

The emerging role of anti-CD25 directed therapies as both immune modulators and targeted agents in cancer

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Summary

CD25 forms one component of the high-affinity heterotrimeric IL2 receptor on activated T cells. Its affinity for IL2 and cellular function are tightly regulated and vary in different cell types. The high frequency of CD25 on the surface of many different haematological tumour cells is now well established and apart from its prognostic significance, CD25 may be present on leukemic stem cells and enable oncogenic signalling pathways in leukemic cells. Additionally, high CD25 expression in activated circulating immune cells and Tregs is a factor which has already been exploited by IL2 immunotherapies for treatment of tumours and autoimmune disease. The relative clinical safety and efficacy of administering anti-CD25 radioimmunoconjugates and immunotoxins in various haematological tumour indications has been established and clinical trials of a novel CD25-directed antibody drug conjugate are underway.

Keywords

CD25, interleukin-2 receptor, regulatory T cells, CD25-expressing malignancies, anti-CD25 targeted therapy

Biology of IL2 and IL2R signalling in the maintenance of normal physiology

IL2 and the components of the IL2R

In the 1970's, IL2 emerged as a key cytokine in regulating the activation and proliferation of immune cells (Malek, 2008). The structure and function of IL2 (T cell growth factor) was refined over the next decade. The antigen in isolation to which IL2 binds with the highest affinity, IL2RA (CD25), was first cloned in T cells of the MT-1 Adult T cell leukemia lymphoma (ATL) cell line (Nikaido et al., 1984) using an anti-Tac (T-cell activation antigen) monoclonal antibody generated a few years previously by Uchiyama *et. al* (Uchiyama et al., 1981). Assembly of the IL2/IL2R is initiated by the interaction of IL2 with IL2RA, followed by sequential recruitment of IL2RB (CD122) and IL2RG (CD132) (Liparoto and Ciardelli, 1999), although a hypothesis for pre-assembly of the receptor components prior to IL2 binding is also feasible (Rickert et al., 2004). It is even more likely that the assembly mechanisms both occur to varying degrees (Rickert et al., 2004). IL2RA on its own constitutes the low-affinity IL2 receptor (dissociation constant $K_d = 10 \text{ nM}$). IL2RA and IL2RB form the pseudo-high-affinity receptor ($K_d = 30 \text{ pM}$) whilst the heterotrimeric IL2R complex forms the high-affinity receptor ($K_d = 10 \text{ pM}$) (Rickert et al., 2005). In cells in which they are expressed in high levels, IL2RB and IL2RG may associate with IL2 ($K_d = 1 \text{ nM}$) (Rickert et al., 2004) and initiate signalling independently.

CD25 expression in immune and non-immune cells

High levels of the trimeric IL2R are transiently expressed by CD4+ and CD8+ T cells following T cell receptor (TCR) activation (Malek, 2008). Unlike other T cell subsets, the majority of regulatory T cells (Tregs) express high levels of CD25, with intermediate levels of CD122 and CD132 (Boyman and Sprent, 2012). Shortly after anti-Tac synthesis and purification, studies describing CD25 expression in tissues other than tumour cells and activated T cells started to emerge. Waldmann *et al.* described its expression in immature B

cells activated with T cell co-culture in the presence of pokeweed mitogen, a potent immunological stimulant (Waldmann et al., 1984). It was discovered on activated natural killer cells and activated cells of the monocyte-macrophage series, including cultured cell lines capable of monocytic differentiation, lung macrophages, liver Kupffer and skin Langerhan's cells (Holter et al., 1987, Herrmann et al., 1985). It was later found to be constitutively expressed on tissue mast cells in patients with inflammatory diseases and in those with no demonstrable pathology (Maggiano et al., 1990). Basophils, often considered a circulating counterpart to tissue mast cells, were shown to express CD25 particularly after activation by the supernatant taken from T cell stimulated cells (Maggiano et al., 1990). Eosinophils extracted from normal subjects and stimulated with granulocyte monocyte colony stimulating factor (GM-CSF) and IL3 *ex vivo* upregulated their CD25 expression (Riedel et al., 1990). Table 1 summarises the expression of IL2R components on different cell types (Boyman and Sprent, 2012).

Table 1. Expression of CD25, CD122 and CD132 in immune and non-immune cells

Cell type	CD25	CD122	CD132
Thymocyte	-/+	-/+	+
Naïve T cell	-	-/+	+
Effector T cell	+++	++	+
Memory T cell	-	+ /+++	+
Treg	+++	+	+
Immature B cell	+	-	+
Mature B cell	-	-	+
NK cell	-	++	+
NK T cell	-/+	-/+	+
Langerhans cell	+	?	+
Endothelial cell	+	+	+

-: background; +: low; ++ high; +++ very high;? unknown

Mechanism of CD25 upregulation in T cells

The mechanism by which CD25 expression is upregulated in T cells occurs by different pathways. Mitogen or allo-antigen binding to T cells activates their T cell receptors initiating a calcium influx leading to phosphorylation of protein kinase C and a cascade resulting in CD25 upregulation (5 to 20 fold greater than the heterotrimeric IL2R as a whole) (Waldmann, 2007). The expression of CD25 by T cells is additionally stimulated by contact with IL2 which induces a positive feedback loop that involves the binding of signal transducer and activator of transcription 5 (STAT5) to the CD25 gene locus³ (Boyman and Sprent, 2012). Analyses have shown the peak expression of CD25 of up to 60,000 molecules per T cell surface occurs 48 to 96 hours after activation, followed by a decline in expression by 80% by 10 to 21 days (Waldmann, 2007, Rubin et al., 1985). Apart from its effects on CD25 gene expression, binding of IL2 to its heterotrimeric receptor stimulates T cell growth and effector function (Malek, 2008). This occurs via signal transduction pathways with the subsequent phosphorylation and translocation of dimerised STAT5 proteins, activated mitogen activated protein kinase (MAPK) or p70 S6 kinase (p70^{S6K}) to the nucleus, stimulating transcription (Figure 1).

