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Cross talk between human regulatory T cells and antigen presenting cells: lessons for clinical applications

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See accompanying review by Giganti et al.

Abbreviations:

AIRE	autoimmune regulator gene
APC	antigen presenting cell
Breg	regulatory B cell
CAR	chimeric antigen receptor

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cTEC	cortical thymic epithelial cells
cDC1	type 1 conventional DC
cDC2	type 2 conventional DC
DC	dendritic cell
GMP	good manufacturing process
GVHD	Graft-vs-Host disease
mTEC	medullary thymic epithelial cells
pDC	plasmacytoid DC
pTreg	peripherally-derived Treg
SMAC	supramolecular activation cluster
Tconv	conventional T cell
Tfr	follicular regulatory T cell
tolAPC	tolerogenic APC
tolDC	tolerogenic DC
Treg	regulatory T cell
tTreg	thymically-derived Treg
Tr1 cell	type 1 regulatory T cell

Abstract

Regulatory T cells (Tregs) have a critical role in maintaining self-tolerance and immune homeostasis. There is much interest in using Tregs as a cell therapy to re-establish tolerance in conditions such as inflammatory bowel disease and type 1 diabetes, with many ongoing clinical studies testing the safety and efficacy of this approach. Manufacturing of Tregs for therapy typically involves *ex vivo* expansion to obtain sufficient cell numbers for infusion and comes with the risk of altering the activity of key biological processes. However, this process also offers an opportunity to tailor Treg function to maximize *in vivo* activity. In this review we focus on the roles of antigen presenting cells (APCs) in the generation and function of Tregs in humans. In addition to stimulating the development of Tregs, APCs activate Tregs and provide signals that induce specialised functional and homing marker expression. Cross talk between Tregs and APCs is a critical, often under-appreciated, aspect of Treg biology, with APCs mediating the key properties of infectious tolerance and bystander suppression. Understanding the natural biology of human Treg-APC interactions will reveal new ways to optimise Treg-based therapeutic approaches.

Introduction

Regulatory T cells (Tregs) maintain immune homeostasis by controlling auto-reactive immune responses and modulating innate and adaptive immunity. Tregs function through several mechanisms of action, including altering the function of antigen presenting cells (APCs), secreting immunosuppressive cytokines (e.g. IL-10, TGF-β), and modulating the metabolic environment (reviewed in [1]).

Treg therapy has shown promise in preventing and treating complications from transplantation or autoimmune conditions in early clinical trials [2, 3]. The majority of these trials have administered polyclonal Tregs to patients with the goal of shifting the balance between effector and regulatory cells in favour of immune tolerance. Although this approach has been successfully implemented with several established clinical manufacturing protocols [4], a drawback to the use of polyclonal Tregs is the large number of cells required per patient, as only a small fraction is expected to recognize disease-relevant antigens. As a result, multiple groups are designing more specialized Treg products, for example, by expanding antigen-specific Tregs or introducing genetic modifications [4].

Designing any T cell therapy requires a concrete understanding of the natural biology of T cells and their desired target(s) in humans. Interactions with APCs shape Treg development, differentiation and migration during natural immune responses in ways that are often not recapitulated in cell manufacturing protocols. Furthermore, it is becoming increasingly clear that modulation of APCs may be a critical suppressive function of Tregs. This review will discuss evidence for the important role of APCs in the development, specialisation, migration and tolerogenic function of Tregs. Unless stated otherwise, the studies reviewed here have been conducted with human cells, and we highlight key aspects of human Treg biology that can be used to develop more efficacious Treg therapy products.

APC-driven Treg development in the thymus

$CD4^+$ T cells can become lineage-committed $FOXP3^+$ Tregs in either the thymus (thymically-derived Tregs; tTregs) or periphery (peripheral-derived Tregs; pTregs), in both cases via interactions with various APCs. tTregs develop from cells encoding TCRs with a relatively high affinity for self-peptides presented in complex with MHC-II (reviewed in [5]). AIRE (autoimmune regulator gene) expression in medullary thymic epithelial cells (mTECs) and/or thymic dendritic cells (DCs) allows presentation of a wide array of self-peptides derived from most human tissues and is critical for the development of a robust tTreg population [6]. Data from mice indicate that TGF-β is critical to tTreg development: TGF-β is released by APCs that phagocytose apoptotic thymocytes generated by negative selection, creating a localized gradient of TGF-β that promotes FOXP3 expression in TCR-engaged tTreg precursors [7]. Although the role of TGF-β in human tTreg development is yet to be elucidated, human tTreg development is known to rely on IL-2 and/or IL-15 in conjunction with strong TCR signaling [5, 8]. Developmentally, the earliest population of human $FOXP3^+$ tTregs is found in the thymic cortex. $CD4^+CD8^+$ double positive thymocytes are guided to the cortex by CXCL12 and CCL25 released from cortical thymic epithelial cells (cTECs), which engage CXCR4 and CCR9, respectively. Differentiation into tTregs is then co-ordinated by cortex macrophages and cTECs. tTregs can also differentiate from $CD4^+$ single positive thymocytes in the medulla, with

localisation guided by CCL17/CCL22 (engaging CCR4) derived from DCs, and CCL19/CCL21 (engaging CCR7) released from mTECs [5, 8].

