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Guidance Factors Orchestrating Regulatory T Cell Positioning in Tissues during Development, Homeostasis, and Response

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Summary

Over their lifetime, regulatory T cells (Treg) recalibrate their expression of trafficking receptors multiple times as they progress through development, respond to immune challenges, or adapt to the requirements of functioning in various non-lymphoid tissue (NLT) environments. These trafficking receptors, which include chemokine receptors (CKRs) and other G-protein coupled receptors (GPCRs), integrins, as well as selectins and their ligands, enable Treg not only to enter appropriate tissues from the bloodstream via post-capillary venules, but also to navigate these tissues to locally execute their immune-regulatory functions, and finally to seek out the right antigen-presenting cells and interact with these, in part in order to receive the signals that sustain their survival, proliferation, and functional activity, in part in order to execute their immuno-regulatory function by altering APC function. Here, we will review our current knowledge of when and in what ways Treg alter their trafficking properties. We will focus on the chemokine system and try to identify specialized, non-redundant roles of individual receptors as well as similarities and differences to the conventional T cell compartment.

Keywords

T regulatory cells (Treg); migration; trafficking; chemokines; tissue environment; tolerance

Introduction

$CD4^+$ $Foxp3^+$ Treg are α T cells specialized in the maintenance of immune homeostasis and the regulation of immune responses. Their majority (~80%) develop in the thymus to produce preferentially self-reactive thymic Treg (tTreg), but some also emerge through extra-thymic conversion of conventional $CD4^+$ cells to generate peripheral Treg (pTreg) that are reactive with commensal, food, and other environmental antigens. In each case, the transcription factor Foxp3 orchestrates gene expression programs that restrain their proinflammatory effector functions and instead endow them with a multitude of immune-regulatory functions. As for all cells of the immune system, Treg have to position themselves appropriately at the organ, at the tissue, and at the microenvironmental level in order to

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execute their regulatory functions appropriately and effectively. Over the past 15 years, numerous studies have provided a wealth of information on the trafficking programs that Treg adopt at different stages of their development, activation, and differentiation. In this review, we first provide a brief overview over the general architecture of the Treg compartment and a short primer on the rules of immune cell positioning. We then review trafficking programs that enable Treg to execute their functions in secondary lymphoid as well as non-lymphoid tissues, whereby we will primarily draw from studies in mice, unless otherwise specified. We will emphasize the roles of several trafficking receptors that are thought to be of particular importance to the function of Treg as opposed to conventional T cells. Finally, we will discuss the mechanisms that Treg use to navigate to and within tumor tissue in order to restrain beneficial immune responses that can otherwise facilitate control of malignant growths, and conclude with a brief outlook on therapeutic opportunities that arise from knowledge of the guidance mechanisms used by Treg.

Guidance mechanisms for immune cell positioning

In order to interpret studies on the guidance cues that help Treg position themselves appropriately, it is helpful to have a general appreciation of the roles of the various molecules involved (Figure 1). The best understood trafficking event is the recruitment of blood-borne immune cells into tissues, which generally occurs in post-capillary venules that express or present ligands for immune cell-expressed trafficking receptors, either constitutively, or inducibly in the context of inflammation (reviewed in Ref. (1) and summarized in Figure 1A). Initial short-lived tethering interactions of leukocytes that are reflected by a rolling motion along the vessel wall are primarily mediated by L-Selectin (CD62L) expressed on leukocytes or E- and P-selectin (CD62E and CD62P) on endothelial cells. Selectins recognize specific carbohydrate modifications of a variety of glycoprotein stalks. Therefore, functional Selectin ligand expression is to a large extent regulated by the activity of the enzymes that produce these modifications during intracellular maturation of different stalk proteins. Rolling interactions expose leukocytes to chemoattractants presented on the endothelial cells (EC) glycocalyx. These can either be expressed by the ECs themselves, or by cells of the underlying tissue and *trans*-presented on the endothelium. Binding of chemoattractants, including chemokines, to GPCRs on leukocytes induces their inside-out-signaling to integrin heterodimers, mostly of the β2 and α4 families, to trigger conformational changes that enable integrin binding to adhesion molecules of the Ig family, such as ICAM-1 or VCAM-1, and thereby stabilize leukocyte-endothelial cell interactions. Following firm adhesion, leukocytes crawl on the luminal endothelium until they find a permissive site for either para- or transendothelial migration, and eventual exit from the subendothelial space past pericytes to complete diapedesis of the vessel wall. These late steps of the process are not as well mechanistically understood, but are also thought to involve the activity of chemoattractants (2). Leukocyte- as well as tissue-specific expression patterns of the numerous members of the three families of trafficking receptors (e.g. >50 chemokines and >20 chemokine receptors (CKRs) have been described) are the basis for combinatorial diversity that allows for the targeted recruitment of selected leukocyte subsets to selected tissues. While the process of leukocyte extravasation has been characterized for

other leukocyte subsets, intravital microscopy studies have indicated that Treg recruitment to tissues is facilitated by the same multistep cascade of molecular interactions (3, 4).

Beyond the vessel wall, chemokines and other ligands for GPCRs regulate the positioning of immune cells in discrete tissue environments, such as T cell and B cell areas in lymph nodes (LNs), as well as their tissue exit, typically via the lymphatics (Figure 1B). Moreover, they also guide lymphocyte migration in these tissue environments more discretely to optimize their interactions with appropriate antigen presenting cells (APCs) that may regulate their proliferation and survival (Figure 1C). When interpreting studies on the role of Treg-expressed trafficking receptors, it is therefore important to recognize that the accumulation of Treg in a given tissue may not only reflect the role of a particular receptor in their recruitment from the bloodstream, but also its role in their subsequent positioning in specialized tissue environment and orchestration of APC contacts that ultimately regulate their local population size.

Central and effector Treg

$CD4^+$ $CD25^+$ Treg were first described in LNs and spleen (5). This circumstance, the observation that the majority of Treg express the LN homing receptor L-Selectin/CD62L, and that the $CD62L^+$ subset is most effective at delaying diabetes in NOD mice (6, 7), seemed to imply that their primary role is to regulate the induction of immune responses in secondary lymphoid organs (SLOs). However, responsiveness of subsets of Treg to chemokines associated with non-lymphoid, and especially with inflamed tissues suggested their ability to enter these sites as well (8, 9). Accordingly, Treg were also found to accumulate in prediabetic islets in NOD mice, where they control diabetogenic effector T cells (Teff) (6), or at sites of chronic Leishmania infection, where they prevented $CD4^+$ Teff from eliminating the pathogen (10). Seminal studies established that CKRs such as CCR4 can function non-redundantly to enable Treg trafficking to NLTs, such as skin and lung, and that this is critical to maintain immune homeostasis specifically at these sites (11). A comprehensive examination of trafficking receptors eventually revealed a fundamental dichotomy between tTreg: On the one hand a $CD62L^+$ $CD44^{low}$ subset programmed to recirculate through SLOs by virtue of expressing CD62L as well as CCR7, the receptor for the SLO chemokines CCL19 and CCL21, and, on the other hand, a $CD62L^-$ $CD44^{hi}$ subset capable of entering NLTs by virtue of expressing CKRs such as CXCR3, CCR4, CCR6, CCR2, and CCR5, P- and E-selectin ligands, as well as elevated levels of the LFA-1, αEβ7, and β1-integrins (12) (Figure 2). The former and latter subsets are frequently referred to as resting and activated Treg, respectively. However, elevated expression of the early activation gene Nur77 by resting Treg compared to naive T conv indicates that they continually receive productive TCR signals (13), and are thus not truly resting. The term central Treg (cTreg), which reflects the tropism for SLOs they share with antigen-experienced central memory T cells, may therefore be more fitting. Conversely, the $CD62L^-$ $CD44^{hi}$ activated subset is interchangeably referred to as effector Treg (eTreg). We will use the terms cTreg and eTreg in this review.

