Dawn of Monitoring Regulatory T Cells in (Pre-)clinical Studies: Their Relevance Is Slowly Recognised

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Regulatory T cells (Tregs) have a prominent role in the control of immune homeostasis. Pharmacological impact on their activity or balance with effector T cells could contribute to (impaired) clinical responses or adverse events. Monitoring treatment-related effects on T cell subsets may therefore be part of (pre-)clinical studies for medicinal products. However, the extent of immune monitoring performed in studies for marketing authorisation and the degree of correspondence with data available in the public domain is not known. We evaluated the presence of T cell immunomonitoring in 46 registration dossiers of monoclonal antibodies indicated for immune-related disorders and published scientific papers. We found that the depth of Treg analysis in registration dossiers was rather small. Nevertheless, data on treatment-related Treg effects are available in public academia-driven studies (post-registration) and suggest that Tregs may act as a biomarker for clinical responses. However, public data are fragmented and obtained with heterogeneity of experimental approaches from a diversity of species and tissues.

To reveal the potential added value of T cell (and particular Treg) evaluation in (pre-)clinical studies, more cell-specific data should be acquired, at least for medicinal products with an immunomodulatory mechanism. Therefore, extensive analysis of T cell subset contribution to clinical responses and the relevance of treatment-induced changes in their levels is needed. Preferably, industry and academia should work together to obtain these data in a standardised manner and to enrich our knowledge about T cell activity in disease pathogenesis and therapies. This will ultimately elucidate the necessity of T cell subset monitoring in the therapeutic benefit-risk assessment.

Keywords: regulatory T cells, immunomonitoring, monoclonal antibodies, JAK inhibitors, registration dossiers, biomarkers, (pre-)clinical study recommendations

INTRODUCTION

The mammalian immune system is indispensable for the protection against a broad range of pathogens. For this, immune cells should be able to differentiate between (pathogenic) non-self and self. In addition, immune responses should be fine-tuned to demarcate the localisation and extent of an inflammatory reaction. Preservation of self-tolerance and immune homeostasis is mediated by various immunosuppressive mechanisms, including regulatory T cells (Tregs) (1). These suppressor cells appear to be specifically equipped to control the activation of other immune cells (2).
Human Tregs can be classified in different subtypes. The major subtype consists of the classical CD4+ Tregs that are either differentiated in the thymus (also known as natural Tregs) or peripherally induced from conventional (effector) CD4+ T cells (3, 4). Classical Tregs highly express CD25 [i.e., interleukin (IL)-2 receptor] and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) (5–7). These surface markers, together with the transcription factor forkhead box protein 3 (FoxP3), have essential roles in Treg-mediated suppressive functionality (8–12). Non-classical Tregs include FoxP3+ T regulatory type 1 (Tr1) and T helper (Th)3 cells. These types are depending on IL-10 and tumour growth factor-β production for their suppressive activity (13, 14). Also γδ T cell and CD8+ T cell populations contain suppressive subsets, but their specific roles in regulating the immune system have yet to be identified (15–19).

The negative regulation of an immune response as mediated by Tregs is essential to prevent auto-immune and allergic disorders. On the other hand, this suppressive activity may prevent pathogen clearance during infections and hinder effective immune responses against (mutated) self-antigens in cancer (20). Therefore, in diseases where the balance between immune activation and suppression is skewed, Tregs could be attractive pharmacological targets (21, 22). For Th1- and Th17-dominated auto-immune disorders and Th2-dominated allergies, a therapy increasing Treg suppressive activity is sought (21, 23, 24). In contrast, for malignant diseases reversing an immunosuppressive tumour micro-environment by reducing Treg functionality would be the goal of treatment (21, 24–26). However, targeting Tregs in vivo is challenging, because a single (surface) marker with high specificity and selectivity for Tregs is still lacking (25). In addition, interfering with Treg numbers and/or functionality may also increase the risk for (auto-)immune-related adverse events (8). Examples are autoimmune enterocolitis and myocarditis following treatment with immune checkpoint inhibitors such as anti-CTLA-4 and anti-programmed cell death-1 (PD-1) (27–33). But also therapies against auto-immune disorders, for example tumour necrosis factor (TNF) inhibitors, have been reported to result in paradoxical autoimmunity-related inflammation (34).

Given the role of Tregs in (maintenance of the) immune balance, inclusion of these cells in the investigation of treatment effects on T cell subsets would be expected to be part of the (clinical) development program of medicinal products, at least for therapies targeting the immune system. Comprehensive overviews of immunomodulatory therapy-related effects on the balance between effector and regulatory T cells are available, for example for arthritis and solid organ transplantation (21, 35, 36). They show that general immunosuppressive drugs (such as corticosteroids), which target intracellular signalling pathways, do not only affect conventional T cell activation, but may also affect Treg activity. However, the sensitivity to the pathway-suppressive effects of these products differs between effector and regulatory T cells, and this difference determines whether immunomodulatory products will inhibit or stimulate immune cell activity. Differences in inhibition sensitivity of shared intracellular pathways are also apparent for more selective immunomodulating drug products. For example, blocking TNF has an effect on both TNF receptor-expressing effector T cells and Tregs, although it appears that positive clinical responses in several auto-immune disorders are the result of a greater inhibition of the effector than the regulatory cells (37).

Medicinal products may also disturb the balance between effector and regulatory T cells or the total T cell population more indirectly or even unintendedly (i.e., off-target effects). For example, monoclonal antibody (mAb)-mediated apoptosis results in the tumour tissue infiltration of immune cells, including Tregs. These Tregs can negatively influence the cytotoxic potential of effector cells, which could result in reduced efficacy. Therefore, immunomonitoring in (pre-)clinical studies is a useful tool to elucidate unintended treatment effects (and potential underlying mechanisms) caused by disturbance of the immune balance. In addition, immunomonitoring can provide more insight in the role of specific immune cells in the disease pathophysiology and thereby contribute to the identification of biomarkers predictive for the clinical response (38).

Given the potential clinical impact of Treg modulation, appropriate monitoring of treatment-induced effects on Treg frequency, phenotype and function would be required. We questioned whether Tregs have been investigated in (pre-)clinical studies to support a marketing authorisation application (MAA). Therefore, we surveyed if and when T cells, and Tregs in particular, were evaluated in these studies and whether the data in the registration dossiers corresponded to the available data in the public domain. There are multiple immunomodulatory therapies registered in the EU. We have chosen to restrict the sample size of registration dossiers to MAAs for approved mAb products based on the assumption that for mAbs immunomonitoring studies most frequently have been performed. After all, the majority is indicated for immune system-related disorders. In addition, we assessed T cell monitoring for a few tyrosine kinases inhibitors known to specifically target cytokine signalling pathways in T cells. We conclude this review with our perspective on the value of Treg monitoring and recommendations for their evaluation in (pre-)clinical studies.

SEARCH FOR IMMUNOMONITORING DATA

Selection of Monoclonal Antibodies

We have evaluated the presence of data on T cell immunomonitoring (with the focus on Tregs) reported in published literature and in registration dossiers for MAA. We included all mAb products used as anti-neoplastic agents (anatomical therapeutic chemical classification code L01XC) or as selective immune inhibitor in the context of auto-immunity (L04AA, AB and AC) or asthma (R03DX), which have been EU-registered between 2006 and the first half year of 2019. Products that have been authorised and subsequently withdrawn in this time frame (for commercial, insufficient supply or unfavourable benefit-risk reasons) have been included. Biosimilars were excluded from our evaluation, because it was not expected that information on Tregs would be included in these dossiers (39).

In total, 46 monoclonal antibodies were considered eligible.
Selection of Publications and Registration Dossier Reports

As far as applicable, this study followed the recommendations of PRISMA in conducting and reporting a systemic review. Registration dossier search is summarised in Figure 1A, literature search is summarised in Figure 1B.

We searched in both registration dossiers [common technical documents, CTDs, required to apply for regulatory approval of a new medicinal product (40) and PubMed literature for Treg-related keywords (including: regulatory T cell, suppressor T cell, Treg, FoxP3, CD25, mAb generic, and trade names). Because the Treg field is relatively new and the extent of T cell monitoring in dossiers of mAb products was not known, we decided to search more generally for lymphocyte and T cell populations in the CTDs, but with a focus on Tregs. Therefore, we also included keywords related to the whole T cell population (such as: lymphocyte, T cell, CD4, and CD8). When lymphocytes or T cells were mentioned in an individual study report, we also searched with the Treg-related keywords. Our main focus was on CD4+ FoxP3+ T cells, because these are the major Tregs in the immune system. However, other (non-classical) suppressor T cells—when mentioned in reports—were also taken along.

To explore lymphocyte and T cell immunomonitoring in registration dossiers, we searched the pre-clinical and clinical sections of the CTD (pre-clinical module 4 and clinical module 5, respectively) for each individual monoclonal antibody. Most study reports containing immunomonitoring results were found in the sections about pre-clinical pharmacology (module 4.2.1), toxicology (module 4.2.3), clinical pharmacokinetics (module 5.3.3), efficacy and safety (module 5.3.5).

RESULTS

Evaluation of Immunomonitoring Data Availability

We investigated whether potential mAb treatment-related effects on Tregs reported in published studies are also reported as individual studies presented in registration dossiers. Table 1 describes the immunomonitoring parameters used to determine potential effects of mAb products on lymphocytes, T cell subsets or specifically Tregs in registration dossier reports. In this table, no individual mAb products have been indicated for confidentiality reasons. The effects of the individual mAb products on Treg frequency, phenotype, and function as found in literature are described in Tables 2–4. Table 5 represents all mAb products for which no scientific literature was publicly available. Product-related effects were measured either pre-clinically (in vitro or in vivo in animal models) or clinically (human healthy donors and patients), the latter further subdivided in systemic (in peripheral blood) and local (at the tumour site or in inflamed tissue) effects. Potential (absence of) associations between clinical results and the presence or activity of Tregs prior to or during/after treatment are included.

To gain better insight in the type of products for which T cell monitoring was available, we divided the mAb products in three groups, based on the relevance of the product’s pharmacological target for T cell (subset) function and survival:

1. mAb target is highly relevant for Tregs, i.e., the target is constitutively expressed on most Tregs (e.g., CTLA-4);
2. mAb target is -expected to be- relevant for the T cell population, i.e., the target is expressed on specific T cell and Treg subsets (e.g., α4 integrins) or the mAb product has a more indirect effect on Treg activity, when the target is involved in the balance between T cell subsets (e.g., IL-6 and IL-17A pathways);
3. mAb target is not directly relevant for T cells, i.e., the target is not involved in T cell functionality (e.g., CD20, which is expressed on B cells).

T Cell Immunomonitoring Data in mAb Registration Dossiers

For the majority of registration dossiers of mAbs targeting Tregs or other T cell subsets, only absolute and relative counts of lymphocytes, lymphocyte subsets (i.e., T and B cells, in some cases also natural killer cells) or T cell subsets (CD4+ and CD8+) were determined clinically and pre-clinically (Table 1). T cell functionality testing (i.e., proliferative or cytotoxic capacity) was limited to pre-clinical studies, whereas further differentiation of T cell subsets (such as naïve/memory state) and determination of the CD4+ to CD8+ ratio was primarily found in clinical reports. Most data were derived from samples evaluated via clinical haematology, flow cytometry or immunohistochemistry. In most cases, however, no summarising data or concluding remarks (such as clinical significance) concerning the treatment effects on T cell frequency and functionality were provided.