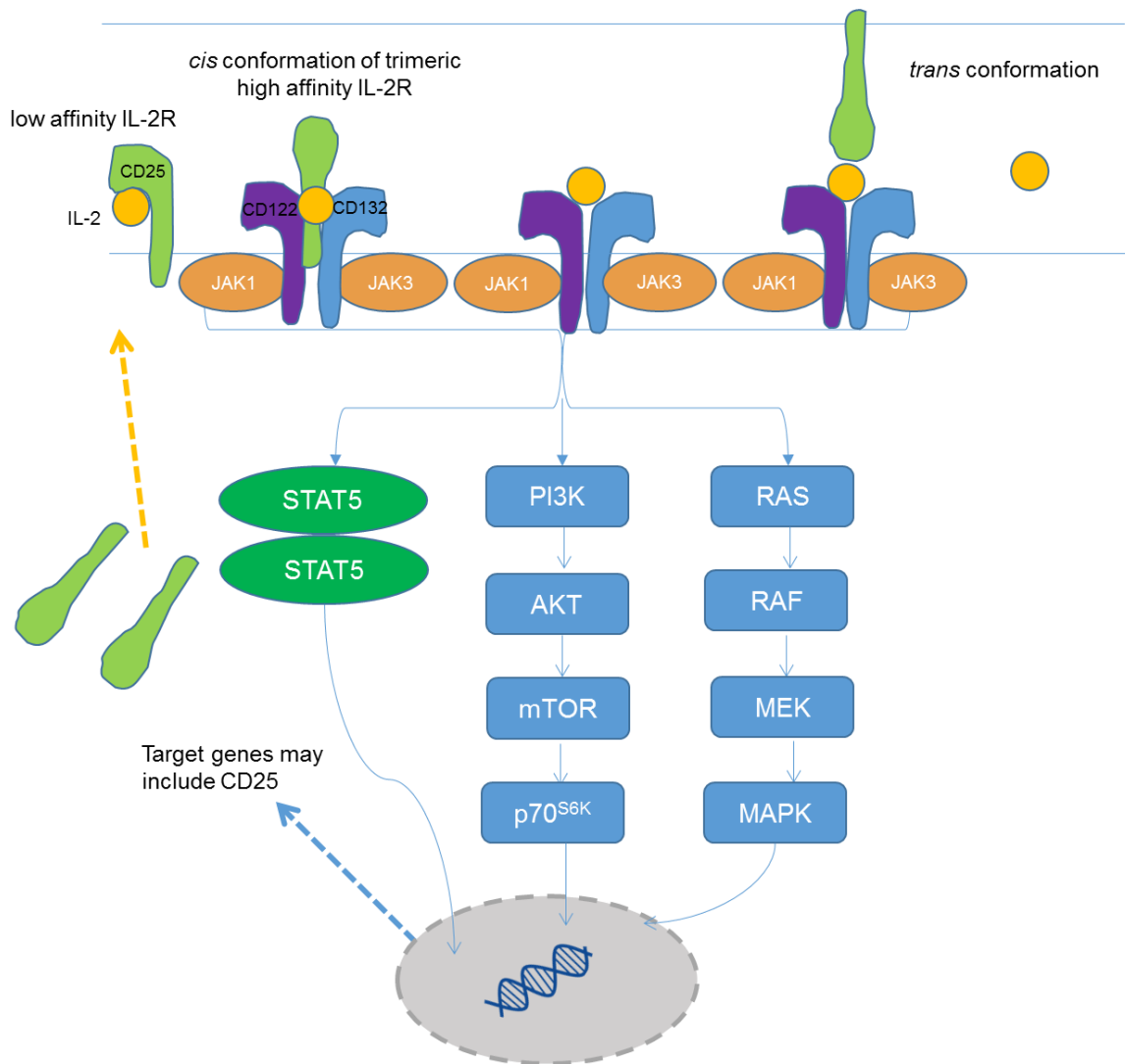


Figure 1. Representation of IL2R activation pathways in a stimulated T cell. Different conformations of the IL2R influence its affinity for IL2 (low affinity CD25 or high affinity trimeric receptor). IL2 can also influence signalling via CD122/CD132 dimers. Additionally, whether the CD25 molecule forming the receptor comes from an adjacent cell (*trans*) or the same cell (*cis*) determines the convention for naming the high affinity heterotrimeric receptor. The activation of pathways is initiated by Janus kinase 1 and 3 (JAK1 and JAK3) phosphorylation, which in turn stimulate STAT5 dimerisation, or the phospho-inositol 3 kinase (PI3K), and rat sarcoma viral oncogene homolog (Ras) pathways which ultimately phosphorylate effector kinases, p70^{S6K} and MAPK.

IL2/IL2R signalling in Tregs

Importantly, upregulated CD25 does not constantly have an activating and proliferating role, with downstream pathways and consequences of IL2 binding to its receptor being modulated to varying degrees in different cell types. Tregs play an important role in immune homeostasis, maintaining immune tolerance to self-antigens. There are at least two Treg subsets in humans. Natural Tregs originate in the thymus and mediate suppression via cell contact-dependent mechanisms playing a major regulatory role in maintaining peripheral tolerance (Raimondi et al., 2007). Inducible Tregs or type 1 regulatory T (Tr1) cells are activated in the periphery in response to cytokines including IL2 and TGF β (Raimondi et al., 2007). Tr1 cells mediate immune homeostasis by the production of growth factors including TGF β and IL10 (Roncarolo et al., 2006). In order to appreciate the complexity of precise Treg monitoring both phenotypically and functionally, it is useful to underscore the heterogeneity and plasticity of and lack of a unique identifying marker on these cells (Camisaschi et al., 2014).

The mechanism by which IL2 facilitates immune tolerance in Tregs is thought to primarily involve the CD25/STAT5 mediated pathway (Malek, 2008). Hickman *et al.* conducted experiments showing that Tregs may quench IL2/IL2R signal propagation (Hickman et al., 2006), one of the factors which upregulate FOXP3 expression and drive their immunosuppressive phenotype (Zeiser and Negrin, 2008). In Hickman's experiments, Tregs isolated from murine lymph node and spleen demonstrated a limited accumulation of second messengers including diacylglycerol which resulted in reduced IL2 transcription. An alternative mechanism of CD25/STAT5 signalling fine tuning occurs via ADP-ribosylation of the CD25 binding site of IL2 (Teege et al., 2015). This inhibits IL2-induced STAT5 phosphorylation and subsequent IL2-dependent cell proliferation. Studies looking at which other signalling pathways may be regulated in Tregs suggest that PI3K activity is also suppressed in these cells (Zeiser et al., 2008) possibly by the upregulation of the inhibitory

effect of PTEN (phosphatase and tensin homolog) on PI3K signalling (Walsh et al., 2006). Regarding the relevance of the third pathway downstream of IL2R, *in vitro* analyses of Treg function have shown downregulation of Ras, mitogen activated protein kinase (MAP2K or MEK1), and extracellular signal-regulated kinase 1/2 (ERK1/2) (Li et al., 2015).

Although pathway redundancy is evident in IL2/IL2R signalling (Rickert et al., 2004), the complex modulation of pathway activity perhaps explains why IL2R signalling dysfunction is implicated in many human autoimmune diseases. For example, polymorphisms surrounding the IL2RA locus at 10p15.1 are associated with Type 1 diabetes or rheumatoid arthritis (Stahl et al., 2010, Lowe et al., 2007). The significance of IL2/IL2R signalling dysfunction in cancer is less well understood.

Tregs and cancer

Patients who demonstrate tumour-specific T cell responses are thought to have a better prognosis than those without such T cell immune responses. Various tumour inhibitory mechanisms account for a tumour's ability to evade immune detection, including presentation of tumour-associated antigens by MHC class I molecules, the suppression of costimulatory ligands for T cell activation and the secretion of immunosuppressive cytokines or upregulation of iTregs (Rabinovich et al., 2007). As one target for antitumour immunotherapy, developing an understanding of their role in both the tumour microenvironment and the peripheral blood, Tregs have attracted much attention.

Tumour-infiltrating Tregs

Many tumours contain significant numbers of Tregs and in particular those which demonstrate a higher ratio of CD4⁺ CD25⁺ FOXP3⁺ Tregs to CD8⁺ cytotoxic T cells are

thought to predict for a poorer prognosis. This may be explained by these infiltrating Tregs suppressing the activation of tumour antigen-specific effector T cells as well as hindering the differentiation and activation of normal and lymphoma B cells (Nishikawa and Sakaguchi, 2014, Grygorowicz et al., 2016) Consequences of this may be illustrated in a study in follicular lymphoma which showed an association of follicular localisation of Tregs with poor survival (Farinha et al., 2010). In this study with median follow up of 17.1 years, FOXP3 upregulation was used as a marker of Treg infiltration and follicular localisation of FOXP3+ cells significantly improved prediction of transformation risk over and above the survival prognostication provided by the established International Prognostic Index.

