Author/s:
Duckworth, BC;Qin, RZ;Groom, JR

Title:
Spatial determinates of effector and memory CD8+ T cell fates*

Date:
2022-03-01

Citation:
Duckworth, B. C., Qin, R. Z. & Groom, J. R. (2022). Spatial determinates of effector and memory CD8+ T cell fates*. Immunological Reviews, 306 (1), pp.76-92. https://doi.org/10.1111/imr.13044.

Persistent Link:
https://hdl.handle.net/11343/299282

DR JOANNA R GROOM (Orcid ID : 0000-0001-5251-7835)

Article type : Invited Review

The spatial determinates of effector and memory CD8$^+$ T cell fates

Brigette C. Duckworth[1,2,3], Raymond Z. Qin[1,2,3] and Joanna R. Groom[1,2].

Affiliations:

[1]Division of Immunology, Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia

[2]Department of Medical Biology, University of Melbourne, Parkville, VIC, Australia

[3]Authors contributed equally

Correspondence should be addressed to Joanna R. Groom

ORCID: http://orcid.org/0000-0001-5251-7835

Email groom@wehi.edu.au

Twitter @groomlab

Running Title: Spatial determinates of CD8$^+$ T cell fate

SUMMARY

The lymph node plays a critical role in mounting an adaptive immune response to infection, clearance of foreign pathogens and cancer immunosurveillance. Within this complex structure, intranodal migration is vital for CD8$^+$ T cell activation and differentiation. Combining tissue clearing and volumetric Light Sheet Fluorescent Microscopy of intact lymph nodes has allowed us to explore the spatial regulation of T cell fates. This has determined that short lived effector (T_{SLEC}) are imprinted in peripheral lymph node

interfollicular regions, due to CXCR3 migration. In contrast, stem-like memory cell (T_{SCM}) differentiation is determined in the T cell paracortex. Here, we detail the inflammatory and chemokine regulators of spatially restricted T cell differentiation, with a focus on how to promote T_{SCM}. We propose a default pathway for T_{SCM} differentiation due to CCR7-directed segregation of precursors away from the inflammatory effector niche. Although volumetric imaging has revealed the consequences of intranodal migration, we still lack knowledge of how this is orchestrated within a complex chemokine environment. Towards this goal, we highlight the potential of combining microfluidic chambers with pre-determined complexity and subcellular resolution microscopy.

Keywords: T cells, Chemokines, Cell Differentiation, Cell Trafficking, Ex Vivo Imaging, Lymph nodes

1. INTRODUCTION

$CD8^+$ T cells are vital effectors of the adaptive immune system and play a crucial role in combating intracellular pathogens and cancers. During primary viral infection, $CD8^+$ T cells form terminally differentiated, short-lived effector cells (T_{SLEC}) to mediate pathogen clearance through production of effector molecules such as interferon-γ (IFNγ), granzyme-B (GZMB) and tumour necrosis factor-α (TNFα). T_{SLEC} also facilitate direct killing of infected cells via perforin/GZMB release or Fas-receptor activation.[1–4] T_{SLEC} differentiation is instructed by the expression the transcription factor T-bet (encoded by Tbx21).[5,6] Alongside T_{SLEC}, memory cells are formed and maintained after pathogen clearance. A potent subpopulation of $CD8^+$ central memory, termed stem-like memory cells (T_{SCM}), defined by expression of L-selection (CD62L) and the transcription factor T cell factor 1 (TCF1, encoded by Tcf7), are programmed to promote self-renewal and repress terminal differentiation.[7,8] Both T_{SLEC} and T_{SCM} offer distinct therapeutic potential. Promotion of effector cells can be utilised during an immediate challenge, such as ongoing infection, or cancer.[9] In contrast, T_{SCM} cells seed the effector cell pool to provide long-term immunity, provide enhanced secondary response to infection and control tumour growth following immunotherapy.[10–13] Thus, harnessing the potential of T_{SCM} offers a new approach to enhance efficacy of vaccines and overcome T cell exhaustion in cancer.[14,15]

The precise positioning, immune interactions and extrinsic factors that control T cell differentiation within lymph nodes is only beginning to be understood. We have recently

combined chemokine reporters with tissue clearing and 3D Light-Sheet Fluorescent Microscopy (LSFM) of intact lymph nodes to determine the spatial requirements of $CD8^+$ T cell fate.[16] In this review we discuss the disparate T cell positioning that imprints T_{SLEC} and T_{SCM} differentiation. In doing so, we highlight imaging advances that have enabled these discoveries. Additionally, we discuss the distinct factors in the effector and memory lymph node niches that promote $CD8^+$ T cell fate bifurcation. We propose a default theory for T_{SCM} differentiation which is established by spatial separation of precursors away from the inflammatory effector niche.

While volumetric imaging has greatly enhanced our understanding of immune cell location and how this specifies function, the fixed nature of this imaging and low-resolution dictates that this method is insufficient to dissect distinct cell migration decisions. Thus, understanding how T cells integrate diverse and competing gradients that underpin intranodal migration and fate decisions is a key ongoing challenge. Towards this goal, we discuss the development and potential of combining microfluidic chambers with pre-determined complexity that mimics the lymph node environment with subcellular resolution microscopy, such as Lattice Light-Sheet Microscopy (LLSM).

2. SPATIAL REQUIREMENTS FOR CD8+ T CELL DIFFERENTIATION

The lymph node is a highly organised structure that serves many roles; to compartmentalize and confine immune cells to distinct regions, facilitate lymphocyte positioning, cellular interactions and presentation of antigen, contain pathogens and maintain immune memory.[17] The lymph node comprises of a collagen rich fibrous outer capsule, beneath which resides the subcapsular sinus, the cortex containing B cell follicles and interfollicular regions (IFRs). Below the cortex lies the cortical ridge, the deep paracortex (T cell zone), and the medulla.[18,19] Within this structure, T cell priming and differentiation occurs in a coordinated and dynamic fashion. These events have been captured using 2-photon imaging which has allowed the nascent events following antigen recognition and T cell priming to be viewed and quantified.[20,21] Following antigen recognition, T cells are primed during close interactions with antigen-specific dendritic cells (DCs)[21–23] As 2-photon microscopy offers limited imaging depth and field of view, the next steps of T cell bifurcation have been complemented with 2D confocal imaging to understand the spatial regulation of this process within the 3D lymph node. This work established that, following viral infection, $CD8^+$ T cells rapidly form clusters with conventional type 2 DCs (cDC2s) in the IFR and cortical ridge of draining

lymph nodes.[24–27] Subsequent to this, $CD8^+$ T cells encounter conventional type 1 DCs (cDC1) in the paracortex and these interactions correlate with differentiation towards memory.[24,25] Combined, this work has revolutionised our understanding of how T cells are activated *in vivo.*[21,28] However, this also raised important questions about how movement around the lymph node is instructed, and ultimately if this positioning was required for the differentiation fates of either effector or memory $CD8^+$ T cells.

2.1 Chemokine receptor requirements for CD8+ T cell differentiation

To address the question of how T cells coordinate intranodal migration to come into contact with distinct DC sources following infection, we adopted an experimental strategy that combined tissue clearing with LSFM.[16] One of the largest issues facing light-based 3D imaging of intact lymph nodes is their complex composition, with biomolecules such as lipids, proteins and nucleic acids causing light absorption and light scattering which interferes with image acquisiton.[29] Optical tissue clearing maintains tissue and cellular morphology, while reducing the lateral scattering of light upon illumination. Light scattering is reduced by minimizing the refractive index mismatches between the immersion media and various tissue components, such as lipids and proteins.[30,31] We and others have used the Ce3D clearing method to conserve endogenously expressed fluorophores and enable additional epitope staining using directly labelled antibodies.[30,32] These features were essential to allow tracking of antigen-specific $CD8^+$ T cells and identify essential lymph node landmarks to identify $B220^+$ B cell follicles and the $CD31^+$ endothelial cell network. Paired with LSFM to optically section intact lymph nodes, these methods provided an integrated, scalable experimental pipeline that overcame the constrains of depth and field of few with 2-photon imaging and reduces tissue damage and loss of landmarks that can be problematic in 2D section-based imaging. As its name suggests, LSFM employs a sheet of light to sequentially illuminate the planes of a sample to provide volumetric imaging.[33] LFSM enables 3D imaging at higher speeds than confocal or multiphoton imaging, with a lower signal to noise ratio.[33] Combining these methods allowed us to determine the regulators of intranodal migration in response to acute, system lymphocytic choriomeningitis virus (LCMV) infection.[16,32,34]

The chemokine receptor CXCR3 is an inflammatory receptor that is rapidly upregulated on T cells following viral infection.[35] CXCR3 is transcriptionally regulated by T-bet following high affinity antigen viral infection.[36,37] CXCR3 had previously been shown to instruct the balance between $CD8^+$ effector and memory fates, making it a prime candidate to investigate

spatial imprinting of $CD8^+$ T cell fate.[38–40] Transferring antigen-specific $CD8^+$ T cells and assessing lymph node positioning six days later, we found a bimodal positioning of cells, with most cells found in the extreme periphery of the lymph node, in the IFRs, while fewer cells were located in the paracortex in the centre of the node. This contrasted to CXCR3-deficient $CD8^+$ T cells, where the majority were retained in the paracortex.[16]

How to quantify cell location in 3D posed a new challenge. Common methods for this adopt strategies similar to those for 2D section-based imaging, such as quantifying cells within a region of a set size and location, or the spatial relationship between cells and structural landmarks within the tissue, such as 'distance to capsule.[24,36,41] To quantify antigen-specific T cell location in 3D, we originally attempted a method similar to Ozga et al. to count cells in specific regions near the lymph node capsule.[36] However, this approach did not consider the non-uniform lymph node shape and expansion over the course of the viral infection. Instead, we adapted the ImageJ script TANGO, a quantification technique based on the measurement of lymph node eroded volume fraction (EVF) to analyse global changes in T cell location in the lymph node. This quantification tool normalizes lymph nodes for to allow for the change in size that occurs during infection. Lymph node images were segmented using manually set global thresholding to define total lymph node volume. The EVF values were then divided into 100 3D layers of equal volumes from 0 (near the lymph node periphery) to 1 (lymph node centre) to create a 3D distance map inside the lymph node.[16] The positioning of antigen-specific T cells was determined within this map. This allowed us to plot the density of $CD8^+$ T cells within the entire lymph node as a normalized curve spanning from centre to periphery. With this tool, we were able to quantify global changes in T cell positioning within an infected lymph node, and directly compare the 3D location of wildtype and CXCR3-deficient $CD8^+$ T cells. This revealed that loss of CXCR3 led to reduced overall T cell migration to the periphery of intact lymph node, with a high proportion of T cells remaining in the T cell paracortex. Combining 2D and 3D imaging with flow cytometry platforms, we established a correlation with the peripheral IFR location and differentiation towards T_{SLEC}, while central paracortex retention, resulted in a specific increase in T_{SCM}.[16] Importantly, we showed that loss of CXCR3 does not lead to an increase in the overall $CD8^+$ central memory population, but rather a specific increase in T_{SCM}. Additionally, we found that WT and CXCR3-deficient T cells diverge in T_{SLEC} and T_{SCM} fates at day six post LCMV infection. This window of opportunity to imprint a specific cell differentiation fate was longer than initially suspected. Combined, a major question for future investigation will involve pinpointing the specific

spatiotemporal requirements for the earliest precursors that seed other members of the heterogeneous memory pool, such as resident memory T cells.[42,43]

Despite distinct positioning and differentiation between WT and CXCR3-deficient T cells, CXCR3 expression alone was insufficient to discriminate T cells that migrate into the IFRs and those that remain in the paracortex.[16] We therefore examined other chemokine receptors that may play a role. The homeostatic chemokine receptor CCR7 is co-expressed on $T_{SCM.}$[44,45] Supporting this role, T_{SCM} cells exhibited greater migration towards the CCR7 ligand, CCL21 than T_{SLEC} *in vitro*. As expected, this migration was unchanged in the CXCR3-deficient cells, suggesting that in the absence of CXCR3, cells were retained in the paracortex, due to CCR7 expression.[16] This work is consistent with previous CCR7 over-expression studies, where the memory fate is expanded.[39] Combined, the use of tissue clearing and LSFM and with reporters to track 3D T cell location established that $CD8^+$ T_{SLEC} and T_{SCM} fates are imprinted in distinct regions and that modulating migration into the lymph node IFR results in increased T_{SCM} differentiation.[16,46]

2.2 Chemokine requirements for CD8+ T cell differentiation

Given the complexity of the CXCR3 chemokine system, where the single receptor, CXCR3, can bind two individual interferon (IFN)-inducible ligands - CXCL9 and CXCL10 - in C57BL/6 mice, it was unclear the precise migration cue that was drawing $CXCR3^+$ cells into the IFR during infection. Here, it is important to note that CXCR3 can also bind CXCL11, however C57BL/6 mice contain a point mutation and a single-base deletion which results in a reading frame shift that introduces a stop codon early within the *Cxcl11* gene, making these mice deficient for CXCL11.[35] Therefore, much of the *in vivo* work on CXCR3 examines the role of CXCL9 and CXCL10 only. To investigate this, we made use of dual reporter mice that identify the ligands of CXCR3, the REX3 transgenic reporters.[47] Generation of these mice have been incredibly useful in visualising the cellular sources of CXCL9 and CXCL10 in multiple infection and inflammatory based settings.[48–50] Additionally, as the expression of CXCL9 and CXCL10 are primarily induced by type I IFNs (IFN-I), this tool has also been used as a spatiotemporal indicator of the IFN inflammatory signature.[51,52]

In our system, use of Ce3D tissue clearing preserved the RFP and BFP expression that identifies the cellular sources of CXCL9 and CXCL10 respectively.[16,47] 3D analysis of REX3 reporter mice following viral infection, revealed that the cells producing these ligands were

positioned in spatially distinct regions of the lymph node. This positioning was quantified using EVF analysis to demonstrate that CXCL10-producing cells positioned in the lymph node periphery in the IFRs. In contrast, CXCL9-producing cells were more evenly distributed through the lymph node with expression in the lymph node paracortex (Figure 1). Using BM chimeras where REX3 cells accounted exclusively for either the haematopoietic compartment or the radio-resistant compartment, we demonstrated that this 3D spatial segregation of the CXCR3 ligands was seen in both compartments.[16] The cellular DC and stromal cell sources of CXCL9 and CXCL10 were identified via flow cytometry. Interestingly, cDC2s primarily produced CXCL10 over the course of infection, while cDC1 cells had a strong preference for CXCL9 expression. The spatial distribution and cellular sources were confirmed using 2D section based confocal microscopy to identify CXCL10-producing cDC2s in the IFR, and CXCL9-producing cDC1s in the paracortex.[16] In the stromal cell compartment, fibroblastic reticular cells and lymphatic endothelial cells showed high expression of both chemokines, but with a preference of higher CXCL10. This was confirmed by confocal microscopy which demonstrated high CXCL10 expression by $CD31^{+}LYVE\text{-}1^{+}$ lymphatic endothelial cells in found in the IFR. Consistent with this spatiotemporal regulation of CXCR3 ligands, analysis of $CD8^{+}$ T cell positioning and differentiation demonstrated that CXCL10 is the ligand responsible for effector cell recruitment into the IFR. Importantly, in the absence of host CXCL10, $CD8^{+}$ T cells accumulate in the paracortex, similar with CXCR3-deficiency, and differentiation of T_{SCM} is promoted. Confirming the connection between 3D $CD8^{+}$ T cell positioning and the imprinting of cell fate in distinct lymph node niches (Figure 1).[16]

The spatially distinct expression of CXCR3 ligands was surprising in several ways. Firstly, these ligands are generally thought to be induced via similar IFN cytokines.[35] Indeed, we have shown in an *in vitro* system that blocking IFN-I suppresses the over 80% of CXCL9 and CXCL10 in cDC1 and cDC2 cells following TLR7 stimulation.[52] Secondly, these chemokine ligands are generally thought to act in a collaborative manner.[35,47,51] Despite this, some differences have been described for the upregulation of CXCL9 and CXCL10.[53] This disparity relates to CXCL9 being more strongly induced by IFNγ, relative to IFN-I.[41] Combined, this suggests that there may be niches within lymph nodes where distinct IFN cytokines accumulate following viral infection, and that this determines the upregulation of individual CXCR3 ligands.