Comparison of Treg Immunomonitoring Between Literature and mAb Registration Dossiers

Targets With High Relevance for Regulatory T Cells

Seven mAb products were classified as affecting targets (here: cell surface receptors) essential for Treg function or survival. In literature, treatment-related effects on frequency and phenotype (other than identity markers such as CD25 and FoxP3) were studied for all these mAb products, whereas effects on suppressive function were evaluated for four mAbs (Table 2). Nevertheless, high variability between mAb products existed in the number of available studies (most on products targeting CTLA-4 and PD-1) and the level of Treg analysis per study. For the majority of the publications, identification of Tregs within the T cell population was based on several markers (mainly a combination of CD25, CD127, and/or FoxP3) to exclude activated effector T cells as much as possible. In four of the seven mAb registration dossiers, effects on the frequency of Tregs (defined as CD4+/CD8+ CD25+ FoxP3+ in most studies) were taken into account (Table 1), although actual results were not always reported. Next to frequency, Treg functionality is an important determinant of the degree of immune suppression and thus requires evaluation. But in none of the dossiers Treg functionality was determined.

In both publications and registration dossier studies, mAb-related effects on Tregs were found. However, comparing these sources elucidated a clear discrepancy. For most mAb products, the public domain contained more studies and within these studies, Tregs were analysed more extensively than in
FIGURE 1 | Flow chart of registration dossiers and literature reports selection process. (A) Registration dossier search and (B) literature search.
TABLE 1 | Overview of lymphocytic parameters evaluated in mAb registration dossiers.

| In vivo target group (# of mAb dossiers evaluated) | Parameters determined on lymphocytes or T cells (# of mAb dossiers) | Treg identification markersa | Parameters determined on Treg frequency and phenotype (# of mAb dossiers) | Parameters determined on Treg functionality (# of mAb dossiers) | Clinical associations (# of mAb dossiers) |
|------------------------------------------------|-------------------------------------------------|-----------------------------|-----------------------------|-------------------------------------------------|-----------------|
| Target highly relevant for Tregs (7) Pre-clinical | Pre-clinical | Pre-clinical | Pre-clinical | Pre-clinical | Prognostic |
| • Amount of total T cell population (1)b | CD4, CD25, CD127, CD4, CD25, FoxP3, CD8, CD25, FoxP3, Unknown | • Binding potential to T suppressor cells (1) | • Amount of (mAb-target+) CD4+ suppressor cells (3) | • Amount of CD8+ suppressor cells (2) | • Association between baseline FoxP3 expression of T suppressor cells and clinical benefit (1) |
| • Amount of (naive/memory) CD4+ and/or CD8+ T cells (6) | | | | | |
| • Activation level of CD4+ and/or CD8+ T cells (3) | | | | | |
| • Cytokine production of CD4+ and/or CD8+ T cells (1) | | | | | |
| • Functionality of CD4+ and/or CD8+ T cells (1) | | | | | |
| Clinical | Clinical | Clinical | Clinical | n.a. | |
| • Amount of total lymphocyte population (1) | CD4, CD25, FoxP3 | • Amount of CD4+ suppressor cells (4)c | | n.a. | |
| • Amount of total T cell population (1)b | | | | | |
| • Amount of (naive/memory) CD4+ and/or CD8+ T cells (6)c | | | | | |
| • Activation level of CD4+ and/or CD8+ T cells (4) | | | | | |
| • Cytokine production of PBMC population (1) | | | | | |
| • Cytokine production of CD4+ and/or CD8+ T cells (2) | | | | | |
| • Functionality of total T cell population (1) | | | | | |
| • Functionality of CD8+ T cells (3) | | | | | |
| Target relevant for the T cell population (28)2 | Pre-clinical | Pre-clinical | Pre-clinical | Pre-clinical | Predictive |
| • Viability of total lymphocyte or PBMC population (2) | Pre-clinical | Pre-clinical | Pre-clinical | Pre-clinical | • Association between amount of CD4+ suppressor cells and clinical response rate (1) |
| • Amount of total lymphocyte population (7)c | FoxP3; CD4, CD25, CD4, CD25, FoxP3, CD4, CD25, CD127; Unknown | • Binding potential to CD45RO+ CD4+ suppressor cells (1) | • Density of tumour-infiltrating suppressor cells (1) | • Amount of T suppressor cells (2)c | |
| • Amount of total T cell population (1)b | | | | | |
| • Amount of (naive/memory) CD4+ and/or CD8+ T cells (14) | | | | | |
| • Activation level of CD4+ and/or CD8+ T cells (4) | | | | | |
| • Cytokine production of PBMC population (1) | | | | | |
| • Cytokine production of CD4+ and/or CD8+ T cells (2) | | | | | |
| • Functionality of total T cell population (1) | | | | | |
| • Functionality of CD8+ T cells (5) | | | | | |
| Clinical | Clinical | Clinical | Clinical | n.a. | |
| • Amount of total lymphocyte population (12)b | Clinical | • Amount of T suppressor cells (2)c | | n.a. | |
| • Amount of total T cell population (4) | | | | | |
| • Amount of (naive/memory) CD4+ and/or CD8+ T cells (9) | | | | | |
| • Amount of CD4+ T cell subsets (2) | | | | | |
| • Activation level of CD4+ and/or CD8+ T cells (3) | | | | | |
| • CD4+ T cell: CD8+ T cell ratio (2) | | | | | |
| • Cytokine production of CD4+ T cells (1) | | | | | |
| • CD4+ T cell: CD8+ T cell ratio (2) | | | | | |
| • Cytokine production of CD4+ T cells (1) | | | | | |

(Continued)
In several public studies, Treg (subset) frequency could be used to differentiate responders from non-responders or to predict the clinical response prior to treatment. A decrease in local or systemic Treg frequency was associated with a better (anti-tumour) treatment response (52, 55–59). A high Treg frequency at baseline was associated with either better or worse clinical outcome, depending on the evaluated Treg phenotype (60, 61, 87–89). These studies indicate that Tregs may be assigned as potential biomarker for disease activity or clinical outcome [see section Value of Treg Monitoring]. Because Treg data would be available much quicker than, for example, long-term clinical responses such as progression-free survival, it could be worthwhile to investigate applicability of such biomarker in product-specific studies (as surrogate clinical end point) (144). Nevertheless, this probably requires more (pre-)clinical experience than available at the time of MAA.

**Targets With Relevance for Tregs or the Balance Between T Cell Subsets**

Most evaluated mAb products [i.e., (28)] were designed to target cell surface receptors or cytokines that have a role in the physiology of T cells. All targets (or their receptors) were known to be expressed on Treg subsets or were earlier defined as involved in maintaining the delicate balance between effector and regulatory T cells.

In general, the number of published studies (Table 3) was related to the level of Treg analysis in the registration dossiers (Table 1), except for Treg functionality. However, where Treg identification in literature was based on a combination of several phenotypic markers (e.g., CD25 and CD127 next to CD4), most dossier reports defined Tregs solely on one marker (if defined at all). Therefore, the latter may have measured therapeutic effects on a mix of Tregs and activated effector T cells, which interferes with correct interpretation of the data [see section Recommendations for (Pre-)clinical Studies]. It was also noted that changes in Treg phenotype (e.g., activation) were analysed more in-depth in public literature compared to registration dossiers.

**Targets Not Directly Relevant for T Cells**

Eleven mAb products were not expected to directly impact T cell function or survival and targets were therefore considered “non-relevant”. Published studies for mAb products targeting non-relevant molecules did indeed not report any Treg monitoring, except for belimumab and trastuzumab (Table 4). Belimumab
| In vivo target and monoclonal antibody (trade mark, year of EU registration) | Treg identification markers* | Effect of mAb on Treg frequency or phenotype | Effect of mAb on Treg functionality | Clinical associations | Key references |
|---|---|---|---|---|---|
| **CCR4**<br>Mogamulizumab (Poteligeo, 2018) | Pre-clinical<br>Peripheral blood<br>CD4, CD45RA, FoxP3 | Pre-clinical<br>Peripheral blood | n.a. | Predictive<br>Peripheral blood<br>↓ % Tregs is associated with induction of skin-related adverse events<br>Association between changes in % eTregs and occurrence of tumour relapse vs. adverse events<br>No relation between changes in % (effector) Tregs and extent of clinical response | (41–51) |
| CD3 and CD19<br>Blinatumomab (Blincyto, 2015) | Pre-clinical<br>In vitro<br>CD4, CD25, CD127 | Pre-clinical<br>In vitro<br>↑ CD25, CD69 and PD-1 protein expression (HC)<br>↑ IL-10 and ↓ IFN-γ and TNF-α production (HC) | Clinical<br>↑ Treg-mediated suppression of T effector cell proliferation and lytic capacity (HC) | Predictive<br>In vitro<br>Depletion of Tregs in non-responders can restore the T effector cell proliferation | (52) |
| CD3 and EpCAM<br>Catumaxomab (Removab, 2009)* | Pre-clinical<br>In vitro<br>CD4, FoxP3 | Pre-clinical<br>In vitro<br>↑ % Tregs (mainly FoxP3 high CD45RA−CD73+ subpopulation) | Clinical<br>n.a. | n.a. | (53, 54) |
| **CTLA-4**<br>Ipilimumab (Yervoy, 2011) | Pre-clinical<br>In vitro<br>CD4, CD25, FoxP3 | Pre-clinical<br>In vitro<br>Treg lysis by mAb-activated CD16+ monocytes | Pre-clinical<br>In vitro<br>Treg-mediated suppressive capacity is not affected | Prognostic<br>Animal model<br>↓ local Treg : Teff ratio is associated with weight loss (HM) | (30, 33, 55–86) |

(Continued)
| In vivo target | Treg identification markers<sup>a</sup> | Effect of mAb on Treg frequency or phenotype | Effect of mAb on Treg functionality | Clinical associations | Key references |
|---------------|----------------------------------|---------------------------------|---------------------------------|----------------------|---------------|
| Clinical | Peripheral blood | CD4, CD25, CD39; CD4, CD25, FoxP3; CD4, CD25, CD127, FoxP3 | Clinical | Peripheral blood | CD4, CD25, CD39; CD4, CD25, FoxP3; CD4, CD25, CD127, FoxP3 | Clinical | Peripheral blood | CD4, CD25, CD39; CD4, CD25, FoxP3; CD4, CD25, CD127, FoxP3 | Clinical | Peripheral blood | CD4, CD25, CD39; CD4, CD25, FoxP3; CD4, CD25, CD127, FoxP3 | Clinical | Peripheral blood | CD4, CD25, CD39; CD4, CD25, FoxP3; CD4, CD25, CD127, FoxP3 | Clinical | Peripheral blood | CD4, CD25, CD39; CD4, CD25, FoxP3; CD4, CD25, CD127, FoxP3 | Clinical | Peripheral blood | CD4, CD25, CD39; CD4, CD25, FoxP3; CD4, CD25, CD127, FoxP3 | Clinical | Peripheral blood | CD4, CD25, CD39; CD4, CD25, FoxP3; CD4, CD25, CD127, FoxP3 |
| Peripheral blood | Tumour site | CD4, FoxP3 | Tumour site | CD4, FoxP3 |

### Effect of mAb on Treg frequency or phenotype

- **Clinical**
  - Peripheral blood:
    - CD4, CD25, CD39; CD4, CD25, FoxP3; CD4, CD25, CD127, FoxP3
  - Tumour site:
    - CD4, FoxP3

### Effect of mAb on Treg functionality

- **Clinical**
  - Peripheral blood:
    - CD4, CD25, FoxP3; CD4, CD25, CD127, FoxP3

### Clinical associations

- **Pre-treatment number of CTLA<sup>-</sup> Tregs (but not CTLA-4<sup>+</sup> Tregs) and overall survival**
  - Positive association between pre-treatment % of CD39<sup>+</sup> Tregs and relapse-free survival
  - Tumour site
    - ↑ number of FoxP3<sup>+</sup> cells in pre-treatment metastatic tumour lesions is associated with clinical response (a.o., overall survival)
  - ↑ % Tregs in pre-treatment tumour lesions is associated with clinical activity

