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Targeting Regulatory T cells in Cancer

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Abstract

Infiltration of tumours by regulatory T cells confers growth and metastatic advantages by inhibiting anti-tumour immunity and by production of RANK ligand, which may directly stimulate metastatic propagation of RANK-expressing cancer cells. Modulation of regulatory T cells can enhance the efficacy of cancer immunotherapy. Strategies include depletion, interference with function, inhibition of tumoural migration and exploitation of T cell plasticity. Problems with these strategies include a lack of specificity, resulting in depletion of anti-tumour effector T cells or global interruption of regulatory T cells, which may predispose to autoimmune diseases. Emerging technologies such as RNA interference and tetramer-based targeting may have the potential to improve selectivity and efficacy.

Keywords

Regulatory T cell; Cancer; Targeted Therapy; Immunotherapy; Tumour Immunology

Introduction

There is renewed optimism that many cancers can be cured or forestalled by immune-based therapies, used either alone or as part of multimodal programmes. This originates from an improved understanding of tumour immune interactions and the availability of gene, cell and ligand-based technologies which promote effector anti-tumour responses. Most tumours develop in the face of normal immune function and anti-tumour responses of varying strength result. A strong immune response against the primary tumour is associated with clearance and induced dormancy of metastatic cancer cells, with a resulting enhanced prognosis. Conversely, global immune deficiencies secondary to disease or therapy are associated with an increased frequency, earlier recurrence, more rapid progression of tumours and poorer prognosis. Responses to chemotherapy and oncolytic virotherapy may in part be immune-determined and there is persuasive evidence that an intact immune system,

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specifically determined by $CD4^+$ T cells, is required for sustained tumour regression following oncogene inactivation therapies (1).

Adaptive anti-tumour immune responses are durable, tumour antigen-specific and acquired through the integrated intercellular responses of the innate and adaptive immune systems (2) (figure 1, centre). Tumour infiltrating T cells, especially $CD8^+$ cytotoxic T lymphocytes (CTLs) and IFNγ-secreting $CD4^+$ (Th1) cells, are central to effective immune containment. Adaptive immune responses are initiated when cells of the innate immune system (NKT, γδ T, NK and macrophages) are recruited to the tumour microenvironment - the continued process of tumour remodelling results in the shedding of cancer cells and debris with a consequent induction of inflammatory signals. The production of IFN-γ (initially from NK and NKT cells) appears critical as it creates a positive feedback loop by inducing some tumour cell death, the further activation of NK cells and macrophages and the production of chemokines and cytokines which are also tumouricidal and anti-angiogenic. Immature dendritic cells (DCs) are activated following uptake of tumour debris/antigens and migrate to the regional lymph nodes where they present the tumour antigens to naive T cells, which can differentiate into Th1, Th2, Th17 or regulatory T cells (T_{Regs}) depending on the cytokine environment. Th1 cells can license DCs to induce tumour-specific CTLs by cross presentation of antigen on MHC class I. Antigen-specific $CD8^+$ T cells traffic to the tumour where cell-mediated killing of tumour cells is augmented by Th1 and Th17 derived cytokines. However these effector responses can be inhibited by T_{Regs}, induced by or recruited to the growing tumour (3).

Immune evasion is a hallmark of cancer that results from both passive and active tolerising conditions that subvert anti-tumour immune responses (4). Passive tolerisation may result from down-regulation of MHC Class I expression on the tumour cells and/or low antigenicity secondary to immune editing and selective cell growth. Other tolerising mechanisms involve inhibition of immune cells in the tumour domain by depletion of tryptophan by the enzyme 2, 3 indoleamine dioxygenase (IDO). Active tolerisation involves suppression of anti-tumour cell-mediated responses by tumour infiltrating T_{Regs} and myeloid derived suppressor cells (5).

Overall T_{Regs} are considered to be the most powerful inhibitors of anti-tumour immunity and the greatest barrier to successful immunotherapy (6). In the early stages of cancer T_{Regs} are concentrated in the tumour mass, resulting in concomitant immunity, whereby the primary tumour can progress due to local inhibition of effector immune responses, but metastatic cells are eliminated by uninhibited systemic anti-tumour immune responses. In advanced stage disease or for poorly immunogenic cancers there are increased T_{Regs} systemically and absence of concomitant anti-tumour immunity (7). While a correlation between increased T_{Reg} number and survival, either negative or positive, remains equivocal, the ratio of T_{Reg} to $T_{effector}$ cells in the tumour mass seems to have greater prognostic significance (8).

