murine IgG1 antibody (RFT5) linked to a deglycosylated ricin A chain (dgA), and LMB-2, a fusion of a single-chain Fγ fragment of the CD25-specific, anti-Tac monoclonal antibody to a truncated form of the bacterial pseudomonas exotoxin A. Although RFT5-SMPT-dgA significantly reduced the numbers of CD4-positive $CD25^{high}$ (by 98%) and CD4-positive, FOXP3-positive (by 71%) cells, one trial was stopped prematurely after treatment of six patients because the elimination of Treg was not judged complete.[63] CD4-positive $CD25^{high}$ and CD4-positive, FOXP3-positive Treg were also eradicated by LMB-2, 79% and 51%, respectively, but in this study no enhancement of immune responses to peptide vaccinations or clinical objective responses were recorded in patients with metastatic melanoma.[62] LMB-2 has an especially short half-life and thereby would reduce the possibility of elimination of activated effector lymphocytes. Whether the lack of immune responses is attributable to an incomplete reduction in Treg or to the failure of the peptide vaccine to induce antigen-specific immune activation is unclear.

Overall, most CD25-targeted methods are able to greatly reduce the numbers of Treg circulating in the blood of patients with melanoma but display variable results for the impact on effector lymphocytes. Clearly, the degree of the reduction in Treg is not straightforwardly indicative of the clinical efficacy of the treatment, and Treg-depleting strategies might be of value for patients with cancer only when combined with immune therapeutic regimens to increase expansion and function of effector T cells. The kinetics of CD25 expression on effector lymphocytes and possibly other cells, such as dendritic cells, could be an important factor determining failure or success of CD25-targeted therapy. The ratio between the Treg and antigen-specific effector cells in the tumour microenvironment might hence be decisive for net efficacy of the combination treatment. How the optimum dose for each individual patient should be determined is difficult to envisage, which restricts the use of anti-CD25 targeting strategies as an effective therapy. Strategies that exclusively target Treg and preferentially deplete Treg before T-cell priming or boosting existing T-effector cells could overcome the adverse effects of CD25 targeting.

FOXP3 vaccination

The discovery of FOXP3 as a crucial transcription factor for the development and function of Treg suggested the elimination of FOXP3-positive cells as a means to delete Treg more specifically and enhance immune responses against cancer. Nair and colleagues[41] postulated that vaccination against FOXP3 would stimulate the generation of specific cytotoxic T lymphocytes that would eliminate all cells expressing FOXP3 peptide MHC class I complexes. Although vaccination of melanoma-bearing mice with *FoxP3* mRNA-transfected dendritic cells did not affect the numbers of *FoxP3*-expressing cells in lymph nodes or spleen, it reduced intratumoral *FoxP3*-expressing Treg by up to 85%.[41] This local elimination of Treg enhanced TRP2-specific cytotoxic T-lymphocyte responses after co-vaccination with TRP2-loaded dendritic cells, such that they were much the same level as recorded for anti-CD25 monoclonal antibody administrations. Why Treg depletion is limited to the tumour site is unclear. One explanation could be that tumour sites contain more activated Treg and can thus be regarded as an accumulation of target antigen for the FOXP3-specific cytotoxic T lymphocytes. Although preferential deletion of activated tumour Treg seems beneficial, whether this afflicts adverse effects in responses where activated Treg are needed to limit damage to self and restore homoeostasis remains to be seen. Moreover, the expression of *FOXP3* in activated effector T cells in human beings could restrict the application in patients. If the preferential deletion of tumour-Treg is indeed caused by the high level expression of *FOXP3* by activated intratumoral Treg, deletion of activated T effector cells would be unlikely.

Treg-modulating strategies

Other molecules have been identified on the surface of Treg, such as GITR, CTLA-4, CD103 or LAG3, and OΩ40, but like CD25, they are not exclusively expressed on Treg.

Cytotoxic T lymphocyte-associated antigen 4

The inhibitory molecule CTLA-4 is constitutively expressed by CD4-positive, CD25-positive Treg and represents one of the mechanisms by which to restrict T-cell proliferation and effector function. Ligation of CTLA-4 on Treg to B7 molecules on the surface of

antigen-presenting cells has been reported to induce IDO, which affects the potency of these antigen-presenting cells to activate other T cells. CTLA-4 is likewise expressed by activated conventional T cells, and its ligation restricts T-cell proliferation and activation. Blocking of antibodies directed against CTLA-4 therefore not only restricts the regulation of dendritic-cell function by Treg but also abrogates suppression of conventional T-cell proliferation after activation.[3] Anti-CTLA-4 increased cytotoxic T lymphocyte infiltration and ratios of CD8-positive to Treg in mice when combined with the GM-CSF-transduced B16/BL6 cell vaccine Gvax.[35] However, chronic exposure caused increased numbers of Treg in lymph nodes of these animals. By contrast, anti-CTLA-4 (tremelimumab, Pfizer, UK) reduced Treg numbers in patients with stage IV melanoma and restored T-cell-receptor dependent T-cell proliferation, which in turn became resistant to Treg-mediated suppression. These responses were related to progression-free survival in seven of ten patients with stage IV melanoma.[3] Importantly, the results of a randomised trial showed an increase in overall survival after anti-CTLA-4 treatment (ipilimumab) with or without additional gp100 peptide vaccine in a large cohort of 676 patients with previously treated metastatic melanoma.[71] This study shows that immune checkpoint modulation provides an important therapeutic strategy against cancer. These results foster in-depth studies into the mechanism and potential biomarkers predicting successful therapy to optimise the clinical efficacy of anti-CTLA-4 treatment.