Adoptive transfer studies have shown that cTreg convert into more highly proliferative eTreg in response to tissue self-antigens under conditions of immune homeostasis (14), while

reversion from eTreg to cTreg is not observed (13). Given that loss of CCR7 and CD62L expression by eTreg likely disables their return to non-inflamed LNs via high endothelial venules upon egress via efferent lymphatics, their frequency in SLOs may either be explained by a constitutively high rate of cTreg to eTreg conversion, influx from NLTs via efferent lymphatic vessels (15, 16), or by their prolonged local retention following conversion. While the LN residence time of naive and activated conventional T cells has been described and is predominantly regulated by their surface expression of S1PR1, a receptor for the sphingolipid S1P, expression of CCR7, and by their MHC-dependent interactions with other LN cells (17, 18), no such information is so far available for Treg. It is possible that expression of inflammatory CKRs not only enables those eTreg that egress from SLOs to enter non-lymphoid and inflamed tissues, but also enables those that may stay behind to reposition themselves to specific LN environments, such as the interfollicular niche, akin to what has been observed for memory and recently activated effector T cells (19, 20), in order to perform their functions locally. Follicular Treg that express the transcription factor Bcl6 as well as CXCR5, the receptor for CXCL13 expressed by follicular dendritic cells in the B cell area, and regulate germinal center reactions, may be just one specific example for the latter (21, 22).

It should be noted that while differentiation of cTreg into eTreg is now firmly established, it is likely that in many studies, including some discussed in this review, Tconv-derived pTreg contribute to varying extent to the eTreg populations examined. As already indicated above, pTreg are reactive to harmless non-self antigens, such as commensal and food antigens, and are most prevalent at sites where these antigens are encountered, such as the small and large intestinal mucosae or the placenta. They may be imprinted during priming of their naive precursors in SLOs to express appropriate trafficking receptors, akin to the imprinting of tissue-tropism during Tconv activation (23). Indeed, pTreg expression of the β7-chain of the gut homing receptor α4β7 that allow T cells to traffic to the lamina propria is required for induction of oral tolerance to food antigens (24). The same has been observed for the gut homing receptor CCR9 (25), although its role on pTreg varies between mouse colonies (26). Similarly, pTreg use the GPCR GPR15 to accumulate in the colon, and deficiency of this receptor, at least in mice, causes exaggerated inflammatory responses at this site (27). Generally, however, whether pTreg follow similar rules of migratory adaptation as Tconv, or whether they adopt differentiation programs more similar to the ones described for eTreg in more detail below, remains unclear at this point.

Thymic origins

T cell development and the generation of a mature αβ TCR repertoire depends on the CCR7 and CCR9-dependent recruitment of blood-borne thymic seeding progenitors (TSPs) into a limiting thymic niche space, followed by maturation and selection based on TCR specificity during a sequence of ordered migration events through discrete cortical and medullary tissue environments and eventual thymic egress of mature T cells back into the bloodstream. This overall process is orchestrated by dynamic changes in the expression of CKRs CCR9, CXCR4, CCR4, and CCR7, the oxysterol receptor Ebi2, as well as the S1P receptor S1PR1 (recently reviewed in (28)). tTreg emerge at a late stage of this process in the thymic medulla through agonist-selection of CD4 SP thymocytes in a cell fate decision that occurs

alternative to deletion in response to intermediate to high avidity cognate encounters with self antigen-presenting APC.

Following positive selection of DP thymocytes in the cortex they rapidly upregulate expression of CCR7 and CCR4 while transitioning to the CD4 SP stage (29, 30). They thus become responsive to CCL19 and CCL21 produced by medullary thymic epithelial cells (mTEC) and CCL17 and CCL22 most highly expressed by medullary dendritic cells (DCs), which guide their migration towards and entry into the medulla (31, 32). Here, interactions with self antigen-presenting mTECs as well as DCs that can present either mTEC-derived or peripherally expressed tissue antigens can divert self-reactive thymocytes into the Treg lineage in a canonical 2-step process of TCR-induced expression of the high-affinity IL-2-receptor α-chain CD25, followed by IL-2-dependent induction of Foxp3-expression. Importantly, only very rare mTEC express a given tissue-restricted self antigen in a stochastic manner (33–35). Although mTEC-derived antigen can also be passed on to and presented by nearby DCs, the opportunity for any individual thymocyte to encounter it would therefore seem low, but it is thought that their high motility in the medulla enables CD4 SP thymocytes to serially scan large numbers of medullary APC in order to maximize their opportunity to engage with their rare cognate self antigen and either be deleted or differentiate into a Treg. Imaging studies in thymic slice cultures have shown that in addition to guiding medullary entry, CCR7 may guide thymocyte interactions with CCL19/CCL21-expressing mTECs, while CCR4 appears to optimize interactions with medullary CCL17/CCL22-expressing DCs (29) (Figure 3A). CCR4-deficient mice release larger numbers of autoreactive conventional and regulatory T cells into the periphery, suggesting that CCR4-mediated optimization of DC interactions in the thymic medulla regulates the balance of Tconv selection versus Treg selection versus clonal deletion (29) (Figure 3B). In addition to chemokines, Ebi2, a GPCR for the chemotactic cholesterol metabolite oxysterol further optimizes CD4 SP thymocyte motility, but its contribution to their medullary accumulation appears rather minor (36). Similarly, although CCR8 is expressed on many mature CD4 SP thymocytes, including on $CD25^+$ Treg precursors, and the CCR8 ligands CCL1 and CCL8 are expressed on mTECs and medullary DCs, CCR8-deficient thymocyte show only a minor defect in medullary accumulation. While CCR8-deficient mice show signs of mild autoimmunity late in life, this may also result from deficits in the function of mature Treg or other CCR8-expressing cells (37).

The study of Treg development was recently complicated by the discovery that mature Treg phenotypically resembling eTreg can return to the thymus, where they constitute a significant fraction of the intrathymic Treg pool and establish a negative feedback loop to regulate *de novo* Treg development (38, 39). Their accumulation in the thymus was reduced upon treatment with the CXCR4 inhibitor AMD3000, suggesting that CXCR4 contributes to the thymic recruitment of Treg from the bloodstream. Recirculating Treg also appear to include cells with $CD25^+$ $Foxp3^-$ and $CD25^-$ $Foxp3^+$ Treg precursor phenotypes, a finding that would necessitate a re-interpretation of some prior observations on Treg development (40). The authors of this study in this regard suggest that expression of CCR6 reliably identifies thymic home-comers and can be used to distinguish them from newly generated precursors. Surprisingly, the same study demonstrated greater thymic Treg cellularity in CCR7-deficient animals, which contrasts with the observed role for CCR7 in medullary

entry required for Treg development. A subsequent study however determined that CCR7-deficiency in hematopoietic cells other than Treg, including in thymic DCs, accounted for this effect by enhancing Treg development via an increased density of SIRPα^{+} DC at the expense of SIRPα^{-} DC (41).

