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Single-cell mass cytometry reveals distinct populations of brain myeloid cells in mouse neuroinflammation and neurodegeneration models

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Neuroinflammation and neurodegeneration may represent two poles of brain pathology. Brain myeloid cells, particularly microglia, play key roles in these conditions. We employed single-cell mass cytometry (CyTOF) to compare myeloid cell populations in the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis, the R6/2 model of Huntington's disease (HD) and the mutant superoxide dismutase 1 (mSOD1) model of amyotrophic lateral sclerosis (ALS). We identified three myeloid cell populations exclusive to the CNS and present in each disease model. Blood-derived monocytes comprised five populations and migrated to the brain in EAE, but not in HD and ALS models. Single-cell analysis resolved differences in signaling and cytokine production within similar myeloid populations in EAE compared to HD and ALS models. Moreover, these analyses highlighted α5 integrin on myeloid cells as a potential therapeutic target for neuroinflammation. Together, these findings illustrate how neuropathology may differ between inflammatory and degenerative brain disease.

The term 'neuroinflammation' has been broadly applied to various neuropathological conditions[1]. A wide spectrum of neurological disorders, ranging from those immunologically driven such as acute disseminated encephalomyelitis and multiple sclerosis[2], to degenerative diseases such as Alzheimer's disease[3] and Parkinson's disease[4], to genetic disorders such as HD[5] and mutant SOD1-driven ALS[6], are often collectively called neuroinflammatory[7]. One rationale behind applying the 'neuroinflammatory' label to these diverse neurological conditions resides in the empirical observation of microgliosis in these conditions[8]. From detection of inflammatory mediators in Alzheimer's disease and Parkinson's disease brain sections at autopsy to genomic and transcriptomic studies of brain specimens[4,9], it has been suggested that neurodegeneration might be promoted in part by microglia responding to inflammation in the CNS[10,11].

Here we used cytometry by time-of-flight mass spectrometry (CyTOF) to enable a high-dimensional analysis of cell surface markers, signaling molecules and cytokines on brain myeloid cells at the single-cell level[12]. We characterized the myeloid cell phenotypes in commonly used mouse models of neuroinflammation and neurodegeneration: EAE, a model of multiple sclerosis[2]; R6/2 mice, a model of HD in which the mice express human mutant HTT exon 1[13]; and mice overexpressing mSOD1, a model of ALS[14].

Results

Phenotypic heterogeneity within the CNS-resident myeloid population. We used CyTOF to analyze the cellular phenotype, signaling properties and cytokine production in single cells of both CNS tissues (brain and spinal cord) and peripheral blood. We first compared different stages of EAE with R6/2 transgenic mice[13] at a late stage of the disease when the R6/2 mice display tremor, irregular gait, abnormal movements and decreased survival[15] (Fig. 1). The CyTOF panel was assembled on the basis of a high-throughput screen of 255 antibodies to integral membrane proteins (Supplementary Table 1), proteins that regulate myeloid cell functions[16], transcription factors and signaling molecules relevant to neuroinflammation (Supplementary Table 2a–c). Single-cell suspensions of CNS tissue and blood were prepared as described previously[17] (Fig. 1).

To explore the phenotypic diversity of immune cell populations in the CNS and blood, we applied a *K*-nearest-neighbor density-based clustering algorithm called X-shift[18]. This algorithm allows the unsupervised clustering analysis of data from single cells[18]. To visualize the phenotypic continuum of cell populations, output is organized into a minimum spanning tree (MST)[18], creating a two-dimensional layout. The size of nodes and the color coding is explained in the methods section.

Unsupervised clustering of 1,800,183 single cells from the CNS and blood of 75 samples created a detailed MST map of distinct cell populations (Supplementary Fig. 1). This analysis revealed three distinct CD11b$^+$ myeloid populations in the CNS that were absent in peripheral blood (Fig. 2a). These populations, identified as CNS-resident myeloid populations, are designated A, B and C (Fig. 2a and Supplementary Fig. 1).