### Predictive

- **Peripheral blood**
  - No difference in number of Tregs between patients with and without immune-related adverse events
  - ↓ number of Tregs is associated with local and systemic clinical benefit
  - ↑ % Tregs is associated with improved progression-free survival
  - ↑ % Treg suppressive function is associated with decreased progression-free survival
  - ↑ % Helios<sup>+</sup> and/or HLA-DR<sup>+</sup> Tregs is associated with decreased clinical response
  - ↓ % Tregs is associated with overall survival
  - No association between changes in % Tregs and clinical response (a.o., overall survival)
  - No association between changes in % Tregs and clinical response (a.o., overall survival)
  - Inverse correlation between number of intra-tumoural Tregs and the degree of tumour necrosis after treatment
  - ↓ % Tregs in post-treatment tumour lesions from responders compared to non-responders
  - No association between intra-tumoural FoxP3 protein expression and clinical response
| mAb | Target and monoclonal antibody (trade mark, year of EU registration) | Treg identification markers<sup>a</sup> | Effect of mAb on Treg frequency or phenotype | Effect of mAb on Treg functionality | Clinical associations | Key references |
|-----|--------------------------------------------------|-------------------------------------|---------------------------------------------|----------------------------------------|----------------------|-----------------|
| PD-1 | Nivolumab (Opdivo, 2015) | Pre-clinical In vitro CD4, CD25; CD4, CD25, FoxP3 Animal model CD4, CD25, FoxP3 | Pre-clinical In vitro ↓ % (K67<sup>+</sup>) Tregs Animal model ≈ number of Tregs (hM) ≈ CD8<sup>+</sup> T cell : Treg ratio (hM) | Pre-clinical In vitro ↓ Treg-mediated suppressive capacity Animal model n.a. | Prognostic Peripheral blood ↓ pre-treatment % Tregs is associated with non-relapsing ↓ pre-treatment % PD-1<sup>+</sup> Tregs is associated with positive clinical response Tumour site ↑ pre-treatment FoxP3 (mRNA) expression is associated with diminished survival ↓ pre-treatment % PD-L1<sup>+</sup> Tregs is associated with diminished clinical outcome | (87–94) |
| Pembrolizumab (Keytruda, 2015) | Pre-clinical In vitro CD4, CD25 | Pre-clinical In vitro ≈ CD15s, CTLA-4, FoxP3, Helios, K67 and LAP protein expression on/in Tregs | Pre-clinical In vitro Treg-mediated suppressive capacity is not affected (HC) | Prognostic Tumour site ↓ pre-treatment % PD-L1<sup>+</sup> Tregs is associated with diminished clinical outcome | (68, 87, 95–97) |

<sup>a</sup>Summary of phenotypic markers used in the studies (in different combinations) to identify cells that are - according to the authors- regulatory cells.

<sup>*</sup>This mAb is now withdrawn from use in the European Union.

↓, decreased level (compared to baseline or control); ↑, increase (compared to baseline or control); ≈, similar level (compared to baseline or control); CCR4, CC-chemokine receptor 4; CD15s, Sialyl Lewis x; EpCAM, epithelial cell adhesion molecule; eTreg, effector (activated) regulatory T cell; HC, healthy controls; HLA-DR, one of the human MHC class II molecules; hM, humanised mice; ICOS, inducible T cell co-stimulator; IFN, interferon; K67, intracellular marker for proliferation; LAP, latency-associated peptide; n.a., not available; PBMC, peripheral blood mononuclear cell.
### TABLE 3 | Overview of published studies for mAbs with a target relevant for the T cell population.

| In vivo target and monoclonal antibody (trade mark, year of EU registration) | Treg identification markers$^a$ | Effect of mAb on Treg frequency or phenotype | Effect of mAb on Treg functionality | Clinical associations | Key references |
|---|---|---|---|---|---|
| α4 subunit of integrins | Pre-clinical | Tissue | Clinical | Predictive | (98–108) |
| | Natalizumab (Tysabri, 2006) | Pre-clinical | Peripheral blood | In vitro | Peripheral blood |
| | α4$^+$ | Pre-clinical | CD4, CD25, FoxP3 | In vitro | CD4$^+$ Th1 cell : CD49d$^+$ Treg ratio |
| | β7 integrin | Tissue | Peripheral blood | | or CD49d$^+$ Th17 cell : CD49d$^+$ Treg |
| | Vedolizumab (Entyvio, 2014) | Pre-clinical | CD4, CD25, CD127, FoxP3 | Pre-clinical | Treg-mediated suppressive capacity is not affected (HC) |
| | α4$^+$β7$^+$ | Pre-clinical | CD4, CD25, CD127 | Pre-clinical | α4$^+$β7$^+$ Treg-mediated suppressive capacity is not affected (HC) |
| | | Pre-clinical | in vitro | Pre-clinical | n.a. |
| | | Tissue | n.a. | Pre-clinical | n.a. |
| | | | | | (102, 109) |

(Continued)
| In vivo target and monoclonal antibody (trade mark, year of EU registration) | Treg identification markers* | Effect of mAb on Treg frequency or phenotype | Effect of mAb on Treg functionality | Clinical associations | Key references |
|---|---|---|---|---|---|
| **C5** | Eculizumab (Soliris, 2007) | Pre-clinical n.a. Clinical Peripheral blood CD4, CD25, FoxP3 | Pre-clinical n.a. Clinical Peripheral blood • ≈ number of Tregs • ≈ number of CXCR4+ Tregs | Pre-clinical n.a. Clinical Peripheral blood • Treg-mediated suppressive capacity is not affected | n.a. (110) |
| **CD30** | Brentuximab vedotin (Adcetris, 2012) | Pre-clinical n.a. Clinical Peripheral blood CD4, CD25, CD127 | Pre-clinical n.a. Clinical Peripheral blood • ↓ % CCR4+ Tregs | n.a. Prognostic Peripheral blood • No correlation between pre-treatment % CD30+ Tregs and clinical response | (91, 111)2 |
| **CD38** | Daratumumab (Darzalex, 2016) | Pre-clinical n.a. Clinical Peripheral blood CD4, CD25, CD127 | Pre-clinical n.a. Clinical Peripheral blood • ↓ number of Tregs • ↓ number of CD38+ Tregs • ↓ % CD38+ Tregs • ↑ CD8+ T cell : Treg ratio | n.a. Prognostic Peripheral blood • Positive correlation between pre-treatment number of CD38+ Tregs (but not total Tregs) and extent of the response Predictive Peripheral blood • No relation between CD8+ T cell : Treg ratio and clinical response | (112, 113) |
| **IL-6R** | Tocilizumab (RoActemra, 2009) | Pre-clinical In vitro CD4, CD25, CD127, FoxP3; CD8, CD25 Animal model CD4, CD25, FoxP3 | Pre-clinical In vitro • ↑ % Tregs, followed by ↓ towards baseline level (probably apoptosis-related decline) • ≈ % Tregs (HC) • ≈ CD4+ Treg : CD8+ Treg ratio (HC) Animal model n.a. | Pre-clinical In vitro • ↑ CD45RA+ Treg-mediated suppressive capacity (after expansion period, HC) | n.a. (114–129) |

(Continued)
| In vivo target and monoclonal antibody (trade mark, year of EU registration) | Treg identification markers<sup>a</sup> | Effect of mAb on Treg frequency or phenotype | Effect of mAb on Treg functionality | Clinical associations | Key references |
|---|---|---|---|---|---|
| **p40 subunit of IL-12 and IL-23** | Ustekinumab (Stelara, 2009) | Pre-clinical n.a. | Clinical Peripheral blood CD4, CD25, CD127, FoxP3 | Pre-clinical n.a. | Clinical Peripheral blood • Treg-mediated suppressive capacity is not affected n.a. | (130, 131) |
| **VEGFR2** | Ramucirumab (Cyramza, 2014) | Pre-clinical In vitro CD4, CD45RA, FoxP3 | Clinical Peripheral blood CD4, CD45RA, FoxP3 Tumour site CD4, FoxP3; CD4, CD45RA, FoxP3 | Pre-clinical In vitro • % eTregs Clinical Peripheral blood • % eTregs Tumour site • % eTregs (in TILs) • % K67<sup>+</sup> Tregs | Prognostic Tumour site • ↑ pre-treatment % eTregs (in TILs) is associated with partial response and longer progression-free survival | (132) |

<sup>a</sup>Summary of phenotypic markers used in the studies (in different combinations) to identify cells that are - according to the authors - regulatory cells.

<sup>b</sup>Study by Romano et al. (133) not taken into account, because of incorrect use of markers to determine Tregs (i.e., CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>+</sup>).

↓, decreased level (compared to baseline or control); ↑, increase (compared to baseline or control); ≈, similar level (compared to baseline or control); C5, complement protein 5; CCR4, CC-chemokine receptor 4; CXCR4, CXC-chemokine receptor 4; eTreg, effector (activated) regulatory T cell; HC, healthy controls; HLA-DR, one of the human MHC class II molecules; hM, humanised mice; K67, intracellular marker for proliferation; n.a., not available; PBMC, peripheral blood mononuclear cell; TIL, tumour-infiltrating lymphocyte.
targets the B lymphocyte stimulator (BLyS) protein, thereby blocking the activation of cells bearing the BLyS receptor. Target cells are primarily B cells, but also T follicular helper cells, which produce IL-21. Belimumab appears to reduce IL-21 production and subsequently restores Treg development at the expense of Th17 expansion (134).

Trastuzumab is indicated for Her2+ breast cancer and does not directly target the immune system. Nevertheless, Treg frequency and phenotype and their association with clinical outcome were evaluated both in human patients and in mice. One reason for assessing Tregs in breast cancer patients and the effect of trastuzumab on these cells may be that disease progression appears to be related to tumour-associated immunosuppression and FoxP3+ cell infiltration (135, 145–147). Indirect effects of the mAb on the balance between pro- and anti-inflammatory immune cells could therefore contribute to a more effective anti-tumour response.

For mAbs with a target outside the T cell population, still the number of the total T population or CD4+ and CD8+ cells were monitored, although no Treg monitoring was performed (Table 1), which is in line with the published reports on these products.

Taken together, we found that the depth of Treg (and T cell subset) immunomonitoring differs between products, depending on the likeliness that the mAb affects T cell functionality or survival. In addition, the extent of Treg evaluation varies between registration dossiers and published studies for individual mAbs. This is most probably because the majority of literature studies were academia-driven and were published only after marketing authorisation. Nevertheless, the involvement of the company in approximately half of these studies reveals that collaboration between industry and academia contributes to increased insight in treatment-related effects on the immune system.

### Other Products Affecting Treg-Relevant Targets

We took a pragmatic approach by evaluating mAb products EU-registered in a time period of 13 years without selecting for products that were actually meant to modulate the immune system. Only seven (of the 46 evaluated) products targeted molecules with high relevance for Treg function and survival. To determine whether other drug products with a Treg-relevant target took these suppressor cells into account in (pre-)clinical studies, we investigating two recently EU-registered Janus kinase (JAK) inhibitors indicated for rheumatoid arthritis. The

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**TABLE 4 | Overview of published studies for mAbs with a target not relevant for T cells.**

| In vivo target and monoclonal antibody (trade mark, year of EU registration) | Treg identification markers*a | Effect of mAb on Treg frequency or phenotype | Effect of mAb on Treg functionality | Clinical associations | Key references |
|---|---|---|---|---|---|
| BlyS (BAFF) Belimumab (Benlysta, 2011) | Pre-clinical In vitro CD4, CD25, CD127 | Pre-clinical n.a. | Pre-clinical n.a. | Predictive Peripheral blood • Inverse correlation between % Tregs and disease activity | (134) |
| | Clinical Peripheral blood CD4, Foxp3 | Clinical Peripheral blood • ↑ % Tregs • ↑ Treg : Th17 cell ratio | Clinical n.a. | Predictive Peripheral blood • Negative correlation between mAb concentration and change in % Tregs • ↑ % Tregs is associated with disease progression • ↓ % Tregs is associated with progression-free survival | (135–142) |
| Her2+ Ado-trastuzumab emtansine (Kadcyla, 2013)b | Pre-clinical Animal model CD4, CD25, Foxp3 | Pre-clinical Animal model • ↑ % Tregs (M) • ↑ Ki67, CTLA-4 and T-bet protein expression in/on Tregs (M) | Clinical Peripheral blood • ≈ number of Tregs • ≈ or ↓ % Tregs • ↑ CD8+ T cell : Treg ratio • ↓ Treg : Th17 cell ratio • Tumour site • ≈ or ↓ number of Tregs | Predictive Peripheral blood • Number of Tregs in post-treatment tumour lesions is associated with clinical response | (134) |

*aSummary of phenotypic markers used in the studies (in different combinations) to identify cells that are -according to the authors- regulatory cells.

