Phenotypic alterations, clinical impact and therapeutic potential of T regulatory cells in cancer

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Abstract

Introduction: T regulatory cells (Tregs) have been characterized in different cancers. They accumulate in peripheral blood and tumour microenvironments where they suppress tumour-specific immune responses, enabling tumours to develop without challenge. This tumour immune evasion represents a major obstacle to successful cancer therapies. Whilst Tregs are generally divided into thymic-derived tTregs and peripherally-induced pTregs, Tregs exhibit a wide spectrum of phenotypes and functional capacity dependent on microenvironment. This phenotypic diversity is also reflected in tumour-infiltrating Treg (TI Treg) populations, which may explain the variable impact of Treg accumulation on prognosis in different cancers. Identifying TI Treg subsets is critical to understand TI Treg biology and for developing effective immunotherapies.

Areas covered: This review discusses the current and potential Treg markers, and the modulation of these markers in cancer. In addition, we systematically review the clinical impact of Tregs in cancer and their potential as a therapeutic target, with a focus on TI Tregs.

Expert opinion: TI Tregs represent dynamic/diverse subsets that are key in promoting tumour progression through their suppressive activities. Targeting specific TI Treg subpopulations and functional TI Treg markers represents a feasible therapeutic strategy that might allow re-establishment of anti-tumour immune responses without affecting physiological immune regulation.

Article Highlights

- Tregs express several surface and intracellular markers; most important among these are those that are not modulated by inflammation or activation:
  - **tTregs:** *$FoxP3^{+}SATB1^{LO/-}CD25^{HI}CD26^{LO/-}4\text{-}1BB^{+}CD40L^{-}$*
  - **pTregs:** *$FoxP3^{+}SATB1^{LO/-}$ $Helios^{+/-}CD25^{HI}CD26^{LO/-}$*
  - **Tr1 cells:** *$FoxP3^{LO/-}CD39^{+}CD73^{+}CD25^{+/-}$ (varies according to method of induction)*
- The clinical impact of $CD4^{+}$ and $CD8^{+}$ Treg subsets in cancer depends on several factors: (i) the ratio at which each subset is present, (ii) tissue localization, (iii) immunogenicity or inflammatory status of tumour and (iv) presence of Tregs during normal physiological function.
- A wide range of Treg mechanisms and markers can be targeted for cancer immunotherapy; utilizing these to deplete or impair specific Treg subsets (i.e. intra-tumoural) without causing autoimmune side effects is an important approach.
- Immune checkpoint blockade offers an exciting novel target for cancer immunotherapy boosting the immune response while inhibiting Treg activity.

1. Introduction

T regulatory cells (Tregs) have come into the spotlight in recent years for their essential role in immune system regulation and as targets for novel immunotherapies. Tregs are immunosuppressive T cells that are key in maintaining immune homeostasis, preventing autoimmunity, and controlling inflammation and allergy [1].

The pathological role of Tregs in cancer is well characterized; Tregs accumulate both in peripheral sites and within the local tumour microenvironment where they suppress anti-tumour immune responses enabling tumour to develop unchallenged [2-4]. This remains a major hindrance to the development of effective anti-cancer vaccines and immunotherapies. Treg depletion prior to treatment has been reported to enhance efficacy of cancer vaccines and immunotherapy [5]. Treg accumulation has been reported to correlate with tumour progression and a worsening prognosis in many cancers [3], although there are exceptions as summarized in Table 1.

In this paper we review and provide updates of the current and potential markers of Treg subsets, and the modulation of these markers in cancer.

2. Treg subsets

Tregs are divided into two major subsets: natural Tregs and adaptive/induced Tregs; or as recently named, thymic-derived Tregs (tTregs) and peripheral-induced Tregs (pTregs), respectively [6]. This updated notation will be adhered to throughout. tTregs and pTregs, have been the subject of a number of recent reviews [7, 8] discussing their function and generation, both *in vitro* and *in vivo*.

tTregs are selected by high-avidity interaction with self-MHC class II-dependent T cell receptors (TCR) in the thymus [8]. tTregs were first defined by their high levels of expression of the IL-2 alpha-chain receptor, CD25, and forkhead box P3 transcription factor, FoxP3 [8, 9]. FoxP3 is important for maintaining the suppressive phenotype; dysregulation of FoxP3 expression

often leads to development of severe autoimmune disorders [1]. pTregs are generated in the periphery from naïve $CD4^+FoxP3^-$ precursors undergoing sub-optimal antigenic stimulation and the majority expresses FoxP3 [8]. The presence of both IL-2 and TGF-β are required both for pTreg generation and thymic development of tTregs [10]. TGF-β signalling greatly enhances FoxP3 expression while IL-2 is critical for maintaining stable pTreg and tTreg levels by driving Treg proliferation.

pTregs also comprise a number of functional $FoxP3^-$ subsets: Type 1 regulatory T cells (Tr1) are induced from $CD4^+$ precursors by the anti-inflammatory cytokine IL-10, and secrete IL-10 and TGF-β [11]. Tr1 cells regulate adaptive immune responses, attacking commensal organisms and maintaining gut homeostasis. Tr1 cells also contribute to immune system regulation *in vivo*, aiding in prevention of autoimmunity, transplantation, and chronic inflammatory diseases [11]. TGF-β dependent T helper 3 cells (Th3) are induced by low doses of antigen and have been associated with a major role in maintaining oral tolerance in the gut, where Th3 cells are often induced by oral antigen [12]. They can be distinguished from Th1 cells by their copious secretion of TGF-β [12].

Selected specifically for self-antigen during thymic development, tTregs are important in preventing autoimmunity. In contrast, pTregs have been considered 'pathological Tregs' in certain scenarios although this is an oversimplified view of their role in the immune system. Antigen-specific pTregs can be generated during inflammation or in tumours where they then inhibit an effective immune response. pTregs are also important for governing immune and inflammatory responses to a variety of microbial and tissue antigens, particularly at mucosal sites such as the gut [7]. Tregs require activation via their T cell receptors (TCR) in response to specific antigens. Following activation, however, Tregs are able to exert both specific and non-specific 'bystander' suppression of $CD4^+$ and $CD8^+$ T cells [13], alter macrophage functionality [14], suppress B cell responses accompanied by inhibition of Ig class switch recombination [15] and

directly inhibit the effector function of natural killer (NK) cells as well as dendritic cell (DC) maturation and function [1].