APC-driven Treg development in the periphery

Tregs can also develop from conventional $CD4^+$ T cells in the periphery following interactions with APCs and are termed peripherally-derived Tregs (pTregs) [9, 10]. There have not yet been any phenotypic markers identified in humans that can reliably discriminate tTregs and pTregs; both tTregs and pTregs contain cells with specificity for self and foreign antigen. An important population of regulatory T cells generated in the periphery are the IL-10-producing $FOXP3^{neg}$ type 1 regulatory T cells (Tr1 cells). In contrast to $FOXP3^+$ Tregs, Tr1 cells primarily function through secretion of cytokines, particularly IL-10, with additional roles for TGF-β, IL-22, IFN-γ and granzyme B, although cell-contact-dependent mechanisms of suppression via PD-1 and CTLA-4 have also been described [9]. Tr1 cells play a critical role in gut homeostasis where they can suppress inflammation through IL-10 secretion and can promote intestinal epithelial cell barrier function through IL-22 secretion [11].

APCs that preferentially suppress inflammatory immune responses while stimulating tolerogenic responses, including induction of Tregs and Tr1 cells, are termed tolerogenic APCs (tolAPC). tolAPCs include monocytes/macrophages, B cells and dendritic cells. However, as conventional DCs (cDCs) are the predominant APC to engage in crosstalk with Tregs they are the focus of this review. cDCs ($CD11c^+$MHC II^+) can be subdivided into two subsets, cDC1 ($CD141^+$) and the more predominant cDC2 ($CD1c^+$). Current evidence shows that cDC2 cells, particularly those expressing high levels of CD5, appear to be the primary tolerogenic APC subtype in humans [12, 13]. It has been suggested that tolerogenic DCs (tolDCs) have an immature phenotype, expressing low levels of MHC-II and CD80/86 and promoting pTreg induction and activation, while mature DCs (MHC-$II^{high}$$CD80/CD86^{high}$) promote inflammatory, Th1-like responses [14]. However, while many tolDCs express relatively lower levels of MHC-II and CD80/CD86, some are phenotypically fully mature [14]. Data from mice show that changes in the DC transcriptional program that drives tolerogenic versus immunogenic responses are surprisingly similar, with both cell types undergoing maturation and relying on NF-κB pathway signalling [15, 16].

Tolerance induction is particularly important in the skin and the gut where immune cells must maintain barrier function despite continuous exposure to pathogenic, commensal, dietary and/or other foreign antigens [17, 18]. In the gut, pTreg induction is enhanced by retinoic acid, a vitamin A metabolite produced by mucosal DCs [19, 20]. Just as TGF-β is important for generation of tTregs, it is also critical to the induction of pTregs in these tissues. In mice, DCs in the gut express integrin αvβ8, which converts latent TGF-β into its bioactive form [21]. Active TGF-β then binds to its receptor on $CD4^+$ T cells and triggers phosphorylation of SMAD2/3, which interacts with the CNS1 region in the *FOXP3* promoter to induce FOXP3 expression [10]. In addition to driving $FOXP3^+$ pTreg generation through TGF-β, mucosal DCs are a source of IL-10, which induces Tr1 cells [9]. Similarly, both dermis-resident DCs and Langerhans cells (a specialized population of DCs populating the epidermis) can induce $FOXP3^+$ pTregs via release of IL-10 and TGF-β [22, 23], with *in vitro* experiments suggesting this process is enhanced by calcitriol (1,25-dihydroxy vitamin D3), the metabolically active form of vitamin D3 [24]. It is known that cutaneous DCs can convert vitamin D into calcitriol [25], and *in vitro* calcitriol has differential effects on human skin-derived APCs, priming dermis DCs to induce Tr1-cells through an IL-10–dependent mechanism, while primed Langerhans cells preferentially induced $FOXP3^+$ Treg cells through a TGF-β–dependent mechanism [24]. Gaining a

better understanding of the distinguishing features of human tolDCs will be useful for the development of new ways to promote the differentiation/expansion of therapeutic Tregs.

