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Global Positioning by Chemokines and other Mediators

Regulators of T cell fate: integration of cell migration, differentiation and function.

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Running title: Chemokine regulation of T cell fate decisions

Summary:
A fundamental question in immunology is how cells decide between distinct T helper, effector or memory differentiation fates. These decisions are paramount to overcome infection and establish long-lasting protection. The impact of cell location for the determination of T cell fate decisions is an emerging field. This review will discuss our current understanding of the migration path that T cells follow, within draining lymph nodes, to steer differentiation down distinct paths of either effector or memory fates. In particular, the regulation of migration and cellular encounters mediated by the chemokine receptor CXCR3 and its ligands will be discussed. The combination of increased antigen density and unique cellular partners play a central role in facilitating the site-specific differentiation of effector T cells, within the interfollicular regions of draining lymph nodes. Recent advances have applied this knowledge to optimize vaccine design to target antigen to lymph nodes. Increased understanding of the regulation of CXCR3 ligands and how T cells integrate multiple chemokine cues will further progress this field and allow further applications to direct cell differentiation outside the lymph node, to enhance memory residency in peripheral tissues and effector anti-tumor responses.

Keywords:
T cell; Chemokine; Migration; Differentiation; Lymph node; CXCR3

1. Introduction

T cells are central orchestrators and effectors of the adaptive immune system. At each stage in the life of a T cell, critical fate decisions are required. Upon activation naïve T cells differentiate down divergent pathways, between distinct T helper lineages and between progenitors with either effector or memory potential. These decisions are paramount to overcome infection and establish long-lasting protection. A fundamental question in immunology is how cells decide between these distinct fates.

T cell differentiation choices are determined by a combination of cell intrinsic factors along with antigen and inflammatory cytokines and the cellular partners that present and provide

these signals. Recent work suggests that alternate T cell differentiation fates likely result from the sum of multiple cellular encounters which occur in specific niches within lymphoid organs. Chemokines act to provide the set of instructions that determine where cells move to and the unique cellular partners they encounter within these sub-organ niches. It is this migration and ultimately these interactions that steer cell fate decisions down distinct paths to produce either effector or memory precursors or differentiation of specific helper effector T cells.

This review will discuss the regulation of T cell positioning that drives effector and memory differentiation decisions within draining lymph nodes, with a focus the requirement for the chemokine receptor CXCR3 and related factors. CXCR3 binds three ligands, CXCL9, CXCL10, CXCL11. While CXCL11 biology is highly relevant to human disease and cancer outcomes, to date the work on the individual role of this chemokine has been hampered as C57BL6 mice contain a frame shift mutation resulting in a null allele [1,2]. Thus, much more work has determined that dendritic cell (DC) and stromal cellular sources of CXCL9 and CXCL10 help mediate T cell fate changes within draining lymph nodes. Together with recent advances, future work will bring the field closer to understanding how to optimize T cell positioning for therapeutic benefit. This will drive the development of targeted vaccines that establish robust memory responses in lymph nodes and peripheral tissues and determine how this may be applied to reinvigorate T cell responses during chronic infection or for cancer immunotherapy.

2. Lymph node organization for antigen capture and presentation

The lymph node is a highly organized structure that permits the efficient transfer of antigen and facilitates interaction between antigen-loaded DCs and naïve T cells. The lymph node microarchitecture is a reticular network which provides a mechanical framework and restricts immune cells into structurally distinct regions. The major structures within lymph nodes include the capsule, subcapsular sinus (SCS), the cortex containing superficial B-cell follicles (B-cell zones), the cortical ridge, deep paracortex (T-cell zone) and the medulla [3,4]. Following infection, antigen is efficiently transferred from the periphery to the lymph node via multiple mechanisms [4]. Migratory DCs carry antigen from peripheral tissues and arrive in lymph nodes via the afferent lymph. Mature DCs can then translocate through the SCS, allowing access to interfollicular regions (IFRs) between B cell follicles. These IFRs are a specialized microenvironment within lymph nodes, they contain innate immune cells and act like a

corridor for migratory DCs to move towards the T cell paracortex in the center of nodes [5-10]. Alternatively, particulate antigens, such as free virions enter the lymph node via the afferent lymphatics and wash over the SCS. Here they are captured and retained by cells immediately lining the sinus, SCS macrophages and DCs, which are the first lymph node resident cells to be infected with virus [8,11-14]. Small viral and protein particles that are not taken up by these phagocytic cells continue to transition through the IFRs and access the reticular conduits. Through these conduits antigen traverses deeper into the paracortex to be taken up and presented by additional DCs [15,16]. Thus, the antigen draining and lymph node organization ensure that are multiple local niches where T cells come into contact antigen, which can be presented by unique DC subsets [4,15,17].

3. T cell migration that determines effector differentiation

In vivo T cell activation drives proliferation and polarized differentiation of naïve T cells, leading to the development of effector cells. This activation is therefore a prerequisite for the acquisition of effector cytokines and provision of help to B cells. Multiple studies have determined that the initial stages of T cell activation occurs within the T cell paracortex [2,18-20]. Recent work has established that newly activated cells undergo a dynamic migration that occurs subsequent to initial antigen encounter. This moves T cells out of the paracortex, into more peripheral regions of draining lymph nodes. This migration directs cells into regions rich in antigen with new opportunities for cellular interactions that are not available within the paracortex. The precise understanding of how this is spatially regulated and how this promotes effector differentiation is only beginning to emerge. This section will detail our current understanding of T cell positioning within lymph nodes and how dynamic movements between specific sub-organ niches determines the outcome of T cell fate decisions for both $CD4^+$ and $CD8^+$ T cells (Figure 1).

3.1 Spatial requirements for the differentiation of effector $CD4^+$ T cells

Following activation $CD4^+$ T cells can differentiate into a heterogeneous population of effector T cells that control immune outcomes. These T helper (Th) subsets, which include Th1, Th2, Th17, T follicular helper cells (Tfh) and induced T regulatory cells (Treg), facilitate the generation of specialized immune responses to mediate clearance of diverse pathogens [21]. The differentiation of $CD4^+$ T cells into Th subsets is driven by cytokines and instructed by master transcription factors. The cytokine factors that control this process have been well elucidated *in vitro* and rely on unique instruction from antigen presenting cells,

primarily DCs *in vivo* [21,22]. Work from us and others has identified how this process is temporally and spatially orchestrated within draining lymph nodes. Initially we found that antigen-specific T cells upregulate the CXCR3 following initial DC engagement. This chemokine receptor was well established to facilitate migration of cells out of lymphoid organs and into peripherally inflamed tissues [23], therefore, we were intrigued to find that cells stayed within draining lymph nodes for over 48 hours following initial CXCR3 expression. By combining T cell tracking with a Th1-inducing vaccine strategy, we found that T cells use CXCR3 to move rapidly out of the T cell paracortex into more peripheral regions of draining lymph nodes, the medulla and IFR [2]. This process was tightly controlled through expression of the CXCR3 ligands, CXCL9 and CXCL10 which we found expressed in these peripheral zones. Importantly, following both vaccination and viral infection protocols, we found that T cell migration to these peripheral zones strongly correlated with increased differentiation into Th1 effectors, as marked by the production of IFNγ, TFNα, and IL-2 [2]. This work was the first to demonstrate the unique spatial requirements for Th1 cell differentiation within draining lymph nodes.