*bStudy by Force et al. (143) not taken into account, because it was not clear which product (pertuzumab or trastuzumab) had effects on Tregs.

*cFor the clinical studies, is was not clear from the description whether trastuzumab (Herceptin) or ado-trastuzumab emtansine (Kadcyla) was used.

↓, decreased level (compared to baseline or control); ↑, increase (compared to baseline or control); ≈, similar level (compared to baseline or control); BlyS (BAFF), B lymphocyte stimulator (B cell activating factor); GD-2, glycolipid disialoganglioside-2; Ki67, intracellular marker for proliferation; M, mice; n.a., not available; PDGFR-α, platelet derived growth factor receptor-alpha; SLAMF7, signalling lymphocytic activation molecule F7.
JAK/STAT (i.e., signal transducer and activator of transcription) pathway is known to play an important role in the activation and survival of immune cells (148). Especially STAT5, a downstream target of the IL-2 receptor, is crucial for FoxP3 induction and Treg differentiation in the thymus (149, 150).

In both registration dossiers, effects of JAK inhibitors on lymphocyte and T cell subsets (cell count, phenotypic markers, cytokine production, and STAT phosphorylation) were determined, both clinically and pre-clinically. Clinically, also effects on Tregs (i.e., frequency) were investigated and a potential association between Tregs and the clinical response was explored. However, the amount of data presented in published studies (i.e., on local and systemic Treg frequency and functionality in mice and men, but also in vitro) was much more extensive than present in the dossier reports, although for one of the JAK inhibitors only one literature study was available (151–158).

**DISCUSSION**

**Study Limitations**

Some limitations in our study need mentioning. First, we specifically selected EU-authorised mAb products (although not restricted to their registered indications). Therapies that did not reach the market or were still under review were not evaluated, although some of these products may target Tregs and thus dossiers could contain valuable information [e.g., isatuximab (159)]. In addition, we acknowledge that several mAb products with direct immune-related or even T cell-related targets are in the late-stage pipeline of several companies (160). Future MAAs containing data on Treg frequency or even functionality may thus be expected.

Second, mAb products authorised before 2006 were excluded, because the main increase in attention for and knowledge about Tregs occurred in the last decade. For several of these older immunomodulatory mAb products with a direct impact on the T cell population (e.g., infliximab, adalimumab, alemtuzumab, daclizumab), recently published immunomonitoring studies involved Treg frequency and functionality, because of their importance in the disease pathophysiology and to (further) elucidate the pharmacological mechanisms of action in lack-of-response issues or for biomarker definition (161, 162). Tregs are also monitored for old mAbs targeting non-T cell receptors, such as CD20 (rituximab) (163, 164). Above-mentioned studies investigating effects of recently authorised products or relatively old products on the immune system could contribute to our knowledge of T cell subsets.

Third, mAb products indicated for infections (e.g., caused by Clostridium difficile or HIV) or immune-related diseases with a distinct pathophysiology (such as paroxysmal nocturnal haemoglobinuria) were also excluded. Only a few mAb products are registered for these indications and it is expected that data concerning Tregs in existing dossiers will be limited.

Fourth, for literature reports, we limited our search to the same products as described for MAA dossiers and thus excluding studies with non-registered human or “mousenised” mAbs against the same target. We acknowledge that these excluded studies would be helpful when more insight in efficacy or safety-related effects of mAb products on specific immune cells would be required. Nevertheless, to establish the relevance of experience with such non-registered products, interpretation of the published data would be needed, which was not the aim of our study.

**Value of Treg Monitoring**

Tregs have a crucial function in regulating immune responses to dampen inflammation, limit tissue damage and prevent auto-reactivity. Pharmacological impact on their number and/or (local) activity, either directly or indirectly, is likely to contribute to (or impair) clinical responses or to adverse events. Therefore, monitoring effects of immunomodulatory products on T cells -including Tregs- should be part of (pre-)clinical studies.

In addition, Tregs or specific Treg subsets may turn out to be predictive biomarkers for specific diseases or patient populations. We noted that in the majority of mAb product dossiers, no clinical relevance was estimated for treatment-induced changes in Treg frequency or phenotype. For only two

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**TABLE 5 | Overview of mAbs for which no published scientific papers were available.**

| In vivo target group | In vivo target | Monoclonal antibody (trade mark, year of EU registration) |
|----------------------|---------------|----------------------------------------------------------|
| PD-1                 |                | Cemiplimab (Libtayo, 2019)                               |
| Target highly relevant for Tregs | EGFR | Nectunumab (Portrazza, 2016) |
| Target relevant for the T cell population | IL-1β | Canakinumab (Iliris, 2009) |
|                      | IL-4R and IL-19R | Dupilumab (Dupixent, 2019) |
|                      | IL-5 | Benraluzumab (Fasenza, 2018) |
|                      | IL-6 | Sarilumab (Kevzara, 2017) |
|                      | IL-17A | Ikizukumab (Taltz, 2016) |
|                      | IL-17RA | Brodalumab (Kyntheon, 2018) |
|                      | IL-23 | Risankizumab (Skyrizi, 2019) |
|                      | TDF | Duvralumab (Imfinzi, 2018) |
|                      | TNF-α | Certolizumab pegol (Cimzia, 2009) |
|                      | CD20 | Ocrelizumab (Ocrevus, 2018) |
|                      | CD22 | Inotuzumab ozogamicin (Besponsa, 2017) |
|                      | CD33 | Gemtuzumab ozogamicin (Mylotarg, 2018) |
|                      | GD-2 | Dinutuzumab (Unituxin, 2015)* |
|                      | Her2 | Pertuzumab (Perjeta, 2013) |
|                      | PDCGF-α | Olaratumab (Lartruvo, 2016)* |
|                      | SLAMF7 | Elotuzumab (Empliciti, 2018) |

*This mAb is now withdrawn from use in the European Union.*
products, an association was determined between Treg frequency and the clinical response. Published reports, on the other hand, frequently mentioned associations between the amount of (local) Tregs and clinical outcome. Thereby, Tregs could act as biomarker for the clinical response (144). Associations with the baseline Treg level prior to treatment may be used as prognostic biomarker, for example to select patients eligible for mAb (anti-tumour) therapy. Changes in Treg level following treatment may act as predictive marker of the mAb-mediated clinical response, in auto-immune as well as in neoplastic indications. Nonetheless, interpretation of clinical associations and treatment-related Treg effects is still rather difficult. For example, differentiation between effects that are a direct consequence of the medicinal product activity or a result of disease remission is usually not accomplished. In addition, the clinical significance of fluctuations in Tregs during or after therapy remains to be established.

In general, immunomonitoring has a substantial value to assess the effectiveness and safety of therapeutic interventions and to select patients eligible to these treatments (2, 165). On the level of T cell subsets, scientific knowledge regarding immune responses is growing exponentially (also for older immunomodulatory treatments) and this knowledge should be taken into account when selecting for specific immunomonitoring parameters. But we consider that the general added value of measuring Tregs in (pre-)clinical studies is not yet sufficiently clear and their contribution to the clinical response requires more extensive analysis. This would include gathering information regarding the Treg role in disease pathophysiology and therapy-related adverse events. Regulatory authorities need this information to estimate the value of Tregs and the necessity to take treatment-related Treg changes along in the benefit-risk assessment. Tregs should therefore be taken into account as exploratory parameter in (pre-)clinical studies, either prior to MAA or post-registration in collaboration with academia.

Despite growing knowledge regarding treatment effects on Tregs, we observed a high variability in data between the different studies, probably due to heterogeneity of the experimental approach. Studies differed in markers used to identify Tregs, in methods to measure their functionality and in assay read-out techniques. Also tissues and species used to monitor Tregs, the time between treatment and analysis points varied between studies. Moreover, the treatment protocol (e.g., administration route, number and quantity of doses and dose intervals, concomitant therapies), therapeutic indication and the number of patients also added to study heterogeneity. D’Arena et al. ran into the same problem of heterogeneity when evaluating the relevance of Tregs as biomarker in the context of hematologic malignancies. Their study also exemplified “the need for more standardised approaches in the study of Tregs” (166). Thus, harmonisation of Treg identification and monitoring is required before these cells can become actual endpoints in clinical investigations or can be used as prognostic or predictive biomarker (25, 144).

Apart from this lack of harmonisation, the scientific knowledge is too limited to demand or guide Treg monitoring in registration dossiers. Nevertheless, we hereby stimulate companies (and academia) to take these cells into account in their investigations or to collaborate with academia to perform T cell subset-specific studies (post-registration).

**Recommendations for (Pre-)clinical Studies**

We will end this review with some specific points-to-consider for Treg (and other T cell subset) monitoring in pre-clinical and clinical studies.

**Sampling**

Treg monitoring (both clinically and pre-clinically) could be restricted to products with a target known to play a vital role in T cell development, differentiation, functionality, or survival. Especially when the target is related to regulation of the immune system and loss-of-function would considerably increase the risk of auto-immunity [e.g., CTLA-4 expression on Tregs (27)], monitoring the frequency and functionality of immune cells closely related to this target may significantly add to the identification of potential safety concerns early in product development. Obviously, for products containing (ex vivo expanded) Tregs or for therapies typically aiming to enhance Treg activity (e.g., tolerogenic dendritic cells), analysis of Treg frequency and/or function will be imperative (24). For immunomodulatory treatments with a target in the non-T cell compartment, a risk-based approach could identify whether monitoring of T cell subset responses would be required to substantiate clinical data.

We suggest to add several Treg-related markers to an existing immune monitoring panel (see below in subsection Identifying Tregs). When this would not be feasible, one could retain clinical samples to be able to retrospectively measure effects on specific T cell or Treg subsets when required [as recently performed for unexpected events with nivolumab (167–169)]. More “standard” Treg monitoring could then be restricted to pre-clinical investigation.

What samples would be most appropriate? In general, in humans peripheral blood is the most accessible compartment for multiple analyses over time. Nevertheless, changes in circulating T cell subsets may not accurately reflect the local environment. Furthermore, it has been reported that the ratio and phenotype of Treg subsets at tumour sites differ substantially from peripheral blood (8, 9, 25, 132, 170). Therefore, when feasible, treatment effects on local T cell subsets may be taken into account as well (26).

We noted that pre-clinical *in vitro* pharmacologic studies are frequently performed with cells from healthy donors. This can be acceptable, but using cells from patients may have added value when the disease has impaired the intrinsic function of the cells. For example, patients with giant cell arteritis can have a defect in their FoxP3 protein, which affects the suppressive capacity of the Tregs, but could be pharmacologically corrected (114).

**Identifying Tregs**

In general, treatment-related effects on the immune system are dose-dependent and difficult to predict. For example, lymphocyte-depleting approaches (such as anti-thymocyte
globulin) do not simply deplete all T cells, but also act as immunosuppressant by, for example, converting effector into regulatory T cells and by preserving or even expanding already existing Tregs. An increased Treg to conventional T cell ratio may therefore be an unexpected effect of T cell-depleting antibodies (21, 36, 171, 172). This indicates that monitoring drug-mediated effects on the whole T cell population may not correctly predict effects on T cell subsets. Therefore, these analyses should preferably discriminate between effector and regulatory T cells, at least for products indicated to specifically target T cells.

