Skin and gut imprinted helper T cell subsets exhibit distinct functional phenotypes in central nervous system autoimmunity

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Multidimensional single-cell analyses of T cells have fueled the debate about whether there is extensive plasticity or ‘mixed’ priming of helper T cell subsets in vivo. Here, we developed an experimental framework to probe the idea that the site of priming in the systemic immune compartment is a determinant of helper T cell–induced immunopathology in remote organs. By site-specific in vivo labeling of antigen-specific T cells in inguinal (i) or gut draining mesenteric (m) lymph nodes, we show that i-T cells and m-T cells isolated from the inflamed central nervous system (CNS) in a model of multiple sclerosis (MS) are distinct. i-T cells were Cxcr6+, and m-T cells expressed P2rx7. Notably, m-T cells infiltrated white matter, while i-T cells were also recruited to gray matter. Therefore, we propose that the definition of helper T cell subsets by their site of priming may guide an advanced understanding of helper T cell biology in health and disease.

Organ-specific autoimmune diseases, including MS, are initiated and maintained by the activation of autoreactive T cells in the systemic immune compartment. In MS, autoreactive T cells are likely activated through molecular mimics of autoantigens acquired at body surfaces. However, it is unclear whether and how the anatomical niche, in which an autoreactive T cell is primed, has an impact on the recruitment, topology and inflammatory effector function of T cells in remote tissues like the CNS. Particularly in MS, the topology and the type of lesions within the CNS are critical determinants of the clinical phenotype, and we hypothesize that lesion development in MS is not random but controlled by immune system intrinsic molecular cues, which in turn might already be imprinted at the site of T cell priming outside the CNS. For instance, the gut microbiome might dictate the susceptibility to and severity of autoimmune diseases, for example, by controlling the adjuvanticity in local lymph nodes, thus inducing distinct helper T cell species.

Helper T cell subsets have been defined by their signature cytokines interferon (IFN)-γ (Teff1), interleukin (IL)-4 (Teff2) and IL-17 (Teff17). This concept has been widely accepted (also in T cell–mediated autoimmune diseases) because cytokines determine the interaction of T cells with other immune cells and thus their function in host defense and inflammation. While these T cell subsets are well characterized on the molecular level after in vitro differentiation of naïve T cells, a large overlap between T cell subsets has been observed in vivo and is explained by the plasticity of helper T cells previously ‘cleanly’ committed to a specific T cell lineage in mice and humans. Alternatively, it is an emerging concept that T cell subsets might be primed as mixed phenotypes from the get-go.

Here, we label antigen-specific T cells in skin draining inguinal lymph nodes (iLN) and gut draining mesenteric lymph nodes (mLN) in vivo, thus establishing a ‘provenance-mapping system’ for the T cells that are later collected in remote non-lymphoid tissues, such as in the CNS, during the course of experimental autoimmune encephalomyelitis (EAE), a preclinical disease model for MS. We characterize activated T cells defined by their anatomical origin in iLN (i-T cells) or mLN (m-T cells) and provide evidence that the priming site is a fundamental determinant of their commitment to a defined helper T cell lineage. Such imprinting ultimately results in the generation of specific effector functions in the CNS, including white matter versus gray matter infiltration. We show that this concept of ‘anatomically’ defined helper T cell subsets is compatible with some aspects of the extensive molecular characterization.
of previously defined helper T cell lineages. However, it also extends beyond rigid lineage definitions and instead provides a framework to accommodate and organize the complex ‘transcriptional continuum’ of single T cells in tissues based on their provenance.

**Results**

Provenance mapping of CNS T cells to distinct priming sites. To track lymphocytes from their priming sites to remote tissues, a photoconvertible protein tagged to mitochondria (mitoDendra2, mD2) was expressed in T cells using a CD4-Cre driver strain (PhAM™ mice). mD2 requires less energy for conversion than the widely used photoconvertible protein Kaede, and tagging to mitochondria increases its half-life.12 By irradiation with violet light (405 nm), we were able to photoconvert all T cells in any lymph node from mD2GREEN to mD2REDS (Extended Data Fig. 1a–c, including gating strategy). mD2REDS T cells could be followed for five cell divisions (and about 3 d) before they ‘lost’ the label (Extended Data Fig. 1d,e). First, we examined the population dynamics of mD2REDCD4 T cells in iLN in steady state (Fig. 1a). While naive T cells (CD4CD45RCD62Llow) decreased within the mD2REDS compartment over time, the relative fraction of antigen-experienced T cells (CD45ROFoxp3+Treg) increased, a process that was entirely blocked by irradiation with violet light (405 nm), which prevents trafficking of lymphocytes out of secondary lymphoid tissue (Fig. 1a). Notably, the fraction of Foxp3+ regulatory T (Treg) cells largely prevailed over conventional T cells in lymph node-resident mD2REDS T cells in steady state (Fig. 1b).

Next, we immunized PhAM™ mice subcutaneously with myelin oligodendrocyte glycoprotein peptide 35–55 (MOG35–55) in complete Freund’s adjuvant (CFA) according to a standard protocol to induce EAE, which reflects many aspects of the autoimmune T cell response in human MS.13 On day 4 after immunization, T cells in iLN were photoconverted, and their population dynamics were monitored. Again, the fraction of naive T cells decreased, and the fraction of antigen-experienced (CD45RO) T cells increased within iLN-resident mD2REDS T cells in an FTY720-dependent manner (Fig. 1c).

In contrast to steady state, the fraction of antigen-experienced effector T cells outnumbered the fraction of Foxp3+ Treg cells in lymph node-resident (mD2REDS) T cells of immunized mice (Fig. 1d). In summary, light-induced labeling of T cells allowed for assessment of the dynamics of lymph node-resident conventional T cells and Treg cells.

Because the gut microbiome might modulate immunopathology in remote tissues,14 we wanted to compare the ability to traffic T cells among all CD4+ T cells directly (day 0; left) and 2 d after photoconversion (right) in iLN. Representative plot from two independent experiments; OVA, ovalbumin.

**Fig. 1 | Provenance mapping of CNS T cells to distinct peripheral priming sites.** a–c, Kinetics of mD2REDCD4 T cells in iLN of FTY720-treated and untreated (no FTy) mice after photoconversion in steady state (a) and on day 4 after EAE induction (c). Representative plot of one PhAM™ mouse per time point and one non-photocverted dark control (bar) from two independent experiments. b, d, Population dynamics of T cell populations 2 d after photoconversion without FTy (b) or with FTy (right) in steady state (b) and on day 6 after immunization (d). Red bars represent the frequencies of mD2REDCD4+ T cells among all CD4+ T cells directly (day 0; left) and 2 d after photoconversion (right) in iLN. Representative plot from two independent experiments. e–g, Flow cytometric assessment of iLN-labeled (e) and mLN-labeled (f) PhAM™ EAE mice 2 d after photocconversion at disease onset in the periphery (e,f) and in the CNS (g). Representative plots from n = 15 mice per group for the periphery and the CNS. The site of photoconversion (iLN or mLN) is indicated (lightning symbol). h, Frequencies of regulatory and activated mD2REDCD4+ T cells in PhAM™ EAE mice for different tissues; n = 2 mice per group; representative plots from three independent experiments. Data are shown as the mean ± s.d. i, Velocity versus Ca2+ indicator ratio (yellow fluorescent protein, YFP)/mCerulean3 (CFP) of TCRMOG Twitch-2B T cells in iLN and mLN imaged on days 3 or 4 after transfer. Cell numbers and Ca2+ ratios: 24 and 883 for iLN (PBS), 25 and 856 for iLN (MOG), 67 and 2,353 for mLN (PBS) and 167 and 5,971 for mLN (MOG), respectively. j, Mean T cell fractions with MOG-specific Ca2+ signals in iLN and mLN; n = 3 experiments. Data are shown as the mean ± s.d. k, IA-A–MOG tetramer binding in mD2REDS i-T cells and m-T cells in the CNS of PhAM™ EAE mice. Pool of n = 3 mice per group; representative plot from two independent experiments; OVA, ovalbumin.
because they showed high levels of cell cycle genes. Single T cells in cluster 10 were also excluded because TCR gene expression was low. We found expanded TCR clones in i-T cells and m-T cells of all clusters (Fig. 2b). To test whether the clones of the 'i-stream' and the 'm-stream' (Extended Data Fig. 2b) were related across organs, we performed hierarchical clustering of CDR3 sequences. The CDR3 repertoires within each stream (but not across streams, for example, according to organs) clustered together, suggesting that the expanded i-T cell and m-T cell clones were distinct (Extended Data Fig. 2c). More i-T cell than m-T cell clones were related among each other in phenotypic clusters 6 and 7, corresponding to the spleen and CNS. By contrast, cluster 0, which extended across organs, contained more related m-T cell clones than i-T cell clones (Fig. 2c).

| T cell antigen receptor β-chain variable (TRBV) families | 5, 12, 19 |

| Control Day 0 | Day 1 | Day 2 |
|---------------|-------|-------|
| CD44intCD62Lhigh (%) | 0 | 25 | 50 |
| 75 | 100 |
| CD44highFoxp3– (%) | 0 | 25 | 50 |
| 75 | 100 |

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and 20 were overrepresented in the spleen and CNS, while TrbV3 was most prominent in cluster 0 (Fig. 2d). Notably, because immune regulatory genes (including Foxp3, Ikaros, CitrA and Girt (Tnfrsf18)) were overrepresented in cluster 0 compared to cluster 6 (Extended Data Fig. 2d), distinct TCR Vβ families appeared to be associated with regulatory and effector functions of T cells, respectively. As i-T cells and m-T cells shared the same TRBV families in the spleen and CNS, we concluded that similar antigens might be recognized by i-T cells and m-T cells, and some of the overrepresented TRBV motifs were consistent with MOG reactivity. However, the lack of public clones between i-T cells and m-T cells suggested separate trajectories from the peripheral immune system to the CNS in both streams without ‘cross-over’.