A major question following this work, is how universal is this migration pathway for other $CD4^+$ T helper lineages? There exists scant information regarding the positioning of newly activated Th2 and Th17 migration within draining lymph nodes. One study has demonstrated the migration of Th2 cells out of the paracortex into the IFRs, directed by CXCR5 following *Heligmosomoides polygyrus* infection [24]. The relocation of cells into this sub-organ niche, provided the signals to increase effector differentiation, in this setting, increased production of IL-4. Another study has demonstrated the commitment of IL-17 producing innate lymphocytes in this region, following interaction with SCS macrophages [7], however, no such studies yet detail this localization for Th17 cells. The most convincing lines of evidence for a shared $CD4^+$ T cell migration pathway, exist for effector lineages that occur in parallel with Th1 cells, the Tfh and Treg fates (Figure 1).

Following viral infection, Tfh and Th1 cells differentiate under similar inflammatory cues and upregulate shared transcriptional programs [25,26]. Notably, this expression includes the Th1 lineage-specifying transcription factor, T-bet, which transcriptionally regulates CXCR3 [23,27-31]. The point of bifurcation between these cell fates is an area of intense research, however the spatial location of where these effector fates diverge has not been fully

reconciled. Several studies have proposed a common precursor that forks late in activation [30,32]. While, others propose these differentiation processes are intrinsically separate prior to initial proliferation events [33]. Intriguingly, the migration pathway for newly activated Tfh cells parallels that described above for Th1 cells, whereby newly activated cells rapidly migrate out of the T cell paracortex and align at the T:B cell boarder. For Tfh cells, this occurs prior to travelling deeper into the follicle to participate in germinal center (GC) reactions [4,34,35]. While not directly tracking T cells following CXCR3-dependent interactions, Woodruff *et al.*, demonstrated that these interactions in the draining lymph node medulla and IFRs correlate with GC formation and protective antibody responses. This work suggests that CXCR3-CXCL10 interactions outside the paracortex are also critical for Tfh cell differentiation following vaccination [36]. Counter to this argument, the upregulation of PD-1 by Tfh cells was shown to directly suppress the expression of CXCR3 [37]. While not specifically discounting a shared requirement for CXCR3-directed migration in the differentiation of Th1 and Tfh cells, this implies that CXCR3-dependent interactions need to be blocked to allow Tfh accumulation within GCs [37]. However, CXCR3 can highly expressed by Tfh cells following viral infection [31], and thus the precise role of CXCR3 on Tfh cell differentiation remains unresolved. Further, several studies have highlighted the potential shared differentiation path for Tfh and Th2 cells, following *Heligmosomoides polygyrus* infection or house dust mite challenge [24,38]. Combined these works suggest that Tfh cells adopt different migration strategies depending on the infectious or allergic onslaught, however this is yet to be fully appreciated. How CXCR3 alter Tfh function is also currently under debate. Several studies have used CXCR3 expression on human circulating Tfh cells as a biomarker of vaccine efficacy and B cell memory [39,40]. In contrast, $CXCR3^-$ TFH have been shown to be superior helpers of B cells compared to $CXCR3^+$ TFH [41-43]. Therefore, it remains unclear how $CXCR3^+$ Tfh cells impact protective humoral responses. Ultimately (as discussed below) the relevance of CXCR3 on Tfh differentiation and function may be impacted by the infection environment.

Another $CD4^+$ T cell subset that whose differentiation is intertwined with Th1 cells, is that of the effector Treg cell. Functional specialization occurs within the Treg lineage to enable context-specific tailoring of suppression [44]. The differentiation of Th1-Treg cells parallels that of Th1 cells and these cells specifically suppresses Th1 cells responses [45,46]. Interestingly, as Treg cells act within lymph nodes to shorten the length of stable T:DC interactions, one mechanism of suppression during early priming events may be to alter the

positioning of cells into different lymph node niches, or suppress the DC-derived chemokines [47]. Th1-Treg cells are chiefly identified by their expression of T-bet and CXCR3, suggesting similar migration and cellular positioning lead to this fate outcome [45,48]. While the specific positioning of Th1-Treg cells has not been tracked during their differentiation, in comparison to circulating Tregs, effector Tregs exhibit lower CCR7 and increased CXCR3 expression, suggesting they are capable of migrating out of the T cell paracortex and into the cortical ridge and IFRs of draining lymph nodes [49]. Th1-Tregs upregulate T-bet via IFNγ stimulation, in a manner similar to Th1 cells [50]. However, they exhibit reduced responsiveness to IL-12, due to deficiency in IL-12Rβ2 expression. Whether the absence of 12Rβ2, which drives this fate decision towards regulatory cells rather than effector Th1, is determined prior to or at the point of initial activation their decision is unclear [50].

3.2 Chemokine receptor control of $CD8^+$ T effector differentiation

While it is undecided if all $CD4^+$ T effector lineages share the intranodal positioning observed for Th1 cell differentiation, several lines of evidence indicate this differentiation pathway for short-lived $CD8^+$ effectors. The direct priming of $CD8^+$ T cells has been shown to occur in the cortical ridge and IFRs of draining lymph nodes [8,12]. Following this, several studies described that CXCR3 expression plays a critical role in the T cell fate decisions of $CD8^+$ effector cells following viral infection. Combined, these studies demonstrated the differentiation of effector cells is curtailed for CXCR3-deficient $CD8^+$ T and differentiation is instead skewed towards the generation of memory precursors [51-53]. In addition, an increase in surface expression of memory (expression of CD127 and reduced KLRG1), CXCR3-deficient T cells exhibited reduced production of $CD8^+$ T-derived inflammatory cytokines IFNγ, TFNα, and IL-2 [53]. These studies correlated the CXCR3-dependent differentiation of effector cells with CXCL9 expression in the marginal sinus of the spleen, where virus was also detected [51,53]. Further, over-expression of CCR7 promoted T cell sequestering in the T cell zone, blocking CXCR3-dependent migration and exacerbating the increase in memory differentiation [51]. However, these studies did not examine the presence of inflammatory gradients and movement of antigen-specific T cells within draining lymph nodes. Subsequent to these studies, T cell priming $CD8^+$ T cells with high affinity antigen was shown to be associated with the rapid relocation out of the lymph node paracortex and into the IFRs, similar to what we have demonstrated for Th1 differentiation [2,54]. This study used an antigen-pulsed DC transfer model without additional inflammatory stimulation, surprisingly

illustrating that viral-induced inflammation was not needed for the intranodal migration of $CD8^+$ T cells. This experimental system was also unable to determine the differentiation between effector and memory precursors or relate intranodal migration to the production of effector cytokines. However, combined with the previous work demonstrating the reliance of CXCR3 for T cell effector differentiation, it is likely these the migration path described by Orga *et al.* in lymph nodes is an essential requirement for this fate outcome [54]. However, this still remains to be confirmed.