There are a number of subtypes of T_{Reg} (8), including natural $CD4^+$ T_{Regs} (nT_{Regs}) which originate in the thymus, express CD25, FOXP3, CTLA-4, LAG3 and GITR and suppress innate and adaptive immune cells. Induced $CD4^+$ T_{Regs} (iT_{Regs}) control immune responses

to tissue antigens, including tumour antigens and include $CD4^+$ nT_{Reg}-like, Tr1 and Th3 cells that suppress through production of IL-10 and TGF-β. The iT_{Regs} develop in the periphery following engagement of the TCR of naive T cells and under the influence of innate IL-10 and TGF-β. Their cell-surface markers are often indistinguishable from those of nT_{Regs} and they differ principally in their mechanism of suppression. Although less well characterised, there are also populations of natural and induced $CD8^+$ T_{Regs}.

While the field is still in its infancy, evidence is emerging that inhibition of T_{Regs} may help in tumour containment, especially when combined with appropriate immunotherapies that activate effector T cells. Systemic T_{Reg} depletion in patients induced regression of melanoma metastases (9) and in mice when combined with immunogene stimulation of intratumoural immune effector cells resulted in cure of 90% of animals who had large and weakly immunogenic sarcomas (10). The clinical objective will be to provide sustained reduction of T_{Reg} function, particularly in the tumour environment, allowing enhancement of anti-tumour effector functions and with minimal risk of developing systemic autoimmune diseases.

Current approaches to T_{Reg} modulation

Regulatory T cell depletion (figure 1 A)

Depletion strategies are not T cell subset-specific but have a selective advantage when the T_{Reg} accumulation provides functional dominance in the tumour environment. T_{Reg} depletion strategies have focused on monoclonal antibodies or ligand-directed toxins targeted to a T_{Reg} cell surface receptor such as CD25. Daclizumab and basiliximab are anti-CD25 antibodies which invoke cell death by cytokine deprivation (IL-2) and also by triggering ADCC or CDC. Results from an ongoing clinical trial have shown that Daclizumab reduces T_{Regs} and thereby enhances cytotoxic T lymphocyte responses to tumour antigen induced by vaccination (11).

Denileukin diftitox (Ontak®) is a fusion protein of human IL-2 and the enzymatically active and membrane-translocating domains of diphtheria toxin. After binding to CD25 and internalisation, release of the toxin is cytocidal. Clinical data on the use of Ontak® for alternative indications has led to its application for CD25 targeting of T_{Regs} and the emergence of similar CD25-targeted immunotoxins LMB-2 and RFT5-SMPT-dgA. With one exception, Ontak® depleted T_{Reg} numbers, albeit transiently, with T_{Reg} nadirs persisting for less than 3 weeks (11). The T_{Reg} elimination was mirrored by a concomitant increase in the prevalence of IFN-γ^+CD3^+ T-cells in the blood and *de novo* appearance of melanoma antigen-specific $CD8^+$ T cells (9). However the clinical benefits were modest. Regression of melanoma metastases in five out of sixteen patients represents the most promising outcome (9). Consistency of response is an issue as two patients who developed antigen-specific T cells failed to show any tumour regression and another study in melanoma patients failed to yield a single objective clinical response (11).

Ontak® is the subject of numerous clinical trials but to date fails to realise its clinical promise. Since CD25 is also expressed on activated $T_{effector}$ cells, Ontak® may also restrain protective anti-tumour immune responses. Ontak® transiently depleted various T subsets

including tumour antigen-specific $CD8^+$ T cells (9). Its indiscriminate effects on $CD4^+CD25^-$ cells are difficult to rationalise.

Low-dose oral metronomic cyclophosphamide induced a profound, selective reduction in T_{Regs} and restored T and NK cell function in advanced cancer patients (12). This invoked temporary disease stabilisation in a number of patients without clinical improvement. The mechanism underpinning its selective toxicity towards T_{Regs} is unexplained. Metronomic cyclophosphamide also has anti-angiogenic and direct cytotoxic effects, which contribute to tumour stabilisation or shrinkage.