There is, however, conflicting evidence on the prognostic significance of Tregs in malignancy (Grygorowicz et al., 2016). Infiltrating Tregs correlated with improved survival in studies of patients with follicular lymphoma (Tzankov et al., 2008, Lee et al., 2008), both subtypes of diffuse large B cell lymphoma (germinal centre and non-germinal centre DLBCL) (Tzankov et al., 2008, Lee et al., 2008) and Hodgkin's lymphoma (Tzankov et al., 2008, Alvaro et al., 2005). A more thorough analysis of this data may help clarify some of the seeming contradictions in the predictive value of tumour infiltrating Tregs. Multivariate analyses were done on the 926 patients included in the *Tzankov et al* study, but only patients in the classical Hodgkin's lymphoma group with higher FOXP3 in affected biopsied lymph nodes demonstrated a statistically significant improvement in survival. For both subtypes of DLBCL analysed by *Lee et al* (2008), a higher proportion of FOXP3 positive infiltrating cells by immunohistochemistry on their initial tumour biopsies predicted for a statistically significant improvement in survival. However, relatively small numbers of patients were retrospectively included and sub-classification of DLBCL subtypes was carried out by less robust tissue microarray analyses in only a proportion of cases (67/96).

In studies predicting for both an improved or worse prognosis, an analysis of the methods by which Tregs were identified in these tissues may suggest that FOXP3+ or CD25+ T cells counted in tissues were not Tregs but rather activated CD4+ or CD8+ effector T cells. The complexity of Treg interactions with other immune cell populations (Alvaro et al., 2009) underlines the importance of validating not only which cell types are infiltrating tumours and in what ratio, but also their specific function.

Circulating Tregs

Circulating Tregs have also been proposed as biomarkers, but quantification of Treg cell number in the peripheral circulation is equally unlikely to provide a simple prognostic tool. In one hepatocellular carcinoma study, survival rate was significantly lower in patients with higher levels of peripheral blood Treg cells compared to those with lower Treg levels (Li et al., 2015). Similarly, in patients with CLL, high Treg numbers were negative prognostic indicators (Giannopoulos et al., 2008). In contrast, a low number of circulating Tregs was associated with poor prognosis in patients with DLBCL (Glowala-Kosinska et al., 2013). For similar reasons highlighted above individual cell populations are unlikely to be helpful as predictive biomarkers. This is illustrated well by *Tarhini et al* (2014), in a study during which biopsies and blood samples in patients with Stage 3 melanoma treated neoadjuvantly with anti-CTLA4 antibody, ipilimumab were collected pre-treatment and at surgery. There was a significant increase in circulating regulatory T cells which corresponded to a significant improvement in progression free survival (Tarhini et al., 2014). These Tregs were identified not only by FOXP3 positivity as in the studies analysing tumour-infiltrating Tregs but by multiparametric flow cytometry: i.e. CD4, CD25^{hi} and FOXP3 positivity. In matched tumours, there was a significant increase in CD8+ T cells after ipilimumab treatment.

CD25 expression and secretion in malignant cells

Almost all patients with human-T cell lymphotropic virus-1 (HTLV-1)-associated adult T cell leukemia/lymphoma (ATL) and hairy cell leukaemia (HCL) constitutively express CD25 on their cell surface (Ambrosetti et al., 1993, Horiuchi et al., 1997). However, the heterogeneity of CD25 expression in many other lymphomas (Strauchen and Breakstone, 1987) and leukemias (Nakase et al., 1994b) is well established. CD25 is expressed in leukemic diseases such as B cell chronic lymphoblastic leukaemia (CLL), acute lymphoblastic leukaemia (ALL) and acute myeloid leukemia (AML) (Nakase et al., 1994a, Nakase et al., 1994b). Both Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL) malignant cells have demonstrated expression of CD25 (Waldmann, 2007, Tesch et al., 1993). NHL tumours with high levels of surface CD25 expression include diffuse large B cell lymphoma (DLBCL) (Hashimoto et al., 2013, Yoshida et al., 2013), follicular lymphoma (Yoshida et al., 2013) and peripheral T cell lymphoma including angioimmunoblastic T cell lymphoma and anaplastic large cell lymphoma (Gualco et al., 2009). Rarer diseases showing CD25 expression include Waldenström's macroglobuliaemia (San Miguel et al., 2003) and systemic mastocytosis which is defined by its CD25 expression (Lim et al., 2009).

A surrogate marker of CD25 expression is the shed surface antigen, soluble CD25 (sCD25). Significantly, release of sCD25 is proportional to its cell surface expression (Junghans and Waldmann, 1996). Table 2 shows examples of diseases which are known to be associated with high sCD25 expression, suggesting elevated levels in any given patient are likely to be multifactorial, arising from different cell types in infection, inflammation and neoplasia. New diseases are being added to this list as the measurement of sCD25 becomes a more accessible laboratory test. For example, the updated guidelines for the diagnosis and prognostication of haemophagocytic lymphocytichistiocytosis (HLH) include sCD25 (Hayden et al., 2016).

Table 2. Disorders associated with elevated sCD25 levels (Bien and Balcerska, 2008)

Autoimmune disease	Neoplasia	Infection	Other
Aplastic anaemia Asthma Idiopathic thrombocytopenia Uveitis Multiple sclerosis	Leukemias Lymphomas Multiple myeloma Solid tumours e.g. sarcomas, head and neck cancer, lung adenocarcinoma	HIV Ebstein Bar Virus Pulmonary tuberculosis Sepsis	Haemophagocytic lymphocytichistiocytosis General anaesthesia End stage renal disease Burns Allograft rejection

Solid tumours in which elevated sCD25 have been described include lung adenocarcinoma (Yano et al., 1996), oesophageal (Wang et al., 2000), and head and neck cancers (Tartour et al., 2001), although it is not clear whether CD25-positive tumour-infiltrating lymphocytes are largely responsible for its secretion. Indeed, as CD25 is expressed both on normal activated lymphocytes and monocytes and on malignant cells, it is sometimes difficult to ascertain which cells within any given tumour are expressing CD25. Although the precise biological role of CD25 expression in these malignant disorders is not clear, it may be involved as a stimulating driver for the proliferation of tumour cells (Waldmann, 2007)

Nakase *et al.* have shown significantly higher sCD25 levels both in haematological and non-haematological cancers compared with healthy volunteers (Nakase et al., 2005). The haematological malignancies thought to secrete very high levels of sCD25 include some of the acute leukemias and non-Hodgkin's lymphomas (Nakase et al., 2005). It is likely that this serum elevation is related to high tumour cell CD25 expression but may have other contributing factors. These increased levels of sCD25 may originate from lymphocytes activated in general inflammation or be released from tumour-infiltrating lymphocytes which also express CD25. The cleavage of surface CD25 may also be accelerated by proteases secreted from tumour-associated macrophages (Sakai and Yoshida, 2014).

Secretion of sCD25 in malignancy is likely to have several biological roles. These may include its role in binding to circulating IL2 which prolongs IL2 half-life and potential bioactivity (Kobayashi et al., 1999), contributing to a maintained state of inflammation in cancer patients. Alternatively, sCD25 secretion may be an adaptive mechanism to mediate tumour immune evasion in hepatocellular carcinoma (Cabrera et al., 2010), melanoma or renal cell carcinoma (RCC) (Gooding et al., 1995). Plasma from patients with melanoma or RCC with elevated sCD25 concentrations produced a significant reduction in cell growth when included in IL2 stimulatory assays (Gooding et al., 1995). Possible mechanisms of tumour immune evasion may be the downregulation of CD25 cell surface expression on effector T cells or immune-inhibitory interactions between sCD25 and Tregs (Cabrera et al., 2010).