3.3 Positioning of T cell memory

Where memory lymphocytes reside within lymph nodes is an area of intense research, often with controversial and conflicting outcomes. Recently, the subcapsular proliferative foci (SPF), a novel structure positioned between the subcapsular sinus (SCS) and B cell follicle, was described in draining lymph nodes [55]. In this site, memory B cells are prepositioned and found together with memory Tfh $CD4^+$ T cells [55,56]. Upon secondary challenge, both these cell types were reactivated and interact within the SPF with further interactions between both cell types and $CD169^+$ SCS macrophages. While a subset of memory B cells residing within the SPF expressed CXCR3, it remains unclear if this receptor is required for this location in the absence of inflammation. Similarly, it is unknown if other $CD4^+$ memory T cells, in addition to Tfh cells, also reside within this niche. One study has described $CD8^+$ T memory cells prepositioned in the cortical ridge of lymph nodes, adjacent to the B cell follicle, albeit not directly adjacent to the SCS as shown for memory B cells and Tfh [18]. Again, while these $CD8^+$ memory T cells also expressed CXCR3, this expression, nor that of other inflammatory chemokine receptors, could explain residency in this zone. Further, this prepositioning was not found in other experimental models, where $CD8^+$ memory T cells have been observed residing within the T cell paracortex alongside naïve cells [20]. Despite these differences in initial positioning of memory cells, these studies both described the CXCR3-dependent recruitment of memory T cells to the IFRs rapidly following secondary challenge [18,20]. Indeed, unlike naïve cells that require time for CXCR3 upregulation, the immediate recruitment of memory cells correlates with increased effector function during memory responses. These studies highlight the ability to rapidly move into the IFR of draining lymph nodes as a key feature of T cell memory allowing prompt eradication of viral pathogens upon secondary challenge. As discussed below, the IFNγ produced by memory cells during secondary challenge leads to a positive feedback whereby CXCL9 expression is increased by

SCS macrophages. This in turn, increases the accumulation of memory cells deep in the IFR, in contact with SCS macrophages [18,20]. At first, these results appear counterintuitive, with the studies suggesting that the absence of CXCR3 promotes memory cell differentiation and function at the expense of effectors [51-53]. However, this work is can be unified in a model where CXCR3 plays a multifaceted role, which appears to be differentially required at each antigen encounter. In support of this model, the increased cell dynamics of migration for memory responses compared to naïve $CD8^+$ T cell priming implies a striking difference in the DCs with which naïve and memory cells interact [18]. This difference geographical priming locations of suggests distinct DC subsets prime naïve and memory cells and would reduce competition between these populations [57]. Further, the differentiation of $CD8^+$ memory T cells in the absence of CXCR3 interactions, may allow compensatory mechanisms by alternative chemokine receptors, such as CCR5, that enables IFR recruitment in CXCR3-deficient cells [58].

4. Why is spatial redistribution important for effector differentiation?

A major and unresolved question for the field is why are IFRs such powerful promoters of effector differentiation? Is it all due to the increased density of antigen? Or are there distinct cellular interactions occurring within this compartment to facilitate this differentiation path? It is likely a combination of both these factors sum to determine T cell effector differentiation.

4.1 Antigen dose and affinity influences cell fate decisions

Both antigen-loaded migratory DCs and free viral particles arrive at draining lymph nodes through the SCS. Here, free antigen particles are sequestered by the cells of the SCS and IFR [4]. Indeed, the capture of virus by $CD169^+$ SCS macrophages is important to prevent systemic dissemination [13,14,20]. The migration of cells into the cortical ridge and IFRs therefore brings them closer to these high antigen deposits. Interestingly, $CD4^+$ and $CD8^+$ cells require different antigen doses for the activation, such that $CD8^+$ T cells require a higher antigen dose to upregulate CD69 [15]. Further, persistent antigen such as that seen during chronic infection pushes $CD4^+$ T cells down the Tfh lineage [33,59]. This feature could help explain the different geographical priming location and DCs responsible for priming these cells early during infection [11,15,60]. CXCR3 drives T cells into lymphoid regions that are highest in antigen [12,51,61]. This increases the contact of between T cells and infected cells and potentially the duration or number of DCs that these cells encounter. Interrupted exposure to antigen

reduces the cytokine production and effector functions, while instead promoting memory formation of both $CD4^+$ and $CD8^+$ T cells [62,63]. Combined, this work suggests that increased antigen dose is a key mechanism for acquisition of effector status following CXCR3-directed migration.

In addition to antigen dose, affinity for antigen also plays a key role for the cell fate decisions of T cells. For $CD8^+$ T cells, the link between high affinity responses and CXCR3-directed migration has been demonstrated in an elegant study by Ozga *et al.* [54]. In this study, DCs loaded with antigen of different affinities were transferred into host mice and antigen-specific T cell positioning was tracked in 3D. High affinity antigen stimulation led to the CXCR3-dependent relocation of T cells into the IFRs. Interestingly, there is conflict over the role of TCR signal strength in the determination of fate choice between Th1 and Tfh cells. While some have shown that high TCR signals favors Tfh, the reverse has also been demonstrated [33,64]. It is likely other environmental factors, including but not limited to IL-2 sensitivity and upregulation of CXCR3 also influence the bifurcation of these cell fates depending on the infection and experimental setting.

4.2 Peripheral lymph node niches: sites for unique cellular interactions

In addition the trapping and increased exposure of antigen, the SCS and IFRs of draining lymph nodes offer T cells an abundant supply of proinflammatory cytokines and unique cellular interactions that may promote T cell effector function. SCS and IFR macrophages are rapidly infected by lymph-borne viruses and can relay antigen into the B cell follicle [14,65]. While these cells participate in the re-activation of memory cells [20,56], it is unclear how they individually contribute towards increased effector differentiation. In mice depleted of all DC subsets, T cells can stably interact with infected macrophages, however this results in suboptimal cell proliferation or IFNγ production [58]. This however, does not discount an additive effect when both lymph node DCs and macrophages are present. Interestingly $CD169^+$ SCS macrophages may play an indirect priming role in IFRs, via the presentation of lipid antigens to NKT cells [66]. The presence of activated NKT cells in these regions may then either act to promote T cell recruitment through induction of CXCR3 ligands, or directly influence T cell activation. Similarly, along with $CXCR6^+$ NKT cells, other innate lymphocytes can be found in IFRs of lymph nodes at steady state [8]. These include $CCR6^+$

IL-17 producing innate lymphocytes, NK cells and γδT cells [7,8]. In contrast, all these innate lymphocytes are sparsely located in the T cell paracortex of steady state lymph nodes [8].

5. T cell cellular partners: chemokine providers and fate regulators

A key strategy to identify the cellular plays important for chemokine-dependent T cell fate decisions has been the generation and study of chemokine reporter mice [2]. Given the complexity of the chemokine superfamily, this approach allows the cellular sources of multiple related chemokine ligands to simultaneously evaluated in time and space. Two chemokine reporter strains have so far been described; the REX3 transgenic reports the expression of CXCR3 ligands, CXCL9 and CXCL10, while the REC8 transgenic that reports the expression of CCR8 ligands, CCL1 and CCL8 [2,67]. This approach is set to be widely adopted for other chemokine families. While these strains have been valuable in describing the positioning of the cellular sources of chemokines *in vivo*, future work will create a map of these cellular sources and describe how they individually and collaboratively impact T cell fate decisions and detail the regulation of these ligands (Figure 2).