Transcriptional signatures of i-T cells and m-T cells are different from classical helper T cell signatures. Unsupervised clustering of single-cell transcriptomes of i-T cells and m-T cells in all organs yielded 10 different clusters. Most strikingly, in the CNS, we observed a large preponderance of cluster 9 in i-T cells and of cluster 5 in m-T cells (Fig. 3a,b). To test whether the transcriptomes of i-T cells and m-T cells were distinct, we used the minimum density hyperplane projection to cluster mD2RED T cells within the CNS using the top 2,000 most variable genes and performed Hartigan’s dip test of multimodality. In isolation, the i-T cell and m-T cell datasets did not show a ‘dip’ (Fig. 3c, left and middle). By contrast, we found a significant dip in the density distribution of the combined set of i-T cells and m-T cells in the CNS, suggesting that the distribution of the combined i-T cell and m-T cell population was at least bimodal (Fig. 3c, right). Therefore, i-T cells and m-T cells in the CNS likely constituted distinct T cell subsets, indicating that their site of priming was a determinant of the segregation of their transcriptomes. RNA velocity analysis illustrated a trajectory from the lymph node to the spleen to the CNS within both the i-stream and the m-stream (Fig. 3d), consistent with the TCR sequencing data. In the CNS, neither i-T cell nor m-T cell phenotypes were congruent with classical helper T cell signatures (Fig. 3e and Extended Data Fig. 4a,b). Accordingly, Tπ17 cells, either current or historic IL-17 producers as marked by mD2 using an IL-17A-Cre driver in PhAM15,17 mice, were primed both in iLN and mLN and trafficked to the CNS in both scenarios (Extended Data Fig. 3b). Despite their mixed phenotypes, however, i-T cells showed an overall greater similarity to Tπ1 cells than m-T cells (Fig. 3e and Extended Data Fig. 3c). Tπ2-ness was evenly distributed among i-T cells and m-T cells. Notably, when using a published pathogenic Tπ17 signature (which is composed of a mixture of Tπ1 and Tπ17 features) as a reference, i-T cells were enriched in the Tπ17 path signature in the lymph node, spleen and CNS compartment (Fig. 3f), arguing in favor of this signature already being pre-imprinted in the systemic immune compartment (that is, in iLN but not in mLN).

The core signatures of i-T cells and m-T cells are distinct. Because i-T cells and m-T cells could not be described as either classical Tπ1 cells or Tπ17 cells, we sought to define the private ‘core signatures’ of i-T cells and m-T cells. We used our scRNA-seq dataset of i-T cells and m-T cells to identify genes uniquely upregulated in i-T cells or m-T cells in all organs (lymph nodes, spleen and CNS) (Fig. 4a,b). The i-T cell and m-T cell core signatures were composed of 94 genes and 50 genes, respectively (Fig. 4b). Examples of i-T cell-associated genes were transcription factors, such as Rora and Runx2, costimulatory molecules, such as Icos and Cd2, cytokine receptors, such as Il1r1 and Il1r1, as well as chemokine receptors and integrins, such as Cxcr6 and Il7r1, respectively (Fig. 4c.e and Extended Data Fig. 4a,b). In the m-T cell core signature, we found Malt1, Ahr as well as Cx3d and P2rx7 and the integrin subunit Igad (Fig. 4d,e and Extended Data Fig. 4a,b). Eventually, to define transcriptional modules unique to i-T cells and m-T cells, respectively, we used the SCENIC algorithm that ranks transcription factors according to the expression levels of known target genes. This transcription factor activity assessment yielded high scores for RORγt, Runx3 and Crem in i-T cells and for Ahr, Foxo1 and EzH2 in m-T cells (Extended Data Fig. 4c). Based on the i-T cell and m-T cell core signatures, we speculated that one of the discriminating features of these T cell subsets might be different trafficking properties. As examples of guidance molecules, we investigated putative transactivators of Cxcr6 and Iga4, respectively. Transcription factors controlling Cxcr6 (including Tbx21, Crem, Rorc and Runx family members) were almost exclusively more active in i-T cells than in m-T cells, consistent with the observation that Cxcr6 was more abundantly expressed in i-T cells than in m-T cells (Extended Data Fig. 4d). By contrast, transcription factors controlling Iga4 were distinct in i-T cells and m-T cells with Klf2 and Ets family members were almost exclusively more active in i-T cells than in m-T cells.

The transcriptomes of i-T cells versus m-T cells are controlled by different transcription factors. To directly assess the transcriptional landscape of i-T cells and m-T cells, we photoconverted T cells in iLN or mLN in immunized mice and sorted i-T cells or m-T cells from the spleen 2 d later for assay for transposase-accessible chromatin with sequencing (ATAC-seq) analysis. When we restricted the RNA core signatures of i-T cells and m-T cells to those loci that also exhibited a homologously differentially open chromatin, the i-T cell signature was reduced to 47 genes and the m-T cell signature to 18 genes (Fig. 5a,b and Supplementary Table 1). Salient examples of loci with differentially open chromatin that were also differentially transcribed included Cxcr6 in i-T cells and Iga4 in m-T cells (Fig. 5c and Extended Data Fig. 5a). Gene Ontology (GO) pathway analysis of the ‘ATAC-seq-validated’ i-T cell and m-T cell core signatures
revealed 'IL-1 receptor activity' and 'purine metabolism' as distinctive between iT cells and mT cells, respectively, while trafficking and adhesion pathways were detected to be active in both subsets (Fig. 5d). Differential open chromatin regions (OCRs) in the iT cell and mT cell core signatures were analyzed for transcription factor activity.22,23 When ranked by transcription factor activity score, transcription factors associated with general activation of T cells, including Tcf1 (encoded by Tcf7), Maf, Irf4, Prdm1 and Foxo1, appeared to be more active in mT cells than in iT cells, while Crem and JunB, but also T-bet and the ROR transcription factors, which have been associated with specific effector T cell commitment, were more operational in iT cells than in...
m-T cells (Fig. 5e). Accordingly, Crem, Fosl2 and T-bet binding motifs were among the top motifs in differential OCR i-T cell signature genes. Conversely, Irf and Ets transcription factor motifs were preferentially found in differential OCR m-T cell signature genes (Extended Data Fig. 5b,c), largely corroborating our SCENIC analysis. Taken together, the ATAC-seq data supported the concept that i-T cells and m-T cells constitute distinct helper T cell subsets that may be controlled by specific transcriptional networks.

Fig. 3 | Imprinting of helper T cell signatures in i-T cells versus m-T cells. a, Unsupervised clustering t-SNE plot of all single mD2RED CD4^+CD44^+ T cells colored according to the cell cluster (left) or i-stream and m-stream (right); n = 2 PhAMT EAE mice per group, n = 4,169 cells. b, Cluster distribution of i-stream (top) versus m-stream (bottom) by tissue. c, Two-sided Hartigan’s dip test of multimodality applied to i-T cells in the CNS (left), m-T cells in the CNS (middle) and all mD2RED (combined i- and m-) T cells per CNS (right) without adjustments for multiple comparisons. d, RNA velocity analysis using ratios of unspliced-to-spliced transcripts plotted in uniform manifold approximation and projection (UMAP) space for single T cells from i-stream (left) and m-stream (right). The directional flow of the velocity arrows between cell clusters shows the projection from the observed state to the predicted future state. e, T cell signature scores based on the expression of key genes; TN1, n = 553 cells, at least three genes out of Csf1, Ccric, Il2rb2, Il18r1, Klrc1 and Hopx; TN2, n = 389 cells, at least two genes out of Il4, Tnfsf13b, Batf, Nfil3 and Atf5; TN17, n = 127 cells, at least four genes out of Il17f, Il17a, Il21, Il2, Tnfsf13b, Ptgfrn, Ahr, Irf4, Rora and Plagl2; Treg, n = 373 cells, at least three genes out of Il12ra2, Nos2, Tnfsf11 and Irf8. f, Gene set enrichment analysis (GSEA) for gene signatures differentially expressed in m-stream versus i-stream tissues; FDR, false discovery rate; NES, normalized enrichment score.
**Fig. 4 | Core signatures of i-T cells versus m-T cells.**