Depleting T_{Regs} may have further consequences aside from an unintended treatment-mediated elimination of activated $T_{effector}$ cells (13). Their depletion leads to an increase in tumour-mediated $T_{effector}$ to T_{Reg} conversion with a diminution in anti-tumour immune responses. This does not seem to occur with the other T_{Reg} modulation approaches.

Suppression of T_{Reg} function (figure 1 B)

Similar to CD25, CTLA-4 is not exclusively expressed on T_{Regs} – it is also found on activated $CD4^+$ and $CD8^+$ T cells (8). CTLA-4 inhibits antigen priming of $T_{effectors}$ by competing with CD28 for the costimulation of CD80/CD86 on APCs. Furthermore, it induces IDO in DCs (14). The consequent depletion of tryptophan and production of tryptophan metabolites, such as kynurenines and picolinic acid, inhibit $T_{effector}$ proliferation and function. The anti-CTLA-4 antibodies ipilimumab (MDX-010) and tremelimumab (CP-675206) are currently undergoing clinical evaluation. Ipilimumab, as monotherapy or in combination with peptide vaccination improved survival in patients with previously treated metastatic melanoma (15). Tremelimumab promotes anti-tumour responses but recently these have been shown to result from $T_{effector}$ activation rather than T_{Reg} modulation (16). This may also be true of Ipilimumab as its clinical mode of action has yet to be fully defined and could be ascribed to direct effects on either $T_{Regs,}$ $T_{effectors}$ or a combination.

The glucocorticoid-induced TNF receptor (GITR) is constitutively expressed on T_{Regs} but also at lower levels on activated $T_{effectors}$. Intratumoural injection of an agonistic antibody to GITR (DTA-1) invoked potent anti-tumour immunity and eradicated established tumours in mice (17). The exact mechanism by which this approach achieves its effects is controversial. One study showed that the benefit of DTA-1 was T_{Reg}-mediated, facilitated by their selective modulation (18). However a more recent study suggested $T_{effector}$ costimulation as the predominant outcome (19). Regardless of mechanism of action, GITR approaches have yet to recapitulate these promising findings in humans. Receptor activator of nuclear factor-kB (RANK) ligand (RANKL) expression on T_{Regs} engages the RANK receptor on cancer cells and promotes metastases (20). Inhibitors of RANK signalling, such as the anti-RANKL antibody denosumab, already used against osteoclastic-mediated bone resorption, may block direct T_{Reg}-induced metastases of certain cancers.

Targeting FOXP3, the essential transcription factor of T_{Regs}, by RNA interference (RNAi) could also modulate their function. Lentiviral-mediated delivery of miR-31 (a negative regulator of FOXP3) to T_{Regs} abolished their suppressor capability (21). Translation to clinical application is challenging, as miR-31 would need to be delivered specifically to

T_{Regs} because FOXP3 is also transiently expressed on activated human $T_{effectors}$. FOXP3 is also expressed (both mRNA and protein) in numerous cancer cell lines (22) but the effects of its down-regulation are unknown and could even be counterproductive.

Further options for disrupting T_{Reg} function include Toll-like receptor (TLR) modulation, OX40 stimulation or interference with the adenosinergic pathway. Exposure of T_{Regs} to the TLR8 ligand, poli-G10 abolished their suppressive influence on $CD8^+$ T cells, leading to improved anti-tumour immunity (23). More recently a synthetic TLR1/TLR2 agonist, an analogue of bacterial lipoprotein, mediated a dose-dependent tumour regression and a long-lasting protective response against tumour rechallenge through a reciprocal downregulation of T_{Regs} and upregulation of CTL function (24). These findings suggest that TLR signaling is a worthwhile pursuit but caution is advised as TLR agonists can promote regulatory as well as effector responses (25). Stimulation of OX40 (a co-stimulatory member of the TNF receptor family) inhibits the suppressive function of T_{Regs} *in vitro* (by downregulation of FOXP3) and abolishes protection against graft-versus-host disease in mice (13). The paradoxical stimulatory effects on $T_{effectors}$ make it an enticing target for cancer immunotherapy. Another potential target on T_{Regs} is ectonucleotidase activity which facilitates local generation of adenosine which has immunosuppressive capability. Ectoenzyme inhibitors such as ARL67156 and other modulators of the adenosinergic pathway, such as inhibitors of the A2A adenosine receptor, have been shown to block T_{Reg}-induced immunosuppression (26).