4.1 Dendritic cell sources of chemokine

DCs are a major source of CXCR3 ligands within draining lymph nodes [2]. Using a DC transfer model, we originally observed that DC-derived CXCL10 acts to increase the number and duration of T cell:DC contacts during T cell priming. This increased contact time was associated with differentiation into effector Th1 cells [2]. The conventional DC (cDC) subset performing this function was not identified and probably consisted of both cDC1 and cDC2 subsets. It is now realized that lymph node resident cDCs subsets are not only functionally specialized, but are uniquely positioned and individually act to facilitate the complex choreography of T cell priming [11,17,60,68]. Initial priming of $CD8^+$ T cells occurs in the IFR and cortical ridge of draining lymph nodes [12,18]. In this area, $CD8^+$ T form clusters with infected cDC2s, although, it is worth noting that during inflammation, the markers of cDC2s overlap with $CD11b^+$ monocyte-derived DCs [17,68,69]. These initial $CD8^+$ T cell:DC clusters did not include $CD4^+$ T cells, which were primed rapidly by migratory DCs, also within the IFR [60,68]. Later in infection, $CD4^+$ and $CD8^+$ T cells cluster with $XCR1^+CD8^+$ cDC1s [60,68]. This subset is known to be the most efficient at cross-presenting antigens to cytotoxic $CD8^+$ T cells, they produce increased levels of IL-12 and act as conduits for $CD4^+$ T cell help and promote $CD8^+$ T cell memory [17,69]. In contrast to the initial T cell priming that occurred in

IFRs, $CD8^+$:$CD4^+$ T cell:cDC1 clusters were spatially distinct, in the T cell paracortex [60,68]. Thus multiple, sequential T cell:DC encounters are required as T cells differentiate and their final fate probably results of the sum of these encounters.

Multiple chemokines and their receptors are upregulated at the time of T cell fate decisions, which may control the spatial and temporal organization of DC interactions [4,12,70-72]. Interactions in the IFR and cortical ridge appear similar to the upregulation of CXCL9 and CXCL10 in this region [2]. Although the precise DC-source of these ligands in this niche has not been elucidated, deficiency in CXCR3 for both $CD8^+$ and $CD4^+$ T cells reduces clustering and contact time with antigen presenting cells, suggesting a critical role of in T cell priming [2,61]. In contrast, CCR5 ligands have been shown to determine the clustering of $CD8^+$:$CD4^+$ T cell:DC within the paracortex and as such may mediate the interactions that promote memory differentiation [12,70,73]. However, $CD8^+$ T cells can differentiate towards a memory fate in the absence of CCR5 [52]. Within tumors, it appears that cDC1s express CXCL9 predominantly, however it is unknown if this is similar within lymph nodes, as the tumor microenvironment can act to suppress and perturb chemokine expression [74,75]. Thus, the dot-to-dot map of chemokines that move T cells between sequential DC encounters has not been articulated. The use of chemokine reporter mice will assist in resolving these questions [2].

For $CD4^+$ T cell effector differentiation, discrepancy has arisen over the primary and required cellular sources that determine priming of Th1 verses Tfh cells. While some have described that Tfh differentiation can occur independent of any priming DC population, others specify the cDC2s at the T:B boarder are required and sufficient for this pathway [76,77]. Intriguingly, this is the same cellular site of $REX3^+$ DCs that induce Th1 [2]. While this is again suggestive a shared pathway for these $CD4^+$ T cell fates in some settings the role of CXCR3 ligands in the differentiation of Tfh has not been tested. Combined, it appears that Th1 verses Tfh fate decisions are far more dependent on inflammatory status and infection type, than decisions regarding $CD8^+$ T cell effector priming.

4.1 Stromal cells

The second major cellular source of CXCR3 ligands is the lymph node stroma [2]. These cells provide the structure and scaffold of lymph nodes to facilitate cell positioning and enable T cell migration and interaction [3]. We have described that the stromal cells located in the IFRs upregulate both CXCL9 and CXCL10 following vaccination [2]. These chemokines work

together to facilitate $CD4^+$ T cell recruitment into this area. Subsequently, CXCL9 was shown to be produced by an activated reticular fibroblast population that resides in both the paracortex and IFR of resting lymph nodes [78]. While the role of the stromal network in facilitating cellular interactions is widely appreciated, fascinating new research has demonstrated that stromal cells can directly alter T cell proliferation and differentiation [79]. Therefore, it is currently unclear if stromal cell-derived chemokines act solely to bring T cells within range of unique DC populations or other cells within the IFRs, or if they these stromal cells direct influence T cell fate changes within lymph nodes.

5 Targeting lymph nodes to optimize T cell effector differentiation

Vaccines have been the single most effective public health intervention ever developed, saving the lives of millions worldwide. Despite successes, considerable challenges remain for the development of vaccines against recalcitrant global infections and to advance anti-cancer vaccines [80,81]. In particular, vaccine development for cancer, AIDS, tuberculosis and malaria, will require cellular immune responses, especially a strong $CD8^+$ T cell response [80,82]. Insights into the spatial regulation of T cell fate decisions has led to strategies that target vaccine delivery to lymph nodes [83]. Several vaccine vehicles can be used to target delivery to lymph nodes [83]. These predominantly fall into two categories of vehicle. Firstly, so called "hitch hiking vaccines" such as those containing an albumin binding site attached to the vaccine cargo. These hijack the role of albumin as a fatty acid transporter, drain through the lymphatics to arrive and be captured in the lymph node [83,84]. This approach applied to a cancer vaccine strategy has demonstrated a 30-fold increase in T cell priming with sustained tumor growth regression [84]. The second approach is via the use of nanoparticles, including silica nanoparticles, for targeted lymph node delivery of subunit vaccines [83,85,86]. These particles are rapidly taken up by lymph node DCs, leading to localization within the IFR and potent activation of primarily $CD8^+$ cDC1s [86,87]. Again, this lymph node targeting strategy leads to improved vaccine efficacy with increased antigen-specific T cell proliferation and effector differentiation resulting in protective immunity. Interestingly, nanoparticle targeting of vaccine components to lymph nodes has been associated with NK cell expansion and activation, along with $CD8^+$ IFNγ induction [87]. Further, nanoparticle delivery targeted to tumor draining lymph nodes is also associated with enhanced efficacy of anticancer vaccines [81]. Compared to soluble vaccines, these methods substantially reduce the systemic side effects of TLR agonist vaccine components, reflecting their low concentrations outside the

lymph nodes [83-86]. The increased efficacy and safety of these vaccine approaches was found to lie in the ability of antigen to deposit and activate DCs within the IFR, in a similar manner to that as shown for viral particles [14,84,85].

In addition to vaccine machinery systems, antigens must be associated with appropriate adjuvant systems to upregulate appropriate chemokine ligands with the IFR. This is essential in order to draw newly activated cells into these regions and promote beneficial T cell:DC interactions. Candidate immunostimulatory methods such as Clec9A, which target antigen to cDC1s for cross presentation, and in Cyclic dinucleotides, which are potent inducers of IFN genes hold promise to act in a targeted fashion and optimize CXCR3-dependent interactions [63,86]. Further, the activation of KALA-MED as a DNA vaccine carrier has duel functionality to increase DNA delivery to DCs and act as an immune-stimulant [88]. This leads to strong induction of DC-derived CXCL9 and CXCL10 and when used as a DNA vaccine packaging system results in reduced tumor burden. Thus, rigorous testing of TLR ligands and adjuvant systems in the context of CXCR3 ligands would be beneficial to determine optimal stimulation that drives T cell migration towards protective T cell responses.