APC-dependent Treg activation and specialisation

Following thymic induction, tTregs (and all T cells exiting the thymus), express CD62L and CCR7 to enable migration to the lymph nodes where they receive signals for proliferation and activation. Within lymph nodes, Treg positioning near DCs is guided by CCL19 and CCL21 produced by fibroblastic reticular cells and DCs [26]. Although some DCs reside permanently in lymph nodes, studies in mice show that migratory DCs, which have acquired antigen in the periphery and trafficked to the lymph node, are the primary activators of Tregs [27].

Treg-APC immune synapse

When a T cell interacts with an APC, they form an immune synapse, which consists of TCRs interacting with peptide-MHC-II complexes, co-stimulatory molecules and adhesion proteins [28]. Immune synapses consist of concentric rings termed supramolecular activation clusters (SMACs). The central SMAC contains the TCR and co-stimulatory molecules and the surrounding peripheral SMAC containing adhesion molecules, creating a "bullseye" formation [28]. The Treg-APC immune synapse is distinct from that of conventional T cells (Tconvs). Most data from mice support the notion that Tregs form longer-lasting, stronger immune synapses with APCs than Tconvs [29-31]. Although less well-studied, human Treg immune synapses have been shown to have higher organisational stability than Tconvs when placed on supported planar bilayers containing intracellular adhesion molecule 1 (ICAM-1) and CD3 [32]. The strength of this interaction is highly dependent on high constitutive expression of a high affinity form of LFA-1, which binds to ICAM-1 on APCs in the peripheral SMAC [33]. Such unique aspects of the Treg-APC immune synapse will be important to apply to *in vitro* Treg expansion approaches, particularly those using artificial antigen-presenting cells. The appropriate synaptic strength and interaction of co-stimulatory/co-inhibitory molecules will be important for effective activation of Tregs.

Treg maintenance

Long-term maintenance of Tregs is also dependent on APC interactions. CD28 engagement by CD80/CD86 is essential for Treg proliferation [34], and accordingly most clinical Treg therapy expansion protocols rely on CD28 agonists [4]. Maintenance of Tregs in the periphery is also influenced by the local microenvironment (reviewed in [17]), with tTregs highly dependent on IL-2 for expansion and FOXP3 stabilisation [35]. Understanding requirements to maintain other specialized Treg subsets will be important to optimize therapeutic approaches and reduce the number and frequency of cell infusions.

Treg differentiation into Th-like subsets

Upon activation by APCs, Tregs can further differentiate into subsets with specialised functions and phenotypes. These include Th1-, Th2- or Th17-like Tregs, driven by the respective Th-polarizing cytokines IFN-γ/IL-12, IL-5 (possibly also IL-4), and IL-6/TGF-β, produced by APCs [36-38]. Th-like Tregs share many phenotypic similarities to their Th1, Th2 or Th17 effector cell counterparts, including expression of the same lineage defining transcription factors (TBX21, GATA3/IRF4, and RORC2 respectively) and chemokine receptors (CXCR3, CCR4/CCR8, and CCR6 respectively), which enable them to home to the corresponding sites of inflammation [38, 39]. These subsets were initially defined in mice, where their individual absences were associated with dysregulated Th1, Th2 or Th17 responses, resulting in conditions such as colitis [40-43]. They have since been identified in a number of tissues in humans, with Th1-like Tregs accumulating in inflamed tissues and Th17-like Tregs being highly prevalent in the gut and skin [44-46]. In addition to Th17-like Tregs, Th1/17-like Tregs, which express both TBX21/CXCR3 and RORC2/CCR6, have been identified [44, 47]. Although not yet directly demonstrated, it is likely this specialisation occurs for both tTregs and pTregs upon encountering antigen in lymph nodes or in the tissues.

Another important Treg subset is T follicular regulatory (Tfr) cells, which modulates T follicular helper (Tfh) cells and B cell germinal centre responses, thus shaping antibody repertoires (reviewed in [48]). Like their Tfh cell counterparts, Tfr cells express the transcription factor BCL6 and chemokine receptor CXCR5, enabling migration towards CXCL13 released from DCs in B cell areas of germinal centres. In mice, both DCs and B cells contribute to the differentiation of Tfr cells. Tfr cell differentiation occurs in two stages; first CXCR5 is upregulated following interactions with DCs to enable trafficking to the T-B cell boundary in the follicle where interactions with B cells induce the full Tfr cell phenotype [49]. Mouse Tfr cells frequently enter the follicle where they exert suppressive function on B cells and Tfh cells [48]; however, a recent study examining human lymph nodes showed that while CXCR5 expression enables migration to the follicle, human Tfr cells reside mainly at the boundary between the T and B cell regions of the follicle, where they modulate the differentiation and function of Tfh cells and B cells and entry of Tfh cells into the germinal centre [50].