To verify that populations A, B and C could be identified by manual gating, we applied a feature of the X-shift called a divisive marker tree (DMT) algorithm[18]. This feature automatically constructs an optimal marker-based classification of clusters[18]. By setting the gates according to computationally defined thresholds, we verified that populations A, B and C were distinguishable by cell surface marker expression of CD45, CD11b, CD317 (BST2/PDCA-1),

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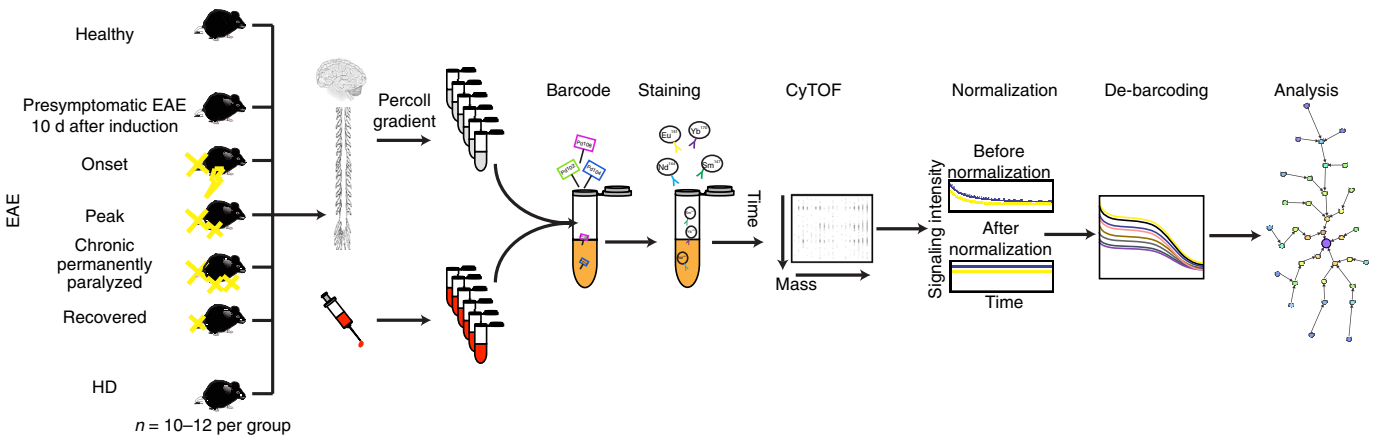

Fig. 1 | Schematic representation of the experimental strategy. Immune response profiles were analyzed in healthy mice, in five different clinical stages of EAE and in end-stage HD. Single-cell suspensions from CNS and whole blood of each condition were prepared as described in Methods. Individual samples were simultaneously processed by using a barcoding strategy involving unique combinations of six palladium isotopes (Methods). Barcoded samples were pooled; stained with a panel of 39 antibodies, each conjugated to a different metal isotope (Supplementary Table 2a–c and Methods); and analyzed by mass cytometry. Raw mass cytometry data were normalized for signal variation over time and de-barcoded and analyzed using the X-shift algorithm, a nonparametric clustering method that automatically identifies cell populations by searching for local maxima of cell event density in the multidimensional marker space. The result is displayed as an MST layout. In each experiment, tissues from ten mice were pooled to provide enough cells (Methods). Each experiment was performed seven to ten times independently.

major histocompatibility complex class II (MHC-II), CD39 and CD86 (Fig. 2b). Population A is CD317$^+$MHC-II$^-$CD39^{low}CD86$^-$; population B is CD317$^+$MHC-II$^-$CD39^{hi}CD86$^+$; and population C is CD317$^+$MHC-II$^+$CD39^{hi}CD86$^+$ (Fig. 2b and Supplementary Fig. 2). MHC-II and CD86, among others, have previously been identified as myeloid activation markers[19], thus suggesting that populations B and C represent activated microglia populations.

Populations A, B and C also expressed several other cell surface markers, including the recently identified microglial markers 4D4 and FCRLS[20] and low to medium levels of CD88 (complement component 5a receptor 1; C5aR), MHC class I (MHC-I, H-2) and TAM receptor tyrosine kinase Mer (MerTK) (Supplementary Fig. 3a).

Populations A, B and C lacked expression of lymphoid lineage markers such as CD3 (T cells), CD45R/B220 (B cells), Ly6C (monocytes) and Ly6G (granulocytes). These three CNS-specific populations were also characterized by the differential expression of several markers. Populations B and C expressed different levels of CD80, TAM receptor Axl, T-cell immunoglobulin mucin protein 4 (TIM4), CD274 (PD-L1), CD195 (CCR5) and CD194 (CCR4), and low levels of CD206 (mannose receptor) and TREM2, while population A lacked expression of all these markers (Supplementary Fig. 3b). The expression level of these markers changed depending on disease conditions (Supplementary Fig. 4a–c).