6 Environmental cues that determine migration

A cornerstone of immunity is its flexibility and adaptability. It is interesting to speculate how diverse immune responses affect cell migration and cell fate decisions. Pathogen-directed diversity likely occurs at both the level of regulation of chemokine receptor and regulation of ligand induction. In this way the pathogen type and cytokine milieu together co-ordinate the outcome of T cell fate decisions by controlling cell location and interactions (Figure 3).

6.1 Environmental control of receptor expression

The molecular control of CXCR3-dependent movement is through the regulation of T-box transcription factors, primarily T-bet (encoded by Tbx21) and a related factor, Eomesodermin (encoded by Eomes) [23,89]. Notably, the defect in effector cell differentiation and reciprocal increase in memory precursor differentiation is similar between mice deficient in CXCR3, T-bet and IFNAR [51-53,90-92]. This suggests that these factors work in concert, whereby viral induction of type-I IFNs leads to the induction of T-bet which transcriptionally regulates CXCR3 expression. The regulation and requirement of T-bet is interesting in this system, as it exhibits graded upregulation, depending on the infectious challenge and cytokine (primarily IL-12 and IFNγ) milieu [91]. Further, in settings where T-bet is over expressed,

either experimentally or due to a loss of $CD4^+$ T cell help, the effector differentiation of $CD8^+$ T cells is reinforced with reciprocal loss of memory precursors [90,91]. While not directly observed, *in vivo* this may be due to greater CXCR3-dependent interactions in the peripheral zones of draining lymph nodes. Although it is difficult to separate the intrinsic role of T-bet in controlling effector differentiation, these results may suggest that CXCR3-dependent interactions reinforce and stabilize T-bet expression [90,91]. The reverse of this model suggests that following pathogen encounter where type-I IFN, IFNγ or IL-12 induction is lacking, such as those skewed to Th2 or Th17 milieus, T-bet and CXCR3 are insufficiently induced and cell positioning and T:DC interactions that would otherwise promote $CD8^+$ or Th1 effector differentiation do not occur. This idea is supported by the heightened Th2 and Th17 responses of T-bet-deficient cells [93-95]. However, it is unclear if other chemokine receptors are induced in these settings *in vivo* leading to the relocation of T cells out of the paracortex into the IFR of draining lymph nodes.

The expression of T-bet is regulated by transcription factors BATF and IRF4 [96]. These act in the first 24-72 hours of initial TCR activation and their upregulation depends on the extended T cell-DC interactions observed with presentation of high affinity cognate antigen [54]. CXCR3 upregulation in this setting drives cells into the IFRs to promote increased effector differentiation [54]. While increased antigen affinity and abundance leads to increased CXCR3 expression and increased effector function, it is interesting to note that this is altered during persistent antigen. In these settings, such as in cancer or chronic viral infection, persistent antigen leads to down regulation of T-bet [97,98]. While overexpression of T-bet restores $CD8^+$ T cells in chronic infection, whether the mechanism for this is primarily through cell intrinsic transcriptional programing or through upregulation of CXCR3 and altered cell positioning, or both, is currently unclear [97].

6.2 Environmental control of chemokine ligands

The upregulation and cellular sources of CXCR3 ligands act as an instruction map, to guide newly activated T cells around lymph nodes. We currently have limited understanding of how these ligands are regulated beyond Th1-inducing vaccination and primary viral infections.

All CXCR3 ligands are strongly induced by IFNγ [1]. In addition each ligand is differentially regulated by type-I IFNs, where CXCL9 is poorly induced by either IFNα or IFNβ, while, both CXCL10 and CXCL11 are induced by these cytokines [1]. During viral infection these differences translate to variances in chemokine expression between primary and secondary challenge. CXCL9 is minimally expressed in primary LCMV infection, but strongly induced in secondary infection due to the increased and rapid induction of IFNγ by memory T cells and NK cells [20,99]. The expression of CXCL9, in addition to CXCL10, in secondary responses further accelerates the rapid relocation of memory cells into the IFRs adjacent to the SCS, thus creating a feed-forward chemokine loop to ensure additional recruitment of memory cells to this site [18,20]. In addition $CD8^+$ T cells themselves may participate in this inflammatory loop, through their own expression of CXCL10 [100]. Importantly, *in vivo*, blocking IFNγ and IFNα has the same effect on T cell location as hosts deficient in CXCL10, illustrating the reliance of these cytokines for ligand induction [20].

Differences in T cell fate changes seen in different infections, such as chronic persistent infections, suggests altered induction and cellular sources of CXCR3 ligands, which varies the migration map for T cells. Indeed it is well established that the lymph node structure is significantly altered in HIV infection [101]. Despite ongoing anti-retroviral therapy, this affects T cell fate decisions following vaccination [102]. Interestingly in this setting CXCR3 is increased $CD4^+$ T cells, and Tfh differentiation was reduced following vaccination [102]. The elevation of IFNα in HIV perturbs CXCL10 expression in a SLAMF7-dependent manner and alters the cellular sources of CXCR3 chemokines [103]. Further, blocking of IFNα during chronic LCMV infection improves $CD4^+$ and $CD8^+$ T cell differentiation defects leading to protection [104]. Combined these studies suggest significant alterations in the regulation of CXCR3 ligands during chronic infection, however this has not be dissected in detail. We have recently studied the inducers of the CXCR3 ligands in DC subsets *in vitro* using REX3 transgenic reporter mice [105]. This work highlighted differences in the potential of cDC1, cDC2 and pDC populations to express either CXCL9 or CXCL10. Specifically, we found that, following TLR7 stimulation, conventional DCs (cDC1 and cDC2s) uniformly upregulate CXCL10. CXCL9 expression was induced in all cDC1s but only in half of the cDC2 cells. Induction of both CXCL9 and CXCL10 in this system was entirely dependent on signaling through IFNAR, as either antibody blocking or receptor deficiency returned chemokine expression to baseline. In contrast to the chemokine induction observed in cDC

populations, production within pDCs was substantially less. No CXCL9 expression was observed in pDCs and stable populations of $CXCL10^{+}$ and $CXCL10^{-}$ were distinguished. Indeed, we show that heterogeneity within DC compartments can be defined by chemokine expression, suggesting that DCs with different chemokine expression potential may differentially contribute to T cell fate decisions *in vivo*. While this is a beneficial approach to understand the induction of chemokine ligands in different cell types, our results also exposed differences in chemokine expression of *in vivo* isolated and *in vitro* derived DC populations [105]. This suggests the critical next step is to determine how these ligands are differentially regulated *in vivo*, following different inflammatory stimulation. These investigations are essential to establish if the induction of these ligands go awry during chronic conditions and contribute to inefficient effector responses during infection, cancer and autoimmune disease. Indeed, perturbations of infection and presence or absence of inflammatory signals not only changes the chemokine map, but will also alter the makeup of chemokine cellular sources [101,103,105,106]. Greater knowledge of the cytokine regulation of chemokine ligand induction in these different settings, may highlight new avenues to return ligand expression to that observed following acute viral infection, where CXCR3 ligands induce a balance of effector and memory T cell differentiation leading to pathogen eradication establish long-lasting protection.