Although these Th-like Treg subsets are required to control their corresponding Th cells *in vivo* in mouse models [40-43, 51, 52], *in vitro* experiments with human cells have not yet identified differential mechanisms of suppression [44, 53]. Thus, their ability to preferentially control different types of Th cells is most likely related to their ability to migrate to the same sites of action, highlighting the importance of controlling the migratory properties of therapeutic Tregs.

APC imprinting of Treg homing capacity

Tregs are present in nearly all organs and tissues throughout the body, where they are involved in responses to pathogens and maintaining tolerance (reviewed in [18]). Homing to tissues allows Tregs to interact with their cognate antigen and exert site-specific functions. In particular, the gut and skin are host to a large number of Tregs, which comprise ~5% and ~20% of human $CD4^+$ T cells in each tissue respectively [54, 55].

As discussed above, Tregs play a critical role in regulating mucosal immune responses [18]. Expression of integrin α4β7 and CCR9 are both required for Tregs to home to the gut [53].

Interactions between Tregs and DCs are particularly important in driving gut-homing phenotypes. Throughout the gut, human DCs can primarily be divided into three subtypes: $CD103^{neg}Sirp\alpha^{+}$, $CD103^{+}Sirp\alpha^{neg}$, and $CD103^{+}Sirp\alpha^{+}$, [19, 20]. Each portion of the gut has varying levels of these DC subsets, with $CD103^{+}Sirp\alpha^{+}$ DCs predominating in the jejunum, ileum and colon. Gut-associated DCs can convert vitamin A to retinoic acid, which promotes expression of CCR9 and integrin α4β7 [53, 56]. Integrin α4β7 can bind both mucosal addressin cell adhesion molecule 1 (MAdCAM-1), expressed on HEVs in the gut and gut-associated lymphoid tissue, and vascular cell adhesion protein 1 (VCAM-1), expressed on endothelial cells in blood vessels, lymph nodes and bone marrow [57]. The ligand for CCR9, CCL25, induces a conformational change to favour binding MAdCAM-1 over VCAM-1 [58], thus combined expression of CCR9 and integrin α4β7 enhances Treg migration to the gut. Retinoic acid also induces expression of CD161, and $CD161^{+}$ Tregs have been shown to promote intestinal wound healing *in vitro* [59].

Co-expression of chemokine receptors and integrins is also critical for Treg homing to the skin. As previously mentioned, cutaneous DCs can convert vitamin D3 to its metabolically active form calcitriol, which also induces CCR10 expression on T cells enabling migration to the skin by sensing CCL27 produced by skin keratinocytes [25, 60]. Although these studies examined total $CD4^{+}$ T cells, upregulation of CCR10 on Tregs has been observed *in vitro* in the presence of calcitriol [61]. In addition, Treg expression of cutaneous lymphocyte antigen (CLA) is required to interact with P- and E-selectin ligands on vascular endothelial cells in the skin [62, 63].

Immunosuppressive effects of Treg-APC interactions

In addition to driving Treg activation and specialisation, the nature of Treg-APC interactions also has important functional consequences. A summary of some key surface proteins important in Treg-APC functional interactions is in **Table 1**. Studies in mice have shown that the strong Treg-APC synapse can block antigen-specific Tconv cell access to the APC [29-31] and reshape the DC actin cytoskeleton to create a refractory period before the DC is able to interact with another T cell [30]. LFA-1 is critical to this synaptic strength and actively contributes to APC suppression. LFA-1 interaction with ICAM-1 on APCs inhibits CD80/CD86 upregulation, suppressing their ability to activate Tconv cells [64]. Furthermore, Tr1 cells [65] and Tregs [66] can kill APCs in a contact, LFA-1-dependent manner, via release of cytotoxic granzymes and perforin. Accordingly, Tregs from patients that have a mutated LFA-1 subunit (CD18; leukocyte adhesion deficiency type 1) cannot suppress Tconv cells [64].

Tregs can also deplete peptide-MHC-II complexes from DCs and incorporate them into their own membrane through an antigen-dependent process termed trogocytosis, thereby further reducing the capacity of DCs to stimulate immunogenic responses [29, 67]. Although this is not a phenomenon restricted to Tregs, mouse Tregs have a higher trogocytic capacity than Tconv cells [29]. Similarly, CTLA-4 is another protein essential to the immune synapse and Treg function, as it reduces the immunogenic potential of APCs [33]. CTLA-4 has higher affinity for CD80/CD86 molecules than CD28, which enables Tregs to 'out-compete' Tconv cells for these ligands. CTLA-4 removes CD86 from APCs via trans-endocytosis, a process that results in target cell CD86 depletion in the absence of Treg membrane integration and is therefore, distinct from trogocytosis [68]. Accordingly, patients with CTLA-4 haploinsufficiency have functionally impaired $FOXP3^{+}$ Tregs and

hyperactivated Tconv cells [69]. CTLA-4-mediated suppression of DCs by Tregs *in vitro* also appears to be a superior predictor of *in vivo* Treg function compared to the more commonly used assay of suppression of effector T cell proliferation [70]. This highlights the ability of Tregs to suppress APCs as an important functional criterion for an effective therapeutic product.