3. Treg markers

To fully understand the role and function of Treg subsets in the physiological and pathological settings, we should be able to accurately define them. The original Treg markers CD25 and FoxP3 are useful but they cannot be relied upon solely. CD25 is highly upregulated on non-regulatory T cells following activation and FoxP3, as an intracellular marker, does not allow isolation of viable cells [9]. While there are no clear phenotypic differences between pTregs and tTregs *in vivo*, a number of markers have proven useful for identifying suppressive Treg subsets, as summarized in Tables 2 and 3. Many of these markers are modulated on Treg and Teff subsets following activation thus preventing their use for identification/isolation of Tregs. Here we discuss a number of surface and intracellular markers that have shown promise for identifying functional Treg subsets.

3.1 Surface markers and chemokine receptors

Neuropilin 1 (NRP1): NRP1and its role on Tregs in cancer has recently been extensively reviewed [16]. NRP1 was identified as a selective tTreg marker in murine studies, where NRP1 expression greatly enhanced Treg immunosuppressive activity through unidentified mechanisms. In addition, NRP1 expression on murine Tregs remained stable following activation both *in vivo* and *in vitro* although NRP1 expression on T cells could be induced during inflammation *in vivo*. In contrast to these findings, in the human immune system significant NRP1 expression has only been observed on small Treg populations in lymph nodes and on plasmacytoid dendritic cells (pDCs). In addition, both human Tregs and non-regulatory conventional T cells (Tconv) have been reported to upregulate NRP1 at varying levels following *in vitro* activation depending to a certain degree on duration of stimuli [7]. The role of NRP1 as a

murine tTreg marker has also been questioned in a very recent investigation where NRP1 was expressed on pTregs and both FoxP3$^{+/-}$ T cells subsets induced *in vivo* in RAG-deficient mice [17]. In cancer, NRP1 has also been reported on Tregs isolated from peripheral blood and tumour-draining lymph nodes (TDLN) of patients; interestingly successful chemotherapy seemed to correlate with a selective decrease of NRP1^{+} Tregs compared to NRP1^{-} Tregs [16]. While NRP1 seems an interesting player within the immune system, possibly representing a novel late T cell activation marker or target for cancer immunotherapies, it does not allow selective isolation of Tregs in humans.

GARP & LAP: Latency-associated peptide (LAP) is a propeptide that is non-covalently associated with the amino-terminal domain of TGF-β. LAP prevents mature TGF-β from binding to its receptor, and from transducing a signal, forming a latent LAP-TGF-β complex. TGF-β activation occurs when TGF-β is released from LAP [18]. A growing body of knowledge now supports a close association between the transmembrane protein Glycoprotein A Repetitions Predominant (GARP) and LAP whereby GARP anchors LAP to the cell membrane [19, 20]. GARP also appears to be useful in identifying Tregs since it was reported to be expressed on freshly isolated human CD4^{+}CD25^{HI} Tregs but not Th subsets, and was also selectively upregulated on Tregs following *in vitro* activation [19, 20]. Similar findings were reported with LAP where LAP provided a specific surface marker that was selectively upregulated on Tregs and accurately distinguished Tregs from Teff following *in vitro* activation [20, 21]. LAP expression has also been characterized on small populations of CD8^{+} Tregs in mice and on TGF-β secreting Th3 cells induced by oral antigen administration in the gut [12, 22].

GARP and LAP expression on TI Tregs has not been explored in depth; however given their selective expression on activated Tregs, TI Tregs might be predicted to express GARP and LAP especially in highly inflammatory or immunogenic tumours.

CD39 & CD73: Ectonucleoside Triphosphate Diphosphohydrolase 1 (ENTPD1), also known as CD39, is a rate-limiting ectoenzyme that catalyses the degradation of ATP into AMP. CD73 is often co-expressed with CD39 and further drives degradation of AMP into adenosine, an anti-inflammatory and immunosuppressive molecule that has been characterised in promoting T cell anergy and protecting tumours from immune-mediated destruction [23]. CD39 expression has been reported on human $CD4^+$ and $CD8^+$ Tregs [24, 25]. Co-expression with CD73 defines an even more accurate Treg subset, since together CD39 and CD73 contribute to a Treg suppressive mechanism [23]. Both CD39 and CD73 have also been described on B cells, activated NK cells, endothelial vasculature and potentially as a late activation marker for T cells [26].

More recent work identified CD39-expressing tumour-infiltrating lymphocytes and Tregs in the peripheral blood and tumour tissue of cancer patients, particularly in human head and neck squamous cell carcinomas (HNSCC) [24, 25, 27-29]. $CD39^+$ Tregs were expanded in cancer patients compared to healthy donors, with greater CD39 expression being observed on TI Tregs than circulating Tregs in patients [25, 27, 29]. In addition, CD39 blockade significantly impaired suppressive activity of tumour-infiltrating and circulating $CD8^+CD39^+$ Tregs isolated from HNSCC patients. This suggests an important role for CD39 in $CD8^+$ Treg suppressive function [24].

CD26: Dipeptidyl peptidase-4 (DPP4) or CD26 is an enzyme that binds with adenosine deaminase (ADA) providing an ADA-CD26 mediated co-stimulatory signal, enhancing T cell activation and proliferation. Recent work identified CD26 as an effective negative marker for human Tregs, defined as $CD4^+CD25^+CD26^{LO/-}FoxP3^+$ [25, 30]. Importantly, the $CD26^{LO/-}$ phenotype of $FoxP3^+$ Tregs was reported to be stable following TCR triggering and activation *in vitro*, while CD26 was upregulated on Teff following activation. This is in contrast to another negative Treg marker, CD127, which is downregulated on Teff following activation preventing effective identification of $CD127^-$ Tregs. Elevated levels of $CD4^+CD26^{LO/-}$ Tregs have been

reported in studies of Hodgkins Lymphoma and head and neck squamous cell carcinoma (HNSCC) [25, 31].