Collectively, these studies point towards partial, but not complete redundancy between CCR7, CCR4, CCR8, and Ebi2 in positioning CD4 SP thymocytes for medullary APC interactions. Further in depth studies of TCR repertoires in Treg that develop in their absence and more subtle defects in their function, especially in models of Treg-specific genetic perturbation, may shed additional light on their individual roles in optimal thymocyte positioning for Treg development.

Central Treg

In adult mice, the overwhelming majority of newly generated tTreg egress from the thymus as CD62L^{hi} CD44^{low} cTreg, as judged from the higher content of T cell receptor excision (12), expression of GFP in RAG2^{GFP} reporter mice (13), as well as tracking studies of thymocytes tagged through intrathymic FITC injection (42). cTreg resemble naive Tconv in their expression of CCR7 and CXCR4, but in addition to CD25 and Foxp3, are distinguished by elevated expression of proteins associated with regulatory function, such as CTLA-4 and CD73. As mentioned above, expression of the early activation marker Nur77 suggests that cTreg continually engage with their cognate antigen in SLOs (13). Nevertheless, they maintain their cTreg differentiation state, proliferate only slowly, if at all, and continually recirculate through SLOs. Similar to naive and central memory Tconv, cTreg depend on CCR7 for migration to SLOs (43, 44). CCR7 expression in cTreg is maintained by the transcription factor Foxo1, which resides in the nucleus of resting T cells and is exported to the cytosol and degraded following phosphorylation by Akt during TCR- and CD28-mediated T cell activation that leads to eTreg differentiation. In addition to CCR7, Foxo1 also controls expression of CD62L and the tissue egress receptor S1PR1, either directly or indirectly, via its target gene Klf2 (45). CCR7-dependent recruitment of Treg to LNs may not only facilitate their self antigen-driven expansion and conversion into eTreg at these sites in order to augment their suppressive activity in NLTs, but also enables them to limit the induction of effector T cell responses already in LNs (43, 46–49).

Beyond positioning Treg at the organ level by recruitment from the bloodstream, CCR7 also positions them specifically in the T cell area of SLOs, where the CCR7 ligands CCL19 and CCL21 are abundantly produced by both fibroblastic reticular cells as well as DCs (13). CCR7-mediated access to the T cell area is important in cTreg, but not eTreg homeostasis, because it provides them with access to their primary survival cytokine IL-2, produced by conventional T cells in this LN environment. While IL-2 is generally thought to be produced by activated Tconv during the induction of immune responses, driving the concurrent expansion of CD25^{+} Treg (50), some autoreactive CD4^{+} Tconv are also activated on a regular basis during immune homeostasis and up to a point where they secrete IL-2. A fine-grained imaging analysis of resting LNs has shown that Treg expressing phosphorylated STAT5 proteins cluster around IL-2-secreting Tconv in a TCR-dependent fashion, suggesting that both Treg and autoreactive Tconv recognize their respective cognate antigen

on the same or on closely juxtaposed DCs, and that Treg continually control incipient autoimmune responses in the context of these clusters (51). $STAT5^{+}$ Treg expressed elevated amounts of suppressive factors such as CTLA4 and CD73 compared to cTreg, and may have been recruited into clusters as bona fide eTreg, or as cTreg that then rapidly upregulated these proteins. In the latter case they may subsequently return to a cTreg state, or they may continue to differentiate into $CD44^{hi}$ eTreg, which could explain the large fraction of $CD62L^{-}$ eTreg in LNs. It will be interesting to examine if and which chemokines are involved in the formation of homeostatic Treg clusters that appear to be the sites where we keep autoimmunity in check on a continual basis.

Th1, Th2, and Th17 effector Treg

It remains unresolved whether cTreg directly contribute to immune homeostasis in SLOs, but they can rapidly differentiate into eTreg in response to TCR signals and IL-2. While cTreg likely encounter on a regular basis the tissue-restricted self antigens they were selected on, it is only when they are simultaneously exposed to IL-2 produced by activated Tconv that they are driven to expand and differentiate into eTreg early during immune responses (50), in a process that is optimized by CD28 co-stimulation (52, 53). eTreg then switch from a dependence on IL-2 to a requirement for ICOS signals, allowing them to downregulate expression of CCR7, depart form the IL-2-rich T cell zone of SLOs, and conduct their regulatory functions at other sites, such a germinal centers, or outside of SLOs in NLTs, where frequent interactions with ICOSL-expressing APC may sustain their survival and function through PI3K-driven metabolic adaptation.

Helper T cell responses can be functionally adapted to the specific requirements of responding to different classes of pathogens. These adaptations are orchestrated by lineage-specifying transcription factors and characterized by the expression of specific cytokines and CKRs: Th1 cells express IFN-γ and CXCR3 under the control of the transcription factor T-bet, and help to eradicate intracellular pathogens; GATA3-dependent Th2 cells produce IL-4, IL-5 and IL-13 and express CCR4 and CCR8, coordinating the immune response to large extracellular pathogens including helminths; Th17 cells depend on RORγt, express CCR6 and protect from extracellular bacteria and fungi through IL-17 and IL-22. The differentiation of eTreg that regulate these distinct responses appears to be coupled to and in several ways resembles the respective Th response type, which optimizes their ability to traffic to as well as to co-inhabit and function in the same tissue environments.

Koch et al. were the first to observe that exposure of Treg to IFN-γ induces their expression of T-bet and its target gene CXCR3, and renders them more adept at controlling Th1 auto-inflammatory disease and at accumulating at sites of mycobacterial replication (54). Accordingly, Treg in prediabetic pancreatic islets in mice, as well as in chronically inflamed livers and kidneys in human are enriched for expression of CXCR3 (55–57). Beyond these correlative observations, Treg-specific deletion of CXCR3 has also directly demonstrated its role in Treg accumulation and function at inflamed sites such as inflamed kidneys in a mouse model of glomerulonephritis (57). Given that CXCR3 optimizes efficient interactions of Th1 cells with DCs in LNs (20), further studies will be needed to dissect the relative importance of this receptor in recruitment versus local tissue positioning of Th1-biased

eTreg at sites of Th1 inflammation. Similarly to Th1 cells (58), Treg cells do not require T-bet for expression of CCR5 (59), another receptor commonly associated with Th1 responses, which may explain in part the comparably mild phenotypes resulting from lack of CXCR3 in Treg.

