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Clinical & Translational Immunology (2017) 6, e154; doi:10.1038/cti.2017.43
Official journal of the Australasian Society for Immunology Inc.
www.nature.com/cti

REVIEW

Regulatory T-cell heterogeneity and the cancer immune response

Kirsten A Ward-Hartstonge and Roslyn A Kemp

The frequency of circulating or tumour-infiltrating regulatory T cells (Tregs) has been associated with poor patient survival in many cancers including breast, melanoma and lung. It has been hypothesised that Tregs impact the anti-tumour function of effector T cells, resulting in worse outcomes for patients. However, high infiltrates of Tregs have been associated with a positive outcome of patients in a minority of cancers including colorectal, bladder and oesophageal. In addition, many studies have shown no impact of Tregs in patient outcome. Traditionally, research has identified Tregs as forkhead box P3 (FOXP3$^+$) T cells in order to make such associations. Recently, it has become evident that regulatory populations are very heterogeneous, and this heterogeneity is essential for Treg function. Treg heterogeneity likely affects predictions of patient outcome, and different Treg populations may have different influences on tumours. The study of Tregs in cancer must include a better definition of the cells analysed. This review will focus primarily on colorectal cancer in humans, due to mixed data on the impact of Tregs on patient outcome in this disease.

Clinical & Translational Immunology (2017) **6,** e154; doi:10.1038/cti.2017.43; published online 15 September 2017

INTRODUCTION

Regulatory T cells (Tregs) are essential for the suppression of self-reactive T cells in the periphery and the inhibition of immune responses at the resolution of infection.[1] Historically, Tregs were divided into two groups, those formed in the thymus (natural), and those differentiated in the periphery (induced). The transcription factor, Helios, has been used as a marker to distinguish natural and induced Tregs, but expression of Helios has been identified on both populations in humans.[2]

Tregs are characterised by high expression of the interleukin two receptor (IL-2R) alpha, CD25 and expression of the transcription factor, FOXP3,[1] both of which are essential for their regulatory functions. The expression of CD25 on FOXP3$^+$ Tregs is believed to facilitate sequestration of IL-2 from the environment, thereby reducing effector T-cell access to IL-2.[3] *FOXP3* expression is critical for the suppressive function of Tregs: mutations in the *FOXP3* gene cause fatal autoimmune disease and immunopathology in humans.[4] CD25 and FOXP3 alone cannot be used to identify Tregs, as conventional T-cell populations can upregulate both of these markers after activation.[5] However, demethylation of the *FOXP3* locus occurs only in Tregs with stable expression of FOXP3 and not conventional T cells that upregulate FOXP3 transiently after activation.[6] Tregs in humans, at least in the blood, also express low levels of CD127 and use of this molecule alongside FOXP3 and CD25 has been proposed as a more accurate means of identifying Tregs.[7,8]

In addition to the inhibition of effector T-cell activation and proliferation via the sequestration of IL-2, Tregs can suppress effector T cells using other mechanisms. Tregs secrete suppressive cytokines, such as IL-10, IL-35 and transforming growth factor (TGF)-β, express inhibitory markers such as cytotoxic T-lymphocyte-associated protein (CTLA)-4 and inducible T-cell costimulator (ICOS) that inhibit the ability of antigen presenting cells to activate effector T cells, and cause apoptosis of effector cells via the production of granzyme (reviewed in Vignali *et al.*[9]). In cancer patients, tumour-infiltrating Tregs often express higher levels of inhibitory markers and are much more suppressive than Tregs from peripheral blood mononuclear cells (PBMCs) or from non-tumour tissue. This is most likely due to factors within the tumour microenvironment.[10]

Tregs are often identified using FOXP3 alone in studies of people with cancer, sometimes without including a T-cell marker. This approach is often dictated by the sample type and availability, however, it is clear that this is not sufficient to identify heterogeneous T-cell populations with regulatory function. For example, we recently showed no association with Tregs and outcome in a cohort of colorectal cancer (CRC) patients when identified by T-cell expression of FOXP3 alone. However, when the transcription factor, B lymphocyte-induced maturation protein-1 (Blimp-1), was used alongside FOXP3 to identify a subpopulation of Tregs, there was a positive association with patient outcome.[11] In a cohort of patients with B-cell lymphoma, a high infiltrate of Tregs identified by expression of FOXP3 alone was associated with good patient outcome, but CTLA-4$^+$FOXP3$^+$ double-positive Tregs were associated with poor patient outcome.[12] These results emphasise the importance of using more than one marker to identify Tregs in human cancer tissue.

Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand
Correspondence: Dr RA Kemp, Department of Microbiology and Immunology, University of Otago, Po Box 56, Dunedin 9054, New Zealand.
E-mail: roslyn.kemp@otago.ac.nz
Received 29 June 2017; revised 14 August 2017; accepted 14 August 2017

EFFECTOR TREGS

FOXP3$^+$ Tregs have been proposed to exist in multiple differentiation states, similar to effector T-cell populations: naive, effector and memory.[13] Two functionally distinct phenotypes of suppressive FOXP3$^+$ Tregs have been identified in human blood: CD45RA$^+$FOXP3^{lo} 'naive' Tregs and CD45RA$^-$FOXP3^{hi} 'effector' Treg populations.[14] It is likely that these naive-like Tregs were thymic derived, resting Tregs that once activated, converted to effector-like activated Tregs.

In mice, the term 'effector Tregs' (eTregs) has been given to a similar population of FoxP3$^+$ Tregs that express Blimp-1 (reviewed in Cretney *et al.*[15]). These eTregs have high expression of many Treg activation markers including CTLA-4, ICOS, CD45RO, and are highly suppressive. Blimp-1 and interferon regulatory factor (IRF)-4 were essential for the development of FoxP3$^+$ eTregs, and Blimp-1 was essential for IL-10 production in T cells.[16] FoxP3$^+$ eTregs were present in the gut of mice[16] and have been identified in human CRC.[11] We have recently demonstrated that Blimp-1 can be used to identify a similar population of Tregs in humans with an effector phenotype (unpublished).

Memory Treg populations are not well understood. In mice, FoxP3$^+$ Tregs specific for self-antigen,[17] viral antigen[18,19] or foetal antigen[20] showed similar characteristics to effector memory T cells: an expansion during primary response, a small remaining population, and then expansion during a secondary response. In humans it is more challenging to study an antigen-specific Treg response over time to measure memory, but using phenotypic markers based on effector memory T cells, a population of memory-like FOXP3$^+$CD25^{hi} Tregs (CD45RO$^+$ICOS$^+$CD27$^+$CTLA-4$^+$) has been identified in human skin.[21]

The tumour microenvironment contains Tregs with an effector phenotype in many human cancers, and this is likely in response to inflammatory factors produced by other cells interacting with Tregs within this environment (reviewed in Chaudhary and Elkord[10] and Norton *et al.*[22]). As outlined above, higher frequencies of Blimp-1$^+$ FOXP3$^+$ eTregs were associated with longer disease-free survival.[11] However, other studies demonstrate conflicting evidence. Saito *et al.*[23] indicated that FOXP3^{hi} eTregs, but not FOXP3^{lo} Tregs, were associated with poor patient outcomes in CRC. Lin *et al.*[24] demonstrated that Tregs with an effector phenotype (FOXP3^{hi}CD45RA$^-$), but not a resting phenotype (FOXP3^{lo}CD45RA$^+$), were associated with late-stage CRC. Nakayama *et al.*[12] classified 'effector Tregs' as FOXP3$^+$ CTLA-4$^+$ in a B-cell lymphoma cohort and high infiltrates were associated with poor patient outcomes. These studies indicate that using different markers to define eTreg populations may result in different associations with patient outcomes, and that eTreg infiltrates may have different roles across human cancers.

HELPER-LIKE TREG SUBSETS

Treg phenotypes can differ as a result of the environment in which they differentiate, similar to effector T cells. FOXP3$^+$ Tregs upregulate transcription factors that can mirror effector T-cell populations differentiated under the same conditions and influence immune responses (reviewed in Cretney *et al.*[15]). The transcription factor, signal transducer and activator of transcription (STAT)-3 is essential for the suppression of (T_H17) inflammatory IL-17 responses in mice.[25] The expression of T-bet in FoxP3$^+$ Tregs is essential for the suppression of (T_H1) IFN-γ effector responses in mice.[26,27] Finally, the expression of IRF4 is essential for the suppression of T_H2 responses in mice.[28]

FOXP3$^+$ Tregs expressing canonical T effector transcription factors, especially RORγt, have been identified in healthy human PBMCs.[29] In mice, RORγt$^+$FoxP3$^+$ Tregs and RORγt$^+$ T_H17 cells coexisted in a healthy state *in vivo*,[30] and RORγt$^+$FoxP3$^+$ Tregs could suppress intestinal inflammation.[31] RORγt$^+$FOXP3$^+$ Tregs have been identified in CRC patients and were enriched in patients with late-stage cancer.[32] RORγt$^+$FOXP3$^+$ Tregs were also upregulated in the blood from people with pancreatic cancer, compared to age-matched healthy controls.[33] RORγt$^+$FOXP3$^+$ Tregs in CRC and pancreatic patients produced both IL-10 and IL-17.[32,33] These data indicate that perturbations in these populations in cancer may influence disease and potentially contribute to poor outcomes for patients.