We distinguished CNS-resident myeloid cells from infiltrating myeloid populations with a genetic tool[21]. In addition to defining populations A, B and C as CNS-resident myeloid cells on the basis of their presence in only the CNS (not in peripheral blood) coupled with the expression of other markers, including low CD45 (traditionally believed to mark microglia in the CNS), 4D4 and FCRLS (Supplementary Fig. 5), we further confirmed them as CNS-resident myeloid cells by analyzing CNS tissues of conditional *Cx3cr1*^{CreER} *Rosa26-YFP* mice[21]. In this model, YFP is conditionally expressed exclusively in long-lived myeloid cells of the CNS, such as microglia after tamoxifen withdrawal, as a result of the irreversible recombination event mediated by Cre recombinase[21]. We were able to identify these populations in conditional *Cx3cr1*^{CreER} *Rosa26-YFP* healthy mice and confirm that they express YFP (Supplementary Fig. 6a). In this paper, we avoid calling these cells microglia and refer to them instead as CNS-resident myeloid cells, which include microglia, meningeal macrophages and perivascular macrophages.

Neuroinflammatory and neurodegenerative mouse models display congruent CNS myeloid cell populations. To investigate whether disease-specific cues modulate the presence and the frequency of the three CNS-resident myeloid cell populations, we analyzed the MSTs and confirmed the findings by manual gating in healthy mice, R6/2 mice modeling HD, and five different states of EAE: presymptomatic, onset, peak, chronic and recovered. Quantification of each population (Fig. 2c) and representative nodes in the MST (Fig. 2d) in independent biological replicates of healthy samples compared to each disease state demonstrated that population C was generated during disease (both EAE and HD disease conditions) but was barely detectable in the healthy CNS (Fig. 2c,d and Supplementary Fig. 6b). In EAE, population C continued to expand from the presymptomatic stage (frequency of 1.8%) to the peak of disease (frequency 9.7%). Thereafter, the frequency of population C declined in chronic EAE animals with permanent paralysis and in recovered EAE mice (to 0.9% and 1.7%, respectively) (Fig. 2d). Of note, of the three CNS-resident populations, only population C expresses CD11c. While very low expression of CD11c is detectable in the R6/2 HD model, CD11c expression levels are increased as EAE progresses to peak disease and then becomes downregulated in both the EAE chronic and recovered groups (Supplementary Fig. 7a). Given that population C expresses MHC-II and upregulates CD11c during EAE, a T-cell-driven inflammatory CNS disease, antigen presentation may be a key role for population C, a CNS-resident population[22].

To capture the expansion rate of each population under each disease condition, we also analyzed the percentage of cells positive for the proliferation marker Ki67. The percentage of Ki67$^+$ cells in all three CNS-resident myeloid populations increased as EAE disease progressed from presymptomatic through the chronic stages and then decreased during recovery (Supplementary Fig. 7b). Of note, the percentage of Ki67$^+$ cells in these population in the HD model was markedly lower than in the different clinical stages of EAE. This suggests that populations A, B and C are all expanding at different rates, depending on each disease condition.