STAT3, which drives Th17 differentiation in response to IL-6 or IL-23, is required for the formation of a Th17-biased Treg population that regulates intestinal inflammation (60). However, STAT3 activity in these cells was driven by IL-10, rather than by IL-6, and it remained at first unclear if they derived from differentiation of thymic cTreg or from conversion of $CD4^+$ Tconv into pTreg (60, 61). Subsequent studies showed that IL-6 can also promote the formation of Th17-biased eTreg of thymic origin that express RORγt and CCR6, akin to the effects of effector-cell derived IFN-γ on T-bet and CXCR3 expression in Th1-biased eTreg, as described above. However, while lack of RORγt abolished the formation of $CCR6^+$ Th17 cells in EAE, it only partially reduced the expression of CCR6 on eTreg, and the course of EAE was not altered through Treg-specific deletion of RORγt (62). Both the role of Th17-biased tTreg and specifically the role of CCR6 in their function therefore remain to be further clarified.

Th2 polarization is characterized by expression of CCR4 and CCR8 and depends on the transcription factor GATA-3. In addition, T cell and NK development depend on this transcription factor. In Treg, rather than merely specifying a Th2-bias, GATA-3 plays a much more fundamental by controlling expression levels of Foxp3 through binding to the CNS2 region of the Foxp3 gene in cooperation with Foxp3 itself. In its absence in Treg, mice develop late-onset but a fatal autoimmune inflammatory syndrome that is not limited to Th2 pathology (63, 64).

IRF4 is another transcription factor with, not unlike GATA-3, broad functions in the immune system, including as an important organizer of Th2 differentiation, and has also been proposed to play a more specific role in producing a Th2 bias in eTreg, since in its absence in Treg, mice develop an Th2-dominated autoimmune inflammatory disease (65). Fittingly, CCR8 was among several Th2-associated genes down-regulated in IRF4-deficient Treg. However, subsequent studies identified more fundamental roles of IRF4 in controlling eTreg differentiation (66), and it remains unclear to what extent expression of CCR4 and CCR8 result from active polarization of eTreg during Th2 responses.

Already early work showed that large proportions of Treg in the peripheral blood of healthy humans respond to CCR4 and CCR8 ligands (8). Interestingly, ectopic expression of Foxp3 in activated $CD4^+$ Tconv cells suffices to induce their expression primarily of CCR8 and CCR4, but not of CXCR3 or CCR6 (67), and expansion of human thymic Treg under non-polarizing conditions produces a predominantly $CCR4^+$ population (68), suggesting that this aspect of Th2 polarization may to some extent represent a default pathway of eTreg differentiation. In support of this notion, CCR4 and CCR8 are also among the genes most differentially expressed by Treg compared to Tconv in tumor tissue (69–71), arguably not a prototypic Th2-, but more variably a Th1, Th2, or Th17-polarizing environment. Moreover, CCR8 expression enables Treg to interact with donor APCs and control GvHD in

experimental bone marrow allotransplantation, a characteristically Th1-dominated pathogenic process (72).

More recent examination of peripheral blood Treg in healthy human indicates that unlike $CD45RA^+$ cTreg, the vast majority of $CD45RO^+$ eTreg (referred to as naive and memory Treg, respectively, in these studies) express CCR4 (73, 74). The latter contained cells with $CXCR3^+$ Th1-like, $CCR6^+$ Th17-like, $CXCR3^+/CCR6^+$ Th1/Th17-hybrid, and $CCR8^+$ Th2-like differentiation states. Furthermore, a $CCR10^+$ subset of Th22-like eTreg expressing the skin homing receptor CLA was prevalent among $CCR6^+$ cells (73). Thus, discrete eTreg polarization states can be defined based on patterns of CKR expression, and at least in the case of Th1- and Th17-biased eTreg, cytokine-regulated signal transducers STAT1 and STAT3 as well as the canonical Th1 and Th17 transcription factors T-bet and RORγt appear to play important roles in their differentiation, while Th2-bias may arise in eTreg without requirement for extrinsic polarizing factors (Figure 4). Treg-specific deletion of subset-specifying CKRs will help to further define not only the role of polarized Treg responses in controlling the corresponding Th responses, but also to reveal their precise roles in appropriate Treg positioning.

Treg in healthy tissues

Migration of eTreg to NLTs is facilitated by productive immune responses against pathogens. However, Treg are also found in NLTs at steady state, where they adapt to the specific environmental condition of the tissue they reside in and not only maintain immune tolerance, but also perform more general functions in tissue homeostasis. In newborn humans, microbial colonization of the lung promotes recruitment of pTreg (as indicated by their lack of expression of the transcription factor Helios), which reduce sensitivity to lung allergen in later life (75). Similarly, colonization of hair follicles in neonatal mice with commensals triggers the recruitment to skin of a wave of commensal antigen-specific Treg that subsequently strengthen commensal tolerance. Surprisingly, given the generally assumed preferential self-reactivity of tTreg, these Treg appear to originate directly from the thymus, since pharmacological blockade of lymphocytes egress from lymphoid tissue, which prevented commensal tolerance, causes Treg to accumulation in the thymus but not in skin-draining LNs (76). Neonatal skin seeding with Treg was optimized by their expression of CCR6 and its ligand CCL20 produced by keratinocytes in response to microbial colonization of hair follicles that form at this time (77). Additional evidence for a “temporally layered” Treg compartment came from an elegant fate-mapping approach that revealed the neonatal development of a unique population of tTreg with a TCR repertoire that diverges from that of Treg formed later in life, but that remains important for peripheral immune tolerance throughout adulthood. While the requirement and the identity of guidance cues for migration of neonatal Treg to NLTs were not examined, they expressed CXCR3 and CCR2 more frequently in SLOs expresses that adult Treg, suggesting an enhanced propensity for continual tissue seeding (78). CCR2 was indeed enriched in Treg found in muscle tissue compared to SLO Treg during recovery from muscle injury, along with CCR8 and CCR5, expression of which was shared with muscle-infiltrating Tconv. A recent integrated transcriptomic analysis of Treg from visceral adipose tissue (VAT), muscle, and colon in addition identified CCR1 and CXCR6 as part of the tissue Treg signature (79). Interestingly,

CCR1, which shares many of its ligands with both CCR2 and CCR5, was preferentially expressed by Treg in healing muscle compared Tconv at the same site (80), as was also observed for Treg in VAT (81), indicating a potentially common role for this receptor specifically in tissue Treg positioning.

While Treg do not emerge from thymic development before day 3 in mice (82), they can be detected outside of the thymus in human fetuses starting between week 13 to 14 post gestation (83), and both small and large intestine are colonized by week 23 (84). At birth, neonatal Treg obtained from cord blood have predominantly a $CCR7^+$ $CD62L^+$ cTreg phenotype, but also express the gut homing receptors $\alpha4\beta7$ and respond to CCL25, the ligand for the gut homing receptor CCR9, suggesting a preferential capacity for recirculation or colonization of the intestine at this stage of life. Only beyond three years of age, $CCR4^+$ eTreg start to predominate in peripheral blood, similarly to human adults (85).