In contrast to CXCL10, the expression of CXCL9 in either the haematopoietic compartment or the radio-resistant compartment did not significantly contribute to $CD8^+$ T cell positioning or determination of fate. These observations led to our investigation of CCR7, which binds the ligands CCL19 and CCL21.[54] As discussed below, these ligands are presented in the lymph node distinctly. While we tested the responsiveness of T_{SCM} to CCL21 *in vitro*, we did not define if one or both ligands was required for T cell location *in vivo*. The requirement for these chemokines in retaining memory may also explain observations in the *plt/plt* (paucity of lymph node T cells) mice, which lack CCR7 ligands but demonstrate increased effector differentiation.[55] Presumably these T cells would be positioned deep in the IFRs of draining nodes of *plt/plt* mice.

2.3 Transcriptional requirements for intranodal T cell position

The upregulation of the transcriptional regulator, T-bet has long been associated with antigen-specific priming of T cells.[56] More recently, its role as a molecular switch to specify $CD8^+$ effector cell differentiation has been established.[5,6] To investigate this, we used the ZsGreen_T-bet reporter to track effector differentiation in the 3D lymph node.[57] This showed that three days post infection, the very first cells committed to effector differentiation were already located within the IFR at the periphery of the 3D lymph node.[16] CXCR3 is a key transcriptional target of T-bet and deficiency in either of these phenocopies the other, suggesting a shared mechanism in the regulating $CD8^+$ effector differentiation.[5,35,39,40] In searching for a mechanism for T_{SCM} retention in the lymph node centre, we followed a similar path of investigation to identify that the transcriptional regulator of T_{SCM}, TCF1, also regulated the chemokine receptor, CCR7 that reinforced this differentiation.[16,58] Together this work identified the transcriptional regulation for T cell location within distinct lymph node niches.[46]

3. SPATIAL MODULATION OF CELL FATE

Our work highlights a tension between the differentiation of T_{SLEC} and T_{SCM}, such that in the absence of effector differentiation, there is a reciprocal increase in memory formation. We identified multiple levers for this process; CXCR3 expression, CXCL10 and IFN-I expression.[16] Deficiency in each of these migration and cytokine cues results in retention of cells within the T cell paracortex and increases in T_{SCM}. In contrast, the migration to and positioning within the IFR facilitates differentiation towards T_{SLEC} differentiation. Many of the potential cellular contacts and inflammatory cues that specify effector differentiation of

both $CD4^+$ and $CD8^+$ T cells in the IFRs have been reviewed elsewhere.[18,53,59] Given the therapeutic potential of T_{SCM} and their role in both chronic infection and cancer, a more critical question is to understand the fate decisions occurring in the lymph node centre. In this section, we discuss the comparison of the modulators of T cell fate in spatially distinct lymph node regions, with a focus on understand the factors that imprint T_{SCM}.

3.1 Modulation of fate by antigen load and inflammation.

During viral infection, antigen arrives to the lymph node via two distinct, but not mutually exclusive paths. Firstly, virions can directly drain into the lymph node subcapsular sinus (SCS) via the afferent lymphatics. This leads to uptake by SCS macrophages and lymph node resident DCs that enter the IFRs to travel deeper into the lymph node through reticular conduits to the paracortex.[18,26,60,61] In the case of acute LCMV infection with promiscuous host tropism, these events lead to local infection of the in the IFR, increased viral load and local inflammation.[60] Alternatively, migratory DCs sample antigen in the peripheral tissues and traffic to the lymph node. DCs transmigrate though the SCS, into the IFRs via CCL21 gradients.[62] Both of these events concentrate the initial location of viral antigens to the IFRs.[27] Increased antigen dose and affinity have been shown to yield a larger effector pool.[36,63] Specifically, Ozga et al. demonstrated that $CD8^+$ T cells migrate towards IFR in a manner that is dependent on high antigen affinity.[36] While our study used T cell receptor (TCR) transgenic $CD8^+$ T cells, such that all cells responded with the consistent affinity for antigen, migration into the IFR may similarly correlate with antigen dose. Combined, these previous studies and ours suggest that early deposition of viral antigens in the IFR acts to imprint effector differentiation in this location.

During infection, the IFR is an inflammatory site with high antigen load and inflammatory cytokines.[26,51,60] We investigated the role of IFN-I as a predominant inducer of CXCR3 ligands that directs $CD8^+$ differentiation away from T_{SCM}.[16,44] LCMV-induced $CD8^+$ T cell differentiation was skewed away from T_{SLEC} and towards T_{SCM} differentiation in host mice deficient for the IFN-I receptor, IFNAR. Cleared, LSFM imaged lymph nodes identified this altered differentiation was associated with increased retention of $CD8^+$ T cells in the central paracortex of $IFNAR^{-/-}$ lymph nodes. This further demonstrates that T_{SLEC} and T_{SCM} fates can be directed by extrinsic factors, and that limiting the inflammatory environment of T cells promotes T_{SCM}.[16] Importantly, we demonstrated that restricting IFN-I during primary viral infection imprints increased stemness and robust secondary responses, even when IFNAR

was intact during these proliferative and secondary challenges. Thus, further demonstrating the therapeutic potential of tuning specific T cell precursors blocking IFN-I, increases both the numbers of T_{SCM} and their functional capacity.[16]

Along with IFN-I, other inflammatory cytokines balance $CD4^+$ and $CD8^+$ fates. [59,64] Type II IFN, IFNγ along with IL-2 and IL-12 promote effector differentiation and limit the expansion of $CD8^+$ memory.[65,66] IL-2 decreases the formation of memory $CD8^+$ T cells in a dose dependent manner, and IL-2R deficiency leads to a reduced effector pool.[67,68] IL-12 elicits a similar graded response between $CD8^+$ T effector and memory formation.[65,69] IL-2 and IL-12 and possibly other inflammatory cytokines, such as IL-12, IL-6, and IFNγ may act together at the time of T cell priming, or shortly after, to impair the formation of memory.[70,71] The before mentioned increase in local viral load in IFRs suggests that multiple inflammatory cytokines may be produced in distinct niches, within inflamed lymph nodes. The identification of an axis between spatially separated fates leads to an intriguing hypothesis; that differentiation to T_{SCM} is established as a default state due to decreased inflammation via the retention in the paracortex, rather than any specific memory-inducing network.[46] Therefore, the continued expression of CCR7 may act primarily to keep T_{SCM} precursors separated from the IFR. Thus, T cell differentiation orchestrated in distinct regions, would establish T_{SCM} differentiation as a default pathway that is amplified in the absence of inflammatory cues (Figure 2). This concept would establish spatiotemporal regulation as a necessary source of heterogeneity within the $CD8^+$ T cell response, and highlights similarities with B cells, where a default pathway for memory differentiation has also been proposed.[72]

3.2 Modulation of fate by distinct cell contacts.

The discovery that $CD8^+$ T cell fates are imprinted in distinct lymph node regions, leads to questions regarding the nature of dynamic interactions that may take place each niche. The cellular partners that directly interact with $CD8^+$ T cells in a niche-dependent manner have not yet been identified.[16] However, we did demonstrate that the chemokine-producing conventional DC populations that dominate in the IFR and paracortex are distinct as are their preferential expression for either CXCL9 or CXCL10. In addition to different chemokine expression, these DC populations also deliver distinct co-stimulation.[73]

We observed that while cDC2 cells present in the IFRs predominantly expressed CXCL10, it was stromal cells lacking CXCL10 that promoted T_{SCM}. It is likely that these stromal cells

produce the bulk of chemokine to draw T cells into the IFR. Thus, this observation doesn't discount direct interaction between $CD8^+$ T cell effectors and CXCL10-expressng cDC2 cells. Indeed, several studies have used dynamic 2-photon imaging to demonstrate that direct clustering of $CD8^+$ and cDC2s promotes effector differentiation.[24,25] Furthermore, using a DC transfer model, we previously observed that the number and duration of T cell:DC interactions during priming was increased by DC derived CXCL10.[47] Although this study pre-dated defining the DC-cellular sources of CXCR3 ligands, given our recent findings, it is likely that these interactions were with cDC2 cells. Together this highlights the importance of cDC2 derived CXCL10 in T cell priming effector cells. In contrast, cDC1s in the lymph node paracortex predominantly produced CXCL9. Hor et al. and Erickson et al. demonstrated direct clustering of memory precursors around cDC1s, however it currently remains unclear if this interaction is essential for memory formation or if cDC1-CXCL9 expression plays any role in mediating these interactions.[24,25] Certainly, we found no evidence of a direct role for CXCL9 in mediating $T_{SCM.}$[16] There are several experimental strategies that could be used in future work to identify the direct interaction between T_{SCM} precursors and cDC1 cells in the paracortex.[46] The ligands of CCR5 have also been shown to promote $CD8^+$ T cell:DC interactions that promote memory differentiation.[26,74,75] However, similar to our observations with CXCL9, disrupting these does not alter memory differentiation, suggesting either no role, or redundant roles for chemokines in mediating these interactions.[76]

In addition to DC interactions, innate lymphocytes are found within distinct regions of the lymph nodes. NKT, NK, and γδT and innate lymphocytes that produce IL-17-producing innate cells can all be found principally in the IFRs.[77,78] Although the role of these in $CD8^+$ bifurcation is largely unexplored, it is worth noting that these innate lymphocytes sparse in the T cell paracortex, again suggesting disparate levels of inflammatory mediators in this region.[79]

The stromal cell compartment is a major producer of lymph node chemokines. How stromal cells support $CD8^+$ T cell differentiation is still relatively unclear. Some studies indicate a context-dependent role for fibroblastic reticular cells, where they can either promote $CD8^+$ T cell differentiation to effectors or to support long lived memory formation.[80] This area would benefit from the identification of distinct populations of cells where defined functions can established. Towards this goal, several studies have used scRNA to dissect the lymph node stromal cell compartment.[48,81] One of these characterised a distinct population of fibroblastic

reticular cells that are identified by *Grem1* expression and are positioned in the lymph node cortical ridge and IFR.[68] Deletion of this population impacted *in vivo* $CD8^+$ T cell proliferation, although no markers of differentiation were included in this study. This result may be indirect regulation, as deficiency in *Grem1*-stromal cells directly supported DC homeostasis and lymph node retention. Indeed, given the essential role of the fibroblastic network in supporting interactions between T and DCs, this plays a primary role in influencing T cell fate.[82–84] However, a population of CCL21-expressing $gp38^+$ fibroblastic reticular cells located in the splenic T cell zone can directly contact $CD8^+$ memory cells.[85] Our work suggests that this, or a similar population, is also present in the lymph node to support T_{SCM}.

Although multiple factors including antigen deposition, DC subsets and innate partners, and inflammatory cytokines can skew $CD8^+$ effector and memory differentiation, those that amplify T_{SCM} are predominantly ones that block inflammatory effector formation, resulting a default memory pathway (Figure 2). Given this, the interactions between CCR7 and stromal-produced CCL19 or CCL21 are the most likely to directly foster the differentiation towards T_{SCM}. Therefore, it is essential to understand how T cells integrate and respond to the multiple chemokine signals present in the lymph node during infection.

4. T CELL INTERGRATION OF COMPLEX MIGRATION CUES

Our work suggests that the CXCR3 axis and the cytokines that induce CXCR3 ligands can be used as levers to modulate T cell fate in a therapeutically tractable manner. Still, many essential points remain unknown regarding how complex migration cues are integrated by individual T cells to drive migration to distinct locations.

A key issue in understanding how cells interpret migration cues to make decisions on cell positioning is the inherent complexity of the chemokine superfamily. Combined there are approximately 20 chemokine receptors that bind 50 ligands.[54] An additional layer of complexity is added by the existence of four atypical chemokine receptors that act in part to establish chemokine gradients.[86–88] It has been proposed that this complexity leads to redundancy of many chemokine ligands, and why, despite promise, limited chemokine therapeutics have reached clinical usage.[54] However, redundancy may not be the only outcome of chemokine superfamily complexity. An alternative hypothesis, that there is exquisite specificity between ligands and their receptors has also been proposed.[89] This

specificity can be achieved by distinct spatial and cellular sources of chemokines, or individual structural elements that determine how a chemokine is presented and gradients are formed within tissues. As discussed, we have demonstrated that CXCL9 and CXCL10 are expressed in spatially distinct lymph node regions, by disparate cell types.[16] Much of this work has been based on observations using REX3 reporter mice.[47] These reporters precisely identify the individual cells that produce both CXCL9 and CXCL10, but how these chemokines are distributed across the tissue is unknown (Figure 1). Furthermore, mechanistically, why T cells destined to become $CD8^+$ T cell effectors preferentially follow CXCL10 gradients, is unclear.