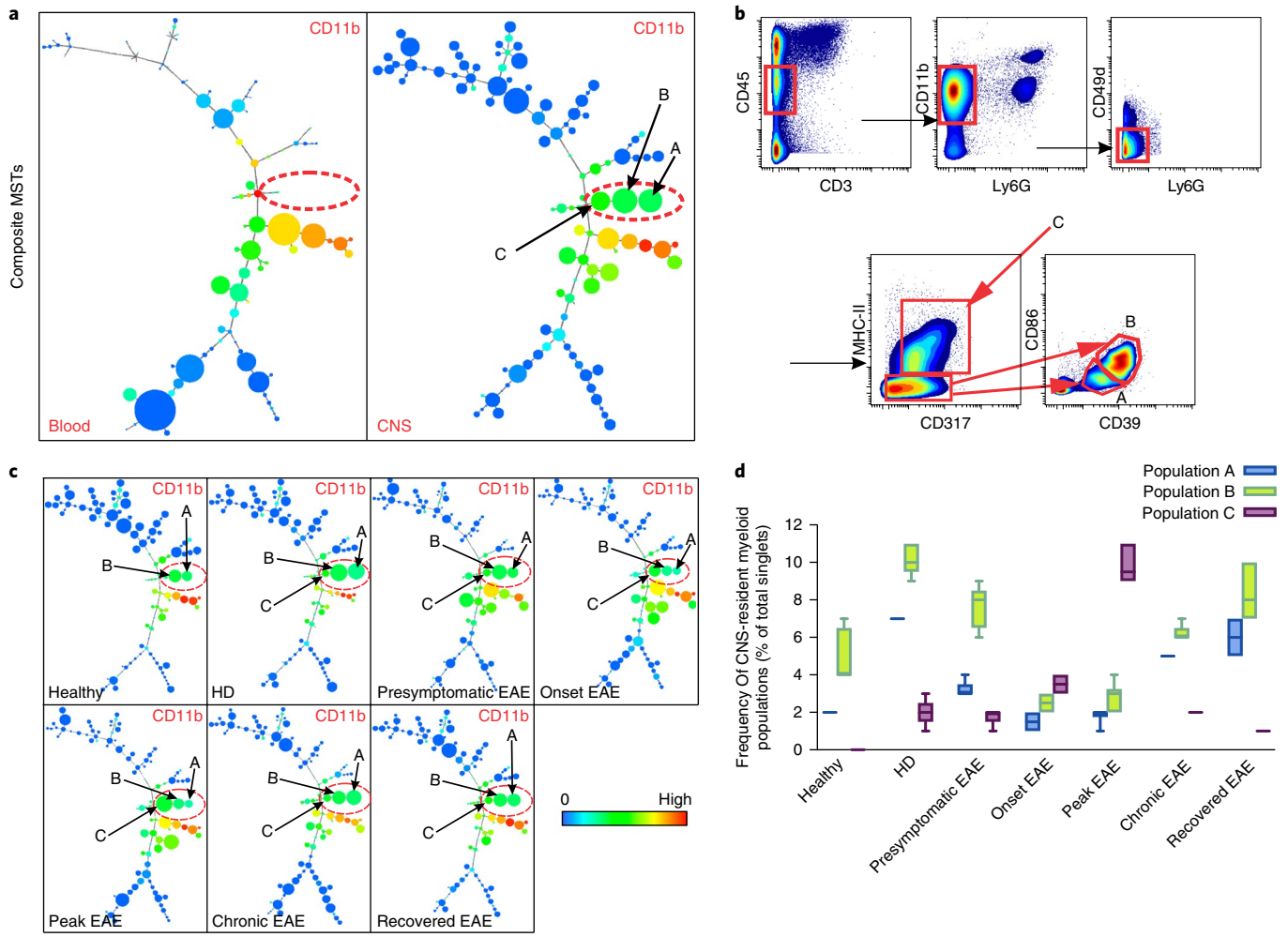

Fig. 2 | Data-driven, unsupervised clustering defines three distinct CNS-resident myeloid populations. a, Composite CNS MST of X-shift clusters constructed by combining CNS samples from all the conditions and their biological replicates ($n=37$) in comparison to composite MST from peripheral blood samples ($n=37$) reveals three myeloid (CD11b$^+$) populations (A, B and C) that are unique to CNS. All samples were barcoded and analyzed together by CyTOF. The color code shows the expression level of CD11b (blue, no expression; green, yellow and red, increasing levels of expression). **b**, Manual gating based on markers and threshold defined by the X-shift DMT algorithm confirmed the existence of populations A, B and C. Live cells are identified by the lack of cleaved poly-(ADP)-ribose polymerase (c-PARP) binding (Supplementary Fig. 13b and Methods). This panel represents data from peak EAE, where 7 independent experiments confirmed a similar gate. **c**, Visualization of clusters under different clinical conditions demonstrates that populations A, B and C are present in both EAE and HD models. The color code shows the expression levels of CD11b ($n=5$). **d**, Frequency of populations A, B and C based on manual gating confirms that all three populations are present in both EAE and HD models. Center line is average; boxes extend to 25th and 75th percentiles; whiskers extend to 5th and 95th percentiles ($n=5$ independent experiments for healthy, end-stage HD, presymptomatic EAE, chronic EAE; $n=6$ independent experiments for onset EAE, peak EAE).

Signaling phenotypes distinguish CNS-resident myeloid cells in neuroinflammatory versus neurodegenerative models. To understand the signaling differences in CNS-resident myeloid populations A, B and C in the HD model versus the different stages of EAE, we next focused on transcription factors and signaling markers such as phosphorylated STAT transcription factors pSTAT1, pSTAT3 and pSTAT5; phosphorylated cAMP response element-binding protein (pCREB); phosphorylated kinase pMAPKAPK2 (pMAPKAPK2); NF-κB (p65) and CCAAT/enhancer-binding proteins and α and β (C/EBPα, C/EBPβ) (Supplementary Table 2a).