Accurately defining Tregs is, however, a challenge. Although there are several useful reviews available that highlight different markers and cytokines that may help identifying Tregs, there is no unique Treg marker (7, 22, 24, 173–175). Expression of FoxP3, the master regulator of classical CD4+ Tregs, is not limited to human regulatory cells: effector T cells transiently upregulate FoxP3 expression after activation and also other immune cells and even tumour cells may express this transcription factor (176–181). In addition, not all regulatory T cell subtypes express FoxP3. Moreover, FoxP3 cannot be used to isolate Tregs alive for ex vivo functionality testing. Combined use of several (surface) markers will therefore be needed to identify and purify Tregs. In contrast to murine CD4+ CD25+ regulatory T cells, only CD4+ cells with a high level of CD25 expression have a suppressive capacity in humans. The other CD4+ CD25+ T cells are activated effector T cells. According to the vision of several experts in the field, CD3, CD4, CD25, CD127, and FoxP3 are the minimally required markers to define human Treg cells in flow cytometric samples and addition of Ki67 and CD45RA/RO could provide information on the activation status of Tregs (173) or improve selection of pure Treg fractions (182). Such Treg panel would also allow for monitoring of different effector T cell subsets (naive/memory state of both CD4+ and CD8+ T cells). The experts also emphasised that a proper flow cytometric gating strategy will improve the reliability and purity of the defined Treg population, and in the meantime diminish inter-assay variability (173, 183). Recently, Pitoiset et al. provided a standardised protocol to monitor Tregs in multicentre clinical trials, using above-mentioned markers (184).

One should, however, keep in mind that there are phenotypic differences between circulating Tregs and Tregs at sites of inflammation (173). A more precise discrimination of Treg subtypes may thus be needed, especially in the peripherally-induced (heterogeneous) population (25, 185). And differentiating between naive and activated Treg subsets may be needed to find treatment-related effects or clinical associations (186, 187). Nevertheless, distinguishing between Treg subsets (especially thymus- vs. periphery-derived cells) is rather challenging (3–5, 188–190). Measuring the amount of demethylation of the FoxP3 gene may provide insight in the stability of FoxP3 expression and thereby distinguish thymus-derived Tregs from peripherally-induced Tregs and activated conventional T cells (191, 192). Nevertheless, this demethylation status analysis requires a highly pure lymphocyte sample.

Functionality Testing of Tregs
There are various methods to analyse the suppressive capacity of Tregs. Most of these assays aim to measure inhibition of effector T cell proliferation or cytokine production, although cytotoxicity inhibition may also be used as read-out (193, 194). The requirement of a rather large amount of autologous cells for such co-cultures would make these types of assays less suitable for patient samples (195). Indeed, we found in several registration dossiers that such testing was considered clinically, but impossible to perform. In addition, the in vivo functionality may be impacted by the tissue environment, which is difficult to mimic in vitro (26). Moreover, impaired in vivo suppressive function is not always reflected by results from an in vitro assay (20).

Treg functionality was only (pre-clinically) analysed in one of the 46 mAb dossiers evaluated. Lack of functionality testing is probably the result of difficulties with assay design. Nevertheless, there are literature examples where Treg functionality testing appeared possible (62). We therefore would like to draw attention to different approaches that may enhance the possibility of Treg function analysis. Instead of using autologous cells, a mixed lymphocyte reaction may be considered (196, 197). In addition, to prevent long co-culture periods, a surrogate read-out (i.e., inhibition of activation marker expression instead of proliferation) could be used (198–200). Identification of functional Tregs via marker gene analysis (e.g., FoxP3, CTLA-4, and IL-10) may also be a simple and quick method, although the level of mRNA expression does not necessarily reflect protein expression and this read-out is also considered surrogate for Treg functionality (201). Simply distinguishing between resting and activated Tregs and effector T cells can also provide information about the presence or absence of suppressive T cells in a sample (182, 187). More considerations and technical challenges for Treg functionality assays can be found in the public domain (25, 115, 183, 194, 202–204).

Future Perspective and Conclusion
We are now starting to understand the role of different T cell subsets in disease pathogenesis and immunotherapeutic mechanisms of action. This provides the opportunity to selectively target specific subpopulations rather than a whole T cell population to improve the effectiveness and safety of immunomodulatory therapies. In addition, monitoring the activation status, function and amount of specific T cell subsets could assist in identifying the patients that would most likely benefit from therapy (2). A risk-based approach is considered helpful to select products that would require T cell subset monitoring to more reliably assess the product’s benefits and risks.

Immunomonitoring, as proposed in this review, will also help to enrich our knowledge about Tregs and their association with the clinical response. This will, however, require accurate phenotypic identification of regulatory subsets and further investigation of the clinical relevance of treatment-induced changes in their levels. To obtain and report such information in a systematic way, a collaboration between industry and academia will be required (205).
We believe there are still many issues to address before Tregs can be used as biomarkers for targeted therapies, but gathering knowledge about Treg subpopulations in health and disease will eventually shed more light on the (pre-)clinical value of these regulatory cells. This will ultimately result in more concrete regulatory guidance for T cell (and particularly Treg) monitoring in studies used for marketing authorisation.

**DATA AVAILABILITY STATEMENT**

The datasets generated for this study will not be made publicly available because they contain confidential information from registration dossiers. Requests to access the datasets should be directed to mh.hoefnagel@cbg-meb.nl.

**REFERENCES**

1. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor a-chains (CD25): breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*. (1995) 155:1151–64.

2. Whiteside TL, Gulley JL, Clay TM, Tsang KY. Immunologic monitoring of cellular immune responses in cancer vaccine therapy. *J Biomed Biotechnol*. (2011) 2011:370374. doi: 10.1155/2011/370374

3. Lee HM, Bautista JL, Hsieh CS. Thymic and peripheral differentiation of regulatory T Cells. *Adv Immunol*. (2011) 112:25–71. doi: 10.1016/B978-0-12-387827-4.00002-4

4. Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. *Nat Rev Immunol*. (2003) 3:253–7. doi: 10.1038/nri1032

5. Schmitt EG, Williams CB. Generation and function of induced regulatory T cells. *Front Immunol*. (2013) 4:152. doi: 10.3389/fimmu.2013.00152

6. Ohkura N, Kitagawa Y, Sakaguchi S. Development and maintenance of regulatory T cells. *Immunity*. (2013) 38:414–23. doi: 10.1016/j.immuni.2013.03.002

7. Chen X, Oppenheim JJ. Resolving the identity myth: key markers of functional CD4+FoxP3+ regulatory T cells. *Int Immunopharmacol*. (2011) 11:1489–96. doi: 10.1016/j.intimp.2011.05.018

8. Chen X, Oppenheim JJ. Resolving the identity myth: key markers of functional CD4+FoxP3+ regulatory T cells. *Int Immunopharmacol*. (2011) 11:1489–96. doi: 10.1016/j.intimp.2011.05.018

9. Takeuchi Y, Nishikawa H. Roles of regulatory T cells in cancer immunity. *Int Immunol*. (2016) 28:901–9. doi: 10.1093/intimm/dxw025

10. Zheng Y, Rudensky AY. Foxp3 in control of the regulatory T cell lineage. *Nat Immunol*. (2007) 8:457–62. doi: 10.1038/nia455

11. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. (2003) 299:1057–61. doi: 10.1126/science.1079490

12. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*. (2003) 4:330–6. doi: 10.1038/nri904

13. Roncarolo MG, Gregori S, Battaglia M, Bachetta R, Fleischhauer K, Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev*. (2006) 221:28–50. doi: 10.1111/j.0105-2896.2006.00420.x

14. Weiner HL. Induction and mechanism of action of transforming growth factor-β-secreting Th3 regulatory cells. *Immunol Rev*. (2001) 182:207–14. doi: 10.1034/j.1600-065X.2001.1820117.x

15. Peters C, Kabelitz D, Wesch D. Regulatory functions of γδ T cells. *Cell Mol Life Sci*. (2018) 75:2125–35. doi: 10.1007/s00018-018-2788-x

16. Siegmund K, Rückert B, Ouaked N, Burgler S, Speiser A, Akdis CA, et al. Unique phenotype of human tonsillar and *in vitro*-induced FOXP3+CD8+ T cells. *J Immunol*. (2009) 182:2124–30. doi: 10.4049/jimmunol.0802271

17. Najafian N, Chitnis T, Salama AD, Zhu B, Benou C, Yuan X, et al. Regulatory functions of CD8+CD28− T cells in an autoimmune disease model. *J Clin Invest*. (2003) 112:1037–48. doi: 10.1172/JCI17935

18. Hayday A, Tigelar R. Immunoregulation in the tissues by γδ T cells. *Nat Rev Immunol*. (2003) 3:233–42. doi: 10.1038/nri1030

19. Neo S, Tokura Y, Takigawa M, Egawa K. Depletion of IL-10- and TGF-β-producing regulatory γδ T cells by administering a daunomycin-conjugated specific monoclonal antibody in early tumor lesions augments the activity of CTLs and NK cells. *J Immunol*. (1999) 163:242–9.

20. Plitas G, Rudensky AY. Regulatory T cells: differentiation and function. *Cancer Immunol Res*. (2016) 4:721–5. doi: 10.1158/2326-6066.CIR-16-0193

21. Furukawa A, Wisel SA, Tang Q. Impact of immune-modulatory drugs on regulatory T cell function. *Transplantation*. (2016) 100:2288–300. doi: 10.1097/TP.0000000000001379

22. Ratekbandt N, Littringer K, Joller N. Regulatory T cells: balancing protection versus pathology. *Swiss Med Wkly*. (2016) 146:w14343. doi: 10.4414/smw.2016.14343

23. Palomares O, Akdis M, Martín-Fontecha M, Akdis CA. Mechanisms of immune regulation in allergic diseases: the role of regulatory T and B cells. *Immunol Rev*. (2017) 278:219–36. doi: 10.1111/imr.12555

24. Perdigoto AI, Chatenoud L, Bluestone JA, Herold KC. Inducing and administering tregs to treat human disease. *Front Immunol*. (2016) 6:654. doi: 10.3389/fimmu.2015.00654

25. Whiteside TL. The role of regulatory T cells in cancer immunology. *Immunotherapies Ther*. (2015) 4:159–71. doi: 10.2147/ITT.S55415

26. Jacobs JFM, Nierkens S, Figdor CG, de Vries IJM, Adema GJ. Regulatory T cells in melanoma: the final hurdle towards effective immunotherapy? *Lancet Oncol*. (2012) 13:e32–42. doi: 10.1016/S1470-2045(11)70155-3

27. Carbonnel F, Soularue E, Coutzac C, Chaput N, Mateus C, Lepage V, van den Bosch, Job van Bragt, and Mitra Dwarkasing, performing contribution to the dossier search work.