4. 1 Chemokine binding and gradient formation

Chemokines can be soluble or insoluble and some exist in both forms.[90] Soluble chemokines that are released from their cellular source passively diffuse across extracellular space and form gradients with higher concentration at the source and lower concentrations with distance. In contrast, insoluble chemokine can be presented on cell surface of its cellular source, or as it diffuses within the tissue, binds to glycosaminoglycan (GAG) extracellular matrix or stromal cells.[91]

Within CXCR3 family, CXCL9 has a separate C-terminus GAG and N-terminus CXCR3 binding domains, whereas CXCL10 has partially overlapping GAG binding and CXCR3 binding domains.[92,93] The third member CXCL11, although not expressed in C57BL/6 mice,, also binds to GAG and induces most potent CXCR3 desensitization.[94–96] In addition, these chemokines are subject to post-translational modifications by matrix metalloproteases, which alters binding affinity to CXCR3 and GAG.[94] The details of these modifications have been reviewed elsewhere.[97] These disparities suggest distinct binding properties of CXCR3 chemokines to the extracellular matrix and lead to individual ligand function.[53] It is suggested that MyD88 signalling interference in liver sinusoidal endothelial cells eliminates CXCL9 gradient by modifying GAG, which consequently disrupts immune niches for resident immune cell localization and function.[50] A compensatory increase in CXCL10 and CXCL11 expression was observed, but cannot re-establish the niches, indicating the importance of GAG-dependent chemokine gradient and specificity. Further research could investigate CXCR3 chemokines binding and gradient in other settings such as the lymph node, where they also play a role in establishing immune niches.[16]

Similar to CXCR3, the ligands of CCR7 bind the extracellular matrix in distinct ways. Early work demonstrated that CCL21, together with CXCL12 and CXCL13, binds to collagen IV at basal lamina of HEVs.[98] This binding promotes naïve T cells adhesion and homing into the lymph node paracortex. CCL21 forms an increasing gradient from the T cell:B cell border and is maintained at similar level within paracortex and cortical ridge.[99] The increasing CCL21 gradient is also established from IFR to the cortical ridge, which is shaped by the atypical receptor ACKR4 (previously CCRL1).[100] Deficiency in ACKR4 allows CCL21 to fill the IFR niche, which interferes with DC migration and entry into the lymph node migration.[100] It is likely this effect extends to defects in T cell differentiation, as transgenic overexpression of CCL21 results in reduced T cell chemotaxis and defects in LCMV clearance.[101] Further, CCL21 has recently been shown to exist in two forms, a full-length insoluble and a cleaved soluble form. Both of these forms can be scavenged by ACKR4 to establish gradients.[102] In contrast, the other CCR7 receptor ligand, CCL19, is predominantly expressed by lymph node fibroblastic reticular cells.[103,104] Importantly, CCL19 exists only in soluble form, leading to increased CCR7 desensitization and reduced chemotaxis (Figure 1).[105,106] Ablation of CCL19 expressing lymphoid stromal cells results in impaired T cell responses against viral infection.[107] Further studies from the Sixt group directly compared the properties and functions of CCL21 and CCL19, indicating their distinct roles in regulating DC migration and immune responses.[108,109] This work provides the strongest evidence to date that differences between soluble and insoluble chemokine gradients lead to distinct integration of migration cues.

4.2 Chemokine detection

An alternative to visualising chemokine sources using reporter animals, such as REX3 mice, is to use antibody labelling and imaging to directly visualise the chemokine location. While detecting chemokines can be difficult, multiple studies have identified the presence of chemokines in various disease and tissue settings.[110–113] For insoluble CCL21, this has also identified gradients and binding to the extracellular matrix across a large region.[99,100,112,114,115] However, for chemokines that exist only in soluble forms, such as CCL19, the detection is less optimal with antibody staining.[116] Information and quantification with respect to the distribution and location of CXCR3 chemokines in extracellular space is still limited. A recent study shows that CXCL9 and CXCL10 levels increase in the B cell follicle of lymph nodes during influenza infection, which stimulates $CD4^+$ T cells to migrate to this T cell:B cell border to provide B cell help. The authors quantify chemokine concentration by

normalising the fluorescent intensity of CXCL9/10 antibody over time.[115] While other studies have focused on chemokine reporter or chemokine deficient models to understand the functional outcome of chemokines, the spatiotemporal regulation of chemokines established in this study provides a platform to investigate T cell migration within lymph nodes, and how chemokine gradients are formed and impact determine cell fate decisions.[115] This antibody staining method has been adapted to preserve chemokines produced by endothelial cells within the tumour microenvironment. Mikucki et al. used intravital injection of chemokine antibody conjugated to microbeads to identify CXCL9 and CXCL10 expression on tumour blood vessels.[117] Visualisation of CXCR3 chemokines in this context demonstrated their role in T cell transmigration across endothelial cells into tumour.[117] To date, studies detecting chemokine distribution have been acquired without volumetric imaging. Instead, this work provides gradient information across a single plane [83] or along a line[82], however this does not fully reflect distribution in a 3D organ. [100,118,119]

Recent studies have offered new strategies to overcome limitations of chemokine detection. An adapted proximity ligation assay was used to effectively amplify CXCL12 chemokine binding signalling by linking a repetitive DNA sequence to the staining antibody and detecting this sequence with fluorescently labelled oligonucleotides.[120] Promisingly, this approach allows for 3D imaging and analysis in a range of tissues.[120] This protocol allowed for higher sensitivity of detection for each chemokine molecule, but with lower resolution, due to the steric hinderance generated in the proximity ligation assay. Three-dimensional analysis provided additional insight to CXCL12 distribution within the bone marrow. Instead of forming a long-range gradient, CXCL12 molecules were shown to accumulate in distinct niches.[120] Another study used computational modelling to map CXCL13 producing cells in tonsils and define the chemokine gradients in their microenvironment. This showed that due to GAG-CXCL13 interaction, the model with each cell generating insoluble CXCL13 gradient is more similar to physiological environment compared to linear diffusion gradient.[121] Combined these advances in 3D imaging and analysis for chemokine distribution have potential to improve the accuracy and robustness of chemokine detection. Applying these methods to the CXCR3 and CCR7 families within a lymph node, will enable mapping of the complex chemokine patterns from all angles of a cell, to understand or predict migration trajectories during infection or cancer.

Fluorescently labelled chemokines are often employed to visualise chemokine uptake and receptor internalisation by atypical or classical chemokine receptors in tissues.[122] This enables the identification of cells that present and form chemokine gradients and establish the role these cells play in modifying cell chemotaxis in tissues. Binding of fluorescent chemokines to cell surface receptors can also visualise receptor movements and internalisation kinetics in different chemokine and extracellular environments. In epithelial-like CHO-K1 cells, GAG has been identified as an important coreceptor for CCR7 and CXCR4 to facilitate effective chemokine binding to receptor.[122] An adaption of this method is the use of fluorescent-activatable-AND-gate chemokine, which allows detection of functional receptor activity intracellularly. Compared to fluorescently labelled chemokines, fluorescent-activatable-AND-gate chemokines identify only the activated target cell, allowing differentiation of chemokine binding to conventional and atypical chemokine receptors.[123] Combined, fluorescently labelled chemokines provide useful tools to understand chemokine-receptor and GAG-chemokine binding, while preserving chemokine functions on target cells both *in vitro* and *in vivo*.

5. COMPLEXITY OF MIGRATION CUES IN 3D

There exist multiple levels of chemokine complexity within 3D tissues.[53,90,124,125] First, lymphocytes can express multiple chemokine receptors at the same time and each receptor can bind to multiple ligands.[90] The co-expression of CXCR3 and CCR7 on T_{SCM} is an important example of this.[16] An additional layer of complexity is that co-expressed competing receptor expression can also form chemokine receptor heterodimers, which exhibit unique binding, internalization and β-arrestin recruitment compared to individual receptors.[126,127] The cellular sources of CXCR3 and CCR7 chemokines, which total four primary chemokines in C57BL/6 mice, are varied, meaning chemokines are released in different lymphoid regions and establish complex, 3D gradients.[16] In this setting, the highest level of chemokine is predicted to be close to the cellular source of each chemokine. However, as discussed, stromal cells and some immune cells express atypical chemokine receptors that act as scavengers to bind, internalise and degrade respective chemokines without generating a migratory response.[86–88] This efficiently reduces chemokine concentration in a precise region and generates a negative gradient, thus promoting directional cell migration away from that region. In addition, allosteric chemokines such as CXCL14, act in concert with existing chemokine gradients to promote chemotaxis, while having no individual effect.[128] Combined, lymphocytes need to incorporate multiple chemokine signals from different directions and

make a collective decision on migratory direction. It is not well understood, how T cells make these decisions (Figure 1).

More than just the overall number of distinct chemokines and receptors, the distinct presentation of chemokines to cells likely plays an important role in establishing migration response hierarchy. As discussed, these differences exist both with chemokine families that bind the same receptor and between chemokines that bind distinct receptors. GAG-chemokine interactions, such as chemokine binding of heparan sulphate, have increasingly been seen as an important factor for regulating chemokine distribution and oligomerisation.[50,129] However, our understanding of the binding capacities of individual chemokines to GAGs is still in its infancy. Chemokine binding also occurs to other extracellular structural components, such as collagen IV.[98] Chemokine binding alters the gradient distribution from diffusive to partially immobilised, increasing gradient stability and potentially increasing the steepness gradient.[121] Binding to the extracellular matrix also prevents chemokines from cleavage by protease and prolongs their functionality.[130] The presentation of immobilized chemokines by GAGs and other extracellular matrix components creates an additional level of complexity. The cellular dynamics of chemotaxis (migration towards a soluble cue) or haptotaxis (migration towards an immobilised cue) differ.[116,131] This regulation is not static, indeed along with changes in chemokine and chemokine receptor expression, the extracellular matrix of the lymph node is also modified during the course of infection.[132] In turn, this alters both chemokine and antigen binding and diffusion, potentially causing less efficient directional cell migration. Cancer also modifies tissue structure and chemokine composition to a large extent.[133] For example, GAG composition can be altered within some tumours leading to a lower heparan sulphate profile on cell surface.[134,135] This feature disrupts normal chemokine binding and promote cancer cell metastasis.[136–138] In addition to this, within tumours chemokine or chemokine receptors can be additionally influenced by anti-inflammatory cytokines or inhibitory checkpoint signals.[133] Therefore, it is unclear how chemokine distribution is altered in an infection- or disease-dependent manner and promotes lymphocyte migration to either appropriate or inappropriate locations to guide cell-cell interactions for the generation of a tailored or inhibited immune response.[115]

6. DECONSTRUCTION OF LYMPH NODE TO UNDERSTAND T CELL MIGRATION DYNAMICS

Cell migration is a fundamental process in immunology. While other cellular systems are spatially restricted, the immune system inherently relies on chemotaxis to be effective. Upon stimulation by chemokine, the respective receptor initiates a signal cascade that results in cellular cytoskeleton changes. These changes together lead to cell migration or chemotaxis towards the target cell or region within the lymph node.[139,140] The lymph node microenvironment is composed of highly complex tissue and multiple migration cues. This presents many challenges in adapting microscopy to the optically incompatible environment to study cell migration dynamics directly, in high resolution and *in vivo*. The location of critical migration events is beyond the reach of 2-photon imaging. While the development of 3-photon microscopy will greatly increase the depth of imaging, this remains a relatively low-resolution platform. We and others have demonstrated the utility of volumetric imaging to determine the consequence of cell migration (Figure 3).[16,36,141] However, these studies pose questions regarding motility, timing and the integration of complex cues to determine the underlying mechanisms that lead to cell migration. Investigating this will require updated experimental platforms to image high resolution dynamic cell migration. This section will explore cell migration research that deconstructs the lymphatic system using *in vitro* devices and high resolution imaging. Combined, these will allow us to convert what we have learnt from tissue level imaging, to dissect critical migration scenarios in *in vitro* experiments and then apply these learnings back into the tissue.

6.1 Capturing cells in motion

Multiple microscope platforms enable the visualisation and analysis of moving cells in 2D and 3D.[142] Early imaging work by 2-photon microscopy enabled observation of cell behaviours in intact organs or tissues *in vivo* or *ex vivo*.[143] Issues arise when imaging cell motility in whole tissues with a complex network of structures, components and density, such as the lymph node. Due to recent advances in high resolution microscopy, such as the Lattice Light-Sheet Microscopy (LLSM), it is now possible to investigate cell migration dynamics at subcellular resolution, in 4D with minimal phototoxicity (Figure 3).[144] Using the LLSM, Cai et al demonstrated that T cells survey the majority of a DC's surface within one minute to detect specific antigen and potentially form an immunological synapse.[145] Small membrane extensions known as microvilli are observed during this process, formed by actin polymerisation and are essential for contact and effective scanning of the DC surface. As the antigen-TCR interaction establishes, microvilli act as a hub for TCR clustering and recruitment of signalling molecules.[146] Higher antigen specificity forms stronger and shorter

bonds with the respective TCR, leading to extensive clustering of TCR and associated signalling molecules.[147] This result was validated using LLSM and Total Internal Reflection Fluorescence Microscopy (TIRF) to show that TCR clusters indicate T cell activation states and antigen strength. This study used high spatiotemporal resolution to identify the sequence of signal transduction events and precise timing of Ca^{2+} flux.[148] Furthermore, the authors developed a robust and automatic analytical pipeline to establish the correlation discovered in this study. However, how the signal strength affects the efficiency of T cell survey and the mechanism that leads to differentiation outcome is currently unknown. Importantly, these observations are linked to the potential of cells to migrate, as antigen-signal strength, plays a central role in the upregulation of CXCR3 and positioning and intranodal migration.[149] Combined, these studies demonstrate the underexplored utility in combining *in vitro* and *ex vivo* analysis with high and low resolution microscopy platforms to understand complex immune cell interactions (Figure 3). This same combined effort will be fruitful in addressing multiple questions regarding cell morphology or intracellular cytoskeleton organisation as the T cell becomes activated and migrates. Given the different chemokine receptor expression, this dynamic cell migration may be distinct for T_{SLEC} and T_{SCM} cells and how chemokines are presented to them.[150]

The clearance of infected or cancerous tissues also requires antigen recognition and migration. As naïve $CD8^+$ T cells differentiate into effector cytotoxic T cells, they migrate to peripheral tissues and induce apoptosis in infected or cancerous cells. High spatiotemporal *in vitro* imaging by LLSM has detailed the process and timing of antigen recognition and cytotoxic granule secretion.[151] Similar to T cell:DC interactions, the TCR forms clusters at T cell-cancer synapse. The actin cytoskeleton is found to be indispensable for cytotoxic granule secretion and can act as an indicator for antigen binding strength. Although higher antigen specificity can lead to faster Ca^{2+} flux, the sequence of events in antigen recognition is identical regardless of its specificity.[152] This series of studies highlight the essential roles 4D imaging plays in our understanding of effector T cell function, which provides critical information to potentially optimise immunotherapy such as CAR T cell therapy. The exquisitely high resolution of the LLSM platform allows these same imaging and analysis pipelines to be repurposed towards understanding how to cells reach their infected or cancerous targets. However, this approach needs to be paired with a highly reproducible assay to establish complex migration gradients.

6.2 Establishing gradients for cell migration imaging

Chemokine gradients are the major factor in guiding directional cell migration. As discussed above, multiple factors are at play within a tissue to establish complex gradients. Chemokines may diffuse passively in soluble form or bind to the extracellular matrix as an insoluble gradient. In addition, the lymph node is a high-density structure, which creates disunified refractive index for light beams, and limits the resolution and depth of dynamic *in vivo* imaging.[143] Therefore, new methods are required to replicate physiological tissue environments in an *in vitro* system where the environment is controlled and homogeneous. By deconstructing the imaging environment, there is a potential to achieve higher spatiotemporal resolution.