First, this revealed substantial differences in the expression patterns of these signaling proteins across the three CNS-resident myeloid populations. Whereas populations B and C showed high levels of signaling, population A showed low expression levels of signaling proteins, potentially highlighting a different function for these CNS-resident myeloid populations (Fig. 3a–d). These results are consistent with the fact that population A does not express myeloid cell activation markers, such as CD86, MHC-II and CD11c, and could therefore represent a functionally less active population under these conditions (Fig. 2b and Supplementary Fig. 7a).

Second, this analysis revealed the progression of key signaling pathways within the CNS in populations B and C during the development of EAE. In the presymptomatic stage of EAE, substantially increased pCREB and pMAPKAPK2 expression represents the only signaling signature in populations B and C (Fig. 3a,b). At the peak of EAE, a second wave of increased expression of pCREB and pMAPKAPK2 in populations B and C emerged as a hallmark, as we observed in the presymptomatic stage and in agreement with previous studies[23] (Fig. 3a,b). Notably, in chronic EAE, in which animals never recovered from paralysis, we identified upregulation of NF-κB (p65) in concert with C/EBPβ in populations B and C (Fig. 3c,d). These data indicate that there is sequential signaling in EAE.

Third, these inflammatory signaling signatures were notably absent in populations A, B and C in the HD model compared to EAE. This emphasizes the considerable differences in signaling properties in the HD model compared to EAE in CNS-resident myeloid cell populations (Fig. 3a–d). Though similar CNS-resident myeloid cell populations were identified in both models, the nature of their signaling properties under these conditions was vastly different.

Multiple cytokine-producing myeloid cells in models of neuroinflammation versus neurodegeneration. We sought to determine the capacity for cytokine production of these myeloid cells, without imposition of any ex vivo stimulation[24]. The cytokine panel included tumor necrosis factor-α (TNF-α), interferon (IFN)-γ, IFN-α, interleukin (IL)-10, IL-6, IL-17A, granulocyte-macrophage colony-stimulating factor (GM-CSF) and transforming growth factor-β (TGF-β) (Supplementary Table 2b). CNS-resident myeloid populations were manually gated as defined above (Fig. 2b). We calculated the fraction of cells detected that secrete a given cytokine, defined by expression values exceeding the 90th percentile of a healthy sample for each cluster (Supplementary Fig. 8a,b).

Among the eight cytokines evaluated, TNF-α was the most prominently produced in the three identified CNS-resident myeloid populations in both neuroinflammatory and neurodegenerative conditions compared to healthy cells (Supplementary Fig. 8a,b). Most notably, in populations B and C during early stages of EAE (presymptomatic, onset, peak) and, in the case of population C, also during chronic EAE, most cells (up to 80%) produced TNF-α whereas the percentage of TNF-α-expressing cells ranged from 30–50% in the neurodegenerative R6/2 model of HD. In addition to TNF-α, a modest percentage of cells in these three populations expressed GM-CSF, IL-6, IL-10 and TGF-β (Supplementary Fig. 8a,b).

To analyze the multifunctional nature of each population at a single-cell level[25], we applied the X-shift clustering algorithm[18]. Each population was clustered on the basis of expression patterns of cytokines only, and the frequency of cells that produce each cytokine alone or in any combination at the single-cell level in each disease condition was assessed. Seven distinct subsets of cytokine-producing cells were delineated in populations A, B and C at the single-cell level on the basis of either (i) production of single cytokines—TNF-α, IL-6 or TGF-β—or (ii) production of a combination of TNF-α with IL-6, GM-CSF or IL-10, or (iii) a lack of cytokine production (Fig. 3e–g).

Quantifying the fraction of each of these seven subsets within each population under different disease conditions, we found that in a healthy state most of the cells within each population produced no cytokines at all or just a single cytokine (Fig. 3e–g). The frequency