7 Balancing the multiple migration cues that influence T cell fates

Complexity within the chemokine superfamily means that the level of regulation and interactions in three dimensions is difficult to resolve. Indeed, the balance of multiple migration cues is complicated by three separate, but not mutually exclusive, features of the chemokine superfamily (Figure 4).

7.1 Biased ligand signaling within chemokine families

The first major consideration is that the number of chemokines outnumber their receptors leading to competition within individual receptor families [72]. For this reason, it has been somewhat easier to determine overall function of individual chemokine receptors, using knockout reporter mice, rather than pinpointing a specific function for each ligand.

Much of work within individual chemokine systems assumes that multiple ligands for any given receptor act together to promote similar receptor functions. However, in different experimental and disease settings, the role for multiple ligands maybe shared, collaborative, redundant or antagonistic. This is true of the CXCR3 family, where each ligand binds and

interacts with CXCR3 via a unique mechanism and can elicit distinct responses [1]. Therefore, it is the expression of the ligands and not the receptor that ultimately determines T cell differentiation outcomes. As discussed above, this extends the requirement to understand the precise cytokine milieu that differentially upregulates each ligand, by individual cellular players in settings that establish appropriate T cell differentiation [105]. The hierarchy of binding and how this impacts individual, competitive and balanced migration cues within lymph nodes following infection or vaccination is not well understood [1]. The generation of chemokine chimeras in the CCR2 system have proven valuable to determine how selective binding influences [107]. This strategy along with the mutation of receptor binding pockets will proof an elegant way to identify targets of small molecule inhibitors that mediate specific chemokine functions [107,108].

7.2 Bias between chemokine receptors

The complexity of the chemokine superfamily is additionally complicated by the binding of single chemokines to multiple receptors. This means the absence or presence of alternate receptors can impact the signaling and function of chemokine. Recently the impact of this has begun to be appreciated in the context of atypical chemokine receptors. Unlike typical chemokine receptors, these do not couple to G-proteins and therefore do not mediate cell migration. While it was originally proposed that this inhibited migration by mopping up chemokine. It is now clear that atypical chemokines work alongside their typical chemokine receptor counterparts to shape chemokine gradients *in vivo*, in effect improving the directionality of migration[10,109]. Again, a sophisticated approach to determine the function of individual receptors in this setting is the generation of chimeric chemokines with binding specific to an individual receptor. Recently a chimeric chemokine was described that specifically binds to the atypical chemokine receptor ACKR3 (previously CXCR7) [110]. Use of this chemokine both *in vitro* and *in vivo* will help determine the function of this receptor, with the exclusion of interference from both CXCR3 and CXCR4 [110]. Cross talk between CXCR3 and ACKR3 will be important to determine in the context of T cell fate decisions within draining lymph nodes.

7.3 Competitive ligand signaling between multiple chemokine receptors

Finally, T cells do not upregulate chemokine receptors in isolation. During lymph node fate decisions, $CD4^+$ and $CD8^+$ T cells down and upregulate multiple chemokines dynamically following their different cellular encounters. This incompletely understood phenomenon is

critical for our understanding of how T cells sequentially move between different DCs subsets during lymph node priming events. As discussed, often these DC contacts are determined by unique chemokine receptors, making it unclear how T cells move between these DC encounters. Competing receptor expression can also result in the formation of chemokine receptor heterodimers, which exhibit unique binding, internalization and β-arrestin recruitment compared to individual receptors [111,112]. Combined, this complexity in receptor expression may lead T cells to be pushed and pulled toward different cellular interactions depending on the combination of receptors on the cell surface.

Migration of activated T cells out of the paracortex relies on the downregulation of CCR7. This occurs simultaneously with upregulation of inflammatory cells to release ties with the paracortex and facilitate movement into peripheral lymph node zones [4,51,113,114]. Upon activation $CD8^+$ T cells upregulate CXCR3 in concert with CCR5 [52,53,58,70]. How these chemokine receptors either work together (or against each other) to direct cell contacts and determine cell fate outcomes is unclear. Intriguingly, CCR5-dependent interactions may lead to enhanced memory precursor differentiation, while CXCR3-dependent interactions promote short-term effectors [51-53,58,70]. Potentially this indicates that each chemokine family differentially regulates different fate outcomes. Although how this is regulated when both receptors are uniformly upregulated by $CD8^+$ T cells upon activation is unclear. In addition, this differential fate outcomes do not always segregate with each receptor, as Kohlmeier *et al.* has demonstrated a shared pathway to promote effector fate differentiation with a loss of memory [52]. Another example where the expression of multiple receptors play an essential role in the fate decision between Th1 and Tfh cells. Newly activated $CD4^+$ T cells express both CXCR3 and CXCR5 [4,35]. While both may be important to direct cells to the cortical ridge and T:B boarder of draining lymph nodes, what happens beyond this point to reinforce each fate is unclear. As discussed above, work by Shi *et al.* highlights that CXCR3 interactions need to be actively suppressed to allow the Tfh fate [37]. This can occur through PD-1 suppression, however, the presence of $CXCR3^+$ Tfh cells demonstrate this is not always the case. Potentially suppression of CXCR3 ligands in the cortical ridge also allows for entry into GCs some settings.

8. Moving outside the lymph node

While the precise chemokine-expressing cellular partners that facilitate T cell effector differentiation are still be elucidated and optimized for improve vaccine approaches, the opportunity has also arisen to apply our knowledge of lymph node dynamics into peripheral tissues and tumors settings.

8.1 T cell priming in peripheral tissues: generation of efficient memory

Long-term tissue-resident (Trm) cells maintain residence in peripheral tissues at the site of cleared infection and have been identified in the skin, nasal and vaginal mucosa, central nervous system, salivary gland, lung and intestine [115]. This strategic tissue positioning allows Trm an enhanced capacity to fight reinfection over circulating memory T cells [116]. Thus, there is much interest in how to optimize the local differentiation and effector function of Trm cells in order to protect peripheral barrier sites from recurrent infection. While CXCR3-deficient cells show a defect in Trm differentiation and accumulation within the skin following HSV-1 infection, this chemokine system does not appear to play a role in the continued scanning and cellular dynamics following local differentiation [117,118]. However, these study did not determine how CXCR3 may alter local protection upon secondary challenge. However, this has been determined in a study investigating Trm recall responses within the nasal-associated lymphoid tissues (NALT) [119]. While these tissues are not a site for peripheral priming of naïve T cells, recall responses are rapidly induced here in a CXCR3-dependent manner [119]. Interestingly, in addition to providing local protection from reinfection, a recent study demonstrated that Trm cells also play a central role in tumor surveillance and control [120].

8.2 T cell recruitment and priming within tumor microenvironments

A central strategy for the development and improvement of immune checkpoint therapeutics in cancer is to increase the recruitment of NK cells $CD8^+$ T cells to the local tumor microenvironment [121]. Similar mechanisms may regulate the priming of $CD8^+$ effector T by DC populations within lymph nodes and the tumor microenvironment. The targeting of chemokine networks towards to optimize recruitment and NK and T cell effector function within tumors is area of current intense research [121]. The expression of CXCR3 has emerged as a critical regulator of $CD8^+$ T cell recruitment into tumors [122]. Indeed, tumors can down regulate expression of CXCR3 ligands epigenetically, reducing the number of tumor-infiltrating $CD8^+$ T cells and tumor outcomes [75]. However the effect of CXCR3 ligand

expression may depend on the cellular source, as cancer stroma expression of CXCL10 correlates with poor patient survival [123].