Another important concept is that of "infectious tolerance" (reviewed in [71]), where signals from tolAPCs and Tregs create a tolerogenic microenvironment and drive further induction of immunosuppressive cells, including more Tregs. This process is thought to occur via secretion of immunosuppressive cytokines, particularly IL-10 and TGF-β, and ultimately leads to non-specific "bystander suppression" of local inflammatory responses and long-term propagation of the effects of Treg therapy [71]. This concept has been harnessed to negate the requirement for a Treg therapy to have the same antigen specificity as the target of suppression, but also presents a risk of undesired suppression of proximal immune responses.

The role of APCs in the clinical application of Tregs

Adoptive Treg therapy has great potential for the treatment of autoimmune diseases and prevention of allograft rejection [2]. To date, multiple phase I/II clinical trials have provided evidence that Treg therapy is generally well tolerated and safe [72-74]. The source of Tregs and their associated manufacturing protocols will undoubtedly influence the clinical efficacy of Treg therapy. Due to the relative scarcity of Tregs in peripheral blood (5-10% of $CD4^+$ T cells), umbilical cord blood (~5-7.5 x 10^6 cells) or thymus (~500 x 10^6 cells), extensive expansion, without compromising function or stability, is required to meet the high cell dose requirements of most trials [3, 4]. Consideration must also be given to how Tregs will encounter antigen and migrate after infusion. This section will describe how advances in knowledge of Treg-APC interactions can be applied to Treg manufacturing protocols to improve functions of the generated Treg cell product.

Artificial thymuses

In an attempt to avoid the requirements for multiple rounds of cell expansion, methods are being tested for the *in vitro* induction of Tregs. Although methods using APCs and TGF-β and/or IL-10 have not yet been able to generate a Treg product with the same epigenetic stability of *ex vivo*-expanded Tregs [75], more sophisticated approaches are being developed. One potential approach could be using artificial thymuses [76] to bio-manufacture large quantities of Tregs *in vitro*. One could envision that this system could be optimised to incorporate essential components of Treg homing to thymic regions and interactions with specialised APCs to better recapitulate natural Treg development in the thymus.

APC stimulated expansion of therapeutic Treg cells

Most Treg therapies currently being developed require cell expansion, so it is important to consider how to best retain the desired suppressive function. The majority of Treg expansion protocols rely upon use of anti-CD3 and anti-CD28 antibodies covalently linked to magnetic beads

[4], although potentially a single CD28 stimulus could be used [77]. However, multiple groups have reported that APC stimulation expands Tregs more efficiently than antibody-coupled beads. K562 cells are a human erythroleukemic cell line that can be transduced to express CD86 and CD64, a high affinity Fc receptor allowing K562s to be coated with anti-CD3 antibodies. Using this approach, good manufacturing practice (GMP)-compatible K562 cells were used to expand umbilical cord blood Tregs 8 fold more than with anti-CD3/CD28 beads [78]. IL-10 conditioned DCs (termed DC-10) are also being used to induce Tr1 cells for therapy using GMP-compatible protocols. This strategy is being applied to the prevention of Graft-vs-Host disease (GVHD) through expanding hematopoietic stem cell donor-derived $CD4^+$ T cells with patient-derived DC-10 to induce alloantigen-specific Tr1 cells for infusion (ClinicalTrials.gov identifier: NCT03198234).

Synthetic platforms for Treg cell expansion

Although effective, the use of a second cell product as an activation reagent adds considerable complexity to a GMP manufacturing protocol, due to testing and batch validation requirements. Therefore, there is considerable interest in developing methods to present anti-CD3 and anti-CD28 antibodies on synthetic fluid membranes, aiming to capture the superiority of cell-based expansion and the dynamic nature of the Treg-APC immune synapse without the complexity of a cell-based system [76]. A protocol for developing fluid lipid bilayers was recently published, demonstrating "off-the-shelf" convenience over cell lines, superior expansion compared to anti-CD3/CD28 beads, and was also effective for expansion of chimeric antigen receptor (CAR) T cells [79]. Notably for Tregs, the use of fluid, artificial APCs could enable incorporation of additional co-stimulatory molecules, antigens or cytokines depending on the desired cell product.