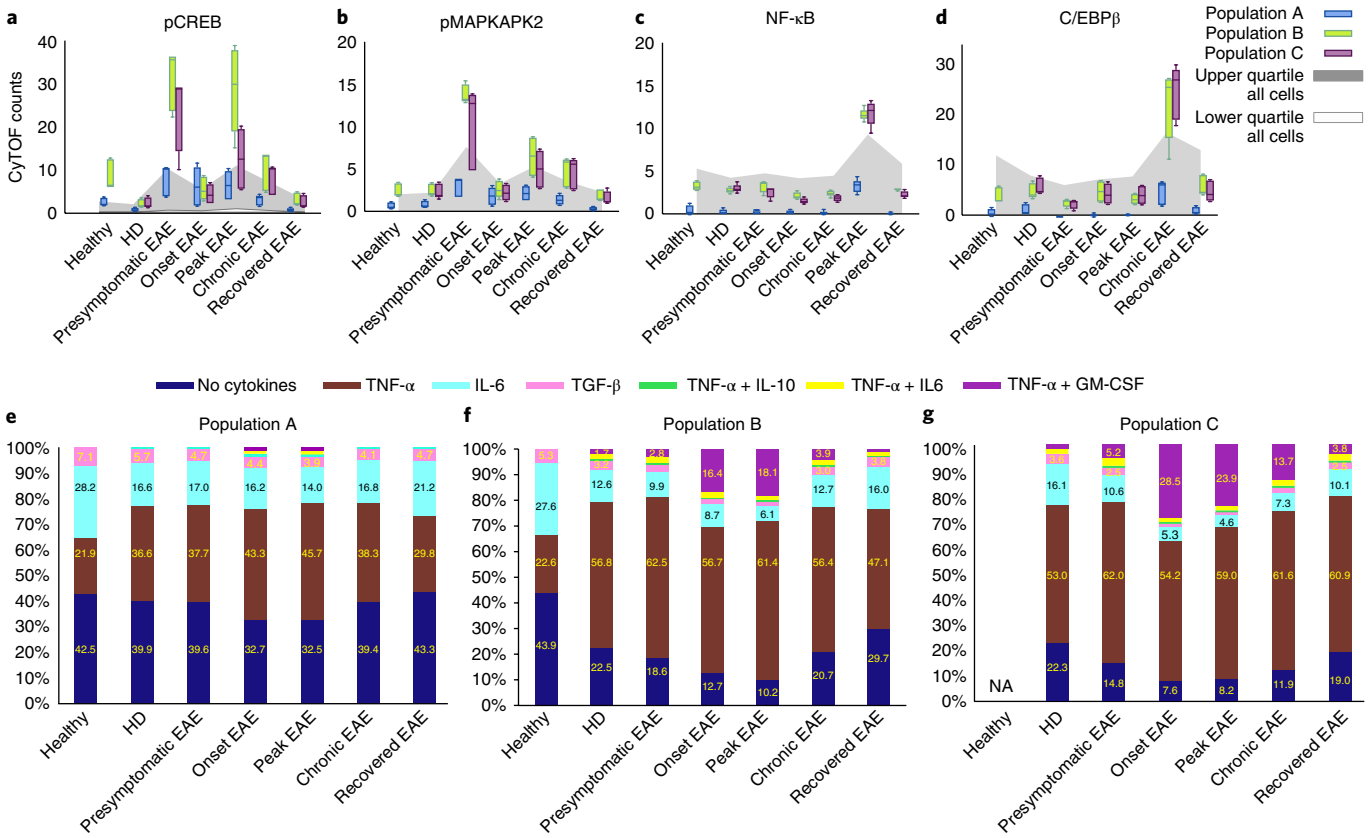

Fig. 3 | CyTOF analysis reveals the signaling and cytokine molecular signatures in the three CNS-resident myeloid populations under different clinical conditions. a–d, Dynamics of key signaling molecules for immune activation pathways in the three CNS-resident myeloid populations. Box-and-whisker plots show median raw CyTOF signal intensity per population. Center line is average; boxes extend to 25th and 75th percentiles; whiskers extend to 5th and 95th percentiles. The gray area represents the interquartile range of the given signaling molecule in all cells in a sample, averaged across replicates, and thus indicates the overall expression range for each marker ($n = 5$ independent experiments for healthy, end-stage HD, presymptomatic EAE, chronic EAE; $n = 6$ independent experiments for onset EAE, peak EAE). e–g, Single-cell analysis of cytokine production by the three CNS-resident myeloid populations in response to different disease conditions. Analysis of cytokine coexpression in CNS-resident myeloid populations in healthy and diseased states demonstrates heterogeneous subsets in each population. Percentages of single cells expressing zero, one or two cytokines are represented in a stacked bar graph ($n = 3$ independent experiments). NA, not applicable.

of single-positive TNF-α-producing cells increased notably in comparison to the healthy state in both neuroinflammatory and neurodegenerative models whereas the frequency of single-positive IL-6 or TGF-β-producing cells decreased (Fig. 3e–g).

We identified three subsets in which more than one cytokine was produced within a single cell: dual TNF-α- and GM-CSF-producing cells, dual TNF-α- and IL-10-producing cells and dual TNF-α- and IL-6-producing cells (Fig. 3e–g). Most noticeably, the frequency of the subset that coexpressed both GM-CSF and TNF-α in populations B and C increased considerably during neuroinflammatory conditions, especially at the onset and peak of EAE disease, making this the second most abundant subset among cytokine-producing cells (up to 18% and 29%, respectively) (Fig. 3e–g).