In the early 1960s, the Boyden Chamber or transwell assay was invented to compare the chemotactic preferences between chemokine concentrations, different chemokines or cellular subsets.[153] However, individual cell trajectory and morphology are not assessable in this device. Microfluidic chambers improve on this system introduce a small amount of fluid and chemical gradient to cell culture or cell migration systems. The development of microfluidic chips and assays allows the deconstruction physiological tissues or organs allows controlled questions about cell migration to be asked in a system with pre-determined complexity.[154,155] These devices can be used to form chemokine gradients that mimic those found in tissues such as the lymph node.[154] This enables the chemokine environment to be precisely controlled *in vitro* so that dynamic T cell responses to gradients one or two chemokines can be assessed.[156,157] These systems use transparent materials that are compatible with high resolution, live imaging, such as the commercially available Ibidi chambers.[158] These are generally highly reproducible devices, which allow researchers to establish a consistent chemokine gradient for cells to migrate in. Microfluidic devices also are compatible with high resolution microscopes by attaching glass coverslip onto the device.[159] The incorporation of polydimethylsiloxane material in the manufacture of microfluidic chambers has further advanced the field by increasing the capacity to alter device shape and dimension.[160,161] This allows bespoke designs that allow individual migration questions to be assessed, such as the use pillar arrays, microchannel devices and the formation of persistent rising gradients.[157,162,163] For other studies, collagen matrix and other hydrogels can be incorporated to form porous 3D structures in chamber through which cells can navigate.

Recent modifications of single chemokine gradients include converting vertical migration

pores to horizontal migration chamber, formation of more consistent gradient and improved compatibility for microscopy imaging.[164–166] Currently, many cell migration studies focus on cell kinetics and migration index towards a chemokine, which can be achieved by using low-resolution imaging.[167] The advent of high spatiotemporal resolution imaging allows subcellular detail that requires intensive image data processing and provides bespoke migration quantification analysis with far more detail than the traditional readouts of migration index, speed and directionality alone. Using TIRF microscopy, Hons et al. analysed naïve T cell 2D morphology in various CCL19 concentrations and surface conditions.[151] The authors found that increased CCL19 concentration is associated with elongated cell shape and higher retrograde actin flow. However, this and other studies are limited to the analysis of 2D T cell migration morphology.[168–170] More complex 3D T cell migration, remains largely unexplored in response to specific chemokine cues.[171] Since understanding 3D morphology and cytoskeleton during T cell antigen recognition has shown promising results, its application to cell migration research is garnering interest. Recent studies examining neutrophils and dendritic cells with a combination of imaging and microfluidics have recently provided significant level of detail.[172,173] The dynamic changes of cell protrusions, the actin cytoskeleton modification and the interplay between cell and extra cellular matrix during cell migration were imaged with subcellular resolution.[172,173] The datasets at this resolution additionally require a bespoke methods to analyse and quantify the data in a hypothesis-driven manner. Parameters for analysis in this field needs to be standardized to produce robust, comparable and statistically significant data.[174,175]

Some work has already been done to investigate redundant, collaborative or antagonistic relationships of chemokines that bind the same receptor.[90,124,125] An interesting study by Haessler et al. investigated DCs migration bias with competing gradient of CCL19 and CCL21.[176] To setup this assay, the authors designed a microfluidic device which established chemokine gradients from two sides of migrating cells. They found that at high chemokine concentrations, DCs showed more efficient migration towards CCL21. However, the mechanism behind this phenomenon was not investigated. Since this study was restricted to wide-field microscopy with low resolution capabilities,[156] using higher resolution and faster imaging, such as LLSM or Spinning-disk Microscopy, would pinpoint the moment when DCs alter their direction towards CCL21 and allow the study of dynamic cytoskeleton changes.[176] Using a similar microfluidic device, another study suggested rising soluble chemokine gradient is critical for persistent DCs chemotaxis due to receptor desensitization.[157] The

spatial and temporal chemokine sensing must both be considered in chemotaxis experimental design and data collection. In contrast, this may not apply to insoluble or GAG-bound chemokine gradients and haptotactic migration as the gradients are spatially more stable.[109,177] Although T cells are not as morphologically variable as DCs[151], this system is still highly adaptable to understand multiple questions surrounding how CXCR3 and CCR7 mediate T cell fate.[171] For instance how a cell expressing CXCR3 decides to follow a CXCL10 gradient, that is spatially distinct from a simultaneous CXCL9 is currently unknown.[113,178] Further, this chemokine hierarchy or migratory preference may be altered between T_{SLEC} and T_{SCM} cells.

A major unanswered question is how T cells respond differentially to either soluble or those immobilized via GAG binding.[116,130,179] This key source of variability may be central to understanding the chemokine specificity. Previously, *in vitro* migration systems were unable to mimic insoluble gradients. Some recent studies have mapped and quantified bound chemokine gradients,[158,159] but limited studies have used this technology to investigate and image haptotactic mechanisms *in vitro*.[180,181] With further advances of microfluidic chambers, chemokines can be "printed" onto cover slip of the device with customised patterns.[180] This can be coupled with soluble chemokine gradients to compare and contrast the mechanisms used for chemotaxis and haptotaxis.

7. OUTLOOK AND FUTURE APPLICATIONS

In vitro migration assays are rapidly evolving. By combining with high resolution imaging, such as LLSM, they can generate near-isotropic 3D data over time.[144] These results provide insights to the mechanism and timing of cell motility events during antigen recognition and elimination.[145,151] It is also important to consider the physiological implications of the findings to better understand cell differentiation and function. Since only limited research has combined light-sheet imaging with microfluidics,[160] further studies may investigate the sequence and timing of cell motion during chemokine sensing and continual migration.[182] The current microfluidic devices can somewhat mimic the diffusive gradients of one or two soluble chemokines. However, as chemokine binding to GAGs or other extracellular matrix molecules changes the way immune cells sense and migrate towards a gradient, this may lead to different chemotactic preferences and cytoskeletal modifications.[116,120,121,180] Hence, establishing of 3D insoluble chemokine gradients is a necessary feature for future microfluidics development.

As discussed throughout this review, the *in vivo* microenvironment is highly complex and dynamic. Although *in vitro* imaging can provide significant details in the dynamic motility and cellular interactions, the physiological microenvironment cannot be fully represented. The 3D stromal cell network, conduit system and functional niches of lymph nodes are not well reflected in *in vitro* microfluidic systems.[53,140] Hence, it is critical to formulate hypotheses from what we learn *in vitro* and take them back to tissues. For 4D intravital imaging, 2-photon microscopy has provided fundamental knowledge in the migration kinetics and cellular interactions (Figure 3).[183] Emerging 3-photon technology will allow visualisation of migration and cellular interactions deeper in the node. However, the subcellular and molecular details and timing of these events are not fully elucidated due to the lack of spatiotemporal resolution. Recent development of Adaptive Optics LLSM, achieve high resolution imaging in live samples and animals.[184–187] The application of these technologies in immunology will produce promising results for years to come.

8. CONCLUSIONS

The powerful combination of chemokine and transcription factor reporters with 2D, 3D and dynamic imaging has clarified our understanding of the spatial requirements for $CD8^+$ T cell fate.[16,41,47,188] Volumetric imaging of intact lymph nodes has revealed the spatial requirements that are instructed by CXCR3 to imprint cellular differentiation.[16] This work highlights the spatially distinct inflammatory axis between T_{SLEC} and T_{SCM} formation that can be leveraged for the promotion of either of these distinct outcomes. Still there is much to understand about how this process is regulated. Key questions surround the inflammatory regulation of chemokine expression, and the distinct cellular partners that instruct fate commitment in spatial distinct lymph node niches. Although volumetric imaging has revealed the consequences of intranodal migration, we still lack knowledge of how this is orchestrated within a complex chemokine environment. Fundamentally, targeting of the chemokine system has been difficult due to the assumption of redundancy and collaboration of ligands that share a single receptor. However, we show that there is specificity in this system, at least at the level of the cellular sources and spatial location of CXCL9 and CXCL10.[16,47] Investigating how T cells integrate distinct and competing chemokine gradients will likely identify further specificities in the chemokine system. This will allow a deeper understanding of the mechanism of T cell migration that determines fate: from tissue level analysis within the lymph node, to analysis of dynamic cell migration at subcellular resolution, and back again.

ACKNOWLEDGEMENTS

Figures were created with BioRender.com. This work was supported by National Health & Medical Research Council Ideas Grant 1182649 and Investigator Grant 2007812. B.C.D. is supported by a WEHI Academic Excellence scholarship. R.Z.Q is supported by Melbourne University research scholarships. This work was made possible through Victorian State Government Operational Infrastructure Support and Australian Government NHMRC IRIISS.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

REFERENCES

1. Cullen SP, Martin SJ. Mechanisms of granule-dependent killing. *Cell Death Differ*. 2008;15(2):251-262. doi:10.1038/sj.cdd.4402244

2. Harty JT, Bevan MJ. Responses of CD8(+) T cells to intracellular bacteria. *Current opinion in immunology*. 1999;11(1):89-93. doi:10.1016/s0952-7915(99)80016-8

3. Zhang N, Bevan MJ. CD8+ T Cells: Foot Soldiers of the Immune System. *Immunity*. 2011;35(2):161-168. doi:10.1016/j.immuni.2011.07.010

4. Groscurth P, Filgueira L. Killing Mechanisms of Cytotoxic T Lymphocytes. *Physiology*. 1998;13(1):17-21. doi:10.1152/physiologyonline.1998.13.1.17

5. Joshi NS, Cui W, Chandele A, et al. Inflammation Directs Memory Precursor and Short-Lived Effector CD8+ T Cell Fates via the Graded Expression of T-bet Transcription Factor. *Immunity*. 2007;27(2):281-295. doi:10.1016/j.immuni.2007.07.010

6. Kaech SM, Cui W. Transcriptional control of effector and memory CD8+ T cell differentiation. *Nature Publishing Group*. 2012;12(11):749-761. doi:10.1038/nm.2232

7. Gattinoni L, Lugli E, Ji Y, et al. A human memory T cell subset with stem cell–like properties. *Nature Medicine*. 2011;17(10):1290-1297. doi:10.1002/cyto.a.21015

8. Kratchmarov R, Magun AM, Reiner SL. TCF1 expression marks self-renewing human CD8+ T cells. *Blood advances*. 2018;2(14):1685-1690. doi:10.1182/bloodadvances.2018016279

9. Rosenberg SA, Restifo NP. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science*. 2015;348(6230):62-68. doi:10.1126/science.aaa4967

10. Gattinoni L, Speiser DE, Lichterfeld M, Bonini C. T memory stem cells in health and disease. *Nature Medicine*. 2017;23(1):18-27. doi:10.1038/nri2657

11. Utzschneider DT, Charmoy M, Chennupati V, et al. T Cell Factor 1-Expressing Memory-like CD8+ T Cells Sustain the Immune Response to Chronic Viral Infections. *Immunity*. 2016;45(2):415-427. doi:10.1016/j.immuni.2016.07.021

12. Im SJ, Hashimoto M, Gerner MY, et al. Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. *Nature*. 2016;537(7620):417-421. doi:10.1038/nature19330

13. Jansen CS, Prokhnevska N, Master VA, et al. An intra-tumoral niche maintains and differentiates stem-like CD8 T cells. *Nature*. 2019;576(7787):465-470. doi:10.1038/s41586-019-1836-5

14. Baharom F, Ramirez-Valdez RA, Tobin KKS, et al. Intravenous nanoparticle vaccination generates stem-like TCF1+ neoantigen-specific CD8+ T cells. *Nat Immunol*. 2021;22(1):41-52. doi:10.1038/s41590-020-00810-3

15. Mo F, Yu Z, Li P, et al. An engineered IL-2 partial agonist promotes CD8+ T cell stemness. *Nature*. 2021;597(7877):544-548. doi:10.1038/s41586-021-03861-0

16. Duckworth BC, Lafouresse F, Wimmer VC, et al. Effector and stem-like memory cell fates are imprinted in distinct lymph node niches directed by CXCR3 ligands. *Nat Immunol*. 2021;22(4):434-448. doi:10.1038/s41590-021-00878-5

17. Grant SM, Lou M, Yao L, Germain RN, Radtke AJ, Lennon-Duménil AM. The lymph node at a glance – how spatial organization optimizes the immune response. *J Cell Sci*. 2020;133(5):jcs241828. doi:10.1242/jcs.241828

18. Groom JR. Moving to the suburbs: T-cell positioning within lymph nodes during activation and memory. Published online March 10, 2015:1-7. doi:10.1038/icb.2015.29

19. Alexandre YO, Mueller SN. Stromal cell networks coordinate immune response generation and maintenance. *Immunol Rev*. 2018;283(1):77-85. doi:10.1111/imr.12641

20. Qi Z, Wang J, Han X, Yang J, Zhao G, Cao Y. Listr1 locus regulates innate immunity against Listeria monocytogenes infection in the mouse liver possibly through Cxcl11 polymorphism. *Immunogenetics*. 2014;66(4):231-242. doi:10.1089/10430349950017734

21. Miller MJ, Wei SH, Parker I, Cahalan MD. Two-Photon Imaging of Lymphocyte Motility and Antigen Response in Intact Lymph Node. *Science*. 2002;296(5574):1869-1873. doi:10.1126/science.1070051

22. Miller MJ, Safrina O, Parker I, Cahalan MD. Imaging the Single Cell Dynamics of CD4+ T Cell Activation by Dendritic Cells in Lymph Nodes. *J Exp Medicine*. 2004;200(7):847-856. doi:10.1084/jem.20041236

23. Stoll S, Delon J, Brotz TM, Germain RN. Dynamic Imaging of T Cell-Dendritic Cell Interactions in Lymph Nodes. *Science*. 2002;296(5574):1873-1876. doi:10.1126/science.1071065

24. Eickhoff S, Brewitz A, Gerner MY, et al. Robust Anti-viral Immunity Requires Multiple Distinct T Cell-Dendritic Cell Interactions. *Cell*. 2015;162(6):1322-1337. doi:10.1016/j.cell.2015.08.004

25. Hor JL, Whitney PG, Zaid A, Brooks AG, Heath WR, Mueller SN. Spatiotemporally Distinct Interactions with Dendritic Cell Subsets Facilitates CD4+ and CD8+ T Cell Activation to Localized Viral Infection. *Immunity*. 2015;43(3):554-565. doi:10.1016/j.immuni.2015.07.020

26. Hickman HD, Takeda K, Skon CN, et al. Direct priming of antiviral CD8+ T cells in the peripheral interfollicular region of lymph nodes. *Nature Immunology*. 2008;9(2):155-165. doi:10.1128/mcb.5.12.3403

27. Gerner MY, Torabi-Parizi P, Germain RN. Strategically Localized Dendritic Cells Promote Rapid T Cell Responses to Lymph-Borne Particulate Antigens. *Immunity*. 2015;42(1):172-185. doi:10.1016/j.immuni.2014.12.024

28. Qi H, Kastenmüller W, Germain RN. Spatiotemporal Basis of Innate and Adaptive Immunity in Secondary Lymphoid Tissue. *Annu Rev Cell Dev Bi*. 2014;30(1):1-27. doi:10.1146/annurev-cellbio-100913-013254