Glucocorticoid-induced tumour necrosis factor receptor

GITR is a type I transmembrane protein constitutively expressed by Treg and upregulated by activated CD4-positive and CD8-positive T cells. GITR stimulation is of particular interest for cancer immunotherapy because it directly inhibits Treg suppression while potentiating responses of CD4-positive and CD8-positive T cells.[72] Anti-GITR agonist induced the proliferation of Treg in vitro and in spleens of B16-bearing mice, while intratumour Treg accumulation was significantly impaired by alteration of Treg stability (loss of *FoxP3* expression) resulting in a greater effector T cell to Treg ratio.[40,57] By contrast with anti-CD25 treatments, GITR stimulation was effective as a monotherapy in eradicating primary B16 but seems more effective after initial priming of the immune response (probably through up-regulation of GITR on effector T cells).[17,40,53,57] Treatment with anti-CD25 combined with GITR stimulation resulted in optimum antitumour immunity, emphasising the potency of Treg deletion in combination immunotherapy.[57] The multimodal targeting of anti-GITR seems a promising strategy in mice but studies exploring the potency of GITR stimulation in patients with melanoma are not available.

Programmed death 1

The transmembrane protein PD-1 is a member of the CD28/CTLA-4 family that is inducibly expressed on CD4-positive (including Treg), CD8-positive, and natural killer T cells, B cells, and monocytes, and negatively regulates T-cell-receptor signals. Monoclonal antibodies that block PD-1 have a dual effect because they bind to the protein on (melanoma-specific) T effector cells, directly downregulating T-cell activation, and on Treg, which reduces Treg suppressive activity.[73] A phase 1 single-agent trial with an anti-PD-1 blocking monoclonal antibody is associated with evidence of antitumoral activity in patients with metastatic melanoma,[74] again suggesting the possible potency of multimodal targeting strategies.

Future perspectives

The synergistic beneficial effect of Treg depletion combined with therapeutic cancer vaccines as observed in murine studies has not been convincingly shown in patients with melanoma. In a multicentre trial we compared the efficiency of three potentially Treg-depleting agents given to patients with melanoma by measuring naturally occurring Treg frequencies in blood with a molecular assay. Our results show that neither daclizumab, nor low-dose cyclophosphamide or denileukin diftitox induced a decrease of more than 50% in blood Treg in most patients.[75] A major obstacle for specific Treg depletion is the lack of well-defined Treg-specific cell-surface markers. One of the most specific Treg markers, FOXP3, is intracellular, so it is difficult to target. The currently targeted Treg-associated surface markers are shared by activated lymphocytes, so these strategies lack specificity. Accurate timing and dosing of the Treg-depleting compound seem crucial in finding the optimum balance between Treg depletion and unwanted effects on activated lymphocytes.

Another line of research focuses on compounds that attenuate the suppressive function of Treg and simultaneously expand effector T cells or augment their effector activity, such as monoclonal antibodies that block CTLA-4 and PD-1, and monoclonal antibodies that are agonists to GITR and OX40.[15,76] Proof-of-concept studies assess the potential clinical benefit of these compounds in patients with melanoma are ongoing. Furthermore, strategies preventing Treg migration to the tumour site or blocking conversion of conventional T cells into a Treg phenotype are being explored.

Treg cells retain an unexpected degree of phenotypic plasticity and display different signalling characteristics than those observed in conventional T cells. Under certain conditions, Treg might lose their suppressor phenotype and become reprogrammed into pro-inflammatory effector cells (producing interferon γ and interleukin 17).[77,78] Future research will focus on whether reprogrammed Treg have a functional role in antitumour immunity.[79]

Search strategy and selection criteria

Data for this Review were identified by searches of PubMed with the search terms "melanoma", "immunotherapy", and "regulatory T cell". References from relevant articles indentified by this strategy were included. Abstracts and reports from meetings were included only when they related directly to previously published work. Only papers published in English were included, without additional limitations. The last search was done in February, 2011.

Conclusions

Accumulation of Treg in patients with melanoma is a major factor in tumour immune escape. As a result, targeting of these cells could provide a mechanism by which antitumour immune function can be restored. In general terms, most of the immunotherapeutic strategies that target Treg in animal studies seem to shift the immune balance in favour of effective anti-cancer immune responses. Up to now, these interventions have not completely fulfilled their promise in human studies. Effective melanoma treatment will depend on a multimodal approach in which immunotherapy is combined with targeted therapy to both stimulate immune effector activity and down modulate the suppressive effects of Treg.

Contributors

JFMJ and SN did the literature searches. All authors contributed to the design, writing, and editing of the Review, and approved the final version.

Conflicts of interest

We declare that we have no conflicts of interest.

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