TIM-3: T-cell immunoglobulin mucin 3 (TIM-3) is expressed on a subset of activated human Th1 cells that negatively regulate T cells [32]. Very recent work suggests TIM-3 might selectively identify Tregs in cancer [33, 34]. In a study of non-small cell lung cancer (NSCLC), TIM-3 was highly expressed on $CD4^+$ and $CD8^+$ T cells isolated from tumour tissue, but not on T cells isolated from adjacent non-tumour tissues or the peripheral blood of patients and healthy donors [33]. Up to 70% of these $CD4^+TIM\text{-}3^+$ T cells isolated from tumour tissue co-expressed FoxP3 and Programmed Death-1 (PD-1) suggesting they were Tregs [33]. Other human studies investigating HNSCC, hepatocellular, cervical, colorectal and ovarian carcinomas, also reported the majority of $CD4^+$ TILs highly upregulated TIM-3 compared to $CD4^+$ T cells isolated from the peripheral blood of both patients and healthy donors [27, 34]. These $CD4^+TIM\text{-}3^+$ TILs exhibited Treg-like features including $CD25^+FoxP3^+CTLA\text{-}4^+CD127^{LO/-}$ phenotype, impaired IFN-γ and IL-2 secretion and suppression of autologous $CD8^+$ Teff, whereas $CD4^+TIM\text{-}3^-$ TILs did not [34]. It has also been reported that TIM-3 could be expressed on both Teff and Treg following *in vitro* activation for up to 72H [32, 33]. The increased TIM-3 expression in TI Tregs could be attributed to constant stimulation with TAA in tumour tissue or inflammatory stimuli associated with immunogenic cancers such as HNSCC. Accordingly, negligible TIM-3 expression was reported on $CD4^+FoxP3^+$ TI Tregs and $CD4^+$ T cells in early-stage hepatocellular carcinoma (HCC) patients while advanced HCC patients showed a greater proportion of $TIM3^+$ TI Tregs and TILs [35].

Chemokine receptors: Treg-tumour trafficking is an important mechanism for intra-tumoural Treg accumulation, and is mainly chemokine receptor-mediated. Several chemokine receptors have been characterized on TI Tregs in cancers; most prominently CCR4, CCR5 and CCR10, that interact with tumour-derived chemokine ligand 22 (CCL22) CCL5 and CCL28 respectively

mediating Treg homing to tumours [36, 37]. A number of other chemokine receptors including CCR6, CCR7, CCR8 and CXCR3 have been implicated in Treg migration, not only into tumour tissue, but to other sites within the body such as mucosal and lymphoid tissues, as reviewed [38]. Chemokine receptor-mediated T cell migration is a feature present in the entire lymphocyte population – the same chemokine receptors utilized by Tregs to infiltrate tumours are expressed on other lymphocytes and have been identified both on tumour-infiltrating Teff and CTLs [39, 40]. Blockade of chemokine receptors or chemokines involved in Treg-homing to tumour tissues is a viable therapeutic strategy that has shown efficacy in mice by reducing intra-tumoural Treg levels [41]. This blockade might however inhibit migration of tumour-specific Teff into tumour tissues, and remains to be tested in humans.

Different tumours have been suggested to secrete specific 'chemokine signatures' potentially allowing for targeted blockade of those chemokines and chemokine receptors playing the most prominent roles in Treg-tumour homing in different cancers [42].

3.2 Intracellular markers

FoxP3: FoxP3 is one of the originally identified phenotypic markers for Tregs [9]. In line with the general inconsistency associated with Treg markers, several studies have reported that FoxP3 expression can be transiently induced in human $CD4^+$ and $CD8^+$ Teff cells upon *in vitro* stimulation, albeit at lower levels than normally expressed in Tregs [43-45]. Interestingly, $CD4^+CD25^-$ T cells stimulated to express FoxP3 did not always acquire suppressive activity, suggesting FoxP3 might not always identify a suppressive T cell subset [43, 45]. Indeed activated $FoxP3^+$ non-regulatory T cells have been reported to secrete the effector cytokines IL-2, IFN-γ and tumour necrosis factor-α (TNF-α) [45]. FoxP3 also upregulates the expression of several other markers including CD25, cytotoxic lymphocyte antigen-4 (CTLA-4) and glucorticoid-induced tumour-necrosis-factor-related protein (GITR) [46].

Increased FoxP3 expression has been reported on TI Tregs [47, 48]. One study reported FoxP3 upregulation on TI Tregs in gastric tumours correlated with a worsening prognosis [48]. These TI Tregs exerted enhanced suppressive effects through increased expression of cyclo-oxygenase 2 (COX2) and prostaglandin E-2 (PGE-2) [48]. This variable expression of FoxP3 on activated T cells can contribute to conflicting reports of the effect of Tregs on clinical outcome. For example, in cancers with inflammatory components, such as HNSCC or colorectal cancers, Treg accumulation has been shown to correlate with a good prognosis [49-52]. Given the inflammatory component of these cancers, CD25 and FoxP3 might be upregulated due to Treg activation status rather than suppressive phenotype. On the other hand, an expanded Treg population might contribute to positive outcome by downregulating the inflammatory effects of these tumours.

Epigenetic modifications in the FoxP3 locus have also been utilized for the identification of Tregs. Demethylation of a conserved DNA sequence within the FoxP3 locus, known as the Treg-specific demethylated region (TSDR) is associated with stable FoxP3 expression and suppressor phenotype in Tregs [53-55]. TSDR demethylation has been reported to be selective to Tregs and not displayed on Tconv [55]. This has been developed into a clinical application whereby TSDR demethylation is utilized for rapid quantitative analysis of Treg levels in blood and solid tumours [56]. The value of TSDR demethylation for distinguishing between tTregs and pTregs is not yet clear – initial reports suggest tTregs exhibit TSDR methylation while *in vitro* TGF-β induced pTregs exhibited low levels of TSDR demethylation and lost FoxP3 expression and suppressive activity following polyclonal stimulation without TGF-β [55, 57]. The demethylation status of pTregs *in vivo* has not yet been established. Although TSDR methylation in TI Tregs has not been studied, it could be expected to reflect the tTreg to pTreg ratio of the TI Treg population. FoxP3 has also been characterized in tumour cells, as recently reviewed [58]. Tumoural FoxP3 expression has been suggested to contribute directly to

suppression of anti-tumour immune responses and to correlate with poor prognosis in several cancers.