Conversely, in the neurodegenerative HD model, the frequency of this GM-CSF and TNF-α subset was very low in all three CNS-resident myeloid populations (0% to 2%). With respect to other multifunctional subsets, a low frequency of TNF-α$^+$IL-6$^+$ and TNF-α$^+$IL-10$^+$ multifunctional cells (2–3%) emerged in both neuroinflammatory and neurodegenerative models. By comparing the cytokine profiles in these neuroinflammatory and neurodegenerative models, we can identify the dual-producing GM-CSF and TNF-α subset as a signature of neuroinflammation (Fig. 3e–g).

Moreover, in contrast to populations B and C, an appreciable fraction of cells in population A produced no cytokines in healthy and disease conditions, and the cytokine-producing subsets were dominated by single-cytokine-producing cells even during disease conditions. Multifunctional subsets producing more than one cytokine comprised only 1% of cells (Fig. 3e–g). This result is consistent with the analysis above, in which population A showed a lack of myeloid cell activation markers and had lower expression of signaling molecules than the other two populations (Fig. 3e–g).

To capture the cellular and cytokine profile of myeloid cells in the earlier stages of the model of HD, before development of severe symptoms (week 13–14), we next analyzed R6/2 mouse CNS tissues at 4 weeks, 7 weeks and 10 weeks. The frequency of CNS-resident myeloid populations (Fig. 2b) showed that populations A, B and C all existed before disease and during the progression of HD (Supplementary Fig. 9a). Over the course of the disease, the frequencies of populations A and B increased, whereas that of population C remained relatively low and unchanged (Supplementary Fig. 9a).

To gain increased resolution of the cytokine profile of each population at the single-cell level, we applied unsupervised X-shift analysis (Fig. 3e–g). This revealed six distinct subpopulations of cytokine-producing cells at the single-cell level, based on the production of IL-10, TNF-α, IL-6, TGF-β or GM-CSF, or their lack (Supplementary Fig. 9b). Of note, these subpopulations had a single-cytokine-only phenotype, and there was a notable lack of cells secreting multiple cytokines (Supplementary Fig. 9b).

Remarkably, during early stages of HD-like pathology, there was a distinct subpopulation that secreted only IL-10. In all three CNS-resident myeloid populations, the fraction of such cells ranged from 30% to 60%, depending on the population (Supplementary Fig. 9b). This IL-10-expressing subpopulation was not detected at a late stage in the HD model (13–14 weeks) (Fig. 3e–g). IL-10 is most often considered an anti-inflammatory cytokine[26].

We then investigated the frequency and cytokine profile in populations A, B and C in a mouse model of a second progressive neurodegenerative disorder, ALS, using transgenic mSOD1 mice[27]. Employing the same CyTOF panel and analysis strategy as above, we analyzed the cellular and cytokine profiles at two time points: onset (95 d of age), when decline in motor performance has been reported, and a late stage (140 d), when mice are completely paralyzed[14]. All three populations A, B and C, defined by the biaxial dot plot as above, were observed at both onset and late stages of the mSOD1 ALS model (Supplementary Fig. 10).

The frequency of populations A and B increased as the symptoms progressed to late-stage disease in the ALS mouse model, which is consistent with previous reports of the increase in the number of microglia in mSOD1 mice[14]. The frequency of population C remained the same during both disease time points (Supplementary Fig. 10a). Analysis of the cytokine profiles of the three CNS-resident myeloid populations at the single-cell level in the ALS model at onset and late stage revealed eight distinct subsets: cells with no cytokine production, cells with individual secretion of either TNF-α, IL-6, IL-10, TGF-β or GM-CSF alone, and those cells expressing combinations of TNF-α and IL-6 or of TNF-α and IL-10 (Supplementary Fig. 10b). The frequency of subsets expressing either no cytokine, GM-CSF alone or TGF-β alone decreased as the disease progressed to the late stage, whereas the frequency of TNF-α-producing cells sharply increased (Supplementary Fig. 10b). As in early stages in the HD model, we observed the presence of IL-10-expressing cells across all three CNS-resident myeloid populations at the onset in the ALS model, albeit at a much lower frequency (2–10%). However, in contrast to the HD model, these IL-10-expressing cells increased (populations A and C) or remained constant (population B) in the ALS model (Supplementary Fig. 10b). Interestingly, as in the end stage in the HD model, there was a very small subset of cells (0.5–2%) producing multiple cytokines in the end-stage ALS model (Supplementary Fig. 10b). Thus, in both HD and ALS models, most cells are not multifunctional cytokine producers.