Irrespective of why sCD25 expression is increased, being able to measure it non-invasively suggests it is worth investigating its clinical utility. The serum level of sCD25 in high-secreting malignancies may directly reflect the disease burden. For example, high pre-treatment sCD25 serum levels have helped identify patients with poorer prognoses in diffuse large B cell lymphoma (Yamauchi et al., 2012) and are used as a prognostic index in ATL (Katsuya et al., 2012). Tumour cell surface CD25 antigen has been shown to be associated with poor outcome in adults with AML and myelodysplastic syndrome (MDS) (Gonen et al., 2012, Terwijn et al., 2009), which may be reflected in sCD25 serum levels. Alternatively, sCD25 has also been tested as a predictive biomarker. In one study, sCD25 levels accurately predicted which melanoma patients would become resistant to anti-CTLA4 antibody ipilimumab (Hannani et al., 2015).

CD25 and oncogenesis

Due to the prognostic significance of CD25, a separate important question is whether its upregulation may in fact play a significant role in oncogenesis or the maintenance of a permissive tumour microenvironment.

Solid tumours

Multiple murine tumour models exhibit decreased tumour growth in B cell deficient animals (Schwartz et al., 2016). B cells are thought to facilitate conversion of T cells to Tregs via the expression of inhibitory ligands on regulatory B cells (Bregs) and the secretion of cytokines IL10 and TGF β (Schwartz et al., 2016). IL10 maintains Bregs and increases the intratumoural Treg subpopulation whilst suppressing Th1/Th17 and has thus been implicated in the promotion of tumour immune evasion in several solid tumours. For example in breast cancer this is thought to impact on tumour aggressiveness (Mohammed et al., 2013) and metastasis (Olkhanud et al., 2011) Tregs are also thought to play a role in the maintenance of an oncogenic phenotype in other solid tumours including hepatocellular carcinoma, lung, oesophageal and ovarian cancers (Schwartz et al., 2016). Other mechanisms by which alterations in CD25 expression may induce oncogenesis include CD25 gene promoter polymorphisms. A case-control study in Chinese patients with one of two polymorphisms in their CD25 gene promoter, showed those with the polymorphism rs7072793 T > C were more likely to develop breast cancer (Li et al., 2013). The authors hypothesised that this polymorphism may alter the function and expansion of CD25 in Tregs and thereby promote tumour immune evasion (Li et al., 2013).

Leukemias

ATL leukemic cells universally overexpress CD25, a phenomenon which may, at least in part, be mediated by the HTLV-1 (Human T cell lymphotropic virus-1) Tax gene product (Waldmann et al., 1988). One hypothesis to explain why a CD25-expressing clone is preferentially selected is that signalling via CD25 permits cell proliferation and tumour survival. Tumour cells from patients who had become resistant to CD25-directed antibody therapy, anti-Tac, showed persistent CD25 expression (Waldmann et al., 1988), suggesting that this antigen remains targetable in refractory ATL.

In acute lymphoblastic and acute myeloid leukemias, research into the role of CD25 has focused on understanding its significance in oncogenic receptor tyrosine kinase signalling and leukemia stem cells (LSCs), respectively.

The fusion product of bcr-abl translocation (Philadelphia chromosome, Ph) is an indicator of unfavourable prognosis in adult B-ALL (Armstrong and Look, 2005). Although an association between Ph-like ALL and CD25 expression has been demonstrated (Geng et al., 2012), the mechanism for this association has not been clearly elucidated. While CD25 does not typically function as a component of the IL2 receptor chain in B cells, it does coordinate pre-B cell receptor (BCR)-dependent signal transduction (Lee JW, 2015). The pre-BCR related tyrosine kinase (BTK) is phosphorylated by bcr-abl in Ph+ ALL and other tyrosine kinase oncogenes in Ph-like ALL. Lee *et al.* measured the relationship between CD25 expression and cell signalling in these mutant cell lines, and showed that overexpression of constitutively active BTK resulted in strong upregulation of CD25 surface expression (Lee JW, 2015). Conversely, BTK-inhibitor ibrutinib abolished CD25 expression, suggesting that feedback control between pre-BCR signalling and CD25 requires BTK (Kersseboom et al., 2006). The authors showed that the mechanism for this feedback control may be via CD25 surface receptor membrane recruitment of phosphatases such as PTEN, which balance

fluctuations in signalling output from a pre-B cell receptor or oncogenic tyrosine kinase and may also provide a mechanism of drug resistance (Lee JW, 2015). Other groups have separately demonstrated that CD25 gene products were significantly overexpressed and hypomethylated in Ph-positive subtype compared to both Ph-negative and normal pre-B cells (Paietta et al., 1997, Geng et al., 2012). Together these data suggest that CD25 may play a role in the evolution of refractory ALL.

AML blasts which express CD25 may be enriched in chemotherapy-resistant LSCs (Gonen et al., 2012). These quiescent cells bear a LSC-like molecular signature (Gonen et al., 2012) and can establish AML in xenograft models (Saito et al., 2010). Reasons for the expression of CD25 on cell-cycle quiescent tumour cells remain obscure but are likely to drive further research into its potential role in the development of multidrug-resistant AML.

Drugs targeting CD25

The main strategies for targeting CD25 are shown in Figure 2 and described in detail below.

Recombinant IL2

The ability of IL2 to activate both Tregs and cytotoxic T lymphocytes (CTLs) brings into question its use as an immunotherapeutic agent (Boyman and Sprent, 2012). However, having at least two approaches to the differential targeting of IL2 to CD25 suggests that its use may be honed for optimal clinical benefit. Although previously thought to be mediated by natural killer cells after IL2 infusion, vascular leak syndrome (VLS) is now thought to occur predominantly via IL2 binding to CD25 low-expressing endothelial cells (Boyman and Sprent, 2012). Studies of genetically modified IL2 (“super-2”) with reduced binding affinity to CD25-positive cells (Levin et al., 2012) showed that compared to IL2, super-2 ameliorated VLS as evidenced by reduced pulmonary oedema *in vivo* (Levin et al., 2012). Super-2 demonstrated a stable crystal structure in free and receptor bound form behaving functionally like IL2 but inducing superior expansion of CTLs and proportionally less expansion of Tregs (Levin et al., 2012). Super-2 infusions resulted in improved anti-tumour responses compared to mice treated with wild-type IL2 (Levin et al., 2012).