29. Tuchin VV. Tissue Optics and Photonics: Biological Tissue Structures. *J Biomed Photonics Eng*. 2015;1(1):3-21. doi:10.18287/jbpe-2015-1-1-3

30. Li W, Germain RN, Gerner MY. High-dimensional cell-level analysis of tissues with Ce3D multiplex volume imaging. *Nature Protocols*. Published online April 25, 2019:1-29. doi:10.1038/s41596-019-0156-4

31. Hofmann J, Gadjalova I, Mishra R, Ruland J, Keppler SJ. Efficient Tissue Clearing and Multi-Organ Volumetric Imaging Enable Quantitative Visualization of Sparse Immune Cell Populations During Inflammation. *Front Immunol*. 2021;11:599495. doi:10.3389/fimmu.2020.599495

32. Li W, Germain RN, Gerner MY. Multiplex, quantitative cellular analysis in large tissue volumes with clearing-enhanced 3D microscopy (C e3D). *Proceedings of the National Academy of Sciences*. 2017;114(35):E7321-E7330. doi:10.1038/nbt1250

33. Power RM, Huisken J. A guide to light-sheet fluorescence microscopy for multiscale imaging. *Nature Methods*. 2017;14(4):360-373. doi:10.1364/oe.18.024229

34. Medaglia C, Giladi A, Stoler-Barak L, et al. Spatial reconstruction of immune niches by combining photoactivatable reporters and scRNA-seq. *Science*. 2017;358(6370):1622-1626. doi:10.1126/science.aao4277

35. Groom JR, Luster AD. CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunology and Cell Biology*. 2011;89(2):207-215. doi:10.1038/icb.2010.158

36. Ozga AJ, Moalli F, Abe J, et al. pMHC affinity controls duration of CD8 +T cell–DC interactions and imprints timing of effector differentiation versus expansion. *The Journal of Experimental Medicine*. 2016;213(12):2811-2829. doi:10.1038/nature07657

37. Hickman HD, Reynoso GV, Ngudiankama BF, et al. CXCR3 Chemokine Receptor Enables Local CD8+ T Cell Migration for the Destruction of Virus-Infected Cells. *Immunity*. 2015;42(3):524-537. doi:10.1016/j.immuni.2015.02.009

38. Kurachi M, Kurachi J, Suenaga F, et al. Chemokine receptor CXCR3 facilitates CD8 +T cell differentiation into short-lived effector cells leading to memory degeneration. *The Journal of Experimental Medicine*. 2011;208(8):1605-1620. doi:10.4049/jimmunol.180.4.2641

39. Hu JK, Kagari T, Clingan JM, Matloubian M. Expression of chemokine receptor CXCR3 on T cells affects the balance between effector and memory CD8 T-cell generation. *Proceedings of the National Academy of Sciences*. 2011;108(21):E118-27. doi:10.1073/pnas.1101881108

40. Kohlmeier JE, Reiley WW, Perona-Wright G, et al. Inflammatory chemokine receptors regulate CD8 +T cell contraction and memory generation following infection. *The Journal of Experimental Medicine*. 2011;208(8):1621-1634. doi:10.1146/annurev.immunol.25.022106.141548

41. Sung JH, Zhang H, Moseman EA, et al. Chemokine Guidance of Central Memory T Cells Is Critical for Antiviral Recall Responses in Lymph Nodes. *Cell*. 2012;150(6):1249-1263. doi:10.1016/j.cell.2012.08.015

42. Kaech SM, Wherry EJ. Heterogeneity and Cell-Fate Decisions in Effector and Memory CD8+ T Cell Differentiation during Viral Infection. *Immunity*. 2007;27(3):393-405. doi:10.1016/j.immuni.2007.08.007

43. Christo SN, Evrard M, Park SL, et al. Discrete tissue microenvironments instruct diversity in resident memory T cell function and plasticity. *Nat Immunol*. 2021;22(9):1140-1151. doi:10.1038/s41590-021-01004-1

44. Wu T, Ji Y, Moseman EA, et al. The TCF1-Bcl6 axis counteracts type I interferon to repress exhaustion and maintain T cell stemness. *Sci Immunol*. 2016;1(6):eaai8593-eaai8593. doi:10.1126/sciimmunol.aai8593

45. Jadhav RR, Im SJ, Hu B, et al. Epigenetic signature of PD-1+ TCF1+ CD8 T cells that act as resource cells during chronic viral infection and respond to PD-1 blockade. *Proc National Acad Sci*. 2019;116(28):14113-14118. doi:10.1073/pnas.1903520116

46. Duckworth BC, Groom JR. Conversations that count: Cellular interactions that drive T cell fate. *Immunol Rev*. 2021;300(1):203-219. doi:10.1111/imr.12945

47. Groom JR, Richmond J, Murooka TT, et al. CXCR3 Chemokine Receptor-Ligand Interactions in the Lymph Node Optimize CD4+ T Helper 1 Cell Differentiation. *Immunity*. 2012;37(6):1091-1103. doi:10.1016/j.immuni.2012.08.016

48. Rodda LB, Lu E, Bennett ML, et al. Single-Cell RNA Sequencing of Lymph Node Stromal Cells Reveals Niche-Associated Heterogeneity. *Immunity*. 2018;48(5):1014-1028.e6. doi:10.1016/j.immuni.2018.04.006

49. Chow MT, Ozga AJ, Servis RL, et al. Intratumoral Activity of the CXCR3 Chemokine System Is Required for the Efficacy of Anti-PD-1 Therapy. *Immunity*. 2019;50(6):1498-1512.e5. doi:10.1016/j.immuni.2019.04.010

50. Gola A, Dorrington MG, Speranza E, et al. Commensal-driven immune zonation of the liver promotes host defence. *Nature*. 2021;589(7840):131-136. doi:10.1038/s41586-020-2977-2

51. Galletti G, Simone GD, Mazza EMC, et al. Two subsets of stem-like CD8+ memory T cell progenitors with distinct fate commitments in humans. *Nature Immunology*. Published online September 25, 2020:1-20. doi:10.1038/s41590-020-0791-5

52. Marsman C, Lafouresse F, Liao Y, et al. Plasmacytoid dendritic cell heterogeneity is defined by CXCL10 expression following TLR7 stimulation. *Immunol Cell Biol*. 2018;96(10):1083-1094. doi:10.1111/imcb.12173

53. Groom JR. Regulators of T-cell fate: Integration of cell migration, differentiation and function. *Immunological Reviews*. 2019;289(1):101-114. doi:10.1158/1078-0432.ccr-18-1139

54. Griffith JW, Sokol CL, Luster AD. Chemokines and Chemokine Receptors: Positioning Cells for Host Defense and Immunity. *Immunology*. 2014;32(1):659-702. doi:10.1146/annurev-immunol-032713-120145

55. Mori S, Nakano H, Aritomi K, Wang CR, Gunn MD, Kakiuchi T. Mice Lacking Expression of the Chemokines Ccl21-Ser and Ccl19 (plt Mice) Demonstrate Delayed but Enhanced T Cell Immune Responses. *J Exp Medicine*. 2001;193(2):207-218. doi:10.1084/jem.193.2.207

56. Sullivan BM, Juedes A, Szabo SJ, Herrath M von, Glimcher LH. Antigen-driven effector CD8 T cell function regulated by T-bet. *Proc National Acad Sci*. 2003;100(26):15818-15823. doi:10.1073/pnas.2636938100

57. Zhu J, Jankovic D, Oler AJ, et al. The Transcription Factor T-bet Is Induced by Multiple Pathways and Prevents an Endogenous Th2 Cell Program during Th1 Cell Responses. *Immunity*. 2012;37(4):660-673. doi:10.1016/j.immuni.2012.09.007

58. Raghu D, Xue HH, Mielke LA. Control of Lymphocyte Fate, Infection, and Tumor Immunity by TCF-1. *Trends Immunol*. 2019;40(12):1149-1162. doi:10.1016/j.it.2019.10.006

59. Sheikh AA, Groom JR. Transcription tipping points for T follicular helper cell and T-helper 1 cell fate commitment. *Cellular & Molecular Immunology*. Published online September 25, 2020:1-11. doi:10.1038/s41423-020-00554-y

60. Junt T, Moseman EA, Iannacone M, et al. Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Proc National Acad Sci*. 2007;450(7166):110-114. doi:10.1089/hum.1998.9.3-367

61. Phan TG, Grigorova I, Okada T, Cyster JG. Subcapsular encounter and complement-dependent transport of immune complexes by lymph node B cells. *Nat Immunol*. 2007;8(9):992-1000. doi:10.1038/ni1494

62. Ulvmar MH, Werth K, Braun A, et al. The atypical chemokine receptor CCRL1 shapes functional CCL21 gradients in lymph nodes. *Nat Immunol*. 2014;15(7):623-630. doi:10.1038/ni.2889

63. Henrickson SE, Perro M, Loughhead SM, et al. Antigen Availability Determines CD8+ T Cell-Dendritic Cell Interaction Kinetics and Memory Fate Decisions. *Immunity*. 2013;39(3):496-507. doi:10.1016/j.immuni.2013.08.034

64. Kaech SM, Wherry EJ, Ahmed R. Effector and memory T-cell differentiation: implications for vaccine development. *Nature Publishing Group*. 2002;2(4):251-262. doi:10.1046/j.1365-2249.2001.01600.x

65. Danilo M, Chennupati V, Silva JG, Siegert S, Held W. Suppression of Tcf1 by Inflammatory Cytokines Facilitates Effector CD8 T Cell Differentiation. *Cell Reports*. 2018;22(8):2107-2117. doi:10.1016/j.celrep.2018.01.072

66. Sercan Ö, Stoycheva D, Hämmerling GJ, Arnold B, Schüler T. IFN-γ Receptor Signaling Regulates Memory CD8+ T Cell Differentiation. *J Immunol*. 2010;184(6):2855-2862. doi:10.4049/jimmunol.0902708

67. Pipkin ME, Sacks JA, Cruz-Guilloty F, Lichtenheld MG, Bevan MJ, Rao A. Interleukin-2 and Inflammation Induce Distinct Transcriptional Programs that Promote the Differentiation of Effector Cytolytic T Cells. *Immunity*. 2010;32(1):79-90. doi:10.1016/j.immuni.2009.11.012

68. Mitchell DM, Ravkov EV, Williams MA. Distinct Roles for IL-2 and IL-15 in the Differentiation and Survival of CD8+ Effector and Memory T Cells. *J Immunol*. 2010;184(12):6719-6730. doi:10.4049/jimmunol.0904089

69. Joshi NS, Cui W, Chandele A, et al. Inflammation Directs Memory Precursor and Short-Lived Effector CD8+ T Cell Fates via the Graded Expression of T-bet Transcription Factor. *Immunity*. 2007;27(2):281-295. doi:10.1016/j.immuni.2007.07.010

70. Pham NLL, Badovinac VP, Harty JT. A Default Pathway of Memory CD8 T Cell Differentiation after Dendritic Cell Immunization Is Deflected by Encounter with Inflammatory Cytokines during Antigen-Driven Proliferation. *J Immunol*. 2009;183(4):2337-2348. doi:10.4049/jimmunol.0901203

71. Xin A, Masson F, Liao Y, et al. A molecular threshold for effector CD8+ T cell differentiation controlled by transcription factors Blimp-1 and T-bet. *Nat Immunol*. 2016;17(4):422-432. doi:10.1038/ni.3410

72. Good-Jacobson KL. Strength in diversity: Phenotypic, functional, and molecular heterogeneity within the memory B cell repertoire. *Immunol Rev*. 2018;284(1):67-78. doi:10.1111/imr.12663

73. Yin X, Chen S, Eisenbarth SC. Dendritic Cell Regulation of T Helper Cells. *Annu Rev Immunol*. 2021;39(1):1-32. doi:10.1146/annurev-immunol-101819-025146

74. Castellino F, Huang AY, Altan-Bonnet G, Stoll S, Scheinecker C, Germain RN. Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell–dendritic cell interaction. *Nature*. 2006;440(7086):890-895. doi:10.1038/nature04651

75. Brewitz A, Eickhoff S, Dähling S, et al. CD8+ T Cells Orchestrate pDC-XCR1+ Dendritic Cell Spatial and Functional Cooperativity to Optimize Priming. *Immunity*. 2017;46(2):205-219. doi:10.1016/j.immuni.2017.01.003

76. Kohlmeier JE, Reiley WW, Perona-Wright G, et al. Inflammatory chemokine receptors regulate CD8 +T cell contraction and memory generation following infection. *The Journal of Experimental Medicine*. 2011;208(8):1621-1634. doi:10.1146/annurev.immunol.25.022106.141548

77. Gray EE, Friend S, Suzuki K, Phan TG, Cyster JG. Subcapsular Sinus Macrophage Fragmentation and CD169+ Bleb Acquisition by Closely Associated IL-17-Committed Innate-Like Lymphocytes. *Plos One*. 2012;7(6):e38258. doi:10.1371/journal.pone.0038258

78. Kastenmüller W, Torabi-Parizi P, Subramanian N, Lämmermann T, Germain RN. A Spatially-Organized Multicellular Innate Immune Response in Lymph Nodes Limits Systemic Pathogen Spread. *Cell*. 2012;150(6):1235-1248. doi:10.1016/j.cell.2012.07.021

79. Valente M, Dölen Y, Dinther E van, et al. Cross-talk between iNKT cells and CD8 T cells in the spleen requires the IL-4/CCL17 axis for the generation of short-lived effector cells. *Proc National Acad Sci*. 2019;116(51):25816-25827. doi:10.1073/pnas.1913491116

80. Brown FD, Sen DR, LaFleur MW, et al. Fibroblastic reticular cells enhance T cell metabolism and survival via epigenetic remodeling. *Nat Immunol*. 2019;20(12):1668-1680. doi:10.1038/s41590-019-0515-x

81. Kapoor VN, Müller S, Keerthivasan S, et al. Gremlin 1+ fibroblastic niche maintains dendritic cell homeostasis in lymphoid tissues. *Nat Immunol*. 2021;22(5):571-585. doi:10.1038/s41590-021-00920-6

82. Bajénoff M, Egen JG, Koo LY, et al. Stromal Cell Networks Regulate Lymphocyte Entry, Migration, and Territoriality in Lymph Nodes. *Immunity*. 2006;25(6):989-1001. doi:10.1016/j.immuni.2006.10.011

83. Worbs T, Mempel TR, Bölter J, Andrian UH von, Förster R. CCR7 ligands stimulate the intranodal motility of T lymphocytes in vivo. *J Exp Medicine*. 2007;204(3):489-495. doi:10.1084/jem.20061706

84. Okada T, Cyster JG. CC Chemokine Receptor 7 Contributes to Gi-Dependent T Cell Motility in the Lymph Node. *J Immunol*. 2007;178(5):2973-2978. doi:10.4049/jimmunol.178.5.2973

85. Jung YW, Rutishauser RL, Joshi NS, Haberman AM, Kaech SM. Differential Localization of Effector and Memory CD8 T Cell Subsets in Lymphoid Organs during Acute Viral Infection. *J Immunol*. 2010;185(9):5315-5325. doi:10.4049/jimmunol.1001948