In vivo expansion of Treg cells

An alternative approach to infusing *ex vivo*-expanded Tregs is to use tolerogenic APCs to expand Tregs *in vivo* by exploiting infectious tolerance, an approach which bypasses the risk of compromising Treg stability during *ex vivo* expansion [80]. Several clinical studies have now assessed tolAPC therapy in treating type 1 diabetes [81, 82], rheumatoid arthritis [83, 84], Crohn's disease [85], multiple sclerosis and neuromyelitis optica spectrum disorders [86], and preventing kidney transplant rejection [74]. These studies used multiple methods to derive autologous, "immature" tolDCs (or regulatory macrophages [74]) from patient monocytes, some involving disease-relevant antigen pulsing prior to injection [82, 84, 86]. Studies to date showed these therapies were well tolerated with small increases in peripheral Treg numbers [81, 84-86].

In vivo antigen-stimulation of Treg cells

Once infused, Tregs must encounter antigen to become activated. Most trials to date have used polyclonal Treg products, containing Tregs with a wide array of specificities. However, a drawback of polyclonal Treg products is that only a fraction of these cells is expected to be specific for disease-relevant antigens. Antigen-specific Treg cell products can be manufactured by isolating

and expanding the small number of cells with the desired specificity (e.g. by stimulating with donor-derived APCs [87]) or by engineering specificity though expression of a transgenic TCR or chimeric antigen receptor (reviewed [88]). Though more complicated to manufacture, antigen-specific Treg cell products have been shown to be more potent than polyclonal Tregs in preclinical models [87] and carry less risk of global immunosuppression. Antigen-specificity has also been shown to promote localisation and retention at the site of a graft in a humanised mouse model [89].

In vivo migration of Treg cells

Appropriate migration to both lymph nodes and the graft/site of inflammation will also be crucial for the efficacy of Treg therapies as shown in preclinical models [90, 91]. To date, expanded clinical-grade Treg products generally maintain high expression of CD62L, CCR7, and CCR4, with varying levels of expression of CXCR3, CCR6 and tissue-homing receptors such as integrin α4β7, CCR9, CCR10 and CLA [92-95]. These data suggest that the infused cells are likely well suited to migrate to lymph nodes but may be less able to migrate to specific tissues or sites of inflammation. One approach to ensuring appropriate migration is to alter manufacturing culture conditions to recapitulate APC-induced expression of homing receptors. This requires knowledge of the key receptors guiding Tregs to tissues and disease-specific sites and how their expression is modulated by APCs *in vivo* (**Table 2)**. For example, it may be desirable to expand tTregs in the presence of IL-12 and IFN-γ to boost CXCR3 expression and create Th1-like Tregs with enhanced capability of migrating to sites of Th1-inflammation [53]. Likewise, to produce gut-homing Tregs to treat inflammatory bowel disease, retinoic acid could be added to promote integrin $\alpha4\beta7$ and CCR9 [53, 56], or calcitriol could be used to promote $CCR10^{+}$ skin-homing Tregs to treat inflammatory skin disorders. Alternatively, fucosylating Tregs to form a sialyl-Lewis X moiety on the P-selectin ligand has been shown to increase their binding to E- and P-selectin, enhancing their persistence and efficacy in a xenogeneic GVHD model [96]. Fucosylated Tregs are now being tested in a Phase I/II clinical trial to prevent GVHD in humans (ClinicalTrials.gov identifier: NCT02423915). Together, modulating Treg specificity and homing receptor expression could recapitulate the *in vivo* requirement for tissue-resident APCs to present antigen and produce chemokines to attract antigen-specific Tregs into the tissues.

Monitoring infused Treg cell products

In addition to modifying Tregs to promote migration to locations of interest, there is a need to track cells *in vivo* to determine cell localisation post-infusion. In clinical trials to date, monitoring of Tregs post-infusion has largely been limited to analysis of peripheral blood by flow cytometry or mass spectrometry [97, 98]. This provides a rough estimate of Treg persistence in the blood, but no information on migration or persistence in tissues. Various labeling and imaging methods are under development to monitor the distribution of cells in tissues throughout the body using non-invasive methods such as MRI or PET (reviewed by [99]). Cells may be labeled directly with contrast agents (e.g. iron oxide, gadolinium, [^{19}F]-fluorine) or indirectly by genetically engineering cells to express receptors that allow them to uptake reporters at the time of imaging [100]. It will be important to understand how these post-infusion migration patterns associate with clinical outcomes.

Conclusion

We have highlighted here some of the critical requirements for Treg induction, migration, activation and function and the role for APCs in orchestrating these processes (**Figure 1**). These aspects of Treg biology are important to consider when designing Treg therapeutic approaches to ensure a cell product that will reach the tissue of interest, express appropriate markers for interaction with, and suppression of, APCs and are equipped to exert the most appropriate immune modulatory functions in the relevant disease-context.