The interplay between PD-1 and CXCR3 expression is highly relevant to the functionality of tumor-infiltrating T cells and their response cancer therapeutics. The before mentioned down regulation of CXCR3 by PD-1 during Tfh differentiation, may also occur within the tumor microenvironment, where PD-1 is highly expressed by exhausted $CD8^+$ T cells [37]. However, this may also be reciprocally regulated, as T-bet can repress the induction of PD-1 altering the responsiveness of $CD8^+$ effector T cells during chronic infection [97]. Recently it was determined that anti-PD-1 treatment acts to increase IFNγ and cDC1-produced IL-12 within the tumor microenvironment. Importantly, this is a critical mechanism of therapeutic success [124]. As described above, both these cytokines are essential regulators of CXCR3 and its ligands. Increasing the IFNγ inflammatory milieu of tumors promotes the CXCR3-directed recruitment of chimeric antigen receptor (CAR) T cells [125]. Further, checkpoint inhibition through TIM3 blockade leads to IL-12 induction of CXCL9 by cDC1s and subsequent CXCR3-dependent $CD8^+$ T cell recruitment. Again, this mechanism synergized with chemotherapy to improve tumor clearance [74]. Combined, this work leads to an attractive hypothesis where CXCR3 and its ligands play a critical role in the effectiveness of checkpoint inhibition during cancer therapy. However, how this revolutionary treatment strategy directly impacts intra-tumoral priming of T cells in a CXCR3-dependent manner remains to be fully elucidated. Approaches that increase the expression of CXCR3 by either natural or CAR tumor-specific T cells, and/or increase the expression of CXCR3 ligands in tumor-resident DC populations are likely to yield additional therapeutic benefit.

9. Conclusion

Multiple studies have now demonstrated that CXCR3 migration cues are required for spatial positioning of T cells within draining lymph nodes. For both $CD4^+$ and $CD8^+$ T cells this migration drives T cell:DC interactions and movement out of the T cell paracortex and into peripheral zones of lymph nodes, primarily the IFRs. This relocation and interactions are critical for T cells to become effectors following viral challenge and vaccination. Development of chemokine reporters has and will continue to advance our understanding of these critical events *in vivo* and provide answers as to why this path is required for effector differentiation. Essential future work will (1) dissect how chemokine ligands work

individually and in concert to influence cell fate decisions, (2) describe how the chemokine map that directs fate decisions varies during unique infectious onslaught, (3) determine how to further exploit lymph node targeting for vaccine design, and (4) apply this knowledge outside the lymph node, to enhance effector function within cancers and memory residency within peripheral tissues.

Figure Legends

Figure 1 Intranodal T cell migration determines T cell effector differentiation. (1) Naïve T cells are located in the T cell paracortex, memory cells may either be located here, or adjacent, in the cortical ridge. (2) $CD4^+$ and $CD8^+$ T cells migrate out of the paracortex and into the interfollicular regions (IFRs), between B cell follicles. (3) IFR migration correlates with increased effector function for $CD8^+$ cytotoxic lymphocytes and $CD4^+$ effectors Th1, Tfh and Th2. Chemokine receptors mediating IFR recruitment for each effector lineage is shown.

Figure 2 The use of chemokine reporters is a key strategy to determine cellular source and location of chemokines. Micrograph shows 3D Light sheet of REX3 transgenic mouse inguinal lymph node following LCMV-Armstrong infection (intravenous route, day 6 of infection). Chemokine cellular sources are shown in red (CXCL9) and blue (CXCL10). Lymphatic vessels are shown in white (CD31 staining).

Figure 3 Shared inflammatory cytokines determine the expression of both CXCR3 and its ligands following viral infection and Th1 skewed vaccination. High affinity antigen, IL-12 and IFNγ lead to the upregulation of the transcription factor T-bet, which then directs expression of CXCR3. Combinations of IFNγ and IFNα/β upregulate CXCL10 and CXCL9, where CXCL9 is more strongly induced by IFNγ. Importantly, in alternate inflammatory settings that lack this cytokine milieu, T-bet and CXCR3 and its ligands are not upregulated. However other migration cues in these settings may still move cells into the IFR of draining lymph nodes.

Figure 4 Complexity within the chemokine superfamily that may influence T cell fate decisions. T cells need to integrate and balance multiple migration cues (1) within the same chemokine family, (2) with multiple ligands binding different receptors, with competing

migration outcomes, (3) between different chemokine families when multiple receptors are simultaneously expressed.

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Conflict of Interest

The author has no conflict of interest to declare.

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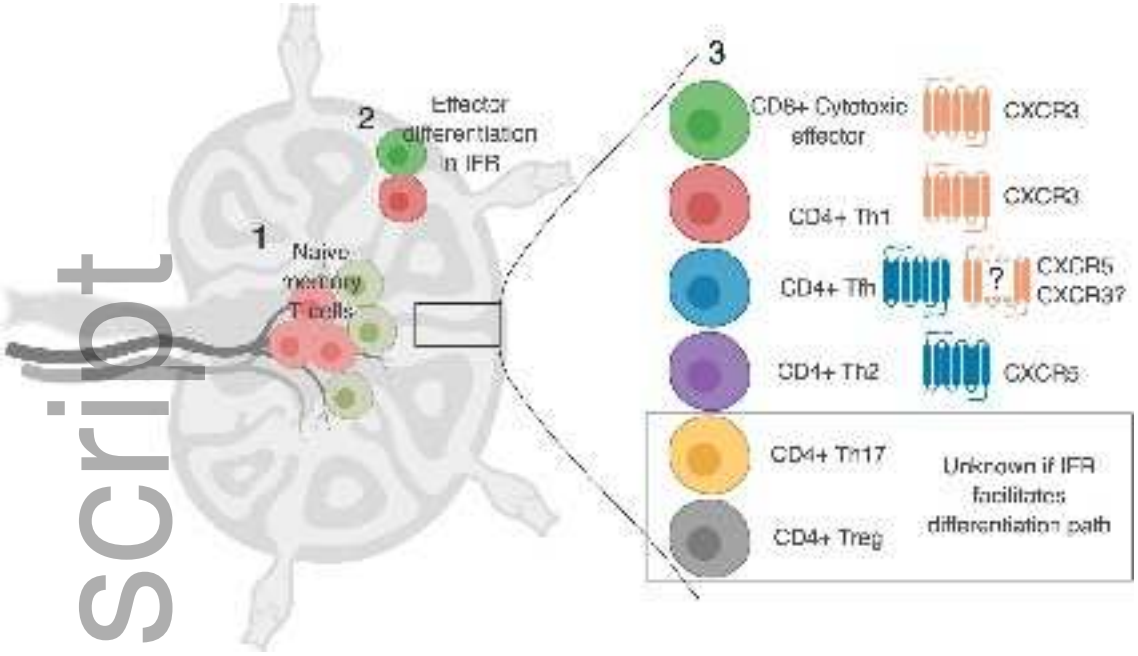