**a.** Unsupervised clustering t-SNE plot of all single mD2RED+CD44high T cells colored according to the lymph node of origin (top), the spleen (middle) and the CNS (bottom) for the i-stream and m-stream; n = 2 PhAMT EAE mice per group, n = 4,169 cells; iLN, n = 1,296 cells; i-Spleen, n = 542 cells; i-CNS, n = 233 cells; mLN, n = 1,404 cells; m-Spleen, n = 525 cells; m-CNS, n = 169 cells. **b.** Core gene set from differentially expressed genes in all three tissues with a P value < 0.05 for the i-stream (top) versus the m-stream (bottom). Two-sided Wilcoxon rank-sum test. **c, d.** Average gene expression for the 94 genes in the i-stream core gene set (c) and the 50 genes in the m-stream core gene set (d) per tissue as indicated. **e.** Flow cytometric assessment of Cxcr6 (top) and P2rx7 (middle) versus CD44 and CD49d (Itgb1) versus CD29 (Itgb1) (bottom) in mD2RED+CD44high i-T cells and m-T cells in the spleens of PhAMT EAE mice 2 d after photoconversion at disease onset. Representative plots from n = 3 mice per group for Cxcr6, P2rx7 and CD49d/CD29.
Fig. 5 | Transcriptional modules of i-T cells versus m-T cells. a, Normalized ATAC signal displaying all peaks for i-T cells and m-T cells from spleen (gray). Dashed lines represent the border of fold enrichment between i-T cells and m-T cells >2; peaks with less than fivefold enrichment over background signal were discarded. Peaks associated with genes overexpressed in CNS i-stream are shown in purple; peaks associated with genes overexpressed in CNS m-stream are shown in orange. Some relevant genes are labeled, including Itga4, Adk, Itgb1 and Cd7 (i-stream) and Ifngr1, Malt1, Icos and Cxcr6 (m-stream). Pool of n = 2 PhAM1 EAE mice per group. b, Average expression of genes (n = 65, 47 i-stream up and 18 m-stream up) that are both in the core gene set of differentially expressed genes and contain differentially open chromatin.

c, Genome browser view of some of the signature genes mentioned in a, which are also associated with differential OCRs. Displayed tracks correspond to ATAC-seq for i-T cells (purple) and m-T cells (orange). In all regions, differential peaks are highlighted with arrows. d, List of the top 20 enriched pathways found for i-stream (left) and m-stream (right) based on genes associated with differential OCRs, which were also differentially expressed (core signature genes). Two-tailed Fisher’s exact test with Benjamini–Hochberg FDR correction. e, Transcription factor activity score as assessed with Regulatory Genomics Toolbox in i-signature genes with differential OCRs as compared to m-T cells and in m-signature genes with differentially OCRs as compared to i-T cells, ranked by transcription factor activity score. The y axis represents the differences in transcription factor dynamics between the two conditions, and the x axis shows the position of transcription factors in the ranking. Transcription factors active in the m-stream and i-stream are positioned on the left and right side of the plot, respectively. Some relevant transcription factors are highlighted.
The i- and m-signatures of antigen-specific T cells are robust under various priming conditions. 

**a** Flow cytometric assessment of mD2^{CD4+CD44^{high}} T cells in the spleens of iLN-labeled (purple) and mLN-labeled (orange) PhAM^{T} mice, 2D2 PhAM^{T} mice and 2D2 × TH PhAM^{T} mice for Cxcr6 (top), P2rx7 (middle) and CD49d (Itga4) versus CD29 (Itgb1) (bottom). PhAM^{T} mice were subjected to either MOG_{35–55}/CFA or OVA_{323–339}/CFA s.c. immunization at the base of the tail or MOG_{35–55}/CFA s.c. immunization at the neck (between the shoulder blades) plus pertussis toxin i.v. on day 0 and day 2 after immunization (as indicated) and analyzed 2 d after photoconversion on day 11 after immunization. The 2D2 PhAM^{T} mice were immunized with 40 μg of MOG_{35–55} i.v., photoconverted on day 2 and analyzed on day 4 after immunization. The 2D2 × TH PhAM^{T} mice were photoconverted at disease onset (approximately at 5 weeks of age) and analyzed 3 d after photoconversion. Representative plots from n = 3 PhAM^{T} mice per group and immunization condition are shown; n = 4 2D2 PhAM^{T} mice and 2D2 × TH PhAM^{T} mice per group. Numbers indicate mean fluorescence intensities.

**b** Gene expression heat map of all differentially expressed genes in the bulk sequencing data (up and down regulated) in i-T cells and m-T cells isolated from the spleens of 2D2 × TH PhAM^{T} mice. Differentially expressed genes (P < 0.05) were detected using the Seurat FindMarkers function based on a two-sided Wilcoxon rank-sum test and adjusted by the Bonferroni method. The positions of Art2b, Itga4, Itgb1, Ifitm2, Cd7, Rarg and Stx11 are indicated.

**c** GSEA of i-T cell and m-T cell core signatures (see Supplementary Table 1) in bulk RNA-seq data of i-T cells versus m-T cells isolated from the lymph nodes (top) and spleens (bottom) of 2D2 × TH PhAM^{T} mice. **d** Waterfall plot of genes expressed in i-T cells (left) versus m-T cells (right) from the spleens of 2D2 × TH PhAM^{T} mice ranked by FC. Significant genes at P < 0.05 are colored. A two-sided Wilcoxon rank-sum test adjusted by the Bonferroni method was used. Some genes are indicated by name.

Fig. 6 | The i- and m-signatures of antigen-specific T cells are robust under various priming conditions. a, Flow cytometric assessment of mD2^{CD4+CD44^{high}} T cells in the spleens of iLN-labeled (purple) and mLN-labeled (orange) PhAM^{T} mice, 2D2 PhAM^{T} mice and 2D2 × TH PhAM^{T} mice for Cxcr6 (top), P2rx7 (middle) and CD49d (Itga4) versus CD29 (Itgb1) (bottom). PhAM^{T} mice were subjected to either MOG_{35–55}/CFA or OVA_{323–339}/CFA s.c. immunization at the base of the tail or MOG_{35–55}/CFA s.c. immunization at the neck (between the shoulder blades) plus pertussis toxin i.v. on day 0 and day 2 after immunization (as indicated) and analyzed 2 d after photoconversion on day 11 after immunization. The 2D2 PhAM^{T} mice were immunized with 40 μg of MOG_{35–55} i.v., photoconverted on day 2 and analyzed on day 4 after immunization. The 2D2 × TH PhAM^{T} mice were photoconverted at disease onset (approximately at 5 weeks of age) and analyzed 3 d after photoconversion. Representative plots from n = 3 PhAM^{T} mice per group and immunization condition are shown; n = 4 2D2 PhAM^{T} mice and 2D2 × TH PhAM^{T} mice per group. Numbers indicate mean fluorescence intensities.

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c, GSEA of i-T cell and m-T cell core signatures (see Supplementary Table 1) in bulk RNA-seq data of i-T cells versus m-T cells isolated from the lymph nodes (top) and spleens (bottom) of 2D2 × TH PhAM^{T} mice. **d** Waterfall plot of genes expressed in i-T cells (left) versus m-T cells (right) from the spleens of 2D2 × TH PhAM^{T} mice ranked by FC. Significant genes at P < 0.05 are colored. A two-sided Wilcoxon rank-sum test adjusted by the Bonferroni method was used. Some genes are indicated by name.
The anatomical lymph node niche dictates the signature of i-T cells and m-T cells independently of the mode of immunization. Next, we asked whether the distinct i-T cell and m-T cell signatures were determined by the antigen dose or route of antigen delivery, which might create differential T cell priming milieus in iLN and mLN. To address this question, we selected Cxcr6 and Itgα4 as well as P2x7 and Itgβ1, which segregated with i-T cells and m-T cells, respectively, and tested their levels of protein expression in splenic CD44hiCD25ip-iT cells or m-T cells 2 d after photoconversion of iLN or mLN of PHAM mice subjected to various immunization regimens. First, s.c. injection of MOG35–55 at remote sites (neck) did not essentially alter the pattern of i-T cell and m-T cell marker expression (Fig. 6a). Second, s.c. immunization with another antigen (OVA23–32,119) in CFA also led to the preferential expression of Cxcr6 and Itgα4hiItgβ1hi in i-T cells and P2x7 and Itgα4hiItgβ1lo in m-T cells (Fig. 6a). When we modified our s.c. immunization protocol by using no adjuvant (IFA) or alternative adjuvants (lipopolysaccharide (LPS) or peptidoglycan (PGN)), we consistently observed that i-T cells had an Itgα4loItgβ1hi phenotype, while m-T cells were Itgα4hiItgβ1lo irrespective of the adjuvant (Extended Data Fig. 6a). Non-immunogenic or moderately immunogenic s.c. immunizations blunted the differential expressions of P2x7 in m-T cells and Cxcr6 in i-T cells (Extended Data Fig. 6a), suggesting that the ‘bedrock’ T cell signature-imprinting capacity of iLN and mLN can be modified (but is not abolished) by different adjuvants. Importantly, we also tested our i-T cell and m-T cell markers in TCRαβBC transgenic PHAM mice (2D2×PHAM) after intravenous (i.v.) administration of MOG35–55, which would not bias the antigen dose in favor of iLN. Two days after i.v. injection of MOG peptide into 2D2×PHAM mice, iLN or mLN were photoconverted followed by analysis of i-T cells and m-T cells in the spleen after another 2 d. Similar to the s.c. immunization protocols, we did not observe cross-trafficking between iLN and mLN (Extended Data Fig. 6b). Again, antigen-specific CD44hi-iT cells had an Itgα4hiItgβ1hi phenotype and expressed more Cxcr6, while m-T cells were Itgα4loItgβ1lo and expressed more P2x7 than their counterparts derived from the alternative lymph node station (Fig. 6a). In an attempt to definitely prove that the i-T cell and m-T cell signature markers were linked to the priming of T cells in iLN or mLN, we bred the PHAM–mice with the double transgenic TCRαβBC and B cell receptor (BCR)αβBC background (2D2×TH). The 2D2×TH PHAM–mice developed spontaneous EAE at around 5 weeks of age (in the absence of any immunization). At 5 weeks, iLN or mLN of 2D2×TH PHAM–mice were photoconverted, and i-T cells and m-T cells were tested in the spleen. Again, we found robust Itgα4loItgβ1hi and Itgα4hiItgβ1lo phenotypes in i-T cells and m-T cells, respectively. P2x7 was higher in m-T cells while Cxcr6 expression was marginally elevated in i-T cells as well as of m-signature genes in m-T cells isolated from the spleens of 2D2×PHAM mice into Rag1−/− secondary host animals. First, we noted that the i-phenotype versus m-phenotype of transferred m-T cells was stable according to the differential expression of Itgα4 (Extended Data Fig. 7b). When we analyzed host animals 2 d into clinical disease, T cell infiltrates in the brain stem were similar irrespective of whether the host mice had received i-T cells or m-T cells (Fig. 7d(ii), arrows). Yet, white matter inflammatory foci in the brain stem were more prominently found in m-T cell recipients (Fig. 7d(ii), arrow heads). In addition, in the cerebellum, only m-T cells, but not i-T cells, infiltrated into the white matter (Fig. 7d(ii), arrows, and (iv) (versus (iii))). In the spinal cord, the infiltration pattern was scattered comprising white matter and gray matter in mice that had received i-T cells. By contrast, similar to the cerebellum, m-T cell recipients exhibited primarily white matter infiltrates in the spinal cord (Fig. 7d(v–viii) and quantified in Fig. 7e). To test whether the differential expression of Cxcr6 was necessary to produce the differential infiltration pattern observed in i-T cells versus m-T cells, we used Cxcr6−/− mice (functional Cxcr6−/−). Following immunization with MOG35–55/CFM, disease severity in Cxcr6−/− mice was comparable to wild-type control mice (Extended Data Fig. 7c). However, T cell infiltrates in the spinal cord of Cxcr6−/− mice were essentially restricted to white matter areas, lymph nodes and spleens of 2D2×TH PHAM− mice by RNA-seq, we identified major i-core and m-core signature genes segregating with i-T cells and m-T cells isolated from spontaneous EAE mice (Fig. 6b), and GSEA confirmed the enrichment of i-signature genes in i-T cells as well as of m-signature genes in m-T cells isolated from lymph nodes and spleens of 2D2×TH PHAM− mice (Fig. 6c,d). In summary, these data support the idea that the specific ecosystems of iLN and mLN are major determinants for the imprinting of distinct transcriptomes in T cells during antigen-specific priming.