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Figure Legend

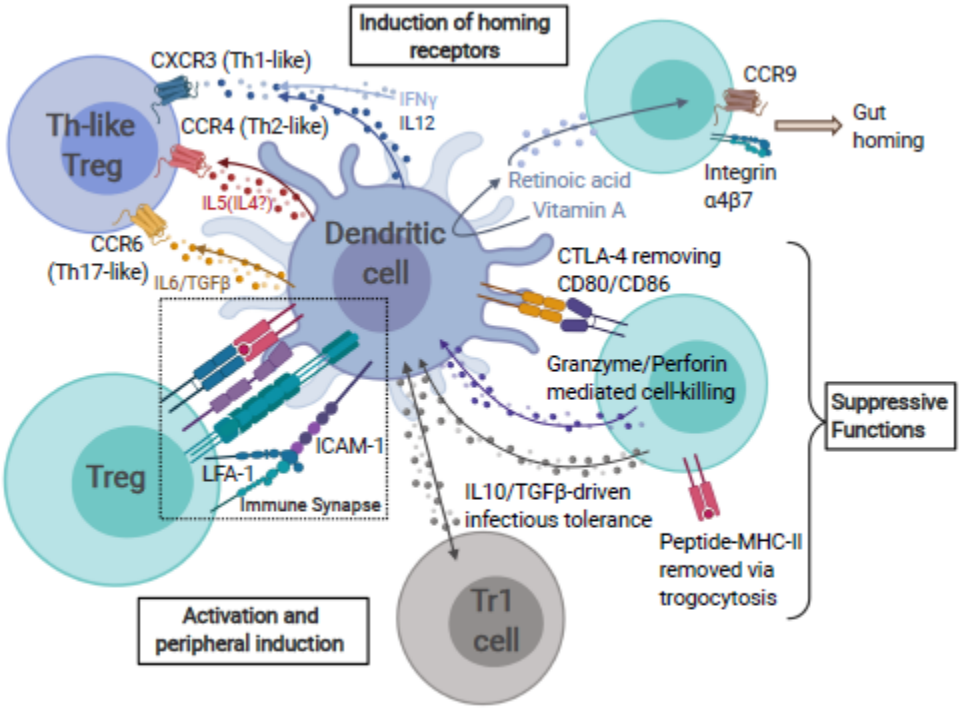


Figure 1. Overview of key interactions between APCs and Tregs.

APCs, such as dendritic cells, are required for Treg development in the thymus and periphery. This figure depicts some of the key mechanisms of how APC-mediated activation (via immune synapse and cytokine secretion) of Tregs in the periphery shapes their subspecialisation (into Th-like Tregs or Tr1 cells) and homing proprieties (gut homing is shown as an example). Tregs also exert suppressive effects on APCs, using a variety of mechanisms, such as the IL-10/granzyme/perforin secretion, transendocytosis of CD80/CD86 and trogocytosis of peptide-MHC complexes depicted here. These actions contribute to immune tolerance by promoting the development of tolerogenic APCs and reducing the capacity of APCs to stimulate effector T cells. Created with BioRender.com

Table 1: Key Treg cell-surface proteins that influence outcomes of APC interactions.

Cell surface proteins which have been studied in the context of interactions between human Tregs and APCs are summarized.