Helios: The role of Helios expression in Tregs and in cancer has been extensively reviewed [2, 7, 59]. Briefly, Helios was initially identified as a murine tTreg marker. While Helios remains effective as a murine tTreg marker, in humans Helios expression has been reported in pTregs, activated T cells and in T cell subsets under inflammatory conditions *in vivo*. One study found that Helios$^-$ Tregs mainly comprised of pTregs in humans [60]. Although the role of Helios as a tTreg marker in humans remains uncertain, it is clear that Helios defines immunosuppressive Treg subsets with distinct phenotypic and functional features [60, 61]. Helios has also been characterized on TI Tregs in human cancers [59, 62]. A recent study investigating Helios function reported that Helios regulated Treg functional stability to a certain extent through binding and repressing the IL-2 gene promoter and enhancing FoxP3 binding affinity for the IL-2 promoter [63]. Loss of Helios expression on Tregs enhanced expression of the IL-2 gene resulting in increased Treg proliferation and secretion of IL-2 following activation, as well as impaired suppressive activity [63]. Helios might also be involved in T cell development; a recent human study found that forcing Helios expression in Tregs induced apoptosis *in vitro*, while Helios knockdown impaired the suppressive activity of human Helios$^+$ Tregs *in vitro* [64]. Helios has been reported to be co-induced with the pro-apoptotic protein Bim1 in negatively selected autoreactive T cell precursors in mice, aiding in establishing self-tolerance [65].

SATB1: Special AT-rich sequence binding protein-1 (SATB1) is a chromatin organizer and a transcription factor that plays a key role in T cell development and maturation. In Tregs, SATB1 is a highly repressed gene [66]. Inducing SATB1 expression in human Tregs led to decreased suppressive activity *in vivo,* while *in vitro* this led to switching from suppressive to effector phenotype through establishment of the Teff transcriptional cell program [66].

FoxP3 has been reported to be key in controlling SATB1 expression acting as a negative regulator [66, 67]. FoxP3 represses SATB1 expression by direct binding to the SATB1 locus, as well as through FoxP3-regulated micro RNAs (miRNAs) [66, 67]. Silencing FoxP3 with small interfering RNA (siRNA) in human Tregs resulted in loss of suppressive phenotype and a significant increase in SATB1 expression. Interestingly, SATB1 has also been shown to downregulate CD25 on T cells [68]. These findings suggest SATB1 repression may be key for maintenance of the Treg suppressive phenotype, while SATB1 expression is key for Teff development and function [66]. SATB1 could perhaps be utilized as a negative Treg marker, in conjunction with FoxP3, to identify Tregs. The expression of SATB1 in TI Tregs has not been explored. However, given studies reporting stable or upregulated expression of FoxP3 on TI Tregs, SATB1 could be expected to remain expressed at relatively low levels.

One other factor to consider is that markers identified on Tregs in murine studies do not always show parallel expression in humans. These include the integrin protein CD103 [69], Galectin-1 (Gal-1) [70], and NRP1. These markers have been induced on human T cells *in vitro* [69, 70] raising an interesting issue regarding Treg phenotypic analysis. *In vitro*-generated Tregs, defined as a separate subset by Abbas et al. [6], often exhibit altered functional and phenotypic characteristics compared to Tregs *in vivo.* Phenotypic studies must take into account the differences between *in vitro*-generated Tregs and Tregs isolated from the dynamic *in vivo* environment and the effects this may have on marker expression.

4. Clinical impact and therapeutic modalities

4.1 Clinical impact

Treg accumulation can have a variable impact on prognosis in different cancers, as summarised in Table 1. This variable impact of Tregs in cancer, although not fully understood, can be attributed to several key factors: (i) Localised Treg accumulation: tumour-infiltrating,

peritumoural or circulating Tregs, (ii) Ratio of $CD8^+$ CTL or $CD4^+$ Teff : Treg; this is important for determining the functional impact of Tregs on immune responses, (iii) Inflammatory status of cancer, (iv) Treg stability and function and (v) Treg subsets. The impact of each of these factors are discussed below.

(i) Localised Treg accumulation

Tregs have been reported to accumulate in the peripheral blood, lymph nodes, peri-tumoural regions and in specific sites within tumour tissue, including the stroma or epithelia. This selective Treg accumulation may contribute to the varying clinical impact of Tregs. A recent breast cancer study, reported that peri-tumoural Tregs decreased significantly following neoadjuvant therapy and correlated with a pathologic complete response. In contrast, intra-tumoural Treg levels remained relatively constant thoroughout, and was an independent prognostic factor correlating with progression-free survival [71]. An earlier breast carcinoma study reported that increased Treg density in lymphoid-enriched sites surrounding tumour tissue correlated with a decreased overall survival (OS) and relapse-free survival (RFS) while Treg levels in tumour tissue showed no correlation with survival [37]. In a colorectal study, Tregs in normal and peri-tumoural tissue correlated with a worse prognosis while a high level of intratumoural infiltration correlated with a better prognosis [52].

Another interesting factor to consider is physiological Treg infiltration. Several tissues, such as the lymph nodes or digestive system, exhibit significant Treg infiltration during normal physiological functioning where Tregs are important for preventing autoimmunity [72]. The gut-associated lymphoid tissue (GALT), for example, is an important location for pTreg generation in response to oral antigen from the environment; in this scenario Tregs prevent excessive immune responses and inflammation in response to envrionmental antigen. The actual contribution and numbers of tumour-induced Tregs in transformed tumour tissues may be

unclear in cancers such as gastric cancer where there is significant level of Tregs during physiological functioning.

(ii) Ratio of $CD8^+$ CTL or $CD4^+$ Teff : Treg

This ratio, especially of $CD8^+$ CTL:Treg, has proven to be a useful tool for determining the functional impact of Tregs in cancers. Tregs contribute to tumour immune evasion by actively suppressing and dampening tumour-specific CTL responses; a greater ratio of Tregs to $CD8^+$ CTL results in increased suppression and predict a worse prognosis as reported in several cases [73-76]. There are, however, exceptions in cancers with inflammatory components. For example, in a colorectal cancer study an elevated Treg:$CD8^+$ CTL ratio was associated with better OS in patients receiving neoadjuvant chemotherapy [77].