The CNS infiltration patterns of i-T cells versus m-T cells are distinct. To test whether the phenotypes of i-T cells and m-T cells resulted in distinct effector functions in the CNS, we photoconverted T cells in the iLN or mLN in MOG35–55/CFM-immunized mice and analyzed the infiltration of i-T cells and m-T cells in the spinal cord and brain stem by histology. While the amount of i-T cells and m-T cells was in the same range in the brain stem, m-T cells were virtually absent in the spinal cord, which was readily targeted by i-T cells (Fig. 7a,b). Because both groups of mice were immunized according to an identical protocol (and then differentially labeled either in iLN or mLN), the disease severity and phenotype was similar (Extended Data Fig. 7a). While at least one-third of i-T cells were negative for α4-integrin (Itgα4, CD49d) expression, essentially all m-T cells expressed the α4-integrin subunit in the CNS (Fig. 7c). Conversely, Cxcr6 was strongly expressed in the majority of CNS i-T cells but not in m-T cells (Fig. 7c). To link this observation with the priming of autoreactive T cells in iLN or mLN, we adoptively transferred MOG-specific i-T cells or m-T cells isolated from the spleens of 2D2×PHAM mice into Rag1−/− secondary host animals. First, we noted that the i-phenotype versus m-phenotype of transferred T cells was stable according to the differential expression of Itgα4 (Extended Data Fig. 7b). When we analyzed host animals 2 d into clinical disease, T cell infiltrates in the brain stem were similar irrespective of whether the host mice had received i-T cells or m-T cells (Fig. 7d(i)(ii)). Yet, white matter inflammatory foci in the brain stem were more prominently found in m-T cell recipients (Fig. 7d(ii), arrow heads). In addition, in the cerebellum, only m-T cells, but not i-T cells, infiltrated into the white matter (Fig. 7d(ii), arrows, and (iv) (versus (iii))). In the spinal cord, the infiltration pattern was scattered comprising white matter and gray matter in mice that had received i-T cells. By contrast, similar to the cerebellum, m-T cell recipients exhibited primarily white matter infiltrates in the spinal cord (Fig. 7d(v–viii) and quantified in Fig. 7e). To test whether the differential expression of Cxcr6 was necessary to produce the differential infiltration pattern observed in i-T cells versus m-T cells, we used Cxcr6−/− mice (functional Cxcr6−/−). Following immunization with MOG35–55/CFM, disease severity in Cxcr6−/− mice was comparable to wild-type control mice (Extended Data Fig. 7c). However, T cell infiltrates in the spinal cord of Cxcr6−/− mice were essentially restricted to white matter areas.
while the T cell infiltrates of wild-type mice extended to the gray matter as well (Fig. 7f and quantified in Fig. 7g). Strikingly, in the cerebellum, T cell infiltrates in Cxcr6$$^{+/−}$$ mice were exclusively observed in the white matter (Fig. 7h), suggesting that Cxcr6 expression contributed to the targeting of autoreactive T cells to gray matter. Therefore, the differential expression of crucial guidance molecules between i-T cells and m-T cells might be responsible for their differential targeting to specific CNS areas.
Representation of i-T cell and m-T cell core signatures in the cerebrospinal fluid (CSF) of individuals with MS. Finally, to translate our provenance-determined T cell signatures into human T cells, we performed scRNA-seq on CSF cells of untreated individuals with relapsing-remitting MS (Supplementary Table 2). Unsupervised clustering of CSF CD4+ T cell identified 4 clusters, with expanded T cells accumulating in clusters 2 and 0 (Fig. 7i and Supplementary Table 3). Next, we projected established helper T cell signatures, including T\textsubscript{H}1, T\textsubscript{H}2, T\textsubscript{H}17 and T\textsubscript{reg} cell signatures, on the transcriptomes of single CSF CD4+ T cells. However, except for T\textsubscript{reg} cells (that localized to cluster 3), helper T cell signatures segregated only insufficiently with CSF T cell clusters (Extended Data Fig. 7d). By contrast, CXCR6 and other molecules that compose the i-T cell signature (including IL2RB, IL18RAP, SYTL2 and CD2) were mainly expressed in cluster 2 T cells (Fig. 7j) and Extended Data Fig. 7e), while m-sigature gene expression (for example, NT5E, DST and AIG1) was overrepresented in T cells mapping to clusters 0, 1 and 3. ITGA4-positive cells were more widely distributed in human CSF CD4+ T cells (Extended Data Fig. 7e). These data suggested that ’provenance-defined’ T cell subsets might be identified in human CSF T cells as well, with some markers being universal across species.

Discussion

In this study, we introduce the concept of provenance-defined T cell signatures that are maintained in remote tissues. Because i-T cells and m-T cells did not cross-traffic to alternative lymph node stations in relevant amounts, it was likely that the specific (anatomically and functionally defined) niches in iLN and mLN were determinants of the distinct transcriptomes and effector phenotypes of i-T cells and m-T cells. In fact, transcription factors such as Crem and Ahr, which were associated with i-T cells and m-T cells, respectively, are known to be activated by environmental cues.25,26 While transcription factor activities were different in i-T cells versus m-T cells, potential non-redundant transcriptional modules of i-T cells and m-T cells remain to be defined.

Primed site-specific imprinting of T cell features has been described, in particular in the context of tissue-selective lymphocyte trafficking.23 For instance, lamina propria-derived dendritic cells (DCs) exposed to vitamin A induce CCR9 and αβ integrin expression in T cells, thus promoting the capacity of those T cells to home back to the gut.27 Conversely, DCs in ILN suppress gut-homing molecules and induce CCR10 as well as cutaneous lymphocyte antigen (CLA) in T cells to enable them to home to the skin.28,29 Moreover, recent papers suggest that ’precursors’ of tissue-resident effector or T\textsubscript{reg} cell subsets are found in the spleen.30,31 Here we propose that the differential imprinting of T cell phenotypes in different lymph node stations is co-opted by autoreactive T cells that then aberrantly home to remote target tissues and exert distinct effector functions according to adjuvant cues they received at their original priming site. The microbiome at body surfaces might provide some of these adjuvant cues. While the significance of the skin microbiome in MS pathology has not been explored to the same extent as the gut microbiome,31 our study might provide a universal framework for the translation of this ‘environmental information’ into immunopathology in remote target tissues.