86. Ulvmar MH, Werth K, Braun A, et al. The atypical chemokine receptor CCRL1 shapes functional CCL21 gradients in lymph nodes. *Nat Immunol*. 2014;15(7):623-630. doi:10.1038/ni.2889

87. Boldajipour B, Mahabaleshwar H, Kardash E, et al. Control of Chemokine-Guided Cell Migration by Ligand Sequestration. *Cell*. 2008;132(3):463-473. doi:10.1016/j.cell.2007.12.034

88. Bonecchi R, Graham GJ. Atypical Chemokine Receptors and Their Roles in the Resolution of the Inflammatory Response. *Front Immunol*. 2016;7:224. doi:10.3389/fimmu.2016.00224

89. Dyer DP. Understanding the mechanisms that facilitate specificity, not redundancy, of chemokine-mediated leukocyte recruitment. *Immunology*. 2020;290:21292. doi:10.1038/s41592-018-0086-z

90. Griffith JW, Sokol CL, Luster AD. Chemokines and Chemokine Receptors: Positioning Cells for Host Defense and Immunity. *Annu Rev Immunol*. 2014;32(1):659-702. doi:10.1146/annurev-immunol-032713-120145

91. Handel TM, Johnson Z, Crown SE, Lau EK, Sweeney M, Proudfoot AE. Regulation Of Protein Function By Glycosaminoglycans—As Exemplified By Chemokines. *Annu Rev Biochem*. 2005;74(1):385-410. doi:10.1146/annurev.biochem.72.121801.161747

92. Campanella GSV, Lee EMJ, Sun J, Luster AD. CXCR3 and Heparin Binding Sites of the Chemokine IP-10 (CXCL10)*. *J Biol Chem*. 2003;278(19):17066-17074. doi:10.1074/jbc.m212077200

93. Vanheule V, Janssens R, Boff D, et al. The Positively Charged COOH-terminal Glycosaminoglycan-binding CXCL9(74–103) Peptide Inhibits CXCL8-induced Neutrophil Extravasation and Monosodium Urate Crystal-induced Gout in Mice*. *J Biol Chem*. 2015;290(35):21292-21304. doi:10.1074/jbc.m115.649855

94. Cox JH, Dean RA, Roberts CR, Overall CM. Matrix Metalloproteinase Processing of CXCL11/I-TAC Results in Loss of Chemoattractant Activity and Altered Glycosaminoglycan Binding*. *J Biol Chem*. 2008;283(28):19389-19399. doi:10.1074/jbc.m800266200

95. Groom JR, Richmond J, Murooka TT, et al. CXCR3 Chemokine Receptor-Ligand Interactions in the Lymph Node Optimize CD4+ T Helper 1 Cell Differentiation. *Immunity*. 2012;37(6):1091-1103. doi:10.1016/j.immuni.2012.08.016

96. Colvin RA, Campanella GSV, Sun J, Luster AD. Intracellular Domains of CXCR3 That Mediate CXCL9, CXCL10, and CXCL11 Function. *Journal of Biological Chemistry*. 2004;279(29):30219-30227. doi:10.1074/jbc.m403595200

97. Metzemaekers M, Vanheule V, Janssens R, Struyf S, Proost P. Overview of the Mechanisms that May Contribute to the Non-Redundant Activities of Interferon-Inducible CXC Chemokine Receptor 3 Ligands. *Frontiers in Immunology*. 2018;8:436-21. doi:10.3389/fimmu.2017.01970

98. Yang BG, Tanaka T, Jang MH, Bai Z, Hayasaka H, Miyasaka M. Binding of Lymphoid Chemokines to Collagen IV That Accumulates in the Basal Lamina of High Endothelial

Venules: Its Implications in Lymphocyte Trafficking. *J Immunol*. 2007;179(7):4376-4382. doi:10.4049/jimmunol.179.7.4376

99. Okada T, Miller MJ, Parker I, et al. Antigen-Engaged B Cells Undergo Chemotaxis toward the T Zone and Form Motile Conjugates with Helper T Cells. *Plos Biol*. 2005;3(6):e150. doi:10.1371/journal.pbio.0030150

100. Ulvmar MH, Werth K, Braun A, et al. The atypical chemokine receptor CCRL1 shapes functional CCL21 gradients in lymph nodes. *Nat Immunol*. 2014;15(7):623-630. doi:10.1038/ni.2889

101. Unsoeld H, Mueller K, Schleicher U, et al. Abrogation of CCL21 chemokine function by transgenic over-expression impairs T cell immunity to local infections. *Int Immunol*. 2007;19(11):1281-1289. doi:10.1093/intimm/dxm098

102. Bastow CR, Bunting MD, Kara EE, et al. Scavenging of soluble and immobilized CCL21 by ACKR4 regulates peripheral dendritic cell emigration. *Proc National Acad Sci*. 2021;118(17):e2025763118. doi:10.1073/pnas.2025763118

103. Link A, Vogt TK, Favre S, et al. Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naive T cells. *Nat Immunol*. 2007;8(11):1255-1265. doi:10.1038/ni1513

104. Rodda LB, Lu E, Bennett ML, et al. Single-Cell RNA Sequencing of Lymph Node Stromal Cells Reveals Niche-Associated Heterogeneity. *Immunity*. 2018;48(5):1-22. doi:10.1016/j.immuni.2018.04.006

105. Bardi G, Lipp M, Baggiolini M, Loetscher P. The T cell chemokine receptor CCR7 is internalized on stimulation with ELC, but not with SLC. *Eur J Immunol*. 2001;31(11):3291-3297. doi:10.1002/1521-4141(200111)31:11<3291::aid-immu3291>3.0.co;2-z

106. Kohout TA, Nicholas SL, Perry SJ, Reinhart G, Junger S, Struthers RS. Differential Desensitization, Receptor Phosphorylation, β-Arrestin Recruitment, and ERK1/2 Activation by the Two Endogenous Ligands for the CC Chemokine Receptor 7*. *J Biol Chem*. 2004;279(22):23214-23222. doi:10.1074/jbc.m402125200

107. Cremasco V, Woodruff MC, Onder L, et al. B cell homeostasis and follicle confines are governed by fibroblastic reticular cells. *Nat Immunol*. 2014;15(10):973-981. doi:10.1038/ni.2965

108. Kiermaier E, Moussion C, Veldkamp CT, et al. Polysialylation controls dendritic cell trafficking by regulating chemokine recognition. *Science*. 2016;351(6269):186-190. doi:10.1126/science.aad0512

109. Weber M, Hauschild R, Schwarz J, et al. Interstitial Dendritic Cell Guidance by Haptotactic Chemokine Gradients. *Science*. 2013;339(6117):328-332. doi:10.1126/science.1228456

110. Campanella GSV, Tager AM, Khoury JKE, et al. Chemokine receptor CXCR3 and its ligands CXCL9 and CXCL10 are required for the development of murine cerebral malaria. *Proc National Acad Sci*. 2008;105(12):4814-4819. doi:10.1073/pnas.0801544105

111. Sarkar SA, Lee CE, Victorino F, et al. Expression and Regulation of Chemokines in Murine and Human Type 1 Diabetes. *Diabetes*. 2012;61(2):436-446. doi:10.2337/db11-0853

112. Sung JH, Zhang H, Moseman EA, et al. Chemokine Guidance of Central Memory T Cells Is Critical for Antiviral Recall Responses in Lymph Nodes. *Cell*. 2012;150(6):1249-1263. doi:10.1016/j.cell.2012.08.015

113. Groom JR, Richmond J, Murooka TT, et al. CXCR3 Chemokine Receptor-Ligand Interactions in the Lymph Node Optimize CD4+ T Helper 1 Cell Differentiation. *Immunity*. 2012;37(6):1091-1103. doi:10.1016/j.immuni.2012.08.016

114. Vaahtomeri K, Moussion C, Hauschild R, Sixt M. Shape and Function of Interstitial Chemokine CCL21 Gradients Are Independent of Heparan Sulfates Produced by Lymphatic Endothelium. *Front Immunol*. 2021;12:630002. doi:10.3389/fimmu.2021.630002

115. Mendoza A, Yewdell WT, Hoyos B, et al. Assembly of a spatial circuit of T-bet–expressing T and B lymphocytes is required for antiviral humoral immunity. *Sci Immunol*. 2021;6(60):eabi4710. doi:10.1126/sciimmunol.abi4710

116. Weber M, Hauschild R, Schwarz J, et al. Interstitial Dendritic Cell Guidance by Haptotactic Chemokine Gradients. *Science*. 2013;339(6117):328-332. doi:10.1126/science.1228456

117. Mikucki ME, Fisher DT, Matsuzaki J, et al. Non-redundant requirement for CXCR3 signalling during tumoricidal T-cell trafficking across tumour vascular checkpoints. *Nat Commun*. 2015;6(1):7458. doi:10.1038/ncomms8458

118. Dyer DP. Understanding the mechanisms that facilitate specificity, not redundancy, of chemokine mediated leukocyte recruitment. *Immunology*. Published online 2020. doi:10.1111/imm.13200

119. Bonecchi R, Graham GJ. Atypical Chemokine Receptors and Their Roles in the Resolution of the Inflammatory Response. *Front Immunol*. 2016;7:224. doi:10.3389/fimmu.2016.00224

120. Kunz L, Schroeder T. A 3D Tissue-wide Digital Imaging Pipeline for Quantitation of Secreted Molecules Shows Absence of CXCL12 Gradients in Bone Marrow. *Cell Stem Cell*. 2019;25(6):846-854.e4. doi:10.1016/j.stem.2019.10.003

121. Cosgrove J, Novkovic M, Albrecht S, et al. B cell zone reticular cell microenvironments shape CXCL13 gradient formation. *Nat Commun*. 2020;11(1):3677. doi:10.1038/s41467-020-17135-2

122. Kawamura T, Stephens B, Qin L, et al. A General Method for Site Specific Fluorescent Labeling of Recombinant Chemokines. *Plos One*. 2014;9(1):e81454. doi:10.1371/journal.pone.0081454

123. Fernandez A, Thompson EJ, Pollard JW, Kitamura T, Vendrell M. A Fluorescent Activatable AND-Gate Chemokine CCL2 Enables In Vivo Detection of Metastasis-Associated Macrophages. *Angewandte Chemie Int Ed*. 2019;58(47):16894-16898. doi:10.1002/anie.201910955

124. Groom JR, Luster AD. CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunology and Cell Biology*. 2011;89(2):207-215. doi:10.1038/icb.2010.158

125. Hauser MA, Legler DF. Common and biased signaling pathways of the chemokine receptor CCR7 elicited by its ligands CCL19 and CCL21 in leukocytes. *J Leukocyte Biol*. 2016;99(6):869-882. doi:10.1189/jlb.2mr0815-380r

126. Isik N, Hereld D, Jin T. Fluorescence Resonance Energy Transfer Imaging Reveals that Chemokine-Binding Modulates Heterodimers of CXCR4 and CCR5 Receptors. *Plos One*. 2008;3(10):e3424. doi:10.1371/journal.pone.0003424

127. Watts A, Lipzig M, Jaeger W, et al. Identification and profiling of CXCR3–CXCR4 chemokine receptor heteromer complexes. *Brit J Pharmacol*. 2013;168(7):1662-1674. doi:10.1111/bph.12064

128. Kouzeli A, Collins PJ, Metzemaekers M, et al. CXCL14 Preferentially Synergizes With Homeostatic Chemokine Receptor Systems. *Front Immunol*. 2020;11:561404. doi:10.3389/fimmu.2020.561404

129. Dyer DP. Understanding the mechanisms that facilitate specificity, not redundancy, of chemokine mediated leukocyte recruitment. *Immunology*. Published online 2020. doi:10.1111/imm.13200

130. Metzemaekers M, Mortier A, Janssens R, et al. Glycosaminoglycans Regulate CXCR3 Ligands at Distinct Levels: Protection against Processing by Dipeptidyl Peptidase IV/CD26 and Interference with Receptor Signaling. *Int J Mol Sci*. 2017;18(7):1513. doi:10.3390/ijms18071513

131. Schumann K, Lämmermann T, Bruckner M, et al. Immobilized Chemokine Fields and Soluble Chemokine Gradients Cooperatively Shape Migration Patterns of Dendritic Cells. *Immunity*. 2010;32(5):703-713. doi:10.1016/j.immuni.2010.04.017

132. Overstreet MG, Gaylo A, Angermann BR, et al. Inflammation-induced interstitial migration of effector CD4+ T cells is dependent on integrin αV. *Nat Immunol*. 2013;14(9):949-958. doi:10.1038/ni.2682

133. Silva PHR da, Borges BC, Uehara IA, Soldi LR, Araújo RA de, Silva MJB. Chemokines and the extracellular matrix: Set of targets for tumor development and treatment. *Cytokine*. 2021;144:155548. doi:10.1016/j.cyto.2021.155548

134. Xiang YY, Ladeda V, Filmus J. Glypican-3 expression is silenced in human breast cancer. *Oncogene*. 2001;20(50):7408-7412. doi:10.1038/sj.onc.1204925

135. Murthy SS, Shen T, Rienzo AD, et al. Expression of GPC3, an X-linked recessive overgrowth gene, is silenced in malignant mesothelioma. *Oncogene*. 2000;19(3):410-416. doi:10.1038/sj.onc.1203322

136. Luster AD, Greenberg SM, Leder P. The IP-10 chemokine binds to a specific cell surface heparan sulfate site shared with platelet factor 4 and inhibits endothelial cell proliferation. *J Exp Medicine*. 1995;182(1):219-231. doi:10.1084/jem.182.1.219

137. Koopmann W, Ediriwickrema C, Krangel MS. Structure and function of the glycosaminoglycan binding site of chemokine macrophage-inflammatory protein-1 beta. *J Immunol Baltim Md 1950*. 1999;163(4):2120-2127.