Treg in muscle tissue, as well as those found in hair follicles, promote tissue regeneration by enhancing the differentiation of stem and progenitor cells (80, 86), indicating a requirement for mechanism promoting their co-localization. Also in bone marrow, which recruits a sizeable population of both Treg via CXCR4 (87), allogeneic stem cells are protected from immunological rejection following bone marrow transplantation by Treg with which they share an endosteal niche (88). It will be of interest to explore if Treg co-opt general guidance cues for cells in stem cell niches, or if the utilize specialized mechanisms to localize to these sites.

Tumor-infiltrating Treg

The slow development of malignant growths and their similarity to the healthy tissues from which they originate favor activation of a plethora of tolerance mechanisms, among which Treg play a central role. It is conceivable that Treg regulating anti-tumor responses derive from both tTreg and peripheral conversion of $CD4^+$ Tconv, and that their relative contribution varies dependent on particular tumor type. However, in mouse models, adoptive transfer studies have so far not produced strong evidence for a sizeable contribution of pTreg (89), and several TCRs of Treg that infiltrated mouse prostate tumors were shown to originate from AIRE-dependent thymic selection on a prostate-specific antigen (90, 91). Irrespective of their origin, understanding mechanisms of Treg function regulating the anti-tumor response holds promise for the development of therapeutic strategies to overcome tumor tolerance.

Solid tumor growth triggers MHC II-dependent expansion in the draining LN of pre-existing, self antigen-specific eTreg, or their accelerated conversion from cTreg, and their deployment to the tumor (92). TCR- and calcineurin-induced NFAT transcription factors have binding sites proximal to many CKR genes, and upregulation of CCR4, CCR5, and CXCR3, all of which are enriched on tumor-infiltrating Treg, requires calcineurin in Treg (53). Inside tumor tissue, Treg require continual TCR activation in order to sustain their immune suppressive activity and interact with $CD11c^+$ APC in order to suppress the function of CTL (93) and (F.M. and T.R.M., in preparation), suggesting a potential role for

chemokine guidance of local cell-cell contacts in the control of anti-tumor immunity (Figure 5).

Initial evidence for a chemokine-mediated mechanism for preferential Treg accumulation in tumors came from a study by Curiel and colleagues, who demonstrated that neutralization of the CCR4 ligand CCL22 significantly reduced the accumulation of adoptively transferred human Treg, but not of Tconv, in human ovarian tumor tissue in a NOD-SCID xenograft model. Although CCL22 was produced by myeloid cells in this model and is generally thought of as an organizer of Treg - APC interactions that could support local Treg proliferation and survival, the short time-frame of the effect in this study (48 hours) hints at a possible role of this chemokine in Treg recruitment from the bloodstream (94). Numerous studies have since examined the role of CCR4 and found that in addition to tumor myeloid cells, the ligands can also be produced by transformed cells in a variety of cancer types, including ovarian cancer, hepatocellular carcinoma, melanoma, head and neck cancer, and lung cancer (95–100). Many more studies have implicated the CCR4 - CCL22 axis in additional cancer types, and a few of these have provided further support for a mechanistic role in Treg accumulation (94, 97, 99, 101–103). A common theme in these studies is that neutralization of CCL22 only partially inhibits Treg accumulation, while the alternative CCR4 ligand, CCL17, only appears to play a role in some cases (103). Thus, additional CKRs are likely relevant for Treg accumulation.

Early studies showed preferential responsiveness of human peripheral blood Treg to the CCR8 ligand CCL1 (8). After a period of understudy, CCR8 has recently regained attention because it is among the genes most differentially expressed in tumor-associated Treg compared to most other tumor-resident immune cells (except for NKT cells) and compared to Treg in peripheral blood (70, 71). Only very recently have murine CCL8 (104) and human CCL18 (105) been recognized as functional homologs and as ligands of CCR8 in addition to CCL1. The role of CCR8 thus has to be reassessed for situations in which a role of CCR8 was excluded on the basis of inefficacy of CCL1 blockade (106), and this includes the recruitment of tumor Treg (103). CCL1 and CCL18 are upregulated in several human tumors including breast, colorectal, and non-small cell lung cancer (70, 71), which may thus recruit or support appropriate positioning of CCR8-expressing Treg in tumor tissue. Intriguingly, Treg infiltrating mammary tumors secrete CCL1 themselves, raising the possibility that Treg accumulation in tumors is amplified through a CCL1/CCR8-based feed-forward loop (107).

Originally implicated in immune cell migration to the skin, the chemokine CCL28 has more recently also been found to be expressed in hypoxic areas of ovarian and liver tumors in an HIF-1α dependent way. CCL28 recruits CCR10-expressing eTreg, possibly resembling the above-mentioned Th22-polarized subset, which, in addition to mediating tumor tolerance, may also support tumor growth by promoting angiogenesis through secretion of VEGFA (108, 109).

The CXCR4 ligand CXCL12 is produced by several tumor types, but appears to have an unconventional role in the regulation of Treg accumulation in tumor tissue. CXCR4 is expressed on cTreg along with CCR7, but in contrast to CCR7, is maintained on eTreg (42), and CXCR4-expressing eTreg are found e.g. in breast cancer (110) and pleural

mesothelioma (111). In the latter, Treg cluster immediately outside of the tumor parenchyma, likely reflective of the frequently observed, general phenomenon of T cell exclusion from this microenvironment (112). While CXCL12 is produced by cancer-associated fibroblasts (CAFs), the CAF-derived protein also coats the surface of tumor cells in mouse models of pancreatic and colorectal carcinoma. Surprisingly, both depletion of CAFs or treatment with the CXCR4 antagonist AMD3100 enables T cell infiltration of the tumor parenchyma (113). While this observation is not yet mechanistically explained, the authors speculated that CXCR4 may act on T cells through cross-desensitization of other CKRs, such as CXCR3. Thus, CXCR4 antagonism is predicted to relieve inhibition of chemokine-mediated intratumoral recruitment mechanisms for T cells (114).

CXCR3-mediated accumulation of CTL is one of the few instances where the role of a chemokine specifically for the recruitment from the bloodstream to tumor tissue has been directly demonstrated through intravital microscopy studies (115). This receptor is induced in tumor-reactive eTreg in tumor-draining LNs (53), and a role in recruitment had previously been proposed based only on the correlative evidence of its expression on Treg in tumor tissue (116). IFN-γ-mediated signaling drives expression of both CXCR3 and its ligands CXCL9, 10, and 11 and it is conceivable that low Treg density in tumors results from weak or absent Th1 inflammation, which also accounts for low CTL density. Any therapeutic measure that elicits Th1 immunity, as for instance chemotherapy of melanoma that increases intratumoral production of CXCL9 and CXCL10 (117), may not only increase infiltration of anti-tumor effector cells, but also of immune-suppressive Treg.