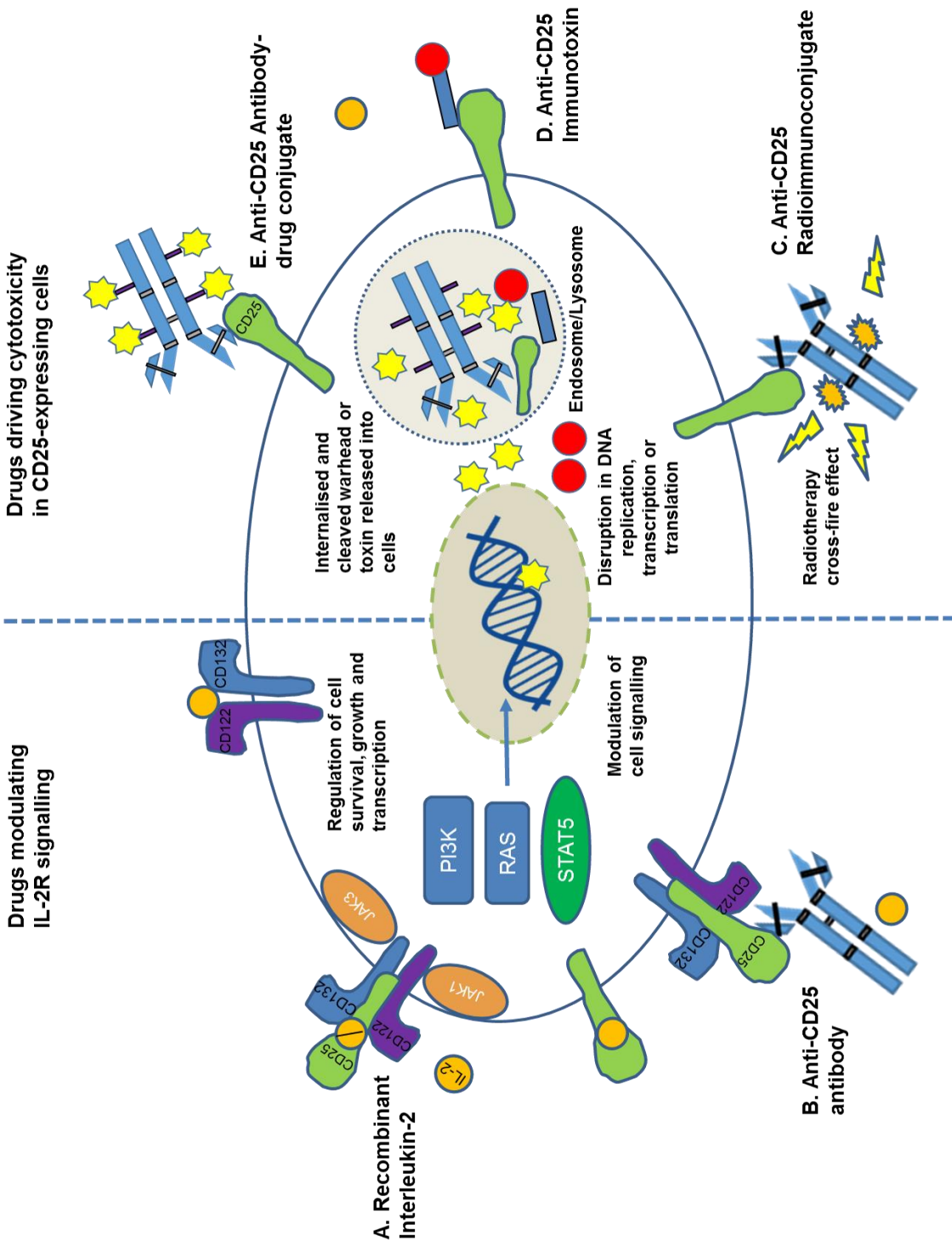


Figure 2. Anti-CD25 therapeutic approaches. A. Recombinant IL2 engineered with altered binding to CD25 may influence tyrosine kinase signalling pathways, PI3K and RAS and STAT5 dimerisation. B. Alternatively, anti-CD25 antibodies modulate cell signalling by binding epitopes on CD25 which compete with IL2. These antibodies may also influence antibody-dependent cell mediated cytotoxicity and complement dependent cytotoxicity. C. Anti-CD25 radioimmunoconjugates may influence signalling but their cytotoxicity is mediated via β -particle emitting radionuclides, which may have a cross-fire effect on neighbouring cells. D. Anti-CD25 immunotoxins are engineered toxin-antibody fragments which are internalised and impair protein synthesis. E. An anti-CD25 antibody-drug conjugate relies on internalisation and warhead release into cells to affect cytotoxicity. ADCT-301, a pyrrolobenzodiazepine (PBD) dimer conjugate, mediates its toxicity via DNA interstrand cross-linking after binding in the DNA minor groove.

Different IL2 dosing regimens may also be expected to preferentially target cell types with varying levels of CD25 expression. For example, using IL2 in low doses may expand high CD25 expressing Treg cells in conditions of relative Treg paucity such as autoimmunity and chronic inflammatory conditions, with preclinical and Phase I clinical trial data supporting this hypothesis (Boyman and Sprent, 2012). In contrast, the results of low-dose IL2 regimens for the treatment of cancer have been disappointing, presumably because of the combined effects of the expansion of the Tregs and the poor stimulation of CD25-negative or low-expressing anti-tumour CTLs (Boyman et al., 2006). High-dose IL2 administration alone, or with tumour vaccines, to patients with metastatic melanoma or renal cell carcinoma has, however, led to significant therapeutic responses and long-term survival in up to 10% of cases (Smith et al., 2008, Klapper et al., 2008).

One rationale for the use of IL2 in addition to therapeutic monoclonal antibody infusions in cancer could be the enhanced stimulation of antibody-dependent cell-mediated cytotoxicity (ADCC) that such a combination would be likely to induce. Indeed the addition of ch14.18, an anti-disialoganglioside (anti-GD2) antibody, GM-CSF, and IL2 to standard of care isotretinoin therapy was associated with improved event-free and overall survival compared to isotretinoin alone (Yu et al., 2010). This Phase 3 trial was undertaken in children with high-risk neuroblastoma who had a response to initial chemotherapy and received treatment

within 100 days after autologous stem-cell transplantation. Prior clinical trials of the addition of cytokines to tumour-reactive monoclonal antibodies that induce ADCC including rituximab, trastuzumab, and cetuximab had not shown any additional benefit over antibody alone (Musolino et al., 2008, Taylor et al., 2009, Weng and Levy, 2003). However, in contrast to the studies of anti-GD2 in the adjuvant, post autologous transplant setting, these antibody/cytokine combinations were all trialled in patients with refractory disease. In a follow-up Phase 3 study of anti-GD2 either with or without IL2 in high risk neuroblastoma, severe infusion reactions and vascular leak syndrome were significant toxicities and no statistically significant improvement in EFS or OS of combination treatment over anti-GD2 alone was demonstrated (Ladenstein R, 2016).

Anti-CD25 antibodies

Basiliximab (Simulect™), a chimeric anti-CD25 antibody, was first licensed as part of an immunosuppressive regimen for induction post renal transplantation in 1998. The humanised anti-CD25 antibody daclizumab (Zinbryta™, formerly Zenapax™) which was initially licensed in both the EU and US for this indication, was subsequently tested in refractory uveitis, and HTLV-1 associated myelopathy/tropical spastic paraparesis (Pfender and Martin, 2014). After completing pivotal Phase III trials in remitting-relapsing multiple sclerosis (Phillips et al., 2016), modified daclizumab High-Yield Process (Biogen Inc) has been approved in this indication.

Crystal structures of daclizumab and basiliximab bound to IL2R- α or the heterotrimeric IL2-IL2R- $\alpha\beta\gamma_c$ complex revealed that both antibodies bind to epitopes that overlap with the regions of CD25 that interact with IL2. Because binding of IL2 to CD25 is significantly less avid than that of daclizumab (0.27 nM) or basiliximab (0.1 nM) to CD25, both these antibodies block the binding of IL2 to CD25 and the subsequent formation of the IL2/IL2R-

$\alpha\beta\gamma_c$ complex, and inhibit IL2 signalling when applied with a sufficient dose (Goebel et al., 2000, Yang et al., 2010). This may explain the efficacy of anti-CD25 antibodies at inhibiting the propagation of T cell activation (Tkaczuk et al., 2002), thereby limiting the damaging effects of further T cell recruitment in autoimmune disease or graft rejection.