Disrupting Tumoural Homing of Regulatory T Cells (figure 1 C)

Chemokine-chemokine receptor and integrin-integrin ligand interactions attract T_{Regs} to the tumour, a phenomenon first observed for the CCL22-CCR4 interaction in ovarian cancer (27). Importantly CCL22 expression was not confined to tumour cells but also included bystander cells such as tumour-associated macrophages. Further chemokines/integrins have been implicated in the selective recruitment and retention of T_{Regs} at tumour sites including CXCR4, CD103 and CCR2 (8). Because chemoattraction is ubiquitous in the immune system efforts to block T_{Reg} recruitment to the tumour mass may be limited by the concurrent effects on $T_{effectors}$. Nevertheless, disruption of CCR5/CCL5 signalling blocks T_{Reg} migration to tumours and inhibits pancreatic tumour growth in mice (28). Methyl gallate has also recently been shown to inhibit infiltration of T_{Regs} into tumours resulting in reduced tumour growth and prolonged survival rates (29).

Immuno-stimulatory therapies may inadvertently promote tumoural homing of T_{Regs}. Therapy with IL-2 can enhance CCR4 expression on T_{Regs}, which stimulates their migration to the tumour mass and an upregulation of CXCR4, the receptor for CXCL12, a chemokine linked to development of organ-specific metastases (8). These findings endorse a more prudent use of IL-2 or perhaps its use in combination with agents such as AMD-3100 which antagonise the CXCR4-CXCL12 interaction..

Exploiting T cell plasticity (figure 1 D)

The origins of iT_{Regs} within the tumour microenvironment are diverse as varying degrees of plasticity exist within the helper $CD4^+$ T cell population (T_{Regs}, Th1, Th2, Th17, Tfh) (30);

Pre-differentiated T_{Regs} may migrate under the influence of chemokines (27), T_{Regs} may arise from *de novo* generation via differentiation and expansion or may derive from conversion of $CD4^+CD25^-$ T cells. The plasticity inherent in each of these processes is a potentially exploitable therapeutic niche.

IL-6 is central to T cell plasticity (30). It helps to convert $FOXP3^+$ T_{Regs} into IL-17 secreting T cells (Th17). It potently abolishes conversion of conventional T cells into iT_{Regs} and in its absence no other cytokine can substitute for this inhibition. Thus, IL-6 merits further investigation as a therapeutic for cancer. TGF-β acts at the axis between T_{Reg} and Th17 differentiation, enhancing the function of FOXP3 and inhibiting the function of RORγt, their essential transcription factors respectively. TGF-β-induced FOXP3 expression is inhibited by proinflammatory cytokines (IL-6 and IL-21 for example) in a Stat-3-dependent manner. Thus Stat-3 may also represent a therapeutic option – indeed forced expression of Stat3 augmented IL-17 production, most likely through increased RORγt expression (30). Re-directing differentiation towards a Th17 phenotype might also be achieved by direct introduction of RORγt, as this has been shown to induce IL-17 expression upon transduction of naive $CD4^+$ T cells (30). Conversely, selective methylation at the FOXP3 locus would likely hinder differentiation along a suppressor pathway. Aside from the epigenetic level, targeting FOXP3 at the mRNA and protein levels would also be worthwhile. Other approaches include antagonists for retinoic acid receptors which facilitate differentiation into Th17 cells over T_{Regs} (30). T_{Reg} differentiation can be redirected towards lineages other than Th17. Specific inactivation of the transcription factor interferon regulatory factor 4 (IRF-4) elevates Th2 cytokine production while IL-4-driven growth factor independent 1 (Gfi-1) facilitates optimal Th2 differentiation (30).

Blocking T_{Reg} proliferation is an obvious goal. This can be achieved by either direct inhibition of TGF-β, inhibition of IDO directly with 1-methyl-D-tryptophan or indirectly by CTLA-4 blockade. Aside from directly stimulating T_{Reg} expansion, COX-2-derived PGE_2 facilitates tolerogenic APC-led T_{Reg} recruitment and is itself a functional instrument of T_{Regs} in certain tumours (8). Thus, use of COX-2 inhibitors like celecoxib may be justified. Alternatively, bevacizumab or blockade of PD1-L on T_{Regs} with MDX-1106 (Phase II) may halt T_{Reg} proliferation.