TREG PLASTICITY

T cells are plastic and the presence of multiple factors can induce changes in T-cell phenotype. The stability of the Treg lineage is complicated and it is still not clear if all Tregs are able to display plasticity, or if this ability is restricted to a selected 'unstable' population.[34] However, there is evidence of the conversion of FOXP3$^+$ Tregs into T helper subsets and vice versa, under suitable conditions (reviewed in Hori[35]). T_H17 cells and Tregs differentiate using closely related pathways and both require TGF-β for differentiation. The flexibility between T_H17 cells and Tregs has been implicated in many diseases including autoimmune diseases and cancers. For example, gut inflammation can cause FoxP3$^+$ Tregs to differentiate into pathogenic inflammatory T cells in mice *in vivo*.[36] In response to an inflammatory bacterial insult in the gut, Tregs lost FOXP3 expression and gained the ability to produce IL-17. Populations of Tregs that secreted effector cytokines such as IL-17 were able to suppress effector T-cell proliferation *in vitro*.[37] It has been proposed that these inflammatory cytokine-producing Tregs may exist in a transitional stage and have the ability to differentiate into FOXP3$^+$ Treg or T_H17 cells depending on the local conditions such as the tumour microenvironment.[38]

The tumour microenvironment is complex and changes during disease progression. It may be hypoxic, contain angiogenic factors and/or suppressive immune cells, all of which contribute to tumour growth and differentiation.[22] Tregs are present in high numbers in tumours and higher infiltrates of Tregs in tumours, compared to surrounding non-tumour tissue, and have been documented numerous times in multiple cancers.[39,40] The recruitment and retention of Tregs into tumours involves the expression of chemokine receptors on Treg populations. In particular, CCR5^{hi}FOXP3$^+$ Tregs were more suppressive than CCR5^{lo}FOXP3$^+$ Tregs in human CRC.[39] Tumours can secrete factors such as vascular endothelial growth factor (VEGF) that recruits Tregs to tumours, aids in the conversion of Tregs to T_H17 cells, induces angiogenesis and enhances the growth of tumour cells (reviewed in Norton *et al.*[22]). Blocking VEGF pathways inhibited the recruitment of FoxP3$^+$ Tregs in a mouse model of CRC, and blocked the proliferation of human Tregs *in vitro*.[41] Tumour-infiltrating Tregs can be more suppressive than those from non-tumour tissue from the same patient, have an eTreg phenotype (FOXP3^{hi}CD45RA$^-$), and upregulate markers associated with enhanced suppression (CTLA-4, ICOS) and activation (CD25, CD69; reviewed in Chaudhary and Elkord[10]). These results indicate that factors within the tumour microenvironment lead to recruitment of Tregs to tumours and enhance the suppressive activity of Tregs.

T-cell subsets can differentiate within a tumour environment; T_H17 cells differentiated into suppressive IL-17$^+$ and IL-17$^-$FoxP3$^+$ Tregs in a CRC mouse tumour model.[42] In this model, tumour-associated TGF-β and prostaglandin E2 promoted the conversion of ex-T_H17 to Foxp3$^+$IL-17$^{+/-}$ cells. IL-17-producing FOXP3$^+$ Tregs have been identified in human CRC.[40,43] IL-17$^+$FOXP3$^+$ Tregs were able to

suppress the proliferation of $CD8^+$ tumour-infiltrating lymphocytes stimulated with CRC antigens in humans.[44] Supernatant from *ex vivo*-cultured IL-17^+ Tregs from human colitis tissue increased IL-6 and IL-1β production in T cells.[43] Tregs that produced IL-17^+ in human CRC tumours lost their ability to suppress mast cell degranulation,[37] and potentially aided growth of cancer initiating cells.[45] Hypoxia-induced $FOXP3^+$ Tregs also produced IL-17 *in vitro*, this may also occur in the tumour microenvironment.[45] These results indicate that IL-17^+ Tregs may be differentiated from T_H17 cells in CRC due to factors in the tumour microenvironment, resulting in the promotion of inflammation and suppression of tumour-specific $CD8^+$ T cells.