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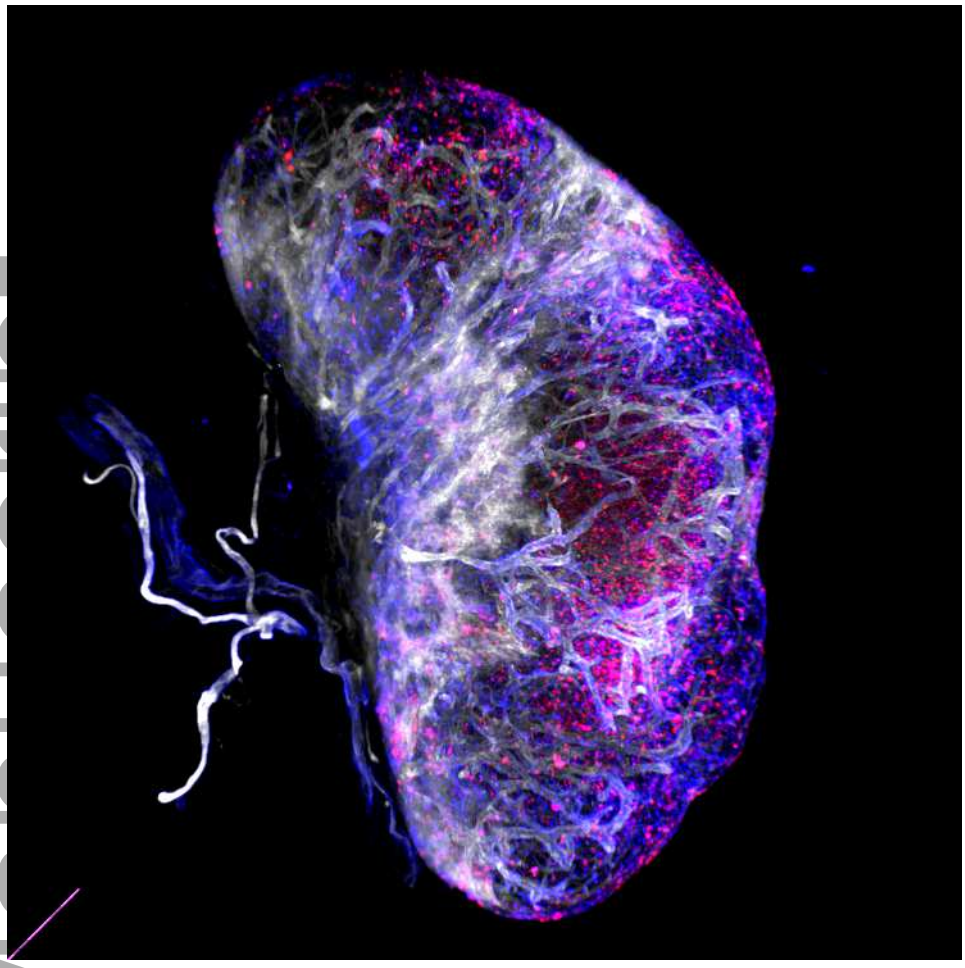

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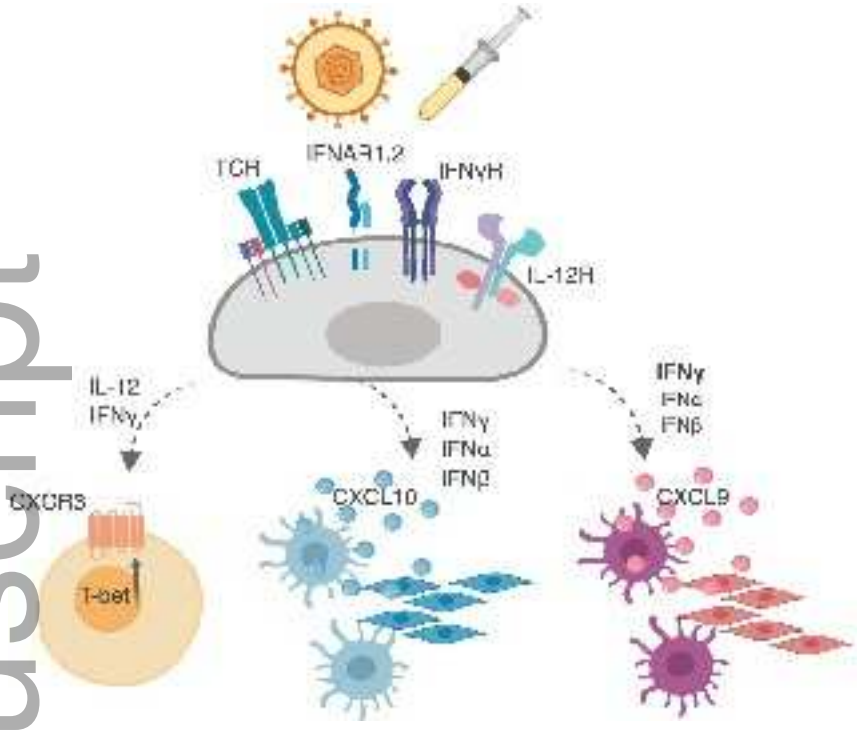

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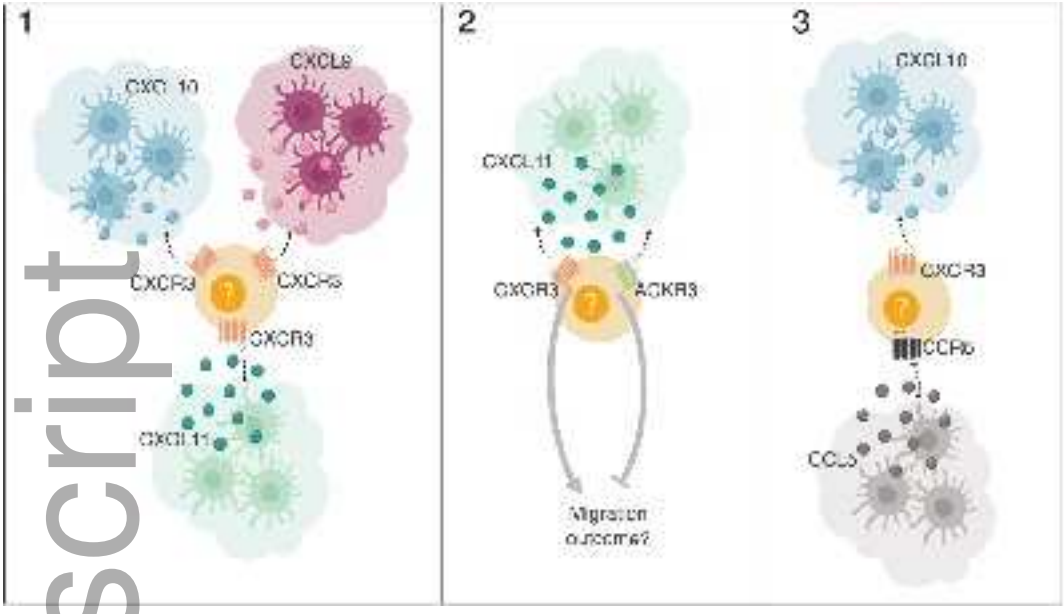

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