Inhibiting the peripheral conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T_{Regs} may be a useful therapeutic approach. The TGF-β-blocking antibody, 1D11 abolished this conversion and reduced tumour burden in mice. Subsequently other TGF-β-modulators including antibodies, soluble TGF-β receptors and the antisense oligonucleotide AP-12009 have reached Phase I/II clinical trials. However systemic TGF-β-blockade may carry the risk of developing autoimmune disorders. Furthermore, under subimmunogenic conditions T cell conversion can occur in the absence of TGF-β; IL-10 and IDO have also been shown to promote induction of T_{Regs} (8).

Novel approaches to T_{Reg} modulation

The multitude of strategies discussed in this review deliver only marginal efficacy. While some strategies have lacked potency the majority flounder on specificity. This dearth of

specificity is understandable given the intersecting differentiation pathways shared by all cells of the T cell lineage. Selective approaches to T_{Reg} modulation are warranted. Simple depletion of T_{Regs} may be naive and the benefit short-lived, while inhibiting their migration to the tumour ignores the *in situ* generation of these cells. Thus strategies focused on negating T_{Reg} function or reprogramming their functional phenotype would seem more meritorious.

A unique cell surface marker which facilitates selective targeting of T_{Regs} has yet to be uncovered. Thus targeting CD25 or CTLA-4 has been encumbered by a concomitant effect on $T_{effectors}$. Introducing a second layer of specificity, so called dual specificity, to receptor targeting would likely be synergistic. This is a strategy under investigation in our laboratory whereby a relatively T_{Reg}-specific gene therapy approach is coupled to ligand selectivity.

A global T_{Reg} modulation is undesirable as it may increase susceptibility to autoimmunity. Tumour-T_{Regs} could be targeted via their antigen-specific T cell receptors (TCRs); antigen-specific T_{Regs} engaged melanoma-expressed LAGE1 and ARTC1 (31) and in colorectal cancer patients CEA, telomerase, HER2/neu and MUC-1 reactive T_{Regs} were detected in the peripheral blood (32). On a practical level this could be achieved by harnessing tetramer technology; Saporin-coupled MHC class I tetramers specifically ablated IGRP-autoreactive T cells and delayed diabetes in NOD mice (33). Identification of $CD4^+$ T_{Regs} specific for a given tumour antigen would facilitate their targeting with MHC class II tetramers by similar means.

While such agents would be specific for a given subset of T_{Regs} they would also target other CD4+ helper cells expressing the same antigen specificity – $CD8^+$ cells would be unaffected. To circumvent this issue the effector component attached to the tetramer could be modified to confer another level of specificity. It could be miR-31 as 100% of target cells internalise the tetrameric complexes (33). Although the consequence of FOXP3 knockdown in non-T_{Regs} is unknown TCR engagement in these cells may simply lead to activation – further augmenting the immune effector response.

Alternatively one could target tumour-T_{Regs} indirectly by modulating dendritic cell activation. This could be achieved by blockade of DC p38 MAPK, COX-2 or PI3K which inhibits innate production of TGF-β and IL-10 and thereby suppresses induction of T_{Regs}. Such strategies enhance the efficacy of TLR agonists or HSPs as immunotherapeutics or adjuvants for DC vaccines and permit an un-restrained development of protective Th1 and Th17 cells (25).

Conclusion

T_{Reg} inhibition in the cancer environment would permit an anti-tumour immune effector competency with containment or elimination of disease. Such responses would be tumour specific and durable and should be effective against systemic disease, particularly micrometastases. There is clinical potential for T_{Reg} inhibitory strategies as part of multimodal programmes or combined with targeted therapies or local immunogene stimulation of anti-tumour immune effector cells. The objective should be to selectively

modulate T_{Regs} within the tumour microenvironment rather than their global depletion in order to minimize the risk of autoimmune manifestations. Strategies targeting T_{Reg} function or differentiation seem currently to be the best option as they are less susceptible to compensatory mechanisms. Emerging technologies such as tetramer or RNA interference approaches should improve specificity and efficacy and thus favour the preferential inhibition of T_{Regs} within the tumour environment.