(iii) Inflammatory status of cancer and T cell activation

Inflammation has been identified as a key contributor to the initial development and establishment of many tumours, including solid tumours. Several cancers, such as HNSCC and colorectal cancers, have significant inflammatory components associated with their pathophysiology [78]. In these cases, tumour-infiltrating lymphocytes and DCs might contribute to establishment of the tumour microenvironment through secretion of inflammatory cytokines while Tregs control this inflammation and inflammation-mediated angiogenesis – hence the positive prognoses associated with Treg infiltration in these cancers (Table 1). In a colorectal cancer study, peri-tumoural Tregs were found to correlate with disease progression whereas intra-tumoural Tregs correlated with a greater OS [52]. Perhaps the TI Tregs suppress inflammatory immune cells while the peri-tumoural Tregs suppress tumour-specific T cells.

The activation status of tumour-associated T cells is also important. Highly inflammatory or immunogenic cancers can induce T cell activation resulting in transient upregulation of FoxP3, as discussed earlier. These non-regulatory $FoxP3^+$ T cells may prevent effective isolation of $CD25^{HI}FOXP3^+$ Tregs thus 'contaminating' study results. Utilising effective Treg markers that

are not modulated by T cell activation, such as GARP, LAP and CD26, will be important to accurately characterise functional Tregs in cancer.

(iv) Treg stability and function

The stability of the Treg lineage under various lymphpenic or inflammatory conditions is an important factor. With the identification of IL-17 secreting Tregs and 'ex-Tregs' – Tregs that lost FoxP3 expression and supressive activity - it seems the functional plasticity of Tregs may contribute significantly to clinical impact [79, 80]. A RORγt-expressing FoxP3$^+$ Treg subset able to suppress T cell responses but with greatly impaired anti-inflammatory activity was identified in colon cancer patients [81]. The regulatory lineage of murine pTregs and tTregs was stable *in vivo*, and was maintained by demethylation of the FoxP3 locus, even in inflammatory or lymphopenic environments [53]. A small population of non-regulatory T cells transiently expressed FoxP3 before developing into FoxP3$^-$ Th cells and were thought to account for the 'unstable Treg' population – again highlighting the need to use more effective Treg markers. It is worth noting that the diversity of Treg subsets has previously been underestimated. The identification of IL-17 secreting Tregs [80], Th/Treg hybrids mirroring Th cell subsets [82], and other Treg subsets may point to a wide range of Tregs induced by varying conditions rather than an unstable *in vivo* lineage. Indeed, a very recent human study using multi-parameter single-cell analysis reported that while Treg diversity was comparable to Teff subsets – specifically with regards to cytokine secretion, chemokine receptor and transcription factor expression – this wide Treg diversity was due to heterogeneity in Treg subsets rather than re-programming of the Treg lineage [83]. Single-cell analysis showed the Treg gene signature was stable in the lymphopenic and inflammatory microenvironment of patients developing graft-versus-host-disease [83].

(iv) Treg subsets

In addition to the previous factors, different Treg subsets may have a distinct effect on clinical impact. Several groups have investigated the specific contributions of pTregs and tTregs in

cancer to determine which is most suitable for targetting in immunotherapies., as recently reviewed [2, 4, 7]. Other Treg subsets, in particular Tr1 cells, have been implicated in cancer pathology. The tumour microenvironment is one particularly suited to the induction of Tr1 cells given abundant amounts of tumour-derived IL-10 and tumour-associated antigen (TAA) present, although this has been shown to be equally conducive to activation and proliferation of $CD8^+$ CTLs [84]. In addition, COX-2, an enzyme upregulated in a number of solid tumours, generates PGE(2) from arachidonic acid. PGE(2) in turn stimulates mature $CD11c^+$ DCs to secrete IL-10, further inducing Tr1 cell generation. This was demonstrated *in vitro* where in the presence of autologous DCs and COX-2-overexpressing HNSCC tumour cells, Tr1 cells were induced from $CD4^+CD25^-$ T cell precursors [85]. Recent findings suggest Tr1 cells may play differing roles from $FoxP3^+$ tTregs in tumour immune evasion. An ovarian cancer study reported that an elevated ratio of Tr1 cells to $FoxP3^+$ tTregs was a positive independent prognostic factor for disease-free survival (DFS) [86]. In one patient who was successfully treated without relapse, the number of $FoxP3^+$ tTregs was reduced and continued to decrease following treatment while the $FoxP3^-$ IL-10 secreting Tr1 cell population was elevated. Similar results were reported in HNSCC studies, where elevated Tr1 cell levels persisted following treatment and remained permanently upregulated in patients with no active disease [87 88]. Finding specific markers for Tr1 cells will aid in further investigations. CD49b/LAG-3 co-expression and CD39/CD73 co-expression have each recently been utilized to identify human Tr1 cells [89, 90]. *C*D39 and CD73 also contribute to a novel immunosuppressive mechanism whereby PGE(2) and adenosine generated from ATP accumulate in tumour tissue impairing Teff functionality [91]. The role of Tr1 cells in tumour immune evasion in different cancers needs to be further investigated in order to determine their potential in therapy.

$CD8^+$ Tregs comprise a subset of Tregs mirroring the $CD4^+$ Treg population in their phenotypic and functional diversity and have increasingly been implicated in cancer pathology and tumour

immunity [92, 93]. The full range of $CD8^+$ Treg interactions and phenotypes warrants an in-depth discussion and has been the subject of a recent perspective [94]. Of note, in a murine study, $CD4^+$ and $CD8^+$ Tregs have been suggested to interact together 'priming' each other for full functionality *in vivo*. Disruption of these *in vivo* interactions between $CD4^+$ Tregs and $CD8^+$ Tregs significantly impaired $CD4^+$ Treg suppressive activity [95] possibly offering another target for Treg depletion and immunotherapies.

4.2 Current and future therapeutic modalities

The aim of cancer immunotherapy is to establish effective tumour-specific immune responses. There are two main approaches: Firstly, an immunostimulatory approach that stimulates the immune system to launch a tumour-specific attack. The second approach involves dismantling tumour immune evasion mechanisms; most prominently targeting Tregs although there is extensive contribution from other cells involved in tumour immunity including TAMs, pDCs and MDSCs. We herein discuss the different strategies to target Tregs in cancer.