Myeloid cells in tumor tissue are a major source of CCL3, 4, and 5, ligands of CCR5. While not only intratumoral Treg, but also CTL and $CD4^+$ Tconv express CCR5, expression is more abundant on Treg, and global deletion of CCR5 reduces the proportion of Treg among tumor-infiltrating T cells and reduces tumor growth (118). Treatment of mice with a CCR5 inhibitor produced similar results (119). CCR5 has been shown to guide interactions of $CD8^+$ T cells to activated DCs during antigenic priming in LNs (120), raising the possibility that it mediates a similar function for $CCR5^+$ Treg in tumor tissue, in addition to or alternative to facilitating their recruitment. Interestingly, the CCR5 ligand CCL5 was recently found to be among a group of chemokines that also included CCL1, CCL7, and CXCL19, and which were overexpressed by epithelial squamous cell cancer cells dependent on the nuclear activity of focal adhesion kinase (FAK). While FAK is better known for its role in integrin-dependent cell adhesion, it can thereby also drive an inflammatory transcriptional program that promotes a tolerogenic tumor environment (121). This observation also illustrates that cancer cells may be selected during their malignant transformation for their ability to enhance Treg accumulation in tumor tissue.

Last, several correlative human studies have suggested a role for CCR6 expressed on Treg in the accumulation in non-small cell lung carcinoma (122), hepatocellular carcinoma (123), and oral cancer (124), but not mechanistic exploration have been conducted on the role of this receptor.

Therapeutic opportunities

Treg are already successfully being used as therapeutic agents in humans, for instance in order to ameliorate graft-versus-host disease (GvHD) in patients receiving umbilical cord blood cell transplantation for the treatment of malignancies. Infusion of large numbers of *in vitro* expanded, graft-matched Treg, is well tolerated and decreases the incidence and severity of GvHD (125, 126). Another clinical study sought to modulate type 1 diabetes through infusion of autologous, *ex vivo* expanded Treg (127).

It is conceivable that the ability to target adoptively transferred to Treg to specific tissues would render this form of immuno-therapy more effective. Retroviral transduction of Treg during their *in vitro* expansion to express appropriate chemokine receptors has been tested in mouse models as a potential clinical approach. Infusion of CXCR3-transduced Treg resulted in amelioration of murine GvHD (128), while transfer of CCR2-transduced Treg mitigated inflammatory disease in a murine model of lupus (129). Alternatively to ectopic expression from transgenes, human Treg can also be induced to stably express CXCR3 through *ex vivo* expansion in the presence of IFN-γ and IL-12, or to express α4β7 and CCR9 by exposure to retinoic acid. The latter enhanced their accumulation in the intestine of humanized mice (68).

Instead of manipulating trafficking receptor expression in order to guide Treg to desired tissues, these surface proteins can also be targeted with the objective to eliminate Treg when their activities are undesired, for instance in cancer patients. Mogamulizumab is a now FDA-approved defucosylated humanized anti-CCR4 IgG1 that effectively depletes CCR4-expressing cells, including normal eTreg and transformed T cells in cutaneous T cell lymphoma (CTCL) (130) and in adult T cell leukemia (ATL) (69). Although mogamulizumab extends the progression-free survival in CTCL patients as compared to standard therapy (130) and also may produce improved outcomes in some ATL patients (131), it is at present difficult to assess the relative contributions of elimination of Treg of of CCR4-expressing tumor cells to the therapeutic benefit. A role of Treg depletion is supported by the observation that those ATL patients who respond best to therapy also develop mild autoimmunity (131). The latter should however also raise awareness of the risk of serious immune-related adverse events (IRAE) resulting from systemic eTreg deletion. A case in point was the development of severe, treatment-refractory colitis in a patient treated with mogamulizumab (132).

The use of anti-CCR4 mAbs for Treg depleting therapy of solid tumors is currently being tested, and it is an important question whether any therapeutic efficacy of anti-CCR4 mAbs results from blockade of CCR4-mediated eTreg accumulation in tumor tissue, or from eTreg deletion. Both humanized IgG1 and IgG4 anti-CCR4 isotypes were produced, and while the IgG4 isotype antibody only blocked the interaction between CCR4 with its ligands and reduced Treg accumulation in a CCL22-expressing ovarian tumor, the IgG1 version depleted eTreg in an *in vivo* human xenograft model. The fact that IgG1 was more effective than IgG4 anti-CCR4 suggests that inhibition of Treg accumulation in tumor tissue may not suffice to elicit a full therapeutic response in itself (95). On the basis of these results, clinical trials using mogamulizumab as mono- or as combination therapy have been initiated

(NCT02476123, NCT02281409, NCT01929486, NCT02705105, NCT02301130, NCT02444793, NCT02867007), and the combination with anti-PD-1 therapy has shown some encouraging results (133).

Conclusion

Following their 'rediscovery' 24 years ago, Treg were at first generally viewed as a specialized, but fairly homogenous subset of T cells. Since then, a tremendous amount of insight has been obtained into their heterogeneity and adaptability to the requirements of the various forms of immune challenge and to different tissue environments. Along the way, some principles have emerged of how dynamically regulated changes in the expression of trafficking receptors enable Treg at different stages of development and differentiation to traffic to the appropriate tissue, position themselves in the appropriate microenvironment, and interact with the appropriate APCs in order to conduct their functions effectively. However, much has yet to be learned about the sometimes unique and sometimes redundant functions of individual receptors. Complementary to a more fine-grained analysis of their expression patterns, modern approaches to genetic perturbation will almost certainly continue to yield surprises and insights that may ultimately inform new strategies to control or leverage their functions in human disease.

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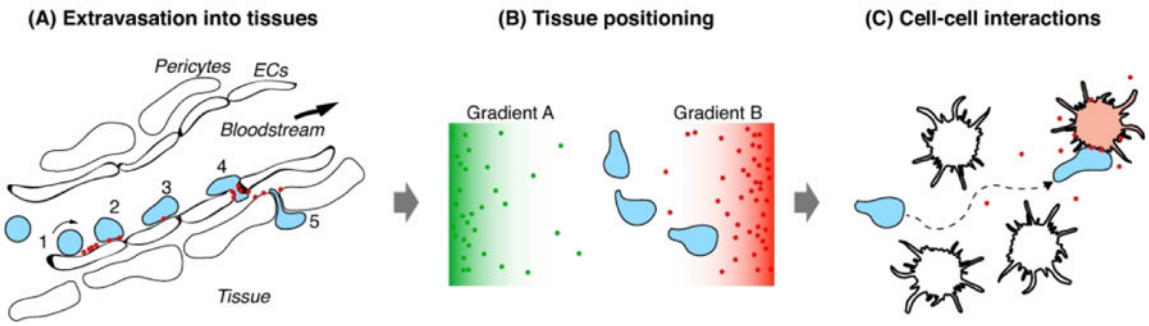

Figure 1.