In vivo studies set up to better understand IL2/IL2R interactions were initially limited by the severe autoimmunity observed in CD25 knock-out mice (Malek, 2008). However, after establishing the tolerability of anti-CD25 antibody administration in immunocompromised mice, the hypothesis that blocking effector T cell activation by competitively blocking IL2/CD25 interactions may be used to prevent graft rejection or treat autoimmunity could be tested in human trials (Church, 2003, Pfender and Martin, 2014). The good clinical safety profile of basiliximab and daclizumab and evidence that CD25 may not be required for Treg maintenance (de Goer de Herve et al., 2010) showed that blocking IL2/IL2R interactions in Treg populations was less likely to have detrimental effects in humans (Pfender and Martin, 2014).

The potential to expand the utility of anti-CD25 antibodies as cancer therapeutics attracted attention after preliminary data showed that administration of these antibodies to mice before they were inoculated with tumours resulted in xenograft eradication (Onizuka et al., 1999). One possible explanation for this efficacy was provided by an experiment which involved the *ex vivo* removal of CD25+ CD4+ Treg cells from human peripheral blood lymphocytes (Nishikawa et al., 2005). The Treg-free mixture permitted the induction of tumour antigen-specific T cells *in vitro*. Clinically, anti-CD25 antibodies have indeed shown the potential of CD25+ T cell depletion with an enhanced anti-tumour immune response when combined with vaccine immunotherapies (Jacobs et al., 2010).

An alternative approach to CD25 targeting is utilising cell-depleting anti-CD25 antibodies to target malignant cells expressing high amounts of this antigen, rather than using these antibodies as immunotherapy and cell signalling modulators. The first-in-human trial of murine anti-Tac, in which some patients with high CD25-expressing ATL demonstrated clinical responses (Waldmann et al., 1988), suggested that this may be a way forward in the treatment of CD25-expressing tumours. However, these responses were not maintained in a follow-up Phase I trial (Waldmann et al., 1993) and Waldmann *et al.* proposed the use of a CD25 directed radioimmunoconjugate (RIC) to enhance the anti-tumour effect and duration of response.

Anti-CD25 RICs

In the first clinical trial of a radiolabelled anti-Tac antibody, ⁹⁰Yttrium-labelled murine anti-Tac was administered at doses between 5- to 15-mCi. ⁹⁰Y is a β -emitter and the rationale for using a β -emitting radiolabelled anti-CD25 antibody was provided in the extended path length of β -particles which these radioactive compounds emit compared to other radioisotopes, causing a “crossfire effect” on nearby non-target expressing cancer cells (Palanca-Wessels and Press, 2014). Results of this trial in ATL were impressive with 2/16 complete responses (CR) and 7/16 partial responses (PR) (Waldmann et al., 1995). Indeed, the responses observed represented improved efficacy in terms of length of remission when compared with previous results with unmodified anti-Tac (Waldmann et al., 1995).

Another anti-CD25 RIC, ¹³¹Iodine-labelled basiliximab (CHT-25) tested in a Phase I trial in 15 lymphoma patients was well tolerated and provided clear evidence of efficacy (Dancey et al., 2009). Remarkably, of nine HL and NHL patients treated at $\geq 1,200$ MBq/m², there were three CR and three PR, with a further two patients showing stable disease (SD), indicating that long term responses can be achieved using this approach (Dancey et al., 2009).

More recently, in a Phase I trial of ⁹⁰Y-daclizumab in HL, there were 14/46 CR and 9/46 PR with a further 14 patients having SD. Responses were observed both in patients whose Reed-Sternberg cells expressed CD25 and in those whose neoplastic cells were CD25-negative provided that associated rosetting T cells expressed CD25 (Janik et al., 2015). Toxicities were transient bone-marrow suppression and myelodysplastic syndrome in six patients who had not been specifically evaluated with bone-marrow karyotype analyses prior to therapy (Janik et al., 2015). The delayed myelosuppression toxicity seen with each of these RIC therapies was likely due to the β -emitting radionuclides having an effect on red marrow rather than an anti-CD25 antibody-specific effect (Dancey et al., 2009).

Despite the promising clinical potential of RICs their use is declining, likely due to the combined emergence of competing targeted therapies, practicalities in transfer of care to nuclear medicine physicians, exorbitant cost and concerns about delayed myelotoxicity (Palanca-Wessels and Press, 2014). Currently there are just two licensed RICs, both with a β -emitting radionuclide, however the manufacture of one of them, ¹³¹I-tositumomab, ceased in 2014 (Palanca-Wessels and Press, 2014). Many laboratories are working on methodologies to improve productivity and renew interest in RICs. One such strategy is multistep pre-targeted radioimmunotherapy designed to improve target-to-organ ratios of absorbed radioactivity (Palanca-Wessels and Press, 2014). However, reducing the logistical hurdles to RIC administration will likely be the key factor for adoption of these and other next generation RICs.

Anti-CD25 immunotoxins (ITs)

Immunotoxins (ITs) are antibodies, or their fragments, fused to a bacterial, plant, fungal or human apoptotic protein toxin, which become internalised in the target cell and induce cytotoxicity (Madhumathi et al., 2016). The clinical development of ITs has been hindered by

several obstacles including low antigen binding specificity of the antibody or antibody fragments and cytotoxicity, immunogenicity and production difficulties.

Some of the problems with immunogenicity are demonstrated in the Phase I dose escalation trial of an anti-CD25 IT, designated as RFT5-SMPT-dgA administered to patients with refractory HL. This IT was constructed by linking the murine anti-CD25 monoclonal antibody RFT5 via a sterically hindered disulfide linker to the potent toxin deglycosylated ricin-A (Engert et al., 1997). Although some patients responded to the IT, including 2/16 PR, many experienced side effects related to VLS with an additional two patients demonstrating a grade 2 allergic reaction (Engert et al., 1997).

LMB-2 is another anti-CD25 immunotoxin that has been tested in a number of human trials. LMB-2, a pseudomonas exotoxin conjugated to anti-CD25 single chain variable fragment (Kreitman et al., 2000) has shown objective responses in CD25-expressing lymphomas including HCL and ATL (Kreitman et al., 2000). The addition of chemotherapy drugs such as fludarabine may enhance efficacy by reducing the development of immunogenicity (Kreitman et al., 2015).

An example of a successful anti-CD25 recombinant fusion immunotoxin is the cutaneous T cell lymphoma licensed denileukin diftitox (Ontak™) which is composed of a diphtheria exotoxin conjugated to an IL2 fragment (Olsen et al., 2001). Refinements in engineering technologies have permitted the development of an identical sequence recombinant immunotoxin, E7777 but with 1.5 to 2 times the bioactivity of denileukin diftitox *in vitro* (Duvic M 2014). The developers put this superior activity down to its improved purity and increased percentage of active monomer species (Duvic M 2014) with a Phase I trial of the IT thus far suggesting good efficacy (Maruyama, 2015).

The immunogenicity and production difficulties of ITs and the development of resistance have, however, been overcome through protein engineering technologies or drug combinations and this remains a promising field of targeted therapies (Wayne et al., 2014).