NON-CLASSICAL REGULATORY T-CELL POPULATIONS IN TUMOURS

LAP^+ Tregs

Tregs that secrete IL-10 and have low or negative expression of FOXP3 have been identified in multiple cancers (reviewed in Chaudhary and Elkord[10]). Of note, populations of $FOXP3^+$ and $FOXP3^-$ T cells that express latency-associated protein (LAP) have been identified in CRC.[46–48] The tumour-infiltrating LAP^+FOXP3^+ cells had 50-fold more suppressive capacity than LAP^-FOXP3^+ Tregs isolated from the peripheral blood.[48] Infiltrates of LAP^+ Tregs were associated with higher tumour, lymph node, metastasis (TNM) stage in this study, indicating that they could be associated with poor patient outcome.

$CD8^+$ $FOXP3^+$ Tregs

There is evidence that $CD8^+$ T-cell populations can also express FOXP3 and have regulatory function. $CD8^+FOXP3^+$ Tregs have been identified in CRC tissue[49] and were able to suppress $CD4^+$ T-cell proliferation and IFN-γ production *ex vivo*.[50] $CD8^+$ $FOXP3^+$ Tregs have also been identified in ovarian cancer patients and higher infiltrates were associated with higher stage of disease.[51] Upon co-culture with ovarian tumour cell lines, $CD8^+$ effector T cells converted into $CD8^+FOXP3^+$ Tregs.[51] These *in vitro*-generated $CD8^+$ $FOXP3^+$ Tregs suppressed $CD4^+$ T-cell proliferation. These data indicate that the tumour microenvironment may induce suppressive $CD8^+$ $FOXP3^+$ Tregs.

TREG FUNCTION IN THE TUMOUR MICROENVIRONMENT

Suppression of effector T cells

Antigen-specific Tregs have been isolated from CRC, pancreatic cancer, bladder cancer and melanoma (reviewed in Chaudhary and Elkord[10]). In advanced melanoma patients, effector T cells and $CD25^{hi}$ Tregs had T-cell receptors specific for the same epitopes.[52] *Ex vivo* analysis of $FOXP3^+$ Tregs from tumour samples of 15 pancreatic cancer patients revealed that Tregs made up 17% of the T-cell clones that were reactive to tumour peptide enolase 1 (ENO1); these Tregs suppressed the proliferation of ENO1-specific T cells.[53] In a cohort of 62 CRC patients, two-thirds of the patients had effector T cells that were reactive to the CRC antigens, 5T4 and carcinoembryonic antigen (CEA). Effector T-cell responses were suppressed by $FOXP3^+$ Tregs from the tumours.[54] In all patients with disease recurrence, $FOXP3^+$ Tregs from the tumours were able to effectively suppress tumour-specific tumour-infiltrating T cells.[54] These results suggest that tumour-specific Tregs and effector T cells co-exist within tumours, and suppression of effector T cells may be associated with poor patient outcome.

Suppression of T_H17 cells

High numbers of infiltrating Tregs have been associated with good patient outcomes in multiple cancers, including CRC. It has been hypothesised that this is at least partly due to $FOXP3^+$ Treg-mediated suppression of T_H17 responses[55] that are known to cause unfavourable outcomes in CRC patients.[56] A higher ratio of Tregs to T_H17 cells was associated with lower metastasis scores, and therefore favourable patient outcomes in a cohort of CRC patients.[57] There is also evidence that, in humans, *ex vivo* naive and memory $FOXP3^+$ Treg populations can inhibit the ability of T_H17 cells to produce the inflammatory cytokines; IL-17 and IL-22.[58]

In mice that lack functional IL-10, severe intestinal inflammation and spontaneous CRC can develop; indicating that IL-10 is involved in the control of inflammation-driven CRC.[59] More recently, others have also demonstrated the importance of IL-10-mediated suppression of inflammatory responses in the colon and in MC38 colon mouse tumour models.[60,61] Blimp-1 is essential for IL-10 production in Tregs; high frequencies of Blimp-1^+FOXP3^+ eTregs were recently associated with improved patient outcomes in human CRC.[11] It is possible Blimp-1^+ eTregs may suppress inflammatory responses in CRC via the production of IL-10.