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**AUTHOR CONTRIBUTIONS**

AW collected the data and wrote the first draft of the manuscript. AW and MH were involved in the interpretation of the data. All authors contributed to the conception and design of the work, manuscript revision, read, and approved the final version.
32. Attia P, Phan GQ, Maker AV, Robinson MR, Quezado MM, Yang JC, et al. Autoimmunity correlates with tumor regression in patients with metastatic melanoma treated with anti-cytotoxic T-lymphocyte antigen-4. J Clin Oncol. (2005) 23:604–3. doi: 10.1200/JCO.2005.06.205
33. Phan GQ, Yang JC, Sherry RM, Hwu P, Topalian SL, Schwartzentruber DJ, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci USA. (2003) 100:8372–7. doi: 10.1073/pnas.1533209100
34. Fiorino G, Danese S, Pariente B, Allez M. Paradoxical immune-mediated inflammation in inflammatory bowel disease patients receiving anti-TNF-a agents. Autoimmun Rev. (2014) 13:15–9. doi: 10.1016/j.autrev.2013.06.005
35. Mijnheer G, Prakken BJ, Van Wijk F. The effect of autoimmune arthritis on regulatory T cells. Curr Opin Rheumatol. (2013) 25:260–7. doi: 10.1097/BOR.0b013e32835d00e4
36. Demirkiran A, Hendriks TK, Baan CC, Van Der Laan LJW. Impact of immunosuppressive drugs on CD4+ CD25+ FOXP3+ regulatory T cell dysregulation in vitro evidence translate to the clinical setting? Transplantation. (2008) 85:783–9. doi: 10.1097/TP.0b013e3181669110
37. Chen X, Oppenheim JJ. The phenotypic and functional consequences of tumour necrosis factor receptor type 2 expression on CD4+Foxp3+ regulatory T cells. Immunology. (2011) 133:426–33. doi: 10.1111/j.1365-2567.2011.03460.x
38. Teniente-Serra A, Ramo-Tello C, Martinez-Caceres EM. Immunomonitoring lymphocyte subpopulations in multiple sclerosis patients. In: Zagon IS, McLaughlin PJ, editors. Multiple Sclerosis: Perspectives in Treatment and Pathogenesis. Brisbane, QLD: (AU) Codon Publications (2017). p. 139–54. doi: 10.15586/codon.multiplesclerosis.2017.ch9
39. EMA/CHMP/BWP/M/403543/2010. Guideline on Similar Biological Medicinal Products Containing Monoclonal Antibodies – Non-Clinical and Clinical Issues. (2012). Available online at: https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-similar-biological-medical-products-containing-monoclonal-antibodies-non-clinical-en.pdf (assessed November 1, 2019).
40. Jordan D. An overview of the Common Technical Document (CTD) regulatory dossier. Med Writing. (2014) 23:101–5. doi: 10.1179/2047480614Z.000000000207
41. Doi T, Muro K, Ishii H, Kato T, Tsuchima T, Takenoyma M, et al. A Phase I study of the Anti-CC chemokine receptor 4 antibody, mogamulizumab, in combination with nivolumab in patients with advanced or metastatic solid tumors. Clin Cancer Res. (2019) 25:6614–22. doi: 10.1158/1078-0432.CCR-19-1090
42. Sato T, Coler-Reilly ALG, Yagishita N, Araya N, Inoue E, Furuta R, et al. Mogamulizumab (Anti-CCR4) in HTLV-I-associated myelopathy. N Engl J Med. (2018) 378:329–38. doi: 10.1056/NEJMoa1704827
43. Ureshino H, Shindo T, Nishikawa H, Watanabe N, Watanabe E, Sato T, et al. Anti-CCR4 mAb selectively depletes effector-Type FoxP3+ regulatory T cells, evoking antitumor immune responses in humans. Proc Natl Acad Sci USA. (2013) 110:17945–50. doi: 10.1073/pnas.1316796110
44. Ichida T, Ito A, Sato F, Kusumoto S, Iida S, Inagaki H, et al. Stevens-Johnson syndrome associated with mogamulizumab treatment of adult T-cell leukemia/lymphoma. Cancer Sci. (2013) 104:647–50. doi: 10.1111/cas.12116
45. Duell J, Dittrich M, Bedke T, Mueller T, Eisele F, Rosenwald A, et al. Frequency of regulatory T cells determines the outcome of the T-cell-engaging antibody blinatumomab in patients with B-precursor all. Leukemia. (2017) 31:2181–90. doi: 10.1038/leu.2017.41
46. Atanackovic D, Reinhard H, Meyer S, Spöck S, Grob T, Luekens T, et al. The trifunctional antibody catumaxomab amplifies and shapes tumor-specific immunity when applied to gastric cancer patients in the adjuvant setting. Clin Transl Med. (2013) 2:333–42. doi: 10.1186/2046-0481-2-333
47. Goërdé D, Flament C, Rusakiewicz S, Poirier-Colame V, Kepp O, Martinus I, et al. Potent immunomodulatory effects of the trifunctional antibody catumaxomab. Cancer Res. (2013) 73:4663–73. doi: 10.1158/0008-5472.CAN-12-4460
48. Davids MS, Kim HT, Bachireddy P, Costello C, Liguori R, Savell A, et al. Ipilimumab for patients with relapse after allogeneic transplantation. New Engl J Med. (2016) 375:143–53. doi: 10.1056/NEJMoa1600202
49. Mozzillo N, Simeone E, Benedetto I, Curvietto M, Giannarelli D, Gentilecore G, et al. Assessing a novel immuno-oncology-based combination therapy: ipilimumab plus electrochemotherapy. OncImmunology. (2015) 4:e1008842. doi: 10.1080/2162402X.2015.1008842
50. Romano E, Kusio-Kobialka M, Fokas PG, Baumgartner P, Meyer C, Ballaben P, et al. Ipilimumab-dependent cell-mediated cytotoxicity of regulatory T cells ex vivo by nonclassical monocytes in melanoma patients. Proc Natl Acad Sci USA. (2015) 112:6140–5. doi: 10.1073/pnas.1417320112
51. Simeone E, Gentilcore G, Giannarelli D, Grimaldi AM, Caracó C, Curvietto M, et al. Immunological and biological changes during ipilimumab treatment and their potential correlation with clinical response and survival in patients with advanced melanoma. Cancer Immunol Immunother. (2014) 63:675–89. doi: 10.1007/s00262-014-1545-8
52. Hodi FS, Butler M, Oble DA, Seiden MV, Halusa FG, Kruse A, et al. Immunologic and clinical effects of antibody blockade of cytotoxic T lymphocyte-associated antigen 4 in previously vaccinated cancer patients. Proc Natl Acad Sci USA. (2008) 105:3065–10. doi: 10.1073/pnas.0712237105
53. Jochens C, Tucker JA, Tsang KY, Madan RA, Dahut WL, Liewehr DJ, et al. A combination trial of vaccine plus ipilimumab in metastatic castration-resistant prostate cancer patients: immune correlates. Cancer Immunol Immunother. (2014) 63:407–18. doi: 10.1007/s00262-014-1524-0
54. Tarhini AA, Edington H, Butterfield LH, Lin Y, Shawr H, Tawbi H, et al. Immune monitoring of the circulation and the tumor microenvironment in patients with regionally advanced melanoma receiving neoadjuvant ipilimumab. PLoS ONE. (2014) 9:e87705. doi: 10.1371/journal.pone.0087705
55. Retseck J, VanderWeele R, Lin HM, Lin Y, Butterfield LH, Tarhini AA. Phenotypic and functional testing of circulating regulatory T cells in advanced melanoma patients treated with neoadjuvant ipilimumab. J Immunother Cancer. (2016) 4:38. doi: 10.1186/s40492-016-0141-1
56. Retseck J, Naar A, Lin Y, Lin H, Mendiratta P, Butterfield LH, et al. Long term impact of CTLA4 blockade immunotherapy on regulatory and effector immune responses in patients with melanoma. J Transl Med. (2018) 16:184. doi: 10.1186/s12967-018-1563-y
57. Ariyan CE, Brady MS, Siegelbaum RH, Hu J, Bello DM, Rand J, et al. Robust antitumor responses result from local...
chemotherapy and CTLA-4 Blockade. Cancer Immunol Res. (2018) 6:189–200. doi: 10.1158/2326-6066.CIR-17-0356

65. Balatoní T, Mohos A, Papp E, Sebestyén T, Liszkay G, Olaj J, et al. Tumor-infiltrating immune cells as potential biomarkers predicting response to treatment and survival in patients with metastatic melanoma receiving ipilimumab therapy. Cancer Immunol Immunother. (2018) 67:141–51. doi: 10.1007/s00262-017-2072-1

66. Yi JS, Ready N, Healy P, Dumbauld C, Osborne R, Berry M, et al. Immune activation in early-stage non-small cell lung cancer patients receiving neoadjuvant chemotherapy plus ipilimumab. Clin Cancer Res. (2017) 23:7474–82. doi: 10.1158/1078-0432.CCR-17-0005

67. Oh DY, Cham J, Zhang L, Fong G, Kwek SS, Kruger M, et al. Increases in absolute lymphocytes and circulating CD4+ and CD8+ T Cells are associated with positive clinical outcome of melanoma patients treated with ipilimumab. Clin Cancer Res. (2016) 22:4484–58. doi: 10.1158/1078-0432.CCR-15-0249

68. Haymaker CL, Wright M, Bierd N, Weckler LH, Rodriguez-Galindo C, Bernstein D, et al. Phase I clinical trial of ipilimumab in pediatric patients with advanced solid tumors. Clin Cancer Res. (2016) 22:1364–70. doi: 10.1158/1078-0432.CCR-15-0491

69. Björk J, Lund Nitschke N, Zeeberg Iversen T, Schmidt H, Fode K, Svane IM. Imunological correlates of treatment and response in stage IV malignant melanoma patients treated with ipilimumab. Oncoimmunology. (2016) 5:e1100788. doi: 10.1080/2162402X.2015.1100788

70. Kwek SS, Lewis J, Zhang L, Weinberg V, Deng S, Preston-Hurlburt P, Torres R, et al. Humanized mice as a model for aberrant immune activation in early-stage non-small cell lung cancer patients receiving ipilimumab therapy. Ann Surg Oncol. (2015) 20:1106–11. doi: 10.1245/s10434-013-3299-1

71. Pico de Coaña Y, Poschke I, Gentilcore G, Mao Y, Nyström M, Hansson V, et al. Preexisting levels of CD4 T cells expressing PD-1 are related to antitumor responses and autoimmunity in patients treated with CTLA-4 blockade. J Immunol. (2005) 175:7746–54. doi: 10.4049/jimmunol.175.11.7746

72. Wu SP, Liao RQ, Tu HY, Wang WJ, Hong ZY, Huang SM, et al. Immune activation in early-stage non-small cell lung cancer patients receiving ipilimumab therapy. Clin Cancer Res. (2011) 17:9204–11. doi: 10.1158/1078-0432.CCR-10-1350

73. Herrera AF, Moskowitz AJ, Bartlett NL, Vose JM, Ramchandren R, Feldman G, et al. The immune checkpoint protein PD-L1 induces and maintains regulatory T cells in glioblastoma. Oncoimmunology. (2018) 7:e148329. doi: 10.1080/2162402X.2018.1448329

74. Irie Y, Sasaoka A, Oshio T, Ito N, Otsuka T, Goto N, et al. Tumor-infiltrating immune cells to shift the T cell response to PD-L1 blockade. J Immunother. (2016) 39:89–97. doi: 10.1097/CLJ.0000000000000341

75. Hamid O, Schmidt H, Nissan A, Rijolli L, Aamdal S, Hansson J, et al. A prospective phase II trial exploring the association between tumor microenvironment biomarkers and clinical activity of ipilimumab in advanced melanoma. J Transl Med. (2011) 9:204. doi: 10.1186/1479-5876-9-204

76. Wu SP, Liao RQ, Tu HY, Wang WJ, Hong ZY, Huang SM, et al. Stromal PD-L1–Positive regulatory T cells and PD-1–Positive CD8-Positive T cells define the response of different subsets of non–small cell lung cancer to PD-1/PD-L1 Blockade Immunotherapy. J Thorac Oncol. (2015) 20:3106–11. doi: 10.1016/j.jtho.2017.11.133

77. Ferris RL, Blumenschein G, Harrington K, Cooke J, Guigay J, Colevas AD, et al. Tumor-associated immune cell PD-L1 expression and peripheral immune profiling: analyses from CHECKmate 141 [abstract]. In: Proceedings of the AACR Annual Meeting 2017; April 1-5; Washington DC, USA. Cancer Res. (2017) 77(Suppl. 13): Abstract nr CT201. doi: 10.1158/1535-7465.AMM2017-CT201