4.2.1 Treg depletion

Treg depleting therapies have shown promise, especially prior to administration of cancer vaccines or adoptive CTL transfer where it greatly enhances their efficacy [3]. These therapies often target the IL-2 receptor chains. The anti-CD25 mAb daclizumab induces cell death by binding to CD25 preventing IL-2 signalling that is necessary for proliferation and activation [96]. Recombinant immunotoxins, such as denileukin diftitox (ONTAK) and LMB-2, target $CD25^{HI}$ cells, causing Treg depletion and impairing Treg function [96, 97]. Unfortunately these therapies also deplete activated $CD4^+CD25^{HI}$ Teff, further impairing anti-tumour immune responses. ONTAK therapy has recently been shown to contribute to tolerance by inducing a tolerogenic DC phenotype and enhancing survival of resting Tregs, although activated Tregs were killed [98]. A recent human clinical trial reported boosted Teff and CTL activity and interestingly, Treg re-programming resulting in secretion of IFN-γ and loss of suppressive activity [99]. High-dose

cyclophosphamide has non-selective cytolytic activity that can cause global Treg disruption while low-dose cyclophosphamide selectively depletes Tregs boosting anti-tumour immune responses without an autoimmune response [100]. Developing specific therapies targeting TI Tregs is a critical approach if Treg depletion is to be utilized as a viable therapy. For example, a recent study identified Semaphorin 4A (SEMA4A)-NRP1 interactions for maintaining intra-tumoural Treg stability in mice – it was not required however for normal immune homeostasis thus providing a specific target for targeting TI Tregs [101].

4.2.2 Immune checkpoint blockade

Immune checkpoint molecules are negative regulators of immune responses and T cell activation. While initially implicated in downregulation of T cell responses, immune checkpoint molecules have also been found to be important in Treg suppressive function and have been reported to be upregulated on TI Tregs in several cancers [34, 102, 103]. Immune checkpoint blockade therefore has the dual effect of activating Teff whilst also depleting or impairing Treg function. Some blocking Abs are currently in development as summarized below:

Anti-CTLA-4: CTLA-4 competes with CD28 for binding to B7, blocking the co-stimulatory signals required for T cell activation. Two blocking antibodies (Abs) have been tested in Phase III trials: Ipilimumab, an IgG1 Ab, currently used in treatment of metastatic melanoma [104] and Tremelimumab, an IgG2 Ab, is being investigated for use in melanomas and solid tumours [105]. Anti-CTLA-4 Abs containing an IgG2a constant region have also been reported to significantly reduce intra-tumoural Treg levels [106]. Although the mechanism of action was not confirmed, the reduction in Tregs was suggested to be due to FcγR binding activity of the Ab inducing activation and proliferation of Teff and CTLs. Increased effector cytokine secretion (IFN-γ and TNF-α) was also reported [106].

Anti-PD-1/PD-L1: Similar to CTLA-4, PD-1 negatively regulates T cell activation. PD-1 also enhances Treg development and is highly upregulated on 'exhausted' anergic T cells that are

unable to contribute to tumour immune responses [107]. Anti-PD1 antibodies have been tested in Phase I-III clinical trials where they showed efficacy against haemotological malignancies and solid tumours [108] – these Abs include Pidilizumab (CT-011), Nivolumab (BMS-936558) Lambrozilumab (MK-3475), as well as PD-ligand 1 blocking Abs. Although the main effect of PD-1 blockade is to reverse T cell anergy, PD-1 blockade can impair Tregs and prevent re-programming of Th into Tregs [109].

Anti-LAG-3: Lymphocyte Activation Gene-3 (LAG-3) has also been implicated in Treg suppressive mechanisms [110]. A number of anti-LAG-3 Abs are being developed. One of these, ImmuFact IMP321, tested in a Phase 1 trial, enhanced $CD8^+$ CTL activation and was also suggested to block Treg function given the lack of IL-10 secretion in an *ex vivo* assay [111, 112]. An antagonist Ab, IMP701, has also been developed and shown to inhibit Treg activity and enhance T cell proliferation.

Anti-TIM3: TIM-3 negatively regulates Th1 and Th17 cells and is also a marker of 'exhausted' $CD8^+$ CTLs, along with PD-1. A TIM3 blocking Ab in pre-clinical development has been reported to enhance anti-tumour immunity and the efficacy of cancer vaccines in murine studies [113-115]. The effect of TIM3 blockade on human T cells and Tregs remains to be confirmed.

Recent studies report blockade of multiple immune checkpoints is a more effective strategy, where blocking Abs have a synergistic effect without causing any additional toxic effects. Co-blockade of PD-1, in particular, with other molecules has shown promise. This strategy works by releasing 'exhausted' T cells and CTLs through PD-1 blockade while blockade of other immune checkpoints such as TIM-3 or LAG-3 inhibits Treg function. TIM-3 and PD-1 co-blockade in murine carcinomas significantly reduced Treg activity and induced complete tumor regression [113]. LAG-3/PD-L1 co-blockade induced complete tumour remission in murine models of recurring melanoma [116]. CTLA-4/PD-1 co-blockade has shown efficacy in murine ovarian and colon carcinomas where it reversed tumour-infiltrating CTL exhaustion and inhibited Treg

activity leading to tumor rejection [103]. Pidilizumab (anti-PD-1) has also been reported to synergise with low-dose cyclophosphamide to prolong TI Treg depletion and enhance cancer vaccine-induced CTL infiltration into tumour tissue in mice [117]. Combing nivolumab plus ipilimumab has been examined in advanced melanoma patients, inducing deep and rapid tumour regression [118]. Taken together, blockade of multiple immune checkpoints might allow improvement of current immunotherapy regimens without the need to develop novel blocking agents.

5. Expert Opinion

Tregs play a pivotal role in dysregulation of anti-tumour immunity and cancer progression. This has made them an attractive target for cancer immunotherapy and the focus of many investigations in recent years. It is also important to note that Tregs do not always have a negative impact on prognosis; in some lymphomas and carcinomas, Treg accumulation correlates with better overall survival. The exact reasons for this are still not clear, highlighting the need to expand our understanding of Treg immunobiology.