Neither i-T cells nor m-T cells fully overlapped with T\textsubscript{H}1 or T\textsubscript{H}17 cells, respectively, but constituted distinct T cell species. IL-7-producing cells were induced in both iLN and mLN and reached the CNS; yet, their transcriptomes and functional phenotypes were distinct. For instance, while m-T cells were primarily recruited to white matter, i-T cells were licensed to infiltrate both gray and white matter. Cxcr6 expression was necessary for this behavior, and Cxcr6 was expressed in i-T cells but much less in m-T cells. Recently, Cxcr6 has been described as a marker of tissue residency in human CSF and brain T cells32-34, even though white versus gray matter distribution has not been analyzed. Cxcr6 is a constituent of the core signature of CD4+ tissue-resident memory T (T\textsubscript{reg}) cells.35 Conversely, while essentially lacking Cxcr6, m-T cells expressed higher levels of P2rx7 than i-T cells. In line with the preferential expression of P2rx7 in m-T cells, retinoic acid has been reported as an inducer of P2rx7 in intestinal T cells with diverse functional consequences.36,37 In CD8+ T cells, sensing of extracellular ATP through P2rx7 is associated with longevity and enhanced metabolic fitness and could represent an alternative means to promote tissue residency by inducing the expression of TGF-βRII (refs. 40-43).

Finally, besides the identification of meaningful functional markers on effector T cells, our approach of classifying helper T cells according to ’provenance’-guided traits might provide a more realistic helper T cell classification than the traditional cytokine-defined helper T cell categorization, in particular in humans where multifunctional and heterogenic cytokine expression by T cells blurs the classification of helper T cells into the T\textsubscript{H}1, T\textsubscript{H}2 or T\textsubscript{H}17 lineage.11 In fact, ex vivo-isolated human memory T cells can be best described according to a graded effectorness model rather than helper T cell subsets by scRNA-seq analysis.23 Our concept of provenance-defined helper T cell subsets embraces the continuity in TCR signaling strength and cytokine production of ex vivo-isolated effector T cells, but still defines distinct helper T cell traits that might be exploited for prognostic and therapeutic individual stratification in autoimmune diseases and perhaps any kind of adaptive immunity.

Online content

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**Methods**

**Human data.** Participants were prospectively recruited in the Ludwigh Maximilian University neuroimmunology outpatient department for diagnostic procedures, including CSF sampling. Inclusion criteria for the additional sequencing analysis was a diagnosis of relapsing-remitting MS according to the revised McDonald criteria and no current treatment with any disease-modifying drug or glucocorticosteroids. The study was approved by the local ethics committee of the Ludwigh Maximilian University Munich (project no. 163-16 and 18-419), and written informed consent was granted by all participants included in the study.

**Animals.** C57BL/6J wild-type mice (000664), PhAMLfloxed reporter mice (018385), C57BL/6J wild-type mice (000664), PhAMLfloxed reporter mice (018385), in the neck (between the shoulder blades where indicated) with 200 μl erythrocyte lysis with BD Pharm Lyse (BD Biosciences, 555899) or ACK solution (see above) followed by treatment with 100-μm cell strainer (Greiner Bio-One, 542000). The cells were pelleted by gravity centrifugation (400g, 4°C, 10 min), followed by Percoll gradient (70%/37%; GE Healthcare, 17-0891-01) centrifugation (460g, 20°C, 22 min). Cells were removed from the interphase, washed and resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS with 2% FCS) for further analysis.

**Antibodies and flow cytometry.** Cell suspensions from lymphoid organs were stained with the following fluorochrome-conjugated antibodies: APC anti-mouse CD3e (145-2C11, BD Biosciences, 553066), 1:100, PerCP-Cy7 anti-mouse CD4 (RM-45, eBioscience, 46-0402-82, 1:400), APC anti-mouse CD4 (RM-5, BD Biosciences, 553051, 1:100, 1:500), B220 (BD Biosciences, 563727, 1:800), APC-Cy7 anti-mouse CD8a (53-6.7, Biolegend, 100714, 1:200), BV786 anti-mouse CD29 (HM-J1-1, BD Biosciences, 740899, 1:100), APC-R700 anti-mouse CD44 (IM7, BD Biosciences, 565480, 1:200), BV421 anti-mouse CD44 (IM7, BD Biosciences, 563970, 1:200), APC anti-mouse CD49d (R1-2, Biolegend, 180622, 1:100), APC anti-mouse CD62L (MEL-14, BD Biosciences, 553152, 1:800), BV575 anti-mouse CD62L (MEL-14, Biolegend, 104440, 1:800), AF647 anti-mouse CD118 (CXCXR6, SA051D1, Biolegend, 151115, 1:100), BV214 anti-mouse CD184 (CXCXR6, SA051D1, Biolegend, 151109, 1:100), BV214 anti-mouse TCR-γ/δ (GL3, Biolegend, 118119, 1:100) and APC anti-mouse P2R7X (1F11, Biolegend, 148706; 1:100). For dead cell exclusion, a LIVE/DEAD fixable near-infrared stain kit (Life Technologies, A10037, 1:100) was used. For cell proliferation analysis, a CellTrace violet cell proliferation kit (Thermo Fisher, C34557) was used.

**Cell culture conditions in 10x Chromium Single Cell 3 Solution**, TotalSeq-Q anti-mouse-Hashtags 1 to 12 (M1/42, 39-F11, Biolegend, 155801, 155803, 155805, 155807, 155809, 155811, 155813, 155815, 155817, 155819, 155821, 155823; 1:100) for all were used. All antibodies were diluted 1:100.

**Induction of EAE.** EAE was induced by s.c. immunization in the base of the tail or in the neck (between the shoulder blades where indicated) with 200 μg of MOG35-55 peptide (MEVGWYRSPFSRVVHLYRNGK; Auspep) in CFA containing 500 μg of Mycobacterium tuberculosis H37Ra (BD Biosciences, 231411) per mouse plus i.v. or intraperitoneal (i.p.) injection of 200 ng of pertussis toxin (Sigma-Aldrich, P2F208-50UG) on days 0 and 2 after immunization. Disease progression and severity were assessed as described before. The onset of disease was typically between 11 and 13 day after immunization, and the peak of disease was typically between 15 and 20 day after immunization.

**Fluorescence-activated cell sorting analysis of T cells.** To perform intracellular staining on mD2REDCD4, two-cell, a two-step fixation protocol was used to conserve the reporter’s fluorescent signal. After fluorescence-activated cell sorting, the cells were fixed with 1% paraformaldehyde (PFA) for 15 min on ice and Fix/Perm from the Foxp3 staining kit (eBioscience, 00-5523-00) for 30 min on ice, followed by permeabilization and staining with e450 anti-mouse Foxp3 (FKJ-16s, eBioscience, 48-5773; 1:200). For IA4-OVA323-339 (MBL, TS-M701-2) and IA4-MOG25-35 (MBL, TS-M704-2) tetramer stainings, cells were treated with 0.7 μl of 0.05% of neumamide (Sigma-Aldrich, N-2133) for 30 min at 37°C and 5% CO2, followed by treatment with 10 μg of dacarbazine (Selleckchem, S1021) for 30 min at 37°C and 5% CO2 and subsequent tetramer staining for 2 h at room temperature.

**Flow cytometric analysis was performed on a CytoFLEX flow cytometer (Beckman Coulter) with CytExpert (v2.3.12.32) software or a FACSAria III (BD Biosciences) with software.** Flow cytometry data were analyzed using FlowJo (v10.5.1) software (Tree Star).

**Fluorescence-activated cell sorting analysis of T cells.** For single-cell suspensions of lymph nodes, spleen and CNS from ILN- or mLN-labeled PhAML-EAE mice (2 day after photoconversion at day 8 after EAE induction) were incubated with LIVE/DEAD fixable near-infrared and mouse Fc Block (BD Biosciences) for 30 min on ice. Cells were washed with PBS and incubated with antibodies against surface markers and cell hashing antibodies for 30 min on ice. Cells were sorted on live mD2REDCD4CD454CD86 into PBS with 2% BSA on a FACSAria III machine (BD Biosciences). Positive mD2RED fluorescence was judged against a non-photoconverted PhAML-EAE mouse.

**For adoptive transfer experiments of photoconverted animals, single-cell suspensions of spleens from ILN- or mLN-labeled PhAML × d2E2 EAE mice (2 day after photoconversion/day 8 after EAE induction) were treated as above except for LIVE/DEAD fixable near-infrared and cell hashing antibody treatment.**

**Preparation of mononuclear cells from the CNS.** Mice were perfused through the left cardiac ventricle with ice-cold PBS. The brain was dissected, and the spinal cord was flushed out with PBS by hydrostatic pressure. CNS tissue was cut into pieces and digested with 1 mg ml−1 collagenase D (Roche Diagnostics, 1108866001) and 40 μg ml−1 DNase I (Roche Diagnostics, 1108886601) at 37°C for 30 min. Mononuclear cells were isolated by passing the CNS through a 100-μm cell strainer (Greiner Bio-One, 542000). The cells were pelleted by gravity centrifugation (400g, 4°C, 10 min), followed by Percoll gradient (70%/37%; GE Healthcare, 17-0891-01) centrifugation (460g, 20°C, 22 min). Cells were removed from the interphase, washed and resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS with 2% FCS) for further analysis.
c culture, T cells were resuspended at a concentration of 4 × 10^6 cells per milliliter in complete DMEM with the concentrated retroviral supernatant at a dilution of 1:10 supplemented with 8 µg ml^−1 polybrene (Sigma-Aldrich, TR-1003-G) and 10 ng ml^−1 IL-2 (PeproTech, 202-02). Cells were plated in 12-well plates with 0.5 ml per well (2,000 g, room temperature, 90 min).