Mechanisms of immune cell guidance to and within tissues. (A) The multistep process for recruitment of blood-borne leukocytes to tissues. Selectins (and in some case integrins) mediate initial tethering interactions and rolling along the vessel wall (1), where exposure to chemoattractants (red symbols) triggers integrin activation and firm arrest of leukocytes (2). Luminal crawling (3) to permissive sites facilitates their para- (or sometimes trans-) endothelial migration migration (4) following chemotactic guidance cues, and eventually exit past pericytes (5) into the interstitial space. (B) Following extravasation, gradients of chemokines and other ligands of leukocyte-expressed GPCRs direct their migration to specific environments defined by the presence of their ligands either in soluble or in cell surface- or extracellular matrix-bound form. (C) Antigen-presenting cells can express chemoattractants that further ‘micro-position’ leukocyte to orchestrate their interactions. ECs - endothelial cells

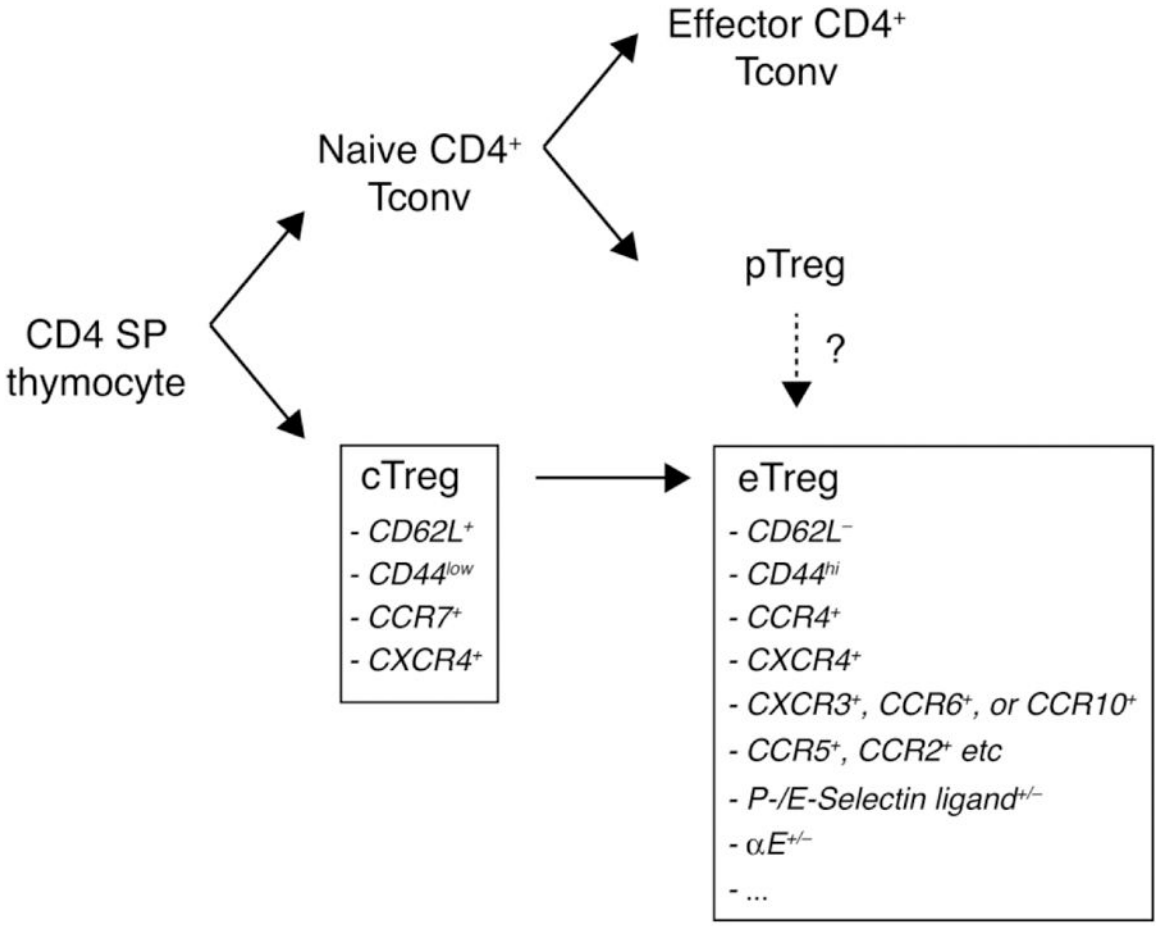

Figure 2.

Developmental paths for the generation of central Treg (cTreg), effector Treg (eTreg), and peripheral Treg (pTreg). The dashed arrow from pTreg to eTreg indicates that their relationship remains unclear since many studies on eTreg do not reveal their developmental origin.

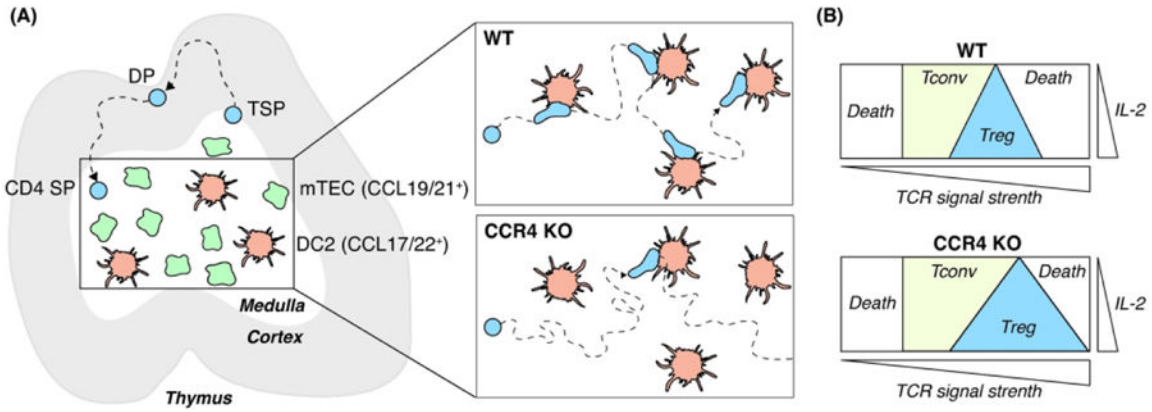

Figure 3.

Thymocyte interactions with medullary APC facilitate tTreg development. (A) Blood-borne thymic seeding progenitors (TSPs) enter the thymus at the corticomedullary junction. Following αβTCR rearrangement and positive selection as DP cells in the thymic cortex, they upregulate CCR4 and CCR7 and enter the thymic medulla in response to CCR7 ligands CCL19 and CCL21 produced by mTECs and CCL17 and CCL22 produced by thymic DCs. In the medulla, CCR4 optimizes their interactions as CD4 SP cells with DCs presenting rare tissue-restricted self antigens expressed stochastically by small subsets of mTECs. (B) A hypothetical model of how CCR4-mediated optimization of medullary CD4 SP - DC interactions, and thereby the frequency and duration of self antigen encounters, regulates the balance of positive selection versus Treg selection versus clonal deletion at different TCR signal strengths and local concentrations of IL-2.