Tregs comprise a diverse range of subsets both in cancer and homeostatic conditions, the full extent of which is just becoming clear. These subsets exhibit varying immunosuppressive activity, *in vivo*-lineage stability and tissue localization. Determining the contributions of different subsets in tumour immunity can allow specific targeting of the most 'pathological' subsets – for example, tumour-infiltrating Tregs – without inducing systemic Treg depletion thus avoiding development of autoimmune side effects. While several novel Treg markers have been identified in recent work (GARP, LAP, CD26, NRP1), many current markers are modulated by activation and also expressed on Teff.

Delineating pTregs and tTregs in humans also remains an unresolved issue. It is likely there is no single 'tTreg or pTreg marker'; rather a combination of markers and analysis of other factors

including TSDR methylation status, epigenetic modifications and *in vivo* microenvironment may allow better characterization of each subset.

Treg depleting therapies could target proliferative and functional pathways: (i) Chemokine and chemokine receptor blockade to block Treg migration, (ii) Inhibiting *in situ* Treg generation or conversion in tumour tissue through blockade of cytokines such as TGF-β/TGF-β receptor blockade and (iii) Blockade of the myriad surface, and intracellular markers that play both functional and redundant roles in Tregs. Most prominently among these are the immune checkpoints. Traditionally implicated in down-regulation of T cell activation, immune checkpoints have also been reported to be relatively stable on tumour-infiltrating Treg subsets where they contribute to suppressive activity. The blockade of immune checkpoint releases 'exhausted' T cells from anergy and also impairs Treg activity; greatly enhancing anti-tumour immune responses. Multi-faceted approaches involving multiple immune checkpoint blockade, chemotherapy, and genetically-modified T-cell adoptive therapy provide exciting therapeutic modalities that are clinically feasible, without requiring development of new drugs or antibodies. Further focused investigations and clinical trials targeting immune checkpoints (CTLA-4, PD-1, LAG-3, TIM-3, IDO), other markers (CD25, FoxP3, NRP1, GITR, ICOS) as well as specific Treg subsets will be crucial to build upon the exciting research and develop new cancer immunotherapies.

Although a considerable progress has been made in understanding the role and function of Treg subsets in different disease settings including cancer, further understandings of the molecular pathways, Treg mechanisms of action and defining surface markers specific for the different Treg subsets should provide chances to use Tregs in the clinic for treating different diseases or to target them to enhance anti-tumour/microbial immune responses.

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Table 1: Clinical impact of Tregs in cancer (a multivariate analyses)

Treg population	Tumour	Clinical Impact	Ref
Elevated tumour-infiltrating FoxP3$^+$ Tregs	HNSCC	Increased DFS	49
		Enhanced locoregional control	50
	Colorectal cancer	Better OS	52
		Lower OS & DFS (VEGFR2$^+$ TI Tregs)	119
	Liver metastasis from colorectal cancer	Lower OS	120
	Classical Hodgkins Lymphoma	Better OS and DFS	121
Elevated circulating FoxP3$^+$ Tregs	Diffuse B cell Lymphoma	Better OS and probability of complete remission	122
	Multiple melanoma	Lower OS* (non-multivariate analysis)	123
Elevated intratumoural CD8$^+$ CTL : Treg ratio	Breast carcinoma	Better OS (with increased peri-tumoural ratio) Better OS & PFS (with increased intratumoural ratio)	76
	HCC	Better OS (following resection)	75
Elevated intratumoural Treg : CD8$^+$ CTL ratio	Breast cancer	Better OS	74
	Colon cancer	Better OS (patients undergoing 5 year adjuvant chemotherapy)	77
	HCC	Lower OS (following resection)	124
Lymph node Treg accumulation	Colorectal cancer	Correlated with disease stage and LN mestastasis	125

Summary of recent studies utilising multi-variable analysis to determine the value of Tregs as an independent prognostic factor in different cancers. This table expands upon the studies included previously on Tregs and their clinical impact in cancer [3].

HNSCC – Head & Neck squamous cell carcinoma, GBM – Glioblastoma multiforme, HCC – Hepatocellular carcinoma, RCC – Renal cell carcinoma, DFS – Disease free survival, OS – Overall survival, PFS – Progression free survival, LN – Lymph node, CTL – Cytotoxic lymphocyte

Table 2: Treg surface markers

Marker	Th1	Treg	TI Treg	T cell activation marker	Ref
CD25	✓ ($CD25^{lo/-}$subsets also exist)	✓	✓	✓	9
CD26	X	Negative marker	Hodgkins Lymphoma and PB of HNSCC	X	25, 30, 31
NRP1 (CD304)	X	✓	??	✓	16
TIM-3	??	✓	Selectively upregulated in TT but not PB of NSCLC HCC, CC, CRC and ovarian carcinomas or NT	✓	33, 34
LAG-3 (CD233)	✓ (co-expressed with CD49b)	✓	In tumour-invaded LN, PB, TT of MM & CRC patients and TILs in Hodkins Lymphoma	✓ Late activation marker also expressed by NK cells and B cells	89, 102
4-1BB (CD137)	X	✓ (combined with $CD40L^{-}$)	✓	??	126
CD39	✓ (when coexpressed with CD73 on $FoxP3^{-}$ T cells)	✓ (when coexpressed With CD73)	✓ (elevated in PB and TT of HNSCC patients)	?? B cell & NK cell activation marker, further studies required	24-27, 29, 31
CD127	X	Negative marker	??	Upregulated on activated Tregs and Teff	127
TNFR2	X	✓	✓ (also expressed on tumourinfiltrating Teff)	✓	
CTLA-4	X	✓	✓ (on TI $CD8^{+}$ Tregs)	✓	
PD-1	✓	✓	✓	✓	103, 128
ICOS	✓	✓	✓	✓	
GARP	??	✓	On TI Tregs in HNSCC patients	✓ Expressed at higher levels on activated Tregs than Teff	19, 20, 28
LAP	??	✓	On TI Tregs in HNSCC patients	✓ Expressed more stably & at higher levels on activated Tregs than Teff	20, 21, 28

Any combination of these markers with CD4 and CD25 allows identification and isolation of highly suppressive and viable Treg subsets.