**Adoptive transfer of T cells.** For all adoptive transfer experiments with photoconverted T cells, 7,000–12,000 splenic T cells or m-T cells (m2D2RED CD4^+ CD69^+ cells) from PhaMΔT × 2D2 mice at day 8 after EAE induction were sorted on a FACSAria III machine (BD Biosciences), washed twice with PBS and transferred into Ringer^−^/EAE-matched recipients (immunized at the same time as the donor mice). The cells of one mouse were transferred into one donor.

For all adoptive transfer experiments with intravital lymph node imaging, 20 × 10^6 to 30 × 10^6 Twitch-2B-transduced T cells were transferred into C57BL/6 wild-type mice i.d after retroviral transduction and on day 10 after immunization.

**Surgical exposure of lymph node for photoconversion and intravital imaging.** Animals were sedated with a cocktail of medetomidine, midazolam and fentanyl, and the fur was removed with depilatory cream (Veet, 07768828). For intravital lymph node imaging experiments, the mice were tracheotomized after anesthesia above. Time-lapse two-photon laser-scanning microscopy was performed using a Nikon Eclipse Ti2 microscope and NIS Elements AR (v.5.20.00) software or at ×20 and ×60 magnifications with a Leica SP8 confocal microscope and the Leica Applications Suite X (v.3.5.6.21594) software.

**scRNA-seq.** Two mouse single-cell RNA experiments were performed. In the first experiment, scRNA-seq and cell hashing libraries were prepared using the 10x Chromium Single Cell 3′ Solution (Chromium Single Cell 3′ v3, 10x Genomics, 1000092) combined with 10x Chromium Human Body Map 3′ v2 (8000047). In the second experiment, scRNA-seq, scTCR-seq and cell hashing libraries were prepared using the 10x Chromium Single Cell 5′ Solution (Chromium Next Gem Single Cell VDJ v1.1 with feature barcoding technology for cell surface protein, 10x Genomics, 1000167, 1000020 and 1000005) as per the manufacturer’s protocol. CSF cells were loaded in total (with a maximum target cell number of 10,000). Libraries were sequenced on an Illumina HiSeq 2500 using read lengths of 28/25 base pairs (bp) read 1, 8 bp index and 91 bp read 2.

Fresh human CSF samples were processed within 1 h after collection. CSF samples (3–6 ml) were centrifuged at 300 g for 10 min. The pellet was then transferred to a 2-ml tube and stained with TotalSeq-C anti-human CD4, CD8A and mouse IgG1 isotype control (Biolegend, 305657, 301071, 400187; 0.3 µg of each) according to the cell surface labeling protocol. All antibodies were purchased from 10x Genomics, with the difference that all centrifugations were done at 300 g for 10 min. The human CSF scRNA-seq and scTCR-seq libraries were prepared using the 10x Chromium Single Cell 5′ Solution (Chromium Next Gem Single Cell VDJ v1.1, 10x Genomics with feature barcoding technology for cell surface protein, 1000006, 1000020 and 1000005) as per the manufacturer’s protocol. CSF cells were loaded in total (with a maximum target cell number of 10,000). Libraries were sequenced on an Illumina NovaSeq6000 54 using read lengths of 150/bp 1 read, 8 bp index and 150/bp 2 read.

**Single-cell sequencing data processing.** Cell Ranger software (10x Genomics, v.3.0.0) was used to denoise and cluster single-cell RNA-seq reads from the mouse mm11 or the human GRCh38 reference genomes and summarize unique molecular identifier (UMI) counts. Filtered gene barcode and hashing barcode matrices that contained only barcodes with UMI counts that passed the threshold for cell detection were used for further analysis. Cell hashing analysis of libraries generated using the Chromium Single Cell 3′ v2 kit was performed using the CITE-seq Count software version 1.3.3 (Zenodo https://doi.org/10.5281/zenodo.2590196). Then, we processed the filtered UMI count matrices using R (v.3.6.1) and the R package Seurat (v.3.1.0) for cell hashing analysis. Raw counts were normalized using centered log ratio (CLR) transformation, where counts were divided by the geometric mean of a hashtag oligonucleotide (HTO) across cells and across samples. To control for HTOs, we filtered out duplicates originating from two cells from different samples (one single barcoded cell was positive for two HTOs) and cells where no HTO was detected. We further discarded cells where the number of detected genes was <200 or >5,000 and/or 10% of the counts belonged to mitochondrial genes. After quality control, only raw gene counts in high-quality singlets were submitted to log normalization, identification of high variable genes by using the VarsePlot method, scaling and regression against the number of UMI s and mitochondrial RNA content per cell.

Two clustering analyses were performed on the mouse scRNA-seq datasets. For single-cell gene expression analysis, only cells analyzed in the 10x Chromium Single Cell 3′ experiment were used for the first clustering analysis and identification of genes differentially expressed in each cluster—organ—stream combination. Additionally, for TCR analysis, both 10x 3′ and 5′ datasets were merged. After merging, integration was performed to remove batch effects, and the integrated assay was used for principal component analysis and unsupervised clustering. After marker identification for each cluster, cells where a TCR clonotype was detected were used for upstream analysis of TCR repertoire.
Single-cell TCR annotation was performed using the 10x cellranger vd pipeline. CDR3 information of the clonotype detected in each single cell and frequency for each clonotype was added to the Seurat meta.data, and the combined dataset was used to generate the input files required for the VDJtools software to perform comparative post analysis of immune CDR3 repertoires. We used velocity\(^{16}\) to determine in which direction cells were changing in the cross-tissue trajectories both in the i-stream and m-stream. For the analysis, we used the R package velocyto and Seurat wrapper package.

For the human CSF scRNA-seq data, only single cells to which a TCR was assigned and in which expression of CD4 but not CD8 was detected were subjected to clustering analysis.

Hartigan’s dip test. A automatic classifier called minimum density hyperplane\(^{16}\) was used to cluster cells based on the top 2,000 most variable genes for several subsets of the 3’ single-cell RNA dataset with the R package PPGI (v.0.1.5). Briefly, the dimensionality reduction merges clusters with contiguous regions and projects the data on two dimensions. The pairwise distances between all cells were calculated for those dimensions, and the multimodality of those distances were tested using Hartigan’s dip test statistic for unimodality\(^{16}\) with the R package dipkit (v.0.75-7).

**RNA-seq.** Total RNA was isolated from sorted i-T cells and m-T cells recovered from iLN and mLN, respectively, as well as from splenic i-T cells and m-T cells (mDiF\(^{12}\)-CD4\(^{+}\)CD44\(^{+}\)-cells) of 2D×TH PhAM\(^{4}\) mice using the RNAeasy Plus micro kit (Qiagen, 74034). Quality and integrity of total RNA was assessed on a Bioanalyzer 2100 (Agilent Technologies). Library preparation for sequencing of poly(A)-RNA was done as described previously\(^{16}\). Briefly, barcoded cDNA of each sample was generated with Maxima RT polymerase (Thermo Fisher, EP0742) using oligo-dT primer-containing barcodes, UMIs and an adaptor. Ends of the CDNA were extended by a template switch oligo (TSO), and full-length cDNA was amplified with primers binding to the TSO site and the adaptor. An NEB EUB II FS kit was used for fragmentmentation. After end repair and A-tailing, a TruSeq adapter was ligated, and 3’-end fragments were finally amplified using primers with Illumina P5 and P7 overhangs. In comparison to previous descriptions\(^{16}\), the P5 and P7 sites were exchanged to allow sequencing of the cDNA in read 1 and barcodes and UMIs in read 2 to achieve a better cluster recognition. The library was sequenced on a NextSeq 500 (Illumina) with 67 cycles for the cDNA in read 1 and 16 cycles for the barcodes and UMIs in read 2. Data were processed using the Drop-seq pipeline (v1.0) to generate reads and gene expression data were processed using R package elements AR (v.5.20.00). Calculations and the generation of graphs were performed using GraphPad Prism v7.05 (GraphPad software).

ATAC-seq reads were aligned to the mouse genome mm10 using Bowtie (v1.1.2) with options \(-q -n 2 -b -c -x -s -u -p 32 -m 1 -S\). Duplicate reads were removed using picard MarkDuplicates, and ATAC peaks over input background were identified using HOMER (v.4.9)\(^{49}\) findPeaks.pl with option \“-style factor\” Differential ATAC peaks were determined based on a FDR > 2, and genomic feature annotation of found peaks was performed using HOMER (v.4.9) annotatePeaks.pl.

Gene expression data from the scRNA-seq dataset were averaged and normalized in reads per million. GSEA (v.4.0.3 Broad Institute) software was used to test for enrichment of gene set signatures. All default options were used except for the permutation type that was set to ‘gene_set’. NES represents the degree to which a gene set is overrepresented accounting for the gene set size using a weighted Kolmogorov–Smirnov-like statistic originally described in\(^{16}\). FDR is the estimated probability that a gene set is a false positive. Gene sets in Fig. 3f were obtained using published data from Gene Expression Omnibus (GEO) accession numbers GSE39820 (ref.\(^{50}\)) and GSE65021 (ref.\(^{51}\)). Differential gene expression was performed using GEO2R (P < 0.05, maximum 200 genes, Benjamin–Hochberg correction) for each condition. Gene sets Fig. 6c were obtained from the core signatures (Supplementary Table 1).