78. Gibney GT, Kudchadkar RR, DeConti RC, Thebeau MS, Czuprym MP, Tetteh L, et al. Safety, correlative markers, and clinical results of adjuvant nivolumab in combination with vaccine in resected high-risk metastatic melanoma. Clin Cancer Res. (2015) 21:712–20. doi: 10.1158/1078-0432.CCR-14-2468

79. Sarnaik AA, Yu B, Yu D, Morelli D, Hall M, Bogle D, et al. Extended dose ipilimumab with a peptide vaccine: Immune correlates associated with clinical benefit in patients with resected high-risk stage III/IV melanoma. Clin Cancer Res. (2011) 17:896–906. doi: 10.1158/1078-0432.CCR-10-2463

80. Carthon BC, Welchok JD, Yuan J, Kamat A, Ng Tang DS, Sun J, et al. Preoperative CTLA-4 blockade: tolerability and immune monitoring in the setting of a presurgical clinical trial. Clin Cancer Res. (2010) 16:2861–71. doi: 10.1158/1078-0432.CCR-10-0569

81. Sarnaik AA, Yu B, Yu D, Morelli D, Hall M, Bogle D, et al. Extended dose ipilimumab with a peptide vaccine: Immune correlates associated with clinical benefit in patients with resected high-risk stage III/IV melanoma. Clin Cancer Res. (2011) 17:896–906. doi: 10.1158/1078-0432.CCR-10-2463

82. Wang C, Thudium KB, Han M, Wang XT, Huang H, Feinigers D, et al. In vivo characterization of the anti-PD-1 antibody nivolumab, BMS-936558, and its ability to target tumors in non-human primates. Cancer Immunol Res. (2014) 2:846–56. doi: 10.1158/2326-6066.CIR-14-0040

83. Sarnaik AA, Yu B, Yu D, Morelli D, Hall M, Bogle D, et al. Extended dose ipilimumab with a peptide vaccine: Immune correlates associated with clinical benefit in patients with resected high-risk stage III/IV melanoma. Clin Cancer Res. (2011) 17:896–906. doi: 10.1158/1078-0432.CCR-10-2463

84. Too M, Saisidharan Nair V, Pfister G, Elkord E. Effect of pembrolizumab on CD4+ CD25+ T Cells and CD4+ CD25+ T Cells and CD4+ CD25+ T Cells. Cancer Immunol Res. (2019) 7:117–25. doi: 10.1158/2326-6066.CIR-18-0910

85. Carthon BC, Welchok JD, Yuan J, Kamat A, Ng Tang DS, Sun J, et al. Preoperative CTLA-4 blockade: tolerability and immune monitoring in the setting of a presurgical clinical trial. Clin Cancer Res. (2010) 16:2861–71. doi: 10.1158/1078-0432.CCR-10-0569

86. Sarnaik AA, Yu B, Yu D, Morelli D, Hall M, Bogle D, et al. Extended dose ipilimumab with a peptide vaccine: Immune correlates associated with clinical benefit in patients with resected high-risk stage III/IV melanoma. Clin Cancer Res. (2011) 17:896–906. doi: 10.1158/1078-0432.CCR-10-2463
69. Toor SM, Syed Khaja AS, Alkurd I, Elkord E. In-vitro effect of pembrolizumab on different T regulatory cell subsets. *Clin Exp Immunol.* (2018) 191:189–97. doi:10.1111/cei.13060

70. Ribas A, Shin DS, Zaretsky J, Frederiksen J, Cornish A, Avramis E, et al. PD-1 blockade expands intratumoral memory T cells. *Cancer Immunol Res.* (2016) 4:694–703. doi:10.1158/2326-6066.CIR-15-0210

71. Balasa RL, Simu M, Voidozan S, Barcutean LI, Bajko Z, Hutanu A, et al. Nalizumab changes the peripheral profile of the Th17 panel in MS patients: new mechanisms of action. *CNS Neurol Drug Targets.* (2017) 16:1018–26. doi:10.2174/171353831666617008170362

72. Rodi M, Dimisianos N, De Lastic A, Sakellarakis P, Deraos G, Matsoukas J, et al. Regulatory cell populations in relapsing-remitting multiple sclerosis (RRMS) patients: changes in disease activity and treatment regimens. *Int J Mol Sci.* (2016) 17:E1398. doi:10.3390/ijms17091398

73. Kimura K, Nakamura M, Sato W, Okamoto T, Araki M, Lin Y, et al. Disrupted balance of T cells under nalizumab treatment in multiple sclerosis. *Neural Neuroinflammation.* (2016) 3:e210. doi:10.12112/NXLI.0000000000000210

74. Kurmaeva E, Lord JD, Zhang S, Bao JR, Kevil CG, Grisham MB, et al. T cell-associated alpha4beta7 but not alpha4beta1 integrin is required for the induction and perpetuation of chronic colitis. *Mucosal Immunol.* (2014) 7:1354–65. doi:10.1038/mi.2014.22

75. Wyant T, Yang L, Fedyk E. *In vitro* assessment of the effects of vedolizumab binding on peripheral blood lymphocytes. *MAbs* (2013) 5:842–50. doi:10.4161/mabs.26392

76. Burman J, Fransson M, Tötterman TH, Fagius J, Mangsbo SM, Loskog J, et al. Disrupted balance of T cells under natalizumab treatment for aggressive relapsing-remitting multiple sclerosis. *Neural Neuroinflammation.* (2016) 3:e210. doi:10.12112/NXLI.0000000000000210

77. Putzki N, Baranwal MK, Tettenborn B, Limmroth V, Kreuzfelder E. Effects of natalizumab on circulating B cells, T regulatory cells and natural killer cells for immune suppression by regulatory T cells in patients with severe rheumatoid arthritis. *Rheumatology.* (2014) 54:601–8. doi:10.1093/rheumatology/keu563

78. Kikuchi J, Hashizume M, Kaneko Y, Yoshimoto K, Nishina N, Takeuchi T. Peripheral blood CD4+ CD25+ CD127low regulatory T cells are significantly increased by tocilizumab treatment in patients with rheumatoid arthritis: Increase in regulatory T cells correlates with clinical response. *Arthritis Res Ther.* (2015) 17:10. doi:10.1186/s13075-015-0526-4

79. Sarantopoulos A, Tselios K, Gkougkourelas I, Pantoura M, Georgiadou AM, Boura P. Tocilizumab treatment leads to a rapid and sustained increase in Treg cells in rheumatoid arthritis patients: comment on the article by Thiolat et al.* *Arthritis Rheum.* (2014) 66:2638. doi:10.1002/art.38714

80. Thiolat A, Semerano L, Pers YM, Biton J, Lemeiter D, Portales P, et al. Interleukin-6 receptor blockade enhances CD39+ regulatory T cell development in rheumatoid arthritis and in experimental arthritis. *Arthritis Rheum.* (2016) 66:273–83. doi:10.1002/art.38246

81. Pesce B, Soto L, Sabugo F, Wurmann P, Cuchacovich M, López MN, et al. Targeting IL-6 signalling in early rheumatoid arthritis is followed by Th1 and Th17 suppression and Th2 expansion. *Clin Exp Rheumatol.* (2014) 32:77–81.

82. Guggino G, Giardina AR, Raimondo S, Giardina G, Sireci G, Dieli F, et al. Targeting IL-6 signalling in early rheumatoid arthritis is followed by Th1 and Th17 suppression and Th2 expansion. *Clin Exp Rheumatol.* (2014) 32:77–81.

83. Pesce B, Soto L, Sabugo F, Wurmann P, Cuchacovich M, López MN, et al. Targeting IL-6 signalling in early rheumatoid arthritis is followed by Th1 and Th17 suppression and Th2 expansion. *Clin Exp Rheumatol.* (2014) 32:77–81.

84. Guggino G, Giardina AR, Raimondo S, Giardina G, Sireci G, Dieli F, et al. Targeting IL-6 signalling in early rheumatoid arthritis is followed by Th1 and Th17 suppression and Th2 expansion. *Clin Exp Rheumatol.* (2014) 32:77–81.

85. Pesce B, Soto L, Sabugo F, Wurmann P, Cuchacovich M, López MN, et al. Targeting IL-6 signalling in early rheumatoid arthritis is followed by Th1 and Th17 suppression and Th2 expansion. *Clin Exp Rheumatol.* (2014) 32:77–81.

86. Guggino G, Giardina AR, Raimondo S, Giardina G, Sireci G, Dieli F, et al. Targeting IL-6 signalling in early rheumatoid arthritis is followed by Th1 and Th17 suppression and Th2 expansion. *Clin Exp Rheumatol.* (2014) 32:77–81.

87. Pesce B, Soto L, Sabugo F, Wurmann P, Cuchacovich M, López MN, et al. Targeting IL-6 signalling in early rheumatoid arthritis is followed by Th1 and Th17 suppression and Th2 expansion. *Clin Exp Rheumatol.* (2014) 32:77–81.
132. Tada Y, Togashi Y, Kotani D, Kuswata T, Sato E, Kawazoe A, et al. Targeting VEGFR2 with ramucirumab strongly impacts effector/activated regulatory T cells and CD8+ T cells in the tumor microenvironment. J Immunother Cancer. (2018) 6:106. doi: 10.1186/s40425-018-0403-1

133. Romano A, Parrinello NL, Chiarenza A, Motta G, Tibullo D, Giallongo C, et al. Immune off-target effects of brentuximab vedotin in relapsed/refractory Hodgkin lymphoma. Br J Haematol. (2019) 185:468–79. doi: 10.1111/bjh.15801

134. Perez SA, Karamouzis MV, Skarlos DV, Ardavanis A, Sotiriadou NN, de Wolf et al. (Pre-)clinical Regulatory T Cell Monitoring trials: emerging lessons from type 1 diabetes. Nat Rev Immunol. (2017) 17:324–34. doi: 10.1038/nrrenu.2016.123

135. Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, et al. Targeting de Wolf et al. (Pre-)clinical Regulatory T Cell Monitoring trials: emerging lessons from type 1 diabetes. Nat Rev Immunol. (2017) 11:307. doi: 10.1038/nri2705

136. Winthrop KL. The emerging safety profile of JAK inhibitors in rheumatoid disease. Nat Rev Rheumatol. (2017) 13:234–43. doi: 10.1038/nrrheum.2017.5

137. Yao Z, Kanno Y, Kerenyi M, Stephens G, Durant L, Watford WT, et al. Nonredundant roles for Stat5a/b in directly regulating foxp. Blood. (2007) 109:4368–75. doi: 10.1182/blood-2006-11-05576

138. Burchill MA, Yang J, Vogehtuber C, Blazar BR, Farrar MA. IL-2 receptor β-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. J Immunol. (2007) 178:280–90. doi: 10.4049/jimmunol.178.1.280

139. Choi J, Cooper ML, Staser K, Ashami K, Vij KR, Wang B, et al. Baricitinib-induced blockade of interferon gamma receptor and interleukin-6 receptor for the prevention and treatment of graft-versus-host disease. Leukemia. (2018) 32:2483–94. doi: 10.1038/s41475-018-0123-z

140. Aguilar-Pimentel A, Graessel A, Alessandri F, Fuchs H, Gailus-Durner V, Hrabe De Angelis M, et al. Improved efficacy of allergen-specific immunotherapy by JAK inhibition in a murine model of allergic asthma. PLoS ONE. (2017) 12:e0178563. doi: 10.1371/journal.pone.0178563

141. Zhou Y, Leng X, Luo S, Su Z, Luo X, Guo H, et al. Tolerogenic dendritic cells generated with tofacitinib ameliorate experimental autoimmune encephalomyelitis through modulation of Th1/Th2 balance. J Immunol. (2016) 196:501537. doi: 10.4049/jimmunol.20150315

142. Yokosawa S, Perera PY, Terawaki S, Watanabe N, Kaminuma O, Waldmann TA, et al. Janus kinase inhibitor tofacitinib shows potent efficacy in a mouse model of Autoimmune Lymphoproliferative Syndrome (ALPS). J Clin Immunol. (2015) 35:661–7. doi: 10.1007/s10875-015-0203-z