Anti-CD25 antibody drug conjugates (ADCs)

The clinical proof of concept and safety for treatment of CD25-positive malignancies using RICs and ITs utilising anti-CD25 antibodies and fragments has been firmly established. The development of antibody drug conjugates (ADCs), however, has arguably been the immunoconjugate class most propelled by the commercial successes of therapeutic monoclonal antibody technologies. ADCs are composed of an antibody conjugated via a linker to a chemotherapeutic drug. The methodology to conjugate the first chemotherapeutics to antibodies was described more than fifty years ago (Decarvalho et al., 1964), although it took another forty years before their design became sufficiently advanced to permit ADC clinical licensing. Since the licensing of anti-CD33 ADC Mylotarg™ in refractory AML (and its subsequent withdrawal), innovations in ADC design have led to an explosion in the number of clinical trials of ADCs with several hundred now open. Currently, just two ADCs, brentuximab vedotin (Adcetris™) and ado-trastuzumab emtansine (Kadcyla™) have been licensed, in 2011 and 2013, respectively.

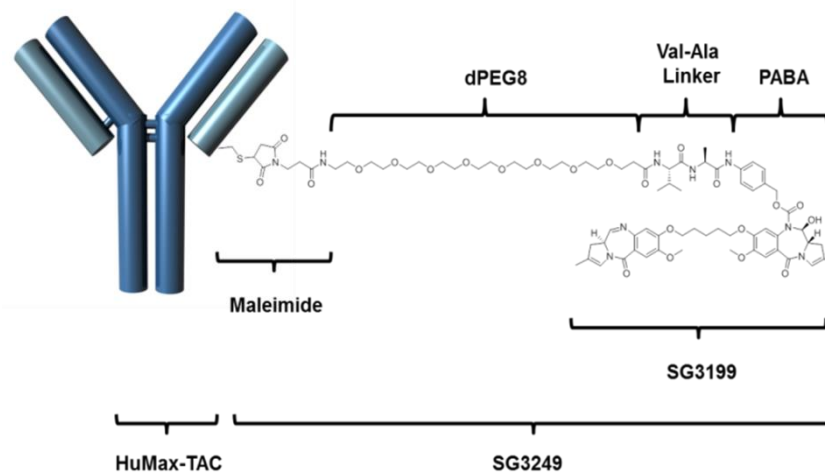


Figure 3. Schematic representation of antibody drug conjugate ADCT-301, consisting of anti-CD25 antibody HuMax-TAC conjugated to tesirine, a drug payload based on the PBD dimer SG3199 and a pegylated val-ala cleavable linker (Tiberghien et al., 2016).

ADCT-301 is the first ADC that targets CD25. ADCT-301 is composed of the human IgG1 HuMax®-TAC against CD25, stochastically conjugated through a dipeptide cleavable linker to a PBD dimer warhead (Figure 3) (Flynn et al., 2016). This drug demonstrated highly potent and selective cytotoxicity against a panel of CD25-expressing human lymphoma cell lines. Although effects on IL2 signalling have been demonstrated for anti-CD25 antibodies, HuMax-TAC did not demonstrate direct cytotoxicity *in vitro*. The mechanism of action of ADCT-301 is instead mediated via the intracellular lysosomal release of a PBD dimer which subsequently forms highly cytotoxic DNA interstrand cross-links (Flynn et al., 2016, Hartley et al., 2004). *In vivo*, a single dose of ADCT-301 resulted in dose-dependent and targeted antitumour activity against both subcutaneous and disseminated CD25-positive lymphoma models (Flynn et al., 2016). Two Phase I clinical trials in refractory lymphomas (NCT02432235) and AML or ALL (NCT02588092), respectively, have been initiated.

Table 3: Anti-CD25 directed therapies and their latest phase of clinical development

Class of drug	Indication	Latest clinical phase of development	References
rIL2	Melanoma RCC	Approval Approval	Smith et al, 2008 Klapper et al, 2008

	Neuroblastoma	Phase 3	Ladenstein 2016
Anti-CD25 ab Anti-Tac	ATL	Phase 1	Waldmann et al, 1988
Anti-CD25 RIC ⁹⁰ Y-anti-Tac	ATL	Phase 1	Waldmann et al., 1995
¹³¹ I-basiliximab	HL, NHL	Phase 1	Dancey et al., 2009
⁹⁰ Y-daclizumab	HL	Phase 2	Janik et al., 2015
Anti-CD25 IT RFT5-SMPT-dgA	HL	Phase 1	Engert et al., 1997
LMB-2	NHL	Phase 2	Kreitman et al., 2000
Ontak™	CTCL	Approval	Olsen et al., 2001
E7777	CTCL	Phase 1	Duvic et al., 2016
Anti-CD25 ADC ADCT-301	NHL, HL AML, ALL	Phase 1 Phase 1	NCT02432235 NCT02588092

The future of anti-CD25 directed therapies

As demonstrated above, there are at least two ways in which targeting CD25 may be utilised in oncology. IL2 and anti-CD25 antibodies modulate IL2 signalling which may influence tumour cell growth and survival. Alternatively, cytotoxins may be delivered to CD25-expressing cells. These cells are either tumour cells, or circulating or infiltrating inflammatory cells which modulate tumour cell survival. It is the targeting of these inflammatory cells which has attracted significant attention as the field of targeted immunotherapy advances.

The efficiency of IL2 signalling in influencing cellular function is suggested by the observation that only a small fraction of CD25 on activated T cells associate with IL2 (Taniguchi and Minami, 1993). Thus, many CD4⁺ CD25⁺ Treg cells may continue to function in the presence of an anti-CD25 antibody in the tissue microenvironment (Huss et al., 2015). This may be due to the anti-CD25 antibody incompletely saturating CD25 (Bien and Balcerska, 2008), or the fact that IL2 can also initiate signalling directly through the CD122/CD132 receptor complex in cells where it is expressed in high levels (Rickert et al., 2004). Indeed, data from a psoriasis trial, in which relapses were seen in patients dosed with daclizumab intermittently, showed that CD25 was incompletely saturated (Krueger et al., 2000). This suggests that anti-CD25 antibodies may have only a limited role as standalone immunotherapy.

Ablation of Tregs rather than modulation of a Treg suppressive effect with a targeted cytotoxin may more directly contribute to tumour regression. Similar approaches to patients with aggressive tumours such as refractory melanoma have included testing denileukin diftitox (Attia et al., 2005) and LMB-2 (Powell et al., 2007). There were no objective clinical responses observed in either trial (Attia et al., 2005, Powell et al., 2007). However, a significant reduction in circulating and intratumoural Treg subpopulations in patients treated with LMB-2 (Powell et al., 2007) supports the idea that inclusion of patients with CD4⁺ CD25⁺ hi Treg sub-populations may yield more promising results. When investigating an anti-CD25 targeted drug for efficacy in patients with CD25-expressing tumours, the influence of the binding characteristics of its anti-CD25 antibody component to Tregs and lower CD25-expressing immune cells may, therefore, also be important. The timing and dosing of anti-CD25 targeted therapy administration might influence the differential control of Tregs and effector T cells involved in tumour immunity (Nishikawa and Sakaguchi, 2014). Future trial

design should prioritise the monitoring of tumour-infiltrating Treg cells before and after therapy to predict the efficacy of Treg depletion in modulation of anti-tumour immunity.