Pathway analysis based on differential signature genes associated with ATAC peaks was performed using the R package SCENIC (v1.1.2-2) and postprocessed using the R package scFunction (v0.0.0.9000) from the scRNA-seq mouse dataset. All genes controlled by a transcription factor form a regulon. A regulon specificity score (RSS) is calculated using Jensen–Shannon divergence and allows for the ordering of regulons by differential activity in inguinal and mesenteric origins.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Next-generation sequencing raw data and processed gene expression data have been deposited into the GEO repository under the accession numbers GSE156718 (scRNA-seq mouse), GSE72903 (scRNA-seq human), GSE172513 (ATAC-seq mouse) and GSE171122 (bulk RNA-seq mouse). Clinical data for human samples can be found in Supplementary Table 2. All other data generated or analyzed during this study are included in the published article or are available from the corresponding author upon reasonable request.

**Code availability**

No custom code or algorithms were used in this study.

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Author contributions

M.H. designed experiments, performed most of the experiments, analyzed data and drafted the manuscript. E.B. performed the human scRNA-seq experiments and analyzed the mouse and human scRNA-seq data. R.K. performed histology and analyzed data. G.L. analyzed the mouse scRNA-seq and bulk RNA-seq experiments. I.D.M. and performed and analyzed the ATAC-seq experiments. S.T., S.K., A.M., C.S., L.A., B.K., G.G., A.M.A., L.A.G. and S.F. performed experiments and analyzed the Ca²⁺ imaging experiments. R.O. performed the mouse bulk RNA-seq experiments and analyzed data. S.G., S.J. and K.S. performed the mouse scRNA-seq experiments and analyzed data. C.G. analyzed the human scRNA experiments. V.R.B., R.R., T. Kumpfel, N.K., B.H., D.H.B., T.M. and K.D. designed experiments and analyzed data. T. Korn conceptualized and directed the study, supervised the experiments, analyzed data and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Site-specific labeling of T cells by photoconversion in inguinal and mesenteric lymph nodes in vivo. a, Schematic of photoconversion of T cells in the iLN and mLN of PhAM<sup>+</sup> mice. b, Flow cytometric gating strategy for mD<sup>RED</sup> or mD<sup>GREEN</sup> CD4<sup>+</sup> T cells. The DUMP channel comprised LIVE/DEAD<sup>®</sup>-Near-IR and CD8α-APC-Cy7. c, Flow cytometric assessment in the indicated LN of iLN-labeled (top) and mLN-labeled (bottom) PhAM<sup>+</sup> mice immediately after photoconversion (photo) or without photoconversion (dark). Representative plots of n = 3 mice per group. d, e, Flow cytometric proliferation readout of in vitro-activated PhAM<sup>+</sup> CD4<sup>+</sup> T cells 4 days after photoconversion (d) and mD<sup>RED</sup> signal over time (e). Representative plots from two independent experiments. f, Experimental design of iLN (top) or mLN (bottom) irradiation in MOG(35-55)/CFA immunized PhAM<sup>+</sup> mice. g, Flow cytometric assessment of mD<sup>RED</sup>CD44<sup>high</sup> frequencies (top) and absolute numbers (bottom) in different tissues in iLN-labeled PhAM<sup>+</sup> EAE mice 2 days after photoconversion at indicated time points. Representative plots from two mice per time point. h, i, Flow cytometric assessment in the spleen of iLN-labeled (left) and mLN-labeled (right) PhAM<sup>+</sup> EAE mice 2 days after photoconversion at disease onset (h) and of the CNS in non-photoconverted (dark) PhAM<sup>+</sup> EAE mice (i). Representative plots from spleen n = 15 mice per group, CNS n = 7 mice. j, Frequencies of regulatory and activated conventional mD<sup>GREEN</sup>CD4<sup>+</sup> T cells in PhAM<sup>+</sup> EAE mice for different tissues. n = 2 mice per group, representative plot from three independent experiments for Foxp3 and CD44 and two for CD69. k, Frequency and duration of Ca<sup>2+</sup> signaling in iLN (top) or mLN (bottom) of mice immunized on days 3 or 4 post transfer, that is days 13 or 14 post immunization. Left panels: individual Ca<sup>2+</sup> signaling durations of TCR<sup>MOG</sup> T<sup>witch-2B</sup> T cells. The dotted line indicates the cut-off (2 minutes) to distinguish between short- and long-term Ca<sup>2+</sup> signaling. Cumulative results from iLN(PBS) and mLN(PBS) n = 2, iLN(MOG) and mLN(MOG) n = 3 mice. Right panels: fractions of T cells presenting short- and long-term Ca<sup>2+</sup> signaling or no Ca<sup>2+</sup> spikes. Data shown as mean. l, Representative images of TCR<sup>MOG</sup> T<sup>witch-2B</sup> T cells from intravital time-lapse two-photon microscopy for Ca<sup>2+</sup> imaging depicted by a fluorescence overlay of T cells (left) and a pseudocolor Ca<sup>2+</sup> ratio image (right) from iLN(PBS) and mLN(PBS) n = 2, iLN(MOG) and mLN(MOG) n = 3 mice (see Supplementary Videos 1–4). Scale bars 25 μm.
Extended Data Fig. 2 | TCR repertoire analysis of i-T cells and m-T cells. **a**, Schematic of single cell sequencing of sorted iLN- or mLN-derived photoconverted T cells from MOG(35-55)/CFA immunized PhAM mice in combination with TotalSeq hashtag barcoding antibodies. **b**, Unsupervised clustering t-distributed stochastic neighbour embedding (t-SNE) plot of all single md2RED CD4+CD44+ T cells analyzed. In the upper plot only single T cells from i-stream are colored (purple). In the lower plot only single T cells from m-stream are colored (orange). n = 5 PhAM mice per group, n = 14621 cells, i-T cells n = 7228 cells, m-T cells n = 7393 cells. **c**, Repertoire overlap analysis using hierarchical clustering. Dendrogram shows weighted clonal overlaps for TRB-CDR3 sequences among mice, analyzed using F pairwise similarity metric in VDJtools. Branch length shows the distance between repertoires. n = 3 PhAM mice per group. **d**, Average gene expression of all single md2RED CD4+CD44+ T cells analyzed for the TOP10 differentially expressed genes of cluster 0 to 7.
Extended Data Fig. 3 | Single cell transcriptome analysis in i-T cells and m-T cells. **a**, Average gene expression of all single mD2RED CD4+CD44high T cells analyzed from i- and m-stream in spleen and CNS grouped into T cell subsets based on key signature genes (see Fig. 3). **b**, Flow cytometric assessment in iLN-labeled (left) and mLN-labeled (right) PhAMT EAE mice 2 days after photoconversion at disease onset. LN (top row), spleen (middle row), and CNS (bottom row). Representative plots of n = 3 mice per group. **c**, Unsupervised clustering t-SNE plot, colored according to i- and m-stream cell group and key gene marker (Tbx21, Rorc, Ccr6, Csf2, Ifng, Il17a, Il10) expression. n = 2 PhAMT EAE mice per group, n = 4169 cells.
Extended Data Fig. 4 | Core signatures and transcription factor regulons of i-T cells and m-T cells. a, b, Gene expression (violin plots) of i-stream signature genes Cxcr6 and Itgb1 and m-stream signature genes P2rx7 and Itga4 in the spleen (a) and CNS (b). c-e, Transcriptional module analysis of scRNAseq data from Fig. 4 based on SCENIC algorithm in i- and m-T cells. All genes controlled by a given transcription factor build a regulon. The regulon specificity score (RSS) was calculated using Jensen-Shannon divergence. c, Genome-wide regulons with RSS > 0.2 in at least one condition are displayed for i-T cells and m-T cells (i-stream and m-stream across all organs). d, e, Regulons that control Cxcr6 (d) and Itga4 (e) with no RSS threshold requirement.
Extended Data Fig. 5 | ATACseq of i-T cells and m-T cells isolated from the spleen. a, Genome browser view of key gene markers (Tbx21, Rorc, Ifng, Csf2, Il21, and Ccr6). Displayed tracks correspond to ATACseq for i-T cells (purple) and m-T cells (orange). In all regions, differential peaks (if existing) are highlighted with arrows. b, c, Ranked list of the top transcription factor motifs predicted by HOMER based on cumulative hypergeometric distribution testing for differential ATAC peaks associated to signature genes for splenic i-T cells (b) and m-T cells (c). For i-T cells 48 transcription factor sequences out of 154 are shown and for m-T cells, 16 sequences out of 30 are shown.
Extended Data Fig. 6 | Robust i-T cell and m-T cell signatures in various immunization protocols. a, Flow cytometric assessment of mD2<sup>RED</sup>CD4<sup>+</sup>CD44<sup>high</sup> T cells in the spleen of iLN-labeled (purple) and mLN-labeled (orange) Pham<sup>+</sup> mice, for Cxcr6 (top), P2rx7 (middle), and CD49d (Itga4) vs CD29 (Itgb1) (bottom). Pham<sup>+</sup> mice were subjected to different s.c. immunization regimens at the base of tail plus pertussis toxin i.v. on day 0 and day 2 after immunization (as indicated) and analyzed 2 days after photoconversion on day 11 after immunization. Representative plots from n = 3 Pham<sup>+</sup> mice per group (iLN- and mLN-labeled) and immunization condition. Numbers indicate mean fluorescent intensities. PGN, peptidoglycan. b, i-T cells and m-T cells do not cross-traffic after i.v. immunization. 2D2 Pham<sup>+</sup> mice were injected with 40 <mu>g MOG(35-55) i.v., photoconverted at iLN (left) or mLN (right) on day 2, and analyzed for the fraction of CD44<sup>high</sup> mD2<sup>RED</sup> T cells in iLN and mLN on day 4 after injection. Representative plots from n = 4 2D2 Pham<sup>+</sup> mice per group.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Distinct functional phenotypes of i-T cells and m-T cells in the CNS compartment both in mice and humans. a, EAE progression in iLN-labeled and mLN-labeled PhAM+ EAE mice. Photoconversion at disease onset and analysis 2 days later. iLN-labeled n = 5 mice, mLN-labeled n = 3 mice, representative plot from five independent experiments. Data shown as mean ± s.d. b, Flow cytometric assessment of CD49d (Itga4) vs CD44 in transferred TCRMOG mD24*CD44+ n i- and m-T cells isolated from the spleen of secondary recipient Rag1−/− mice (approximately 20 days after transfer). n = 3 mice per group. c, EAE progression in wild-type (WT) and Cxcr6−/− mice. WT n = 5 mice, Cxcr6−/− n = 6 mice, representative plot from three independent experiments. Data shown as mean ± s.d. d, Unsupervised clustering t-distributed stochastic neighbour embedding (t-SNE) plot of cerebrospinal fluid (CSF) single CD4+ T cells isolated from untreated MS patients, colored according to T helper cell and Treg cell signature gene expression. Th1 (CSF1, CXCR6, IL12B2, IL18R1, KLRC1, HOPX), Th2 (IL4, TNFSF13B, BATF, NFIL3, ATF5), Th17 (IL17A, IL21, IL2, TNFRSF13B, PTGER5, AHR, IRF4, RORA, PLAGL2), and Treg (FOXP3, IKZF2, NRP1, FO5B, TNFSF11, IRF8). e, Gene marker expression of a selection of i-stream core signature genes (IL2RB, IL18RAP, SYTL2) (top row), and m-stream core signature genes (NT5E, DST, AIG1) (bottom row). n = 6 CSF samples from untreated MS patients, n = 14339 cells.
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BD FACSDIVA (v.8.0.1); CytExpert (v.2.3.1.22); Leica Applications Suite X (v.3.5.6.21594); Leica LCS (v.2.6.1.5173); NIS Elements AR (v.5.20.00)