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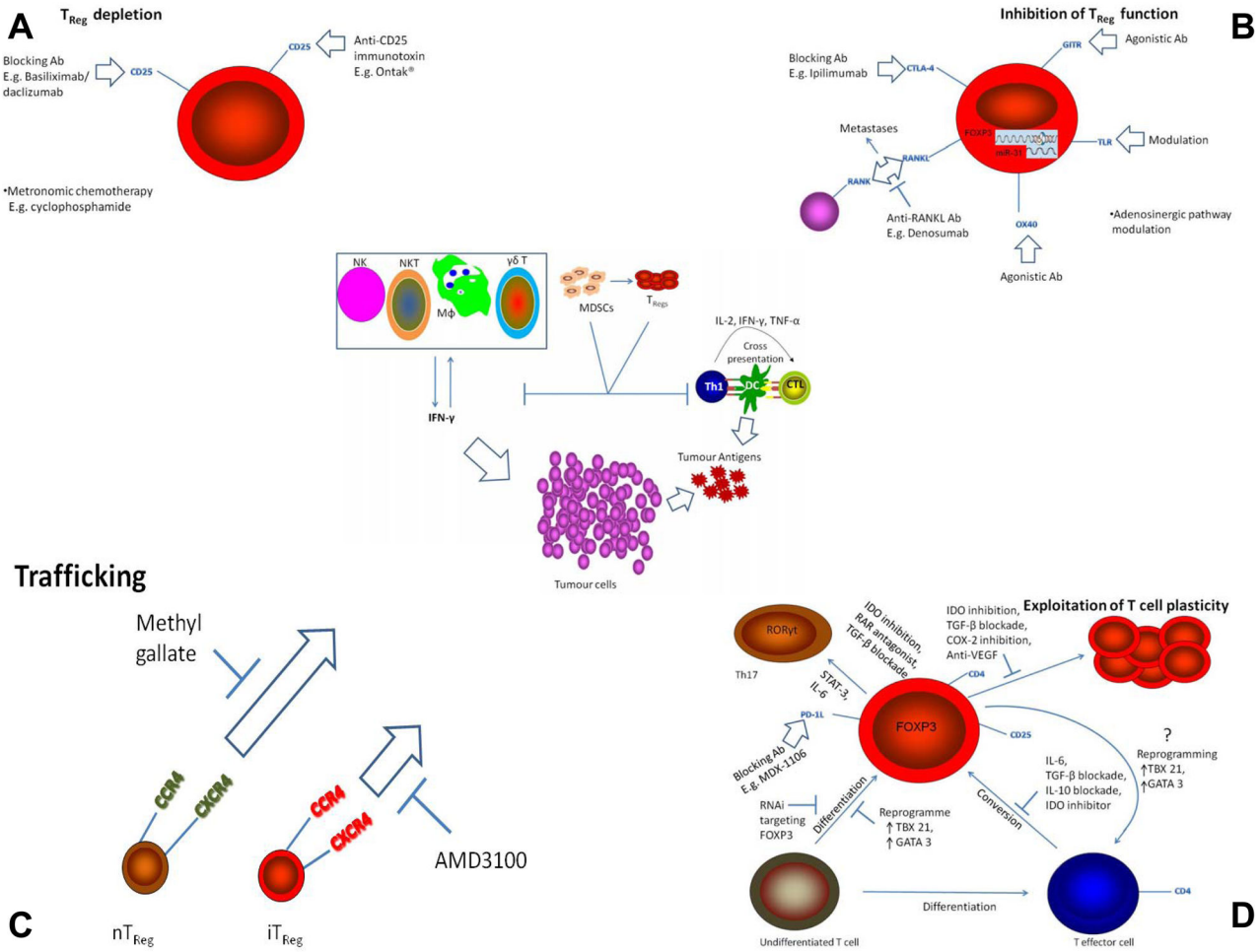

Figure 1.

Targeting regulatory T cells in cancer. The central schematic depicts the main events involved in mounting an immune response to a tumour. Cells of both the innate and adaptive systems contribute (further details are provided in the text). T_{Regs} offer substantial resistance to this immune assault and thus four different approaches for reducing their immunosuppressive contribution are advanced (figure 1; A, B, C and D); depletion, inhibition of function, blockade of trafficking and modulation of T cell plasticity. Within each approach numerous existing and novel options for therapeutic manipulation are forwarded. Ab – antibody; DC – dendritic cell; IDO - indoleamine 2,3-dioxygenase; MDSCs – myeloid-derived suppressor cells; T_{Reg} – regulatory T cell.