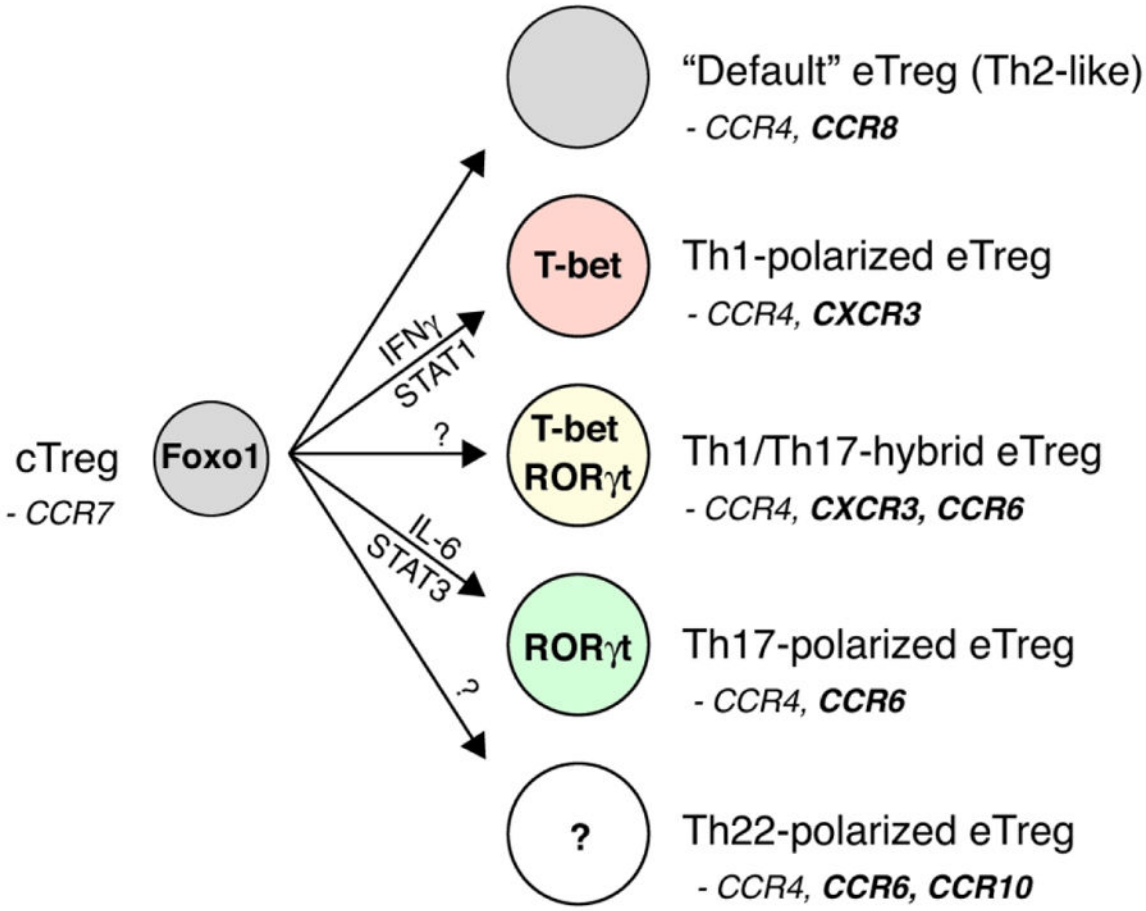

Figure 4.

Diversification of eTreg differentiation. While the trafficking pattern of cTreg is largely governed by the transcription factor Foxo1, TCR-, CD28-, and IL-2-signals enable differentiation of eTreg that resemble Th2 effector cells by virtue of expressing CCR4 and CCR8. However, all eTreg express CCR4, but expression of T-bet or RORγt, driven by IFN-γ or IL-6, respectively, can instruct Th1- and/or Th17-biased CKRs expression patterns. Co-expression of CCR6 and CCR10 may reflect further specialization of skin-tropic Th22-biased eTreg.

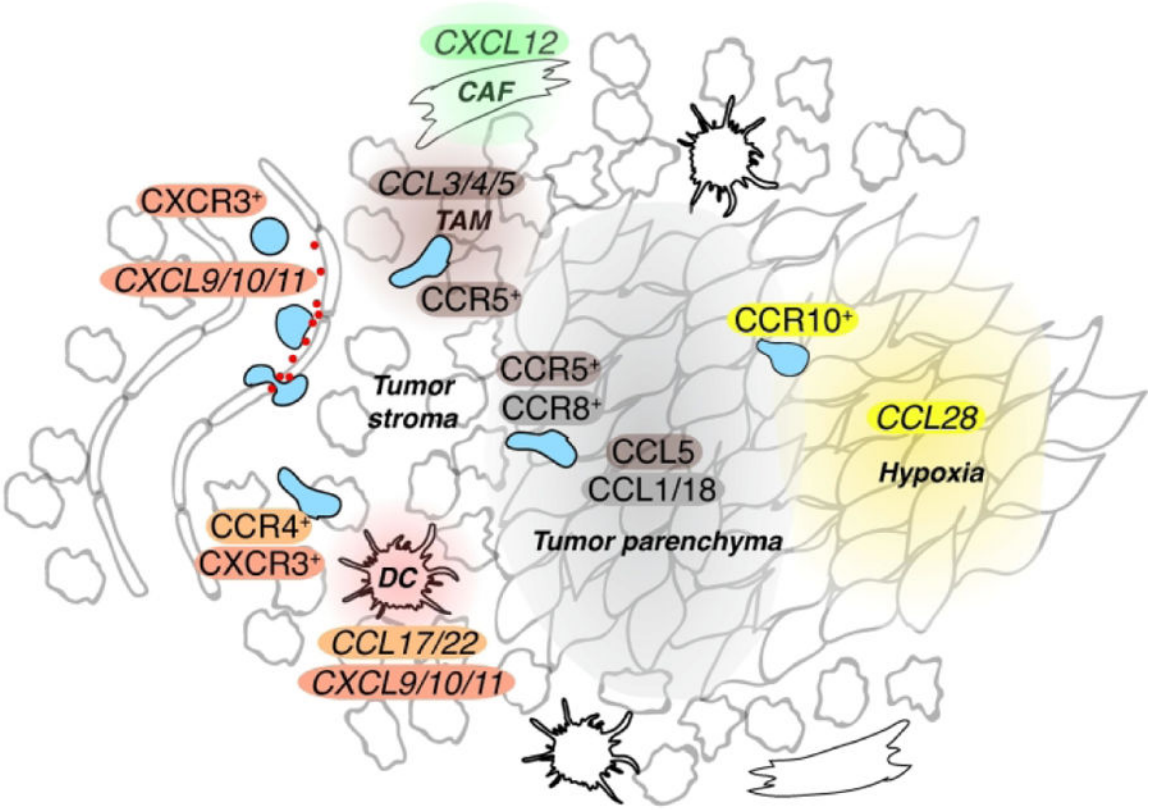

Figure 5.

Summary of sources and suggested roles for chemokines and their receptors in guiding the recruitment and intratumoral positioning of Treg. The CXCR3 chemokine system participates in the recruitment of Treg from the bloodstream with other chemoattractants likely supporting this process. Production of CCR4 and CXCR3 ligands by DC may optimize their interactions with Treg, akin to the function of these chemokines during Treg development and Th1 priming, respectively. A variety of chemokines are expressed by the myeloid tumor infiltrate, including the CCR5 ligands CCL3, 4, and 5. Cancer cells also produce chemokines, such as CCL5 and the CCR8 ligands CCL1 and CCL18, but their precise role in local Treg accumulation remains unclear. CCL28 expressed in hypoxic tumor regions can attract CCR10-expressing Treg, while the CAF-secreted CXCR4-ligand CXCL12 may prevent Treg accumulation in the tumor parenchyma by desensitizing Treg to other chemokines. CAF = Cancer-associated fibroblast, TAM = Tumor-associated macrophage