PB – Peripheral blood, TT – Tumour tissue, NT – Normal tissue, LN – Lymph nodes, MM – Metastatic Melanoma, CRC – Colorectal cancer, HNSCC – Head & Neck Squamous cell carcinoma

Table 3: Treg intracellular markers

Marker	Th1	Treg	TI Treg	Activation marker	Ref
FoxP3	X	✓	✓	✓	[9]
SATB1	??	Negative marker	??	??	[66, 67]
Helios	X	Mu tTreg marker but unconfirmed role as Hu pTreg/tTreg marker	✓	✓ (also expressed on activated $CD8^+$ T cells)	[59, 62, 129]
Foxo1/3	??	✓	✓	Possible-late activation marker	[130]

Several intracellular Treg markers have been identified. They can be useful for flow cytometric analyses; however, the intracellular staining process requires cell fixation that kills cells, thus preventing their use for isolation of viable Tregs. They can still be targeted for immunotherapies, as recently reported where a P300 HDAC inhibitor was utilized to target FoxP3 in mice [131]

(Mu – Murine, Hu – Human)

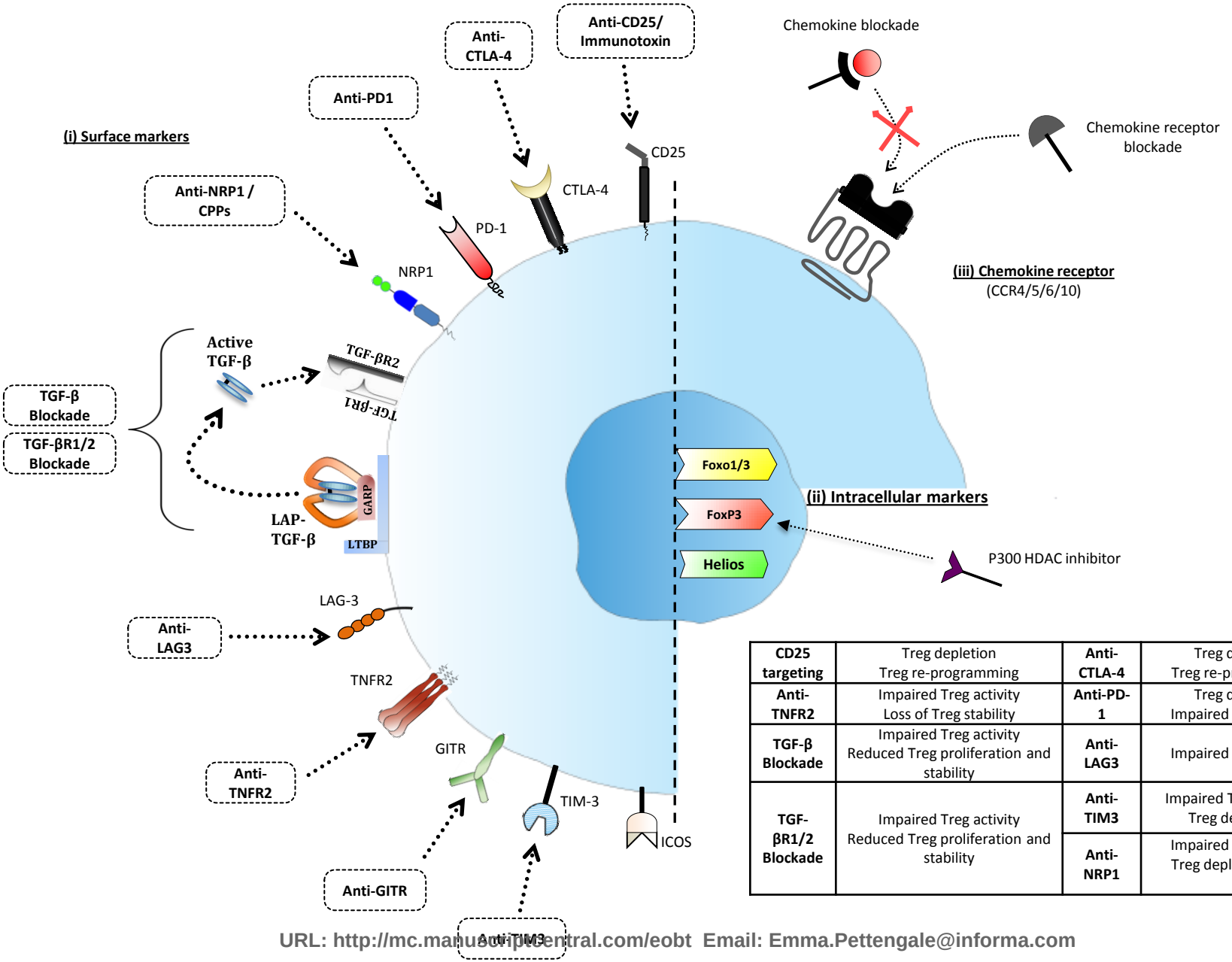
Figure legend

Figure 1: Treg markers and targets for immunotherapy. Tregs express a range of markers that can be utilized for their identification, as summarized in Tables 2 and 3. Importantly, many of these markers can be targeted in immunotherapies.

(i) Surface markers are useful for isolating viable cells and also offer the most accessible target for immunotherapies.

(ii) Intracellular markers, such as FoxP3 and the Foxo transcription factors, do not allow isolation of viable cells. They can be still, however, targeted for immunotherapies for example using a P300 HDAC inhibitor to target FoxP3 causing Treg apoptosis and reduced tumour growth in immunocompetent mice [131]. This could be useful for $FoxP3^+$ Treg depletion although activated T cells also upregulate FoxP3 to a certain degree.

(iii) Chemokine receptors are crucial for Treg migration and can also identify certain Th-specific Treg subsets [82]. Blocking these chemokine receptors can impair Treg migration and has shown efficacy in murine models – it remains to be tested in humans. Chemokine blockade can also be utilized to restrain Treg migration into tumour tissue or other sites.



CD25 targeting	Treg depletion Treg re-programming	**Anti-CTLA-4**	Treg depletion Treg re-programming
Anti-TNFR2	Impaired Treg activity Loss of Treg stability	**Anti-PD-1**	Treg depletion Impaired Treg activity
TGF-β Blockade	Impaired Treg activity Reduced Treg proliferation and stability	**Anti-LAG3**	Impaired Treg activity
TGF-βR1/2 Blockade	Impaired Treg activity Reduced Treg proliferation and stability	**Anti-TIM3**	Impaired Treg activity ? Treg depletion ?
		Anti-NRP1	Impaired Treg activity Treg depletion (CPPs)