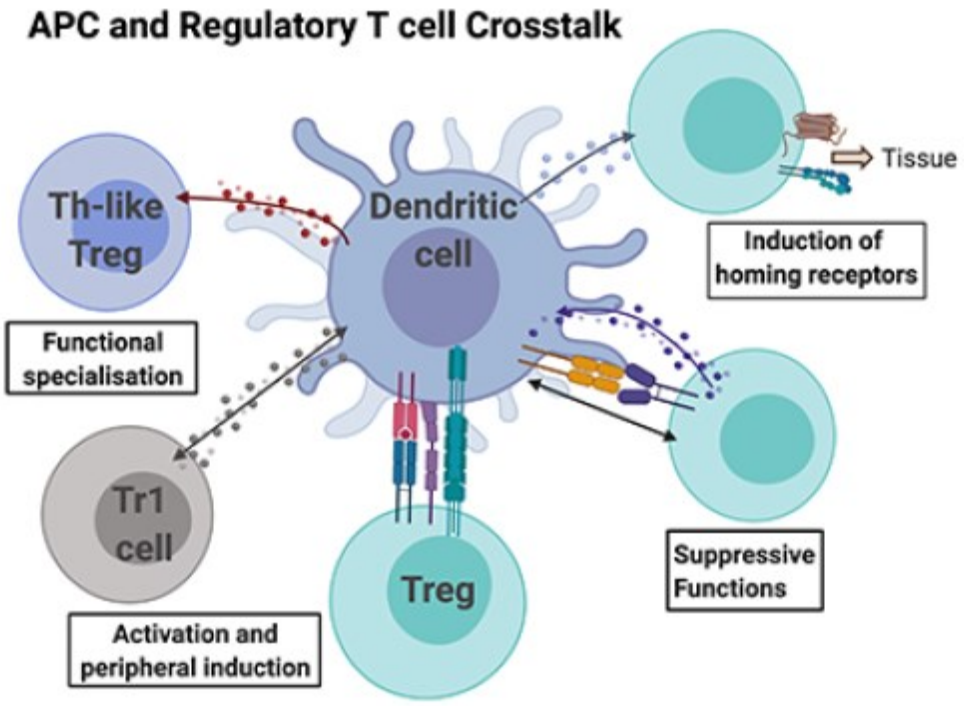
Surface protein	Ligand	Impact of ligation	References
LFA-1	ICAM-1; expressed by APCs	Promotes Treg-APC immune synapse strength Enables APC-targeted cytotoxicity Blocks APC maturation	[64-66]
Immunoglobulin superfamily proteins			
CD28	CD80 and CD86: expressed by APCs	Expansion of stable Tregs	[34]
CTLA-4	CD80 and CD86; expressed by APCs	Critical to Treg-APC immune synapse Physically blocks APC ability to provide Tconv co-stimulation Trans-endocytoses CD80 and CD86 from APCs	[33, 68]
TIGIT (VSTM3)	CD155 (Poliovirus receptor); expressed on DCs	Enhances Treg suppression Increases Treg stability	[101, 102]
LAG-3 (CD223)	HLA class II; expressed by APCs	Associated with enhanced suppression Influences DC maturation	[103, 104]
ICOS (CD278)	ICOS-L (CD275); expressed by APCs, particularly mature pDCs	Enhances Treg suppression Enhances Treg proliferation	[105, 106]
PD-1 (CD279)	PD-L1 (CD274); expressed on APCs, particularly following activation PD-L2 (CD273); expressed to a lesser extent on activated APCs	Reduces Treg suppression Reduces Treg proliferation	[107, 108]
TNF receptor superfamily proteins			
OX40 (CD134)	OX40L (CD252); expressed by APCs, particularly following activation	Abrogates FOXP3$^+$ Treg and Tr1-mediated suppression Inhibits induction of FOXP3$^+$ Treg and Tr1 cells	[109, 110]
GITR (CD357)	GITRL (TNFSF18); expressed at low	Reduces Treg suppressive capacity	[111, 112]

	levels by APCs, upregulated on activated pDCs		
4-1BB (CD137)	4-1BBL (CD137L); expressed by APCs, expression enhanced by activation	Promotes Treg expansion	[113]

Table 2: Homing receptor expression required for Treg migration to tissue or disease sites

Destination	**Receptor on Tregs**	**Ligands**	**Notes**	**References**
Lymph nodes	CCR7 CD62L	CCL19, CCL21 GlyCAM-1, CD34, MAdCAM-1, PSGL-1	Migration to and rolling at lymph nodes	[26]
Th1 inflammation	CXCR3	CXCL9/10/11	Expressed by Th1-like Tregs	[39, 44-46, 53]
Th2 inflammation	CCR4 CCR8	CCL17, CCL22 CCL1	Expressed by Th2-like Tregs	[39, 44]
Th17 inflammation	CCR6	CCL20	Expressed by Th17-like Tregs	[39, 44, 47, 114]
B cell follicles and germinal centres	CXCR5	CXCL13	Expressed by Tfr Migration to T-B cell boundary of follicle	[48, 50]
Intestine	$\alpha4\beta7$ CCR9	MAdCAM-1, VCAM-1 CCL25	Migration to intestine and associated lymphoid tissues	[53]
Skin	CCR4 CCR10 E- and P-selectin ligands	CCL17, CCL22 CCL27	Expressed by skin-homing Tregs	[44, 62, 63, 115]
Transplantation	CXCR3	CXCL9/10/11	Associated with acute and chronic GVHD	[116, 117]

	CCR2 CCR4 CCR5 CXCR3 E- and P-selectin ligands	CCL2, CCL17; CCL22 CCL3/4/5 CXCL9/10/11	Associated with graft rejection and migration to allograft or draining lymph node in mice	[90, 91, 118]



Human regulatory T cell (Treg) induction, activation, suppression and homing capacities are orchestrated by antigen presenting cells (APCs). APCs and Tregs modulate each other's functions via release of soluble mediators, co-stimulatory molecule ligation and immune synapse formation. Understanding the Treg-APC relationship is critical to guiding clinical Treg therapy improvement.