Effect of tumour microenvironment on Treg function

There is extensive evidence that effector T cells upregulate markers of exhaustion and lose their ability to function in tumours. It is not yet clear whether T-cell exhaustion also occurs in Tregs, especially in the tumour microenvironment. In human glioblastoma tissue, $FOXP3^+$ Tregs expressing programmed cell death protein (PD)-1 were enriched in tumours compared to peripheral blood.[62] Mass cytometry was used to determine the phenotype of PD-$1^{hi}FOXP3^+$ Tregs in human glioblastoma and PD-1^{hi} expression correlated with higher expression of ICOS and lymphocyte activation gene (LAG)-3. PD-1-expressing $FOXP3^+$ Tregs had genetic signatures that correlated with exhaustion and Treg activation.[62] Tregs in tumours of CRC patients also upregulated immunosuppressive markers present on populations of exhausted effector T cells, but these $FOXP3^+$ Tregs were proposed to be activated and highly suppressive due to high expression of CD39 and LAP.[63]

Other immune cells from CRC tumours are able to modulate the function of Tregs. $FOXP3^+$ Tregs from CRC tissue, compared to the same cells from non-tumour tissue, had upregulated expression of genes associated with the promotion of inflammation.[64] Blanter *et al.*[37] showed that Tregs from human CRC tumours lost the ability to produce IL-10 and instead produced IL-17. These Tregs, although able to suppress effector T-cell proliferation, lost the ability to inhibit the degranulation of mast cells. In CRC, mast cells contribute to tumour growth and angiogenesis, and higher numbers are associated with advanced stage CRC.[65] *In vitro* stimulation of naive Tregs with mast cells was sufficient for Tregs to secrete IL-17 and become inflammatory.[37] Therefore, mast cells in CRC may convert Tregs to an inflammatory IL-17 phenotype that promotes inflammation in the tumour microenvironment and aids in tumour progression.

The tumour site contributes to Treg heterogeneity

The human gut contains more than 10^{14} bacterial cells. The intestinal epithelium prevents bacteria from entering the mucosa, but this physical barrier can become permeable allowing the entry of bacteria.[66] Once in the mucosa, bacteria encounter immune cells and are able to induce inflammatory responses including T_H17 cells.[67] It has been proposed that the presence of tumours can make the colon more permeable, allowing the infiltration of bacteria-multiple bacterial species have been identified in tumours of CRC patients.[68] Bacteria could promote inflammatory responses in CRC.[69] In mouse models, $CD25^{hi}$ Tregs were able to prevent cancer growth induced by

bacteria.[70] Therefore, Tregs in CRC may suppress bacteria-driven inflammatory responses such as those mediated by T_H17 cells.

POTENTIAL FOR CLINICAL INTERVENTION

The role of Tregs in cancer immunotherapy

Immune checkpoint inhibitors (ICI) are currently being investigated as a cancer treatment and have shown promise in specific cancers such as melanoma. Tregs upregulate multiple ICI receptors (for example, CTLA-4, PD-1) in cancer[10] and the effect of ICI treatment on Treg infiltrates in tumours is unclear. Interestingly, in multiple melanoma clinical trials with ipilimumab (anti-CTLA-4) treatment, Treg numbers in peripheral blood increased and were associated with both poor and favourable patient outcomes.[10] In a small cohort of 10 melanoma patients, the number of $FOXP3^+$ Tregs were measured in response to ipilimumab therapy and infiltrates tended to associate with worse clinical outcomes.[71] The role of Tregs in ICI-treated cancer patients is not clear, but it is possible Treg infiltrates may influence patient outcomes in those treated.

Utilisation of beneficial factors from Tregs to treat cancer

It has been proposed that IL-10 may not be strictly suppressive in the cancer microenvironment, but may also contribute to a favourable anti-tumour T-cell response.[72] In mouse tumour models, treatment with PEGylated IL-10 increased the activation of $CD8^+$ T cells and increased the production of IFN-γ.[73] Phase I clinical trials are underway, using PEGylated IL-10 to treat advanced staged human solid tumours, including CRC (https://clinicaltrials.gov/ct2/show/NCT02009449). Early phase I clinical trial data in 16 CRC patients indicated that IL-10 therapy increased the number of IFN-γ^+ activated T cells while simultaneously decreasing unfavourable inflammatory cytokine production, including IL-17.[74] Therefore, treating cancer patients with IL-10 may be beneficial to patient outcome.

SUMMARY AND CONCLUSIONS

Tregs exist in multiple phenotypes-the heterogeneity and plasticity are common in the tumour microenvironment and may be a contributing factor to disease. This review has summarised Treg sub-populations of interest in human cancer and attempted to link these populations to clinical outcomes. It is clear that the cells and molecules within the tumour microenvironment can alter Treg phenotype and ultimately impact patient survival in cancer patients. A more detailed identification of Treg phenotype is required to fully appreciate the role of Tregs in cancer. We have emphasised that FOXP3 alone is not sufficient to describe Tregs. The addition of more detailed markers, used in conjunction with one another, will allow for a deeper understanding of the role of Tregs in cancer and whether they can be used to improve therapies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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