138. Sasisekharan R, Shriver Z, Venkataraman G, Narayanasami U. Roles of heparan-sulphate glycosaminoglycans in cancer. *Nat Rev Cancer*. 2002;2(7):521-528. doi:10.1038/nrc842

139. Kameritsch P, Renkawitz J. Principles of Leukocyte Migration Strategies. *Trends Cell Biol*. Published online 2020. doi:10.1016/j.tcb.2020.06.007

140. Grant SM, Lou M, Yao L, Germain RN, Radtke AJ, Lennon-Duménil AM. The lymph node at a glance – how spatial organization optimizes the immune response. *J Cell Sci*. 2020;133(5):jcs241828. doi:10.1242/jcs.241828

141. Stoler-Barak L, Biram A, Davidzohn N, Addadi Y, Golani O, Shulman Z. B cell dissemination patterns during the germinal center reaction revealed by whole-organ imaging. *J Exp Med*. 2019;216(11):2515-2530. doi:10.1084/jem.20190789

142. Liu Z, Lavis LD, Betzig E. Imaging Live-Cell Dynamics and Structure at the Single-Molecule Level. *Mol Cell*. 2015;58(4):644-659. doi:10.1016/j.molcel.2015.02.033

143. SOULET D, LAMONTAGNE-PROULX J, AUBÉ B, DAVALOS D. Multiphoton intravital microscopy in small animals: motion artefact challenges and technical solutions. *J Microsc-oxford*. 2020;278(1):3-17. doi:10.1111/jmi.12880

144. Chen BC, Legant WR, Wang K, et al. Lattice light-sheet microscopy: Imaging molecules to embryos at high spatiotemporal resolution. *Science*. 2014;346(6208):1257998. doi:10.1126/science.1257998

145. Cai E, Marchuk K, Beemiller P, et al. Visualizing dynamic microvillar search and stabilization during ligand detection by T cells. *Science*. 2017;356(6338):eaal3118-12. doi:10.1126/science.aal3118

146. Ghosh S, Bartolo VD, Tubul L, et al. ERM-Dependent Assembly of T Cell Receptor Signaling and Co-stimulatory Molecules on Microvilli prior to Activation. *Cell reports*. 2020;30(10):3434-3447.e6. doi:10.1016/j.celrep.2020.02.069

147. Sasmal DK, Feng W, Roy S, et al. TCR–pMHC bond conformation controls TCR ligand discrimination. *Cell Mol Immunol*. 2020;17(3):203-217. doi:10.1038/s41423-019-0273-6

148. Rosenberg J, Cao G, Borja-Prieto F, Huang J. Lattice Light-Sheet Microscopy Multi-dimensional Analyses (LaMDA) of T-Cell Receptor Dynamics Predict T-Cell Signaling States. *Cell Syst*. 2020;10(5):433-444.e5. doi:10.1016/j.cels.2020.04.006

149. Ozga AJ, Moalli F, Abe J, et al. pMHC affinity controls duration of CD8+ T cell–DC interactions and imprints timing of effector differentiation versus expansion. *J Exp Medicine*. 2016;213(12):2811-2829. doi:10.1084/jem.20160206

150. Farsakoglu Y, McDonald B, Kaech SM. Motility Matters: How CD8 + T-Cell Trafficking Influences Effector and Memory Cell Differentiation. *Csh Perspect Biol*. Published online 2021:a038075. doi:10.1101/cshperspect.a038075

151. Ritter AT, Asano Y, Stinchcombe JC, et al. Actin Depletion Initiates Events Leading to Granule Secretion at the Immunological Synapse. *Immunity*. 2015;42(5):864-876. doi:10.1016/j.immuni.2015.04.013

152. Frazer GL, Gawden-Bone CM, Dieckmann NMG, Asano Y, Griffiths GM. Signal strength controls the rate of polarization within CTLs during killing. *J Cell Biol*. 2021;220(10):e202104093. doi:10.1083/jcb.202104093

153. Boyden S. THE CHEMOTACTIC EFFECT OF MIXTURES OF ANTIBODY AND ANTIGEN ON POLYMORPHONUCLEAR LEUCOCYTES. *J Exp Medicine*. 1962;115(3):453-466. doi:10.1084/jem.115.3.453

154. Whitesides GM. The origins and the future of microfluidics. *Nature*. 2006;442(7101):368-373. doi:10.1038/nature05058

155. Sackmann EK, Fulton AL, Beebe DJ. The present and future role of microfluidics in biomedical research. *Nature*. 2014;507(7491):181-189. doi:10.1038/nature13118

156. Keenan TM, Folch A. Biomolecular gradients in cell culture systems. *Lab Chip*. 2008;8(1):34-57. doi:10.1039/b711887b

157. Aronin CEP, Zhao YM, Yoon JS, et al. Migrating Myeloid Cells Sense Temporal Dynamics of Chemoattractant Concentrations. *Immunity*. 2017;47(5):862-874.e3. doi:10.1016/j.immuni.2017.10.020

158. Zengel P, Nguyen-Hoang A, Schildhammer C, Zantl R, Kahl V, Horn E. μ-Slide Chemotaxis: a new chamber for long-term chemotaxis studies. *Bmc Cell Biol*. 2011;12(1):21. doi:10.1186/1471-2121-12-21

159. Sackmann EK, Fulton AL, Beebe DJ. The present and future role of microfluidics in biomedical research. *Nature*. 2014;507(7491):181-189. doi:10.1038/nature13118

160. McDonald JC, Duffy DC, Anderson JR, et al. Fabrication of microfluidic systems in poly(dimethylsiloxane). *Electrophoresis*. 2000;21(1):27-40. doi:10.1002/(sici)1522-2683(20000101)21:1<27::aid-elps27>3.0.co;2-c

161. Fujii T. PDMS-based microfluidic devices for biomedical applications. *Microelectron Eng*. 2002;61:907-914. doi:10.1016/s0167-9317(02)00494-x

162. Renkawitz J, Reversat A, Leithner A, Merrin J, Sixt M. Micro-engineered “pillar forests” to study cell migration in complex but controlled 3D environments. *Methods Cell Biol*. 2018;147:79-91. doi:10.1016/bs.mcb.2018.07.004

163. Reversat A, Gaertner F, Merrin J, et al. Cellular locomotion using environmental topography. *Nature*. Published online 2020:1-4. doi:10.1038/s41586-020-2283-z

164. Zigmond SH. Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors. *J Cell Biology*. 1977;75(2):606-616. doi:10.1083/jcb.75.2.606

165. Zicha D, Dunn GA, Brown AF. A new direct-viewing chemotaxis chamber. *J Cell Sci*. 1991;99(4):769-775. doi:10.1242/jcs.99.4.769

166. Muinonen-Martin AJ, Veltman DM, Kalna G, Insall RH. An Improved Chamber for Direct Visualisation of Chemotaxis. *Plos One*. 2010;5(12):e15309. doi:10.1371/journal.pone.0015309

167. Lämmermann T, Germain RN. The multiple faces of leukocyte interstitial migration. *Semin Immunopathol*. 2014;36(2):227-251. doi:10.1007/s00281-014-0418-8

168. Hons M, Kopf A, Hauschild R, et al. Chemokines and integrins independently tune actin flow and substrate friction during intranodal migration of T cells. *Nat Immunol*. 2018;19(6):606-616. doi:10.1038/s41590-018-0109-z

169. Obeidy P, Ju LA, Oehlers SH, et al. Partial loss of actin nucleator actin-related protein 2/3 activity triggers blebbing in primary T lymphocytes. *Immunol Cell Biol*. 2020;98(2):93-113. doi:10.1111/imcb.12304

170. Pruitt HC, Lewis D, Ciccaglione M, et al. Collagen fiber structure guides 3D motility of cytotoxic T lymphocytes. *Matrix Biol*. 2019;85-86:147-159. doi:10.1016/j.matbio.2019.02.003

171. Schoppmeyer R, Zhao R, Hoth M, Qu B. Light-sheet Microscopy for Three-dimensional Visualization of Human Immune Cells. *J Vis Exp*. 2018;(136). doi:10.3791/57651

172. François J, Kandasamy A, Yeh YT, et al. The interplay between matrix deformation and the coordination of turning events governs directed neutrophil migration in 3D matrices. *Sci Adv*. 2021;7(29):eabf3882. doi:10.1126/sciadv.abf3882

173. Fritz-Laylin LK, Riel-Mehan M, Chen BC, et al. Actin-based protrusions of migrating neutrophils are intrinsically lamellar and facilitate direction changes. *eLife*. 2017;6:437. doi:10.7554/elife.26990

174. Driscoll MK, Zaritsky A. Data science in cell imaging. *J Cell Sci*. 2021;134(7):jcs254292. doi:10.1242/jcs.254292

175. Wait EC, Reiche MA, Chew TL. Hypothesis-driven quantitative fluorescence microscopy – the importance of reverse-thinking in experimental design. *J Cell Sci*. 2020;133(21):jcs250027. doi:10.1242/jcs.250027

176. Haessler U, Pisano M, Wu M, Swartz MA. Dendritic cell chemotaxis in 3D under defined chemokine gradients reveals differential response to ligands CCL21 and CCL19. *P Natl Acad Sci Usa*. 2011;108(14):5614-5619. doi:10.1073/pnas.1014920108

177. Schumann K, Lämmermann T, Bruckner M, et al. Immobilized Chemokine Fields and Soluble Chemokine Gradients Cooperatively Shape Migration Patterns of Dendritic Cells. *Immunity*. 2010;32(5):703-713. doi:10.1016/j.immuni.2010.04.017

178. Duckworth BC, Lafouresse F, Wimmer VC, et al. Effector and stem-like memory cell fates are imprinted in distinct lymph node niches directed by CXCR3 ligands. *Nat Immunol*. Published online 2021:1-15. doi:10.1038/s41590-021-00878-5

179. Petrie RJ, Doyle AD, Yamada KM. Random versus directionally persistent cell migration. *Nat Rev Mol Cell Bio*. 2009;10(8):538-549. doi:10.1038/nrm2729

180. Schwarz J, Bierbaum V, Merrin J, et al. A microfluidic device for measuring cell migration towards substrate-bound and soluble chemokine gradients. *Scientific Reports*. Published online October 26, 2016:1-12. doi:10.1038/srep36440

181. Ricoult SG, Kennedy TE, Juncker D. Substrate-Bound Protein Gradients to Study Haptotaxis. *Frontiers Bioeng Biotechnology*. 2015;3:40. doi:10.3389/fbioe.2015.00040

182. Fan YJ, Hsieh HY, Tsai SF, et al. Microfluidic channel integrated with a lattice lightsheet microscopic system for continuous cell imaging. *Lab Chip*. 2020;21(2):344-354. doi:10.1039/d0lc01009j

183. Pittet MJ, Weissleder R. Intravital Imaging. *Cell*. 2011;147(5):983-991. doi:10.1016/j.cell.2011.11.004

184. Liu TL, Upadhyayula S, Milkie DE, et al. Observing the cell in its native state: Imaging subcellular dynamics in multicellular organisms. *Science*. 2018;360(6386):eaaq1392-15. doi:10.1126/science.aaq1392

185. Gao R, Asano SM, Upadhyayula S, et al. Cortical column and whole-brain imaging with molecular contrast and nanoscale resolution. *Science*. 2019;363(6424):eaau8302. doi:10.1126/science.aau8302

186. Wu J, Lu Z, Jiang D, et al. Iterative tomography with digital adaptive optics permits hour-long intravital observation of 3D subcellular dynamics at millisecond scale. *Cell*. 2021;184(12):3318-3332.e17. doi:10.1016/j.cell.2021.04.029

187. Manley HR, Potter DL, Heddleston JM, Chew TL, Keightley MC, Lieschke GJ. Frontline Science: Dynamic cellular and subcellular features of migrating leukocytes revealed by in vivo lattice lightsheet microscopy. *J Leukocyte Biol*. Published online 2020. doi:10.1002/jlb.3hi0120-589r

188. Cremasco V, Woodruff MC, Onder L, et al. B cell homeostasis and follicle confines are governed by fibroblastic reticular cells. *Nat Immunol*. 2014;15(10):973-981. doi:10.1038/ni.2965

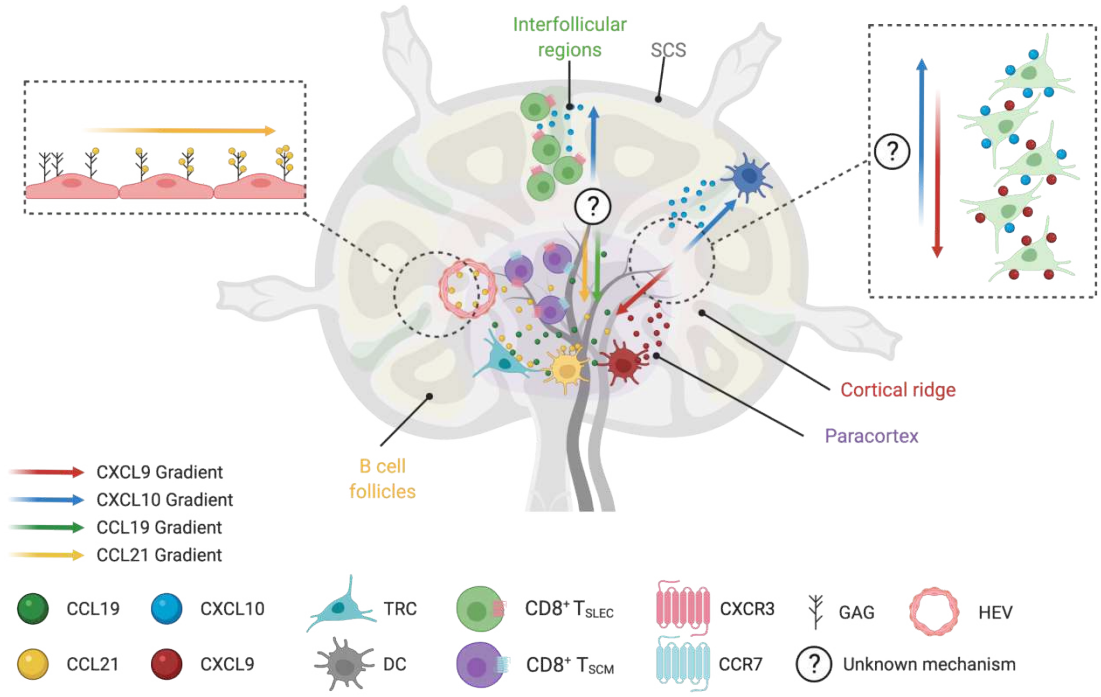


FIGURE 1 Complex chemokine gradients within the lymph node. The lymph node microarchitecture is comprised of the SCS, B cell follicles and interfollicular regions (IFRs), cortical ridge, T cell paracortex and the medulla. T cell migration is coordinated by complex chemokine gradients within the lymph node, and this migration drives T cell differentiation. Following LCMV infection, T_{SLEC} fates are imprinted in the IFR while T_{SCM} cells are retained in the T cell paracortex. CXCR3 ligands, CXCL9 and CXCL10 are produced in distinct regions of the lymph node. CXCL10-producing cells are positioned in the lymph node periphery, in the IFR while CXCL9-producing cells are more evenly distributed through the lymph node, with an increased expression in the lymph node paracortex. T_{SLEC} migration is facilitated by CXCR3 dependent migration towards the CXCL10 producing cells in the IFRs. Together CXCL9 and CXCL10 create competing gradients within the lymph node, but how these chemokines are distributed across the tissue is unknown. CXCL9 and CXCL10 have distinct GAG- and CXCR3- binding domains, suggesting they bind differently to the extra cellular matrix which may lead to individual ligand function. T_{SCM} express both CXCR3 and CCR7, however they remain in the lymph node paracortex, despite CXCL10 gradients in the periphery. T_{SCM} are likely retained in the paracortex via CCR7 ligands, CCL21 and CCL19. CCL21 is produced by fibroblastic reticular cells and T cell stromal cells, and binds to collagen IV at basal lamina of HEVs. This creates a CCL21 gradient across the endothelial

cells, which facilitates entry of CCR7^{+} naïve T cells into the lymph node via HEVs. CCL19 is also expressed by FRCs, but in a lower concentration that CCL21. CCL21 and CCL19 chemokines differ in their ability to bind to heparan sulfate residues. Consequently, CCL21 exists in both a soluble and insoluble form (immobilized by GAGs) while CCL19 exists only in soluble form.