Data analysis

R (v.3.6.1); Seurat (v.3.1.0); Cell Ranger (v.3.1.0); CITE-seq Count (v.1.4.3); GSEA (v.4.0.3); l2p (v.0.0.3); SCENIC (v.1.1.2-2); scFunction (v.0.0.0.9000); bowtie (v.1.1.2); HOMER (v.4.9); PPC (v.0.1.5); diptest (v.0.75-7); python (v.3.6); ImageJ (v.1.49k); Prism (v.7.05); FlowJo (v.10.5.1); Adobe Photoshop CS6 (v.13.0)

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NGS raw data and processed gene expression data have been deposited into the GEO repository under the accession number GSE156718 [scRNAseq mouse], GSE172003 [scRNAseq human], GSE172513 (ATACseq mouse) and GSE171122 [bulk RNAseq mouse]. Clinical data for patient samples can be found in Extended Data Table 2.

Pathway analysis was done using the R package l2p (v.0.0.3) with the following databases: GO, KEGG, PANTH and REACTOME

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Sample size
This study is purely explorative. No statistical methods were used to predetermine the sample size. Sample sizes were determined to be sufficient based on established standards for explorative studies in the field. Importantly, whenever statistical analysis was applied the sample size was ≥3 biological replicates per group.

Data exclusions
Figure 2 scRNA-seq (mouse) data: Single T cells in clusters 8 and 9 were excluded from further analysis because they showed high levels of cell cycle genes. Single T cells in cluster 10 were also excluded because TCR gene expression was low.

Replication
Biological replicates were used in this study to ensure reproducibility. Most of the experiments were repeated at least twice as stated in the figure legends. All attempts at replication were successful.

Randomization
The mice were allocated for ILN- or mlN irradiation within age- and sex-matched groups (no randomization). Randomization was not necessary for this study because mice were allocated to the different treatment groups for ILN- or mlN irradiation based on an EAE disease score of 1 [tail paralysis] two days before the results were obtained and therefore no potential self-selection bias is present and hence there is no impact on the results. Whenever possible littermate controls were used and equal number of mice were allocated to different groups within one cage to rule out cage effects.

Blinding
For mice experiments blinding during data collecting was usually not possible due to required cage labeling. However, scoring of EAE mice was occasionally performed by members of the staff who were not familiar with the experimental design. Furthermore, data analysis used in this study was strictly quantitative (and not subjective) and therefore blinding was not necessary.

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|----------------------------------|---------|
| n/a  | n/a                      |
| ☑ Antibodies  | ☑ Involved in the study |
| ☑ Eukaryotic cell lines  | ☐ ChiP-seq |
| ☑ Palaeontology and archaeology | ☐ Flow cytometry |
| ☑ Animals and other organisms  | ☐ MRI-based neuroimaging |
| ☑ Human research participants |       |
| ☑ Clinical data  |       |
| ☑ Dual use research of concern |       |

Antibodies

The following antibodies were used in flow cytometry:

- APC anti-mouse CD3e (145-2C11, BD Biosciences, #553066, 1:100, RRID: AB_398529);
- PerCP-eF710 anti-mouse CD4 (RM4-5, eBioscience, Cat. #46-0042-82, 1:800, RRID: AB_1834431);
- APC anti-mouse CD4 (RM4-5, BD Biosciences, #553051, 1:1000, RRID: AB_398528);
- BV786 anti-mouse CD4 (RM4-5, BD Biosciences, #563727, 1:800, RRID: AB_2728707);
- APC-Cy7 anti-mouse CD8α (53-6.7, Biolegend, #100714, 1:200, RRID: AB_312753);
- BV786 anti-mouse CD29 (HM β1-1, BD Biosciences, #740899, 1:100, RRID: AB_2740547).
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  
HEK293T [purchased from ATCC, #CR-L3216]

Authentication  
Cell line was authenticated prior to receipt by the commercial vendor using the STR method.

Mycoplasma contamination  
Cells were not tested for mycoplasma after receipt.

Commonly misidentified lines  
No commonly misidentified cell lines were used.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals  
For animal experiments sex-matched 5-12-week-old female and male mice on C57Bl/6j background were used.

- Strains obtained from The Jackson Laboratory:
  - female and male C57Bl/6j wild-type mice (#000664) were used at an age of 8-12 weeks
  - female and male Phamflxed reporter mice (#018385) were used at an age of 8-12 weeks
  - female and male CD4-Cre mice (#022071) were used at an age of 8-12 weeks
  - female and male Il17a-Cre mice (#016879) were used at an age of 8-12 weeks
  - female and male 2D2 mice (#006912) were used at an age of 8-12 weeks
  - female and male Rag1-/- mice (#002216) were used at an age of 8-12 weeks

- Cxcr5fp/gfp mice (#005693) were kindly provided by P. Knolle (Klinikum rechts der Isar, TU Munich, Germany) and female and male mice were used at an age of 8-12 weeks.

- PhAM T mice and PhAM IL17 mice were generated in house by breeding Phamflxed reporter mice with CD4-Cre mice or with Il17a-Cre mice, respectively, and female and male mice were used at an age of 8-12 weeks.

- TH mice were kindly provided by H. Wekerle (Max-Planck-Institut für Neurobiologie, Martinsried) and bred with 2D2 and PhAM T mice to generate 2D2 x TH PhAM T mice that develop spontaneous EAE. Female and male 2D2 x TH PhAM T mice were used at an age of 5-8 weeks.

- Mice were housed with a dark/light cycle of 12 hours, a temperature of 20-24°C, and a humidity of 45-60%.
Human research participants

Policy information about **studies involving human research participants**

Population characteristics  Untreated Patients with Relapsing Remitting MS, Age between 28-49. Please see Extended Data Table 2.

Recruitment  Patients were prospectively recruited in our neuroimmunology outpatient department for diagnostic procedures including CSF sampling. For this study CSF samples were incubated only if the diagnosis of multiple sclerosis was confirmed with detection of inflammatory changes in the CSF including positive oligoclonal bands. In addition, all patients were treatment naive (no prior steroid treatment, no disease modifying treatment) to exclude any treatment bias. In summary, sample selection for study inclusion was based on neurological, MRI, and CSF findings before the study results were obtained and therefore no potential self-selection bias is present and hence there is no impact on the results.

Ethics oversight  The study was approved by the local ethics committee of the LMU Munich (Project-No 163-16).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation  Sample preparation as described in the methods.

Instrument  CytoFLEX (Beckman Coulter); FACS Aria III (BD Biosciences)

Software  BD FACSDIVA (v.8.0.1); CytExpert (v.2.3.1.22); FlowJo (v10.5.1)

Cell population abundance  Cell sorting was performed with the strictest purity setting [4-Way Purity], ensuring a purity of >95%.

Gating strategy  See flow cytometric gating strategy for mD2GREEN or mD2GREEN mD2RED double positive CD4+ T cells in Extended data figure 2b. Debris and dead cells were gated out based on a distinctive FSC and SSC gate specific for lymphocytes as is common practice. Singlets were gated based on FSC-H/FSC-W and SSC-H/SSC-W. Dump channel exclusion of dead cells (LiveDead Near Infrared positive) and CD8a cells (CD8a APC-Cy7 positive). Afterwards gating on CD4 positive cells.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.