Apart from anti-CD25 antibody targeting, alternative attempts of ablating Tregs have met with varied success. Chemotherapeutics such as cyclophosphamide and fludarabine can selectively modulate Tregs (Beyer et al., 2005). This selectivity can be harnessed by for example combining cyclophosphamide with a multiple peptide vaccine. This approach was shown to ablate Tregs and expand multiple clones of effector T cells resulting in improved survival in a group of patients with RCC (Walter et al., 2012). In addition, Tregs express other immunomodulatory antigens apart from CD25 including CCR4, CTLA4, PD1 and glucocorticoid-induced TNF-receptor family related protein (GITR) (Nishikawa and Sakaguchi, 2014). Therapeutic approaches which have been tested include the anti-CCR4 antibody mogamulizumab (Ni et al., 2015), and antibodies targeting GITR, PD1 and CTLA4 (Nishikawa and Sakaguchi, 2014). The effect of these antibodies on other cell types including effector T cells may make efficacy as difficult to predict as they are for anti-CD25 directed approaches.

The drive for the design of clinical trials of combination therapies with anti-CD25 targeted therapies and other modalities is gaining momentum. Combination therapies of ADCs with other therapies are increasingly being tested as a means of overcoming receptor downmodulation or drug efflux and resistance (Loganzo et al., 2015, Chen et al., 2015) which may impede the delivery of potent cytotoxins as well as exercise different mechanisms of cytotoxicity. These include combination therapies of ADCs with standard of care chemotherapies such as the first-line regimen in HL, ABVD (doxorubicin, bleomycin, vinblastine and dacarbazine) (Oflazoglu et al., 2008). The rationale for combining antimetabolite cytarabine with an anti-CD25 directed therapy is suggested in a study by Guo *et al.*, which demonstrated CD25 upregulation on the CD25-negative KG-1 cell line after

cytarabine treatment (Guo et al., 2014). The targeting of dual-expressed antigens like CD25 and CD30 on HL Reed-Sternberg cells (Schnell et al., 1998), makes CD25 targeting after or during treatment with a CD30-targeted agent such as Adcetris an attractive alternative or adjunct, possibly even as a bispecific (anti-CD30 and anti-CD25) ADC. Combinations of ADCs and immunotherapies are also being tested (Gerber et al., 2016). However, the optimal scheduling and dosing required for acceptable tolerability and enhanced efficacy is likely to create significant challenges in trial design.

A timeline of the discovery and clinical development of CD25 as a therapeutic target is shown in Figure 4.

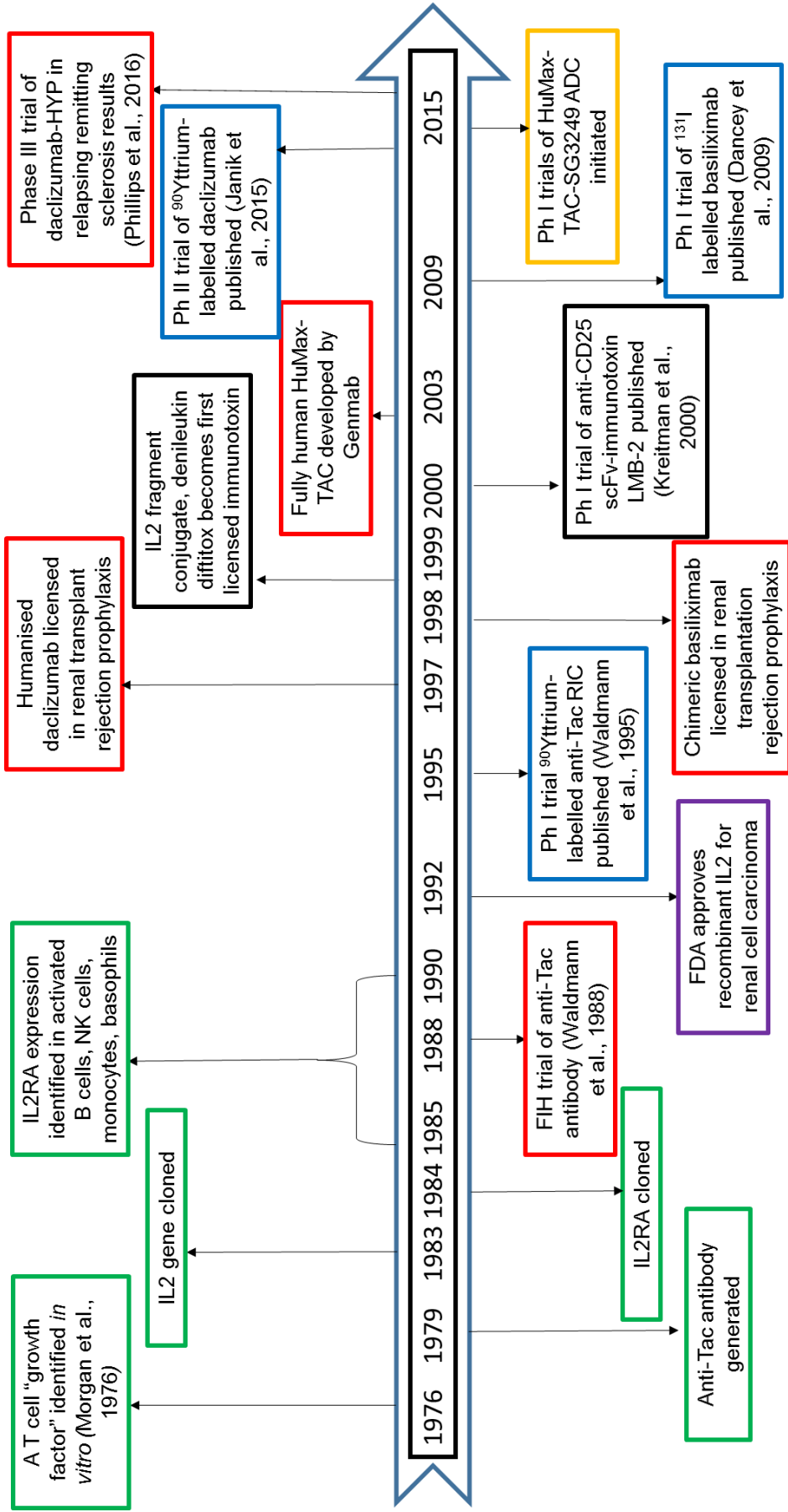


Figure 4. Timeline of IL2 discoveries and targeted anti-CD25 therapeutic approaches. Green boxes track the biological discovery timeline of IL2 and CD25. The purple box shows the first instance of IL2 licensing for the treatment of cancer. Red boxes highlight critical timelines in CD25 therapeutic antibody development. Blue and black boxes enclose radioimmunoconjugate and immunotoxin clinical development timepoints respectively, whilst the timeline for the initiation of anti-CD25 ADC clinical trials is highlighted in a yellow box.

Conclusions

CD25 forms one component of the high-affinity heterotrimeric IL2 receptor on activated T cells. Its affinity for IL2 and cellular function are tightly regulated and vary in different cell types. The high frequency of CD25 on the surface of many different tumour cells is now well established and apart from its prognostic significance, CD25 may be present on leukemic stem cells and enable oncogenic signalling pathways in leukemic cells. Additionally, high CD25 expression in activated circulating immune cells and Tregs is a factor which has already been exploited by IL2 immunotherapies in both malignancy and autoimmune disease. The relative clinical safety and efficacy of administering anti-CD25 radiommuconjugates and immunotoxins has been established and clinical trials of a novel anti-CD25 directed antibody drug conjugate are underway. There are multiple approaches to targeting Tregs as a strategy to reduce tumour immune evasion. The heterogeneity of CD25 and its expression on both immune-modulating and tumour cells makes combination trials of chemotherapies with anti-CD25 directed immunotherapies attractive therapeutic options. Anti-CD25 therapies may therefore add new dimensions to an era of increasingly specific immuno-oncology therapeutics.

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