143. Kubo S, Yamaoka K, Kondo M, Yamagata K, Zhao J, Iwata S, et al. The JAK inhibitor, tofacitinib, reduces the T cell stimulatory capacity of human monocyte-derived dendritic cells. Ann Rheum Dis. (2014) 73:2192–8. doi: 10.1136/annrheumdis-2013-203756

144. Stevenson W, Sadrai Z, Hua J, Kodati S, Huang JF, Chauhan SK, et al. Effects of topical Janus Kinase inhibition on ocular surface inflammation and immunity. Cornea. (2014) 33:177–83. doi: 10.1097/ICO.000000000000019

145. Sewgobind VDKD, Quaedackers ME, Van Der Laan LJW, Kraaijveld M, Korevaar SS, Chan G, et al. The JAK inhibitor CP-690,550 preserves the function of CD4+CD25brightFoxp3+ regulatory T cells and inhibits effector T cells. Am J Transplant. (2010) 10:1875–95. doi: 10.1111/j.1600-6143.2010.03200.x

146. Van Gurp EAJP, Schoorjied-Verschoor W, Klepper M, Korevaar SS, Chan G, Weimar W, et al. The effect of the JAK inhibitor CP-690,550 on peripheral immune parameters in stable kidney allograft patients. Transplantation. (2009) 87:79–86. doi: 10.1097/TP.0b013e3181a87b07

147. Feng X, Zhang L, Acharya C, An G, Wen K, Qiu L, et al. Targeting CD38 suppresses induction and function of T regulatory cells to mitigate immunosuppression in multiple myeloma. Clin Cancer Res. (2017) 23:4290–300. doi: 10.1158/1078-0432.CCR-16-1319

148. Kaplan H, Reichert JM. Antibodies to watch in 2019. MAbS. (2019) 11:219–38. doi: 10.1002/19420862.2018155645

149. Amat F, Tallon P, Foray AP, Michaud B, Lambert N, Saint-Pierre P, et al. Control of asthma by omalizumab: the role of CD4+Foxp3 regulatory T cells. Clin Exp Allergy. (2016) 46:1614–6. doi: 10.1111/cea.12839

150. Talotta R, Berai A, Atzeni F, Batticciotto A, Clerici M, Sarzi-Puttini P, et al. Paradoxical expansion of Th1 and Th17 Lymphocytes in rheumatoid arthritis following infliximab treatment: a possible explanation for a lack of clinical response. J Rheumatol. (2015) 42:550–7. doi: 10.3899/jrheum.1401556

151. Bhattacharjee R, De D, Handa S, Minz RW, Saikia B, Joshi N. Assessment of the effects of rituximab monotherapy on different subsets of circulating T-regulatory cells and clinical disease severity in severe pemphigus vulgaris. Dermatology. (2017) 232:572–7. doi: 10.1159/000478083

152. Roccaletto D, Sciascia S, Di Simone D, Solfetti L, Naretto C, Fenoglio A, et al. New insights into immune mechanisms underlying response to rituximab in patients with membranous nephropathy: a prospective study and a review of the literature. Autoimmun Rev. (2016) 15:529–38. doi: 10.1016/j.autrev.2016.02.014
165. Butterfield LH, Palucha AK, Britten CM, Dhodapkar MV, Häkansson L, Janetzki S, et al. Recommendations from the sBTIC-SITC-FDA/NCI workshop on immunotherapy biomarkers. Clin Cancer Res. (2011) 17:3064–76. doi: 10.1158/1078-0432.CCR-10-2234

166. D’ArenA G, Vitale C, Coscia M, Festa A, Di Minno NMD, De Feo V, et al. Regulatory T cells and their prognostic relevance in hematologic malignancies. J Immunol Res. (2017) 2017:1832968. doi: 10.1155/2017/1832968

167. Okamura K, Fukuda Y, Sado H, Ogawa D, Iwasaki K, Fuchi S, et al. Pulmonary pleomorphic carcinoma with few PD-1-positive immune cells and regulatory T cells that showed a complete response to nivolumab. Thorac Cancer. (2018) 9:193–6. doi: 10.1111/1759-7714.12557

168. Ogawa D, Sado H, Iwasaki K, Suyama T, Taniguchi H, Fukuda Y, et al. Remarkable response of nivolumab-refractory lung cancer to salvage chemotherapy. Thorac Cancer. (2018) 9:195–7. doi: 10.1111/1759-7714.12543

169. Uchida A, Watanabe M, Nawata A, Ikari Y, Sasaki M, Shigemoto K, et al. Tubulointerstitial nephritis as adverse effect of programmed cell death 1 inhibitor, nivolumab, showed distinct histological findings. CEN Case Rep. (2017) 6:169–74. doi: 10.1007/s13730-017-0269-y

170. Lin YC, Mahalingam J, Chiang JM, Su PJ, Chu YY, Lai HY, et al. Activated but not resting regulatory T cells accumulated in tumor microenvironment and correlated with tumor progression in patients with colorectal cancer. Int J Cancer. (2013) 132:1341–50. doi: 10.1002/ijc.27784

171. Mohy M. Mechanisms of action of antithymocyte globulin: T-cell depletion and beyond. Leukemia. (2007) 21:1387–94. doi: 10.1038/sj.leu.2404683

172. Lopez M, Clarkson MR, Albin M, Sayegh MH, Najafian N. A novel mechanism of action for anti-thymocyte globulin: induction of CD4+CD25+Foxp3+ regulatory T cells. J Am Soc Nephrol. (2006) 17:2844–53. doi: 10.1681/ASN.2006050422

173. Santegoets SJAM, Dijkgraaf EM, Battaglia A, Beckhove P, Britten CM, Mohty M. Mechanisms of action for antithymocyte globulin: induction of CD4+CD25+Foxp3+ regulatory T cells expressing the FoxP3 transcription factor. Immunol. (2009) 80:899–911. doi: 10.1006/jimm.2009.03.018

174. Lopez M, Albin M, Sayegh MH, Najafian N. A novel mechanism of action for anti-thymocyte globulin: induction of CD4+CD25+Foxp3+ regulatory T cells. J Am Soc Nephrol. (2006) 17:2844–53. doi: 10.1681/ASN.2006050422

175. Vignali DAA, Collison LW, Workman CJ. How regulatory T cells work. Nat Rev Immunol. (2007) 7:83–9. doi: 10.1038/nri2474

176. Ihara F, Sakurai D, Horinaka A, Makita Y, Fujikawa A, Sakurai T, et al. CD45RA−CD25high Foxp3(high) regulatory T cells have a negative impact on the clinical outcome of head and neck squamous cell carcinoma. Cancer Immunol Immunother. (2017) 66:1275–85. doi: 10.1007/s00262-017-2021-z

177. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity. (2009) 30:899–911. doi: 10.1016/j.immuni.2009.03.019

178. Shevach EM, Thornton AM. iTregs, pTregs, and iTreg similarities and differences. Immunol Rev. (2014) 259:88–102. doi: 10.1111/imr.12160

179. Lin X, Chen M, Liu Y, Guo Z, He Dr. X, Brand D, et al. Advances in distinguishing natural from induced Foxp3+ regulatory T cells. Int J Clin Exp Pathol. (2013) 6:1166–23.

180. Ito T, Hanabuchi S, Wang YH, Park WR, Arima K, Bover L, et al. Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery. Immunity. (2008) 28:870–80. doi: 10.1016/j.immuni.2008.03.018

181. Huehn J, Polansky JK, Hamann A. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? Nat Rev Immunol. (2009) 9:83–9. doi: 10.1038/nri2474

182. Baron U, Floess S, Wieczorek G, Baumann K, Grützkau A, Dong J, et al. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3+ conventional T cells. Eur J Immunol. (2007) 37:2378–89. doi: 10.1002/eji.200735794

183. Zhang L, Manirarora JN, Wei CH. Evaluation of immunosuppressive function of regulatory T cells using a novel in vitro cytotoxicity assay. Cell Biosci. (2014) 4:51. doi: 10.1186/2455-3701-4-51

184. Mcmurchy AN, Levings MK. Suppression assays with human T regulatory cells: a technical guide. Eur J Immunol. (2012) 42:27–34. doi: 10.1002/eji.201146531

185. Whiteside TL. What are regulatory T cells? Semin Cancer Biol. (2012) 22:327–34. doi: 10.1016/j.semcancer.2012.03.004

186. Levitsky I, Miller J, Levenhajl J, Huang X, Faa C, Wang E, et al. The human "treg MLR": immune monitoring for FOXP3+ T regulatory cell generation. Transplantation. (2009) 88:1303–11. doi: 10.1097/TP.0b013e3181bbee98

187. Porter SB, Liu B, Rogosheske J, Levine BL, June CH, Kohl VK, et al. Suppressor function of umbilical cord blood-derived CD4+CD25+ T-regulatory cells exposed to graft-versus-host disease drugs. Transplantation. (2006) 82:23–9. doi: 10.1097/01.tp.0000225842.8931.a

188. Hill D, Eastaff-Leung N, Breszat-Atkins S, Warner N, Ruitenberg J, Krumbiegel D, et al. Inhibition of activation induced CD154 on CD4 CD25 cells: a valid surrogate for human Treg suppressor function. Immunol Cell Biol. (2012) 90:812–21. doi: 10.1007/ibc.2012.18

189. Canavan JB, Afzali B, Scottà C, Fazekasova H, Edozie FC, Macdonald TT, et al. A rapid diagnostic test for human regulatory T-cell function to enable regulatory T-cell therapy. Cytometry B Clin Cytom. (2019) doi: 10.1002/cyto.b.21841. [Epub ahead of print].

190. Camisaschi C, Tazzari M, Rivoltini L, Castelli C. Monitoring the frequency and function of regulatory T cells and summary of the approaches currently used to inhibit regulatory T cells in cancer patients. Methods Mol Biol. (2014) 1139:201–21. doi: 10.1007/978-1-4939-0345-0_18

191. Petiot F, Barbie M, Monneret G, Braudeau C, Pochard P, Pellegrin I, et al. A standardized flow cytometry procedure for the monitoring of regulatory T cells in clinical trials. Cytometry B Clin Cytom. (2018) 94:621–6. doi: 10.1002/cyto.b.21622.
201. Chu ST, Chien KH, Lin HH, Wu WH, Jian JY, Tzeng WF, et al. Using marker gene analysis instead of mixed lymphocyte reaction assay for identification of functional \( \text{CD4}^+ \) \( \text{FOXP3}^+ \) regulatory T cells. *Biotechnol Lett.* (2018) 40:535–42. doi: 10.1007/s10529-017-2998-8

202. Azimi M, Aslani S, Mortezagholi S, Salek A, Javan MR, Rezaianemesh A, et al. Identification, isolation, and functional assay of regulatory T Cells. *Immunol Invest.* (2016) 45:584–602. doi: 10.1080/08820139.2016.1193869

203. Whiteside TL. Regulatory T Cell (Treg) assays: repertoire, functions, and clinical importance of human treg. In: Detrick B, Schmitz J, Hamilton R, editors. *Manual of Molecular and Clinical Laboratory Immunology.* American Society for Microbiology (2016). p. 296–9. doi: 10.1128/9781555818722.ch31

204. Brusko TM, Hulme MA, Myhr CB, Haller MJ, Atkinson MA. Assessing the in vitro suppressive capacity of regulatory T cells. *Immunol Invest.* (2007) 36:607–28. doi: 10.1080/08820130701790368

205. Fuchs A, Gliwinski M, Grageda N, Spiering R, Abbas AK, Appel S, et al. Minimum information about T regulatory cells: a step toward reproducibility and standardization. *Front Immunol.* (2018) 8:1844. doi: 10.3389/fimmu.2017.01844

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