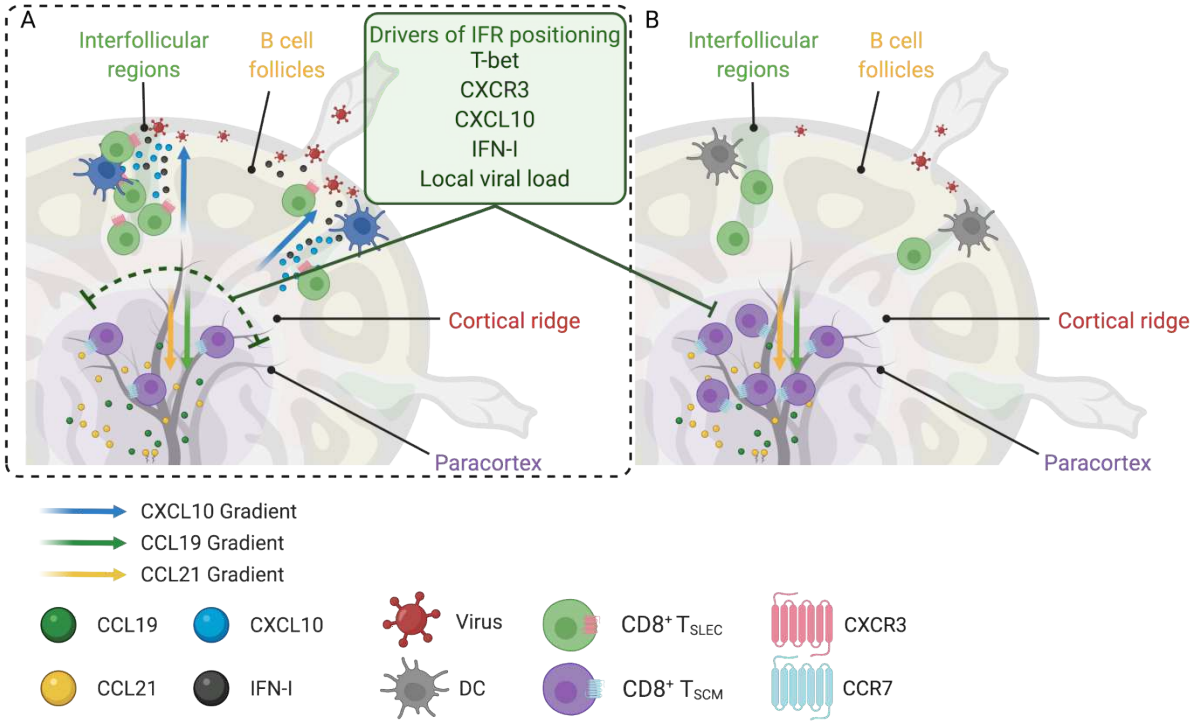


FIGURE 2 Proposed default theory for T_{SCM} generation based on spatial T cell position. A. Within an infected lymph node the IFR is a region with high inflammation, caused by virus draining into and establishing local infection. This upregulates IFN-I, stromal and DC sources of CXCL10, leading to the migration of Tbet^{+} CXCR3^{+} CD8^{+} T cells which are imprinted in this region to form T_{SLEC}. Within the paracortex, cells are segregated from peripheral lymph node inflammation due to duel expression of CCR7. This reduced local inflammation leads to default differentiation towards T_{SCM}. B. In the absence of local lymph node inflammation or reduced migration into the IFR (due to loss of CXCR3, CXCL10, or IFN-I signalling), CD8^{+} T cell retention in the paracortex is increased due to CCR7 expression and default T_{SCM} formation.

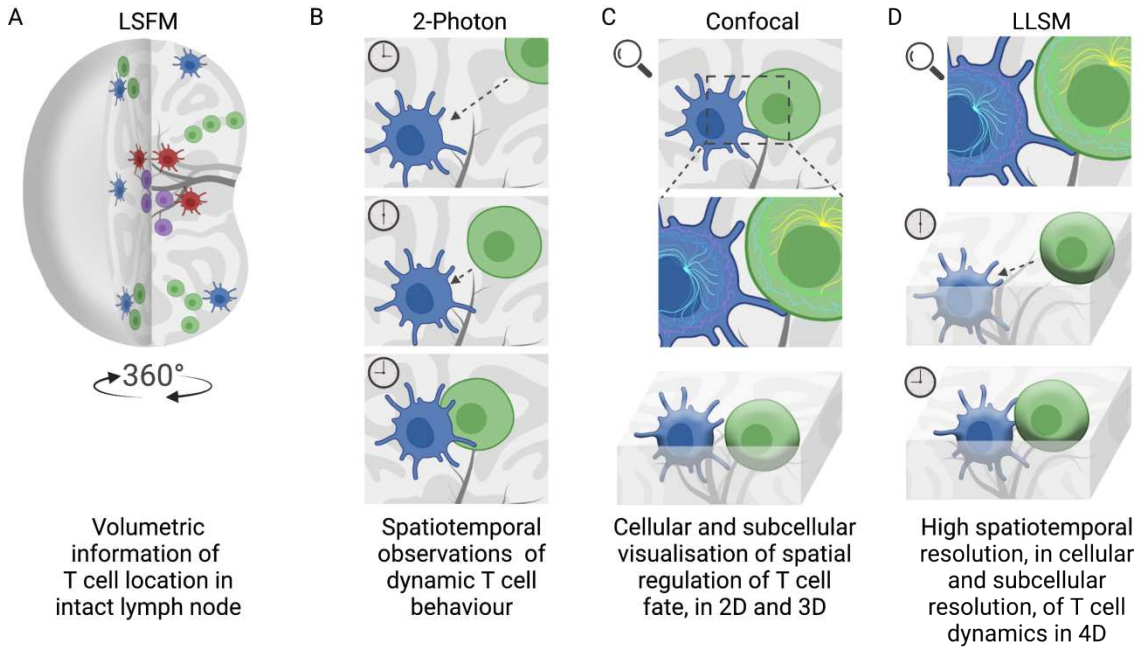
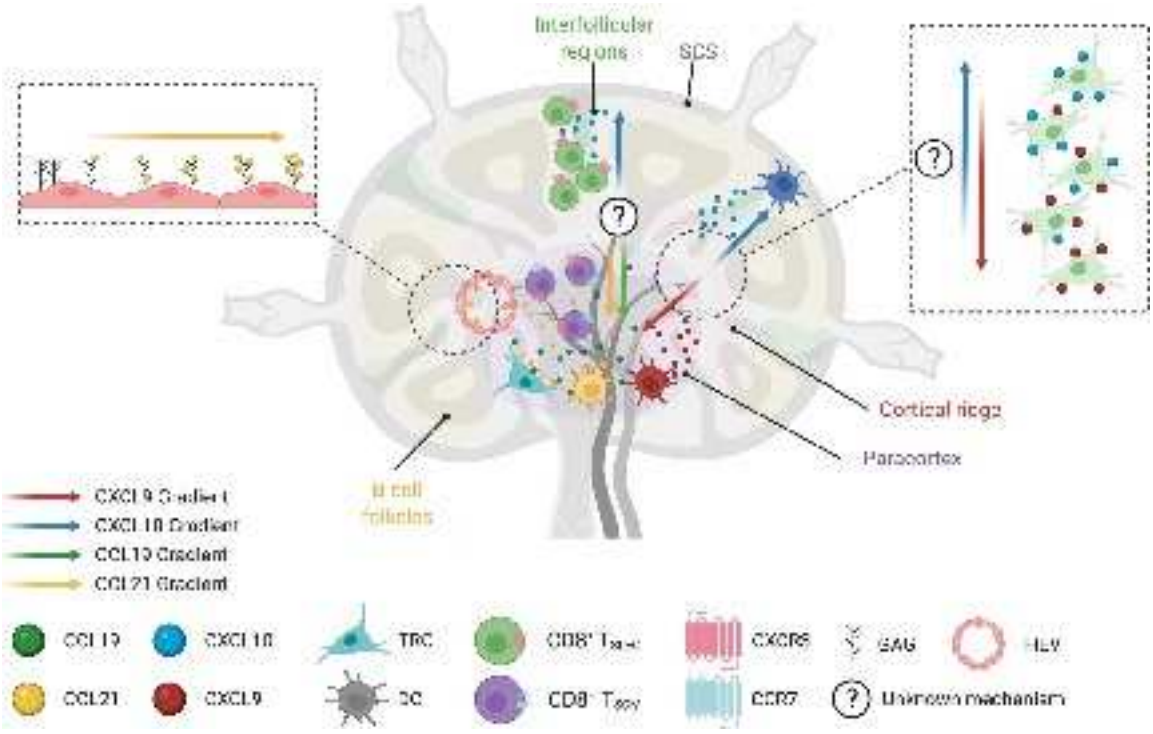
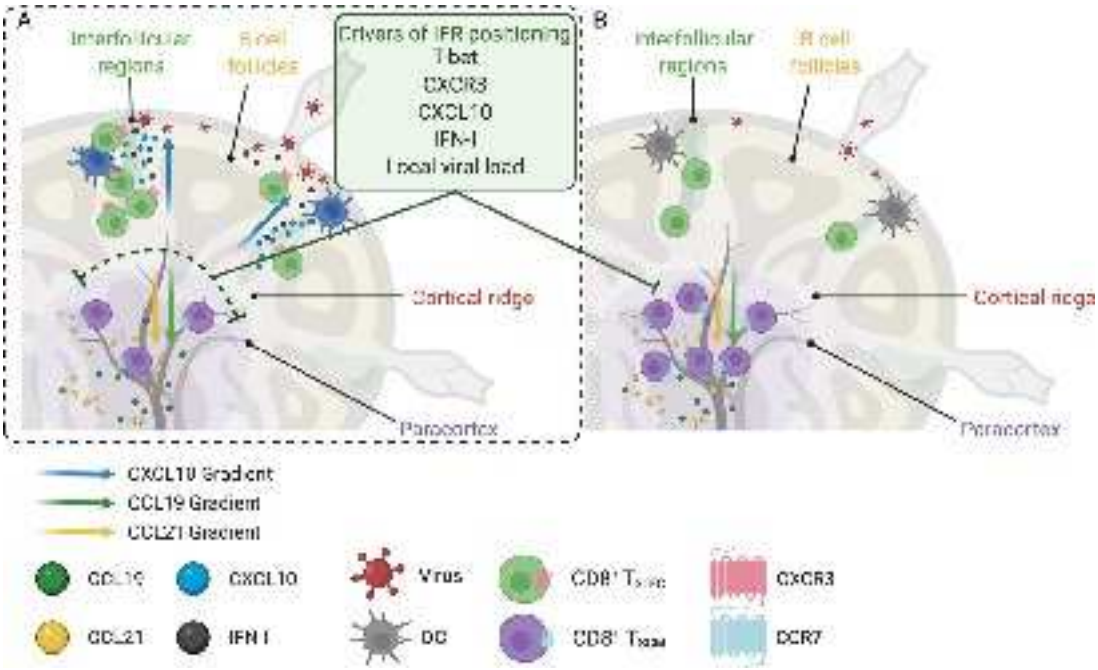


FIGURE 3 Imaging methods to investigate CD8+ T cell fate decisions A. Lightsheet fluorescence microscopy (LSFM), combined with tissue clearing, provides 3D information about antigen-specific T cell location within an intact lymph node. This allowed quantification of $CD8^+$ T cell positioning and cellular sources of chemokine at greater depths than 2P and multiphoton microscopy. B. While LSFM is limited to fixed tissues, 2-photon microscopy has enabled visualisation of dynamic T cell behaviour in intact organs *in vivo*. Intravital 2-photon microscopy has revealed the initial events following antigen recognition and T cell priming. However, the location of other critical T cell migration events in the paracortex are beyond the reach of 2-photon imaging. C. 2D and 3D confocal microscopy complements 2-photon imaging to visualise, in both cellular and subcellular resolution, the spatial regulation of T cell fate within the lymph node. Confocal microscopy was critical in understanding how and where T cells are activated. D. To further dissect the regulators of T cell migration dynamics *in vitro* and *ex vivo*, lattice lightsheet microscopy (LLSM) provides high spatiotemporal resolution with minimal phototoxicity. With this technology, critical T cell behaviours in 4D during antigen recognition and immunological synapse formation have been observed at subcellular resolution. Combined with rapidly evolving microfluidic

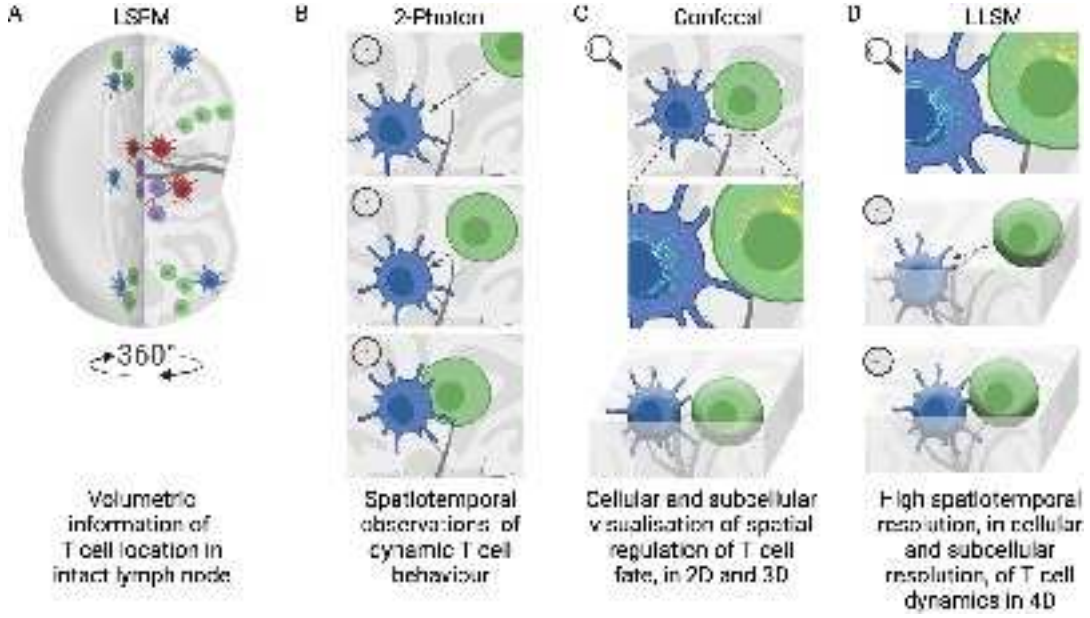
devices, LLSM also provides a platform to examine how $CD8^+$ T cells integrate complex chemokine signals.



imr_13044_f1.png



imr_13044_f2.png



imr_13044_f3.png