Collectively, these data highlight a fundamental property of three identified CNS-resident myeloid cell populations, by demonstrating that each population, although defined as relatively homogeneous by common cell surface markers, in fact contains heterogeneous functional subsets based on their cytokine secretion profile.

Notably, we have identified two distinct cytokine-secreting subsets that represent the signature of neuroinflammatory conditions, in contrast to neurodegenerative models. Although both neuroinflammatory and neurodegenerative models developed double-positive TNF-α- and GM-CSF-producing cells, the high frequency of this subset correlated best with the height of neuroinflammatory conditions in EAE—peak and onset—in populations B and C, whereas the frequency of cells in these same populations was extremely low or absent in both models of HD and ALS. Conversely, both neurodegenerative conditions showed a remarkable subset of cells expressing IL-10, often considered an anti-inflammatory cytokine[26], across all three CNS-resident myeloid populations before late-stage disease symptoms.

Blood-derived monocyte subsets exhibit different kinetics of migration to the CNS in inflammatory versus HD and ALS models. The inflammatory response in the CNS is due in part to the entry of peripherally derived myeloid cells[28,29]. We next characterized the properties of these infiltrating cells in both EAE and HD mice. Peripheral monocytes were distinguished from other myeloid cells (CD11b$^+$ cells) on the basis of expression of their key surface marker Ly6C and lack of Ly6G expression. A composite MST from all samples combined revealed five discrete Ly6C$^+$Ly6G$^-$ cell clusters in CNS samples (Fig. 4a). The X-shift algorithm separated the Ly6C compartment into five separate clusters—D, E, F, G and H—and the DMT visualization identified the main markers driving the separation as CD274 (PD-L1), CD88 (C5aR1), CD217 (IL-17R) and MHC-II (Fig. 4b). Population D is characterized as Ly6C$^+$CD274$^+$MHC-II$^+$ monocytes, population E as Ly6C$^+$CD274$^+$MHC-II$^-$ monocytes, population F as Ly6C$^+$CD274$^-$CD88$^-$CD217$^-$ monocytes, population G as Ly6C$^+$CD274$^-$CD88$^+$CD217$^-$ monocytes and population H as Ly6C$^+$CD274$^-$CD88$^+$CD217$^+$ monocytes.

We analyzed the frequency of each of these five peripheral monocyte populations in the healthy state and in different stages in the disease models (Fig. 4c). In agreement with previous studies[8], we observed no contribution of peripheral monocytes (an average

of less than 0.4%) in the CNS in the HD model. In accordance with earlier reports[28–30], there was a very low frequency of monocytes in the CNS of both healthy and recovered EAE mice (0.8% to 1.2%, respectively), and only population F was detected. In contrast, the inflammatory stages of EAE—presymptomatic, onset and peak—evoked the presence of all five identified peripheral monocyte subsets (Fig. 4c). In chronic EAE we observed a low frequency (0.5% to 0.9%) of monocyte populations F, G and H (Fig. 4c).

Furthermore, the expression pattern of other cell surface markers demonstrated that CD80, CD86, CD38, CD39, MerTK, Axl, CD206 and TREM2 were upregulated in populations D and E. Populations F and G expressed low levels of these markers, and population H expressed intermediate levels of these cell surface markers (Supplementary Fig. 11). With expression of co-stimulatory molecules (CD80, CD86), populations D and E most likely represent activated antigen-presenting cells (APCs). Population E (MHC-II$^-$) could be an independent population or may simply be a population that has transitioned from population D. Both populations express CD274 (PD-L1) following activation, as a natural immunological brake that results in the inactivation of newly activated T cells[31].

Population F (CD49d$^+$CD88$^-$CD217$^-$MHC-II$^-$) is upregulated in onset and recovered EAE. Populations G and H, present predominantly during presymptomatic EAE and further distinguished by CD49d$^+$CD274$^-$CD88$^+$MHC-II$^-$ expression, are likely activated monocytes or macrophages generated during the innate and adaptive inflammatory response following EAE immunization. CD88 (C5aR1), the receptor for the complement peptide C5a, is expressed in EAE on activated monocytes or macrophages infiltrating into the CNS[32]. Population G (CD217$^-$) may arise predominantly from activation of T_H1 cells that is sustained during EAE onset whereas population H (IL-17R$^+$) is generated in response to the activation of T_H-17 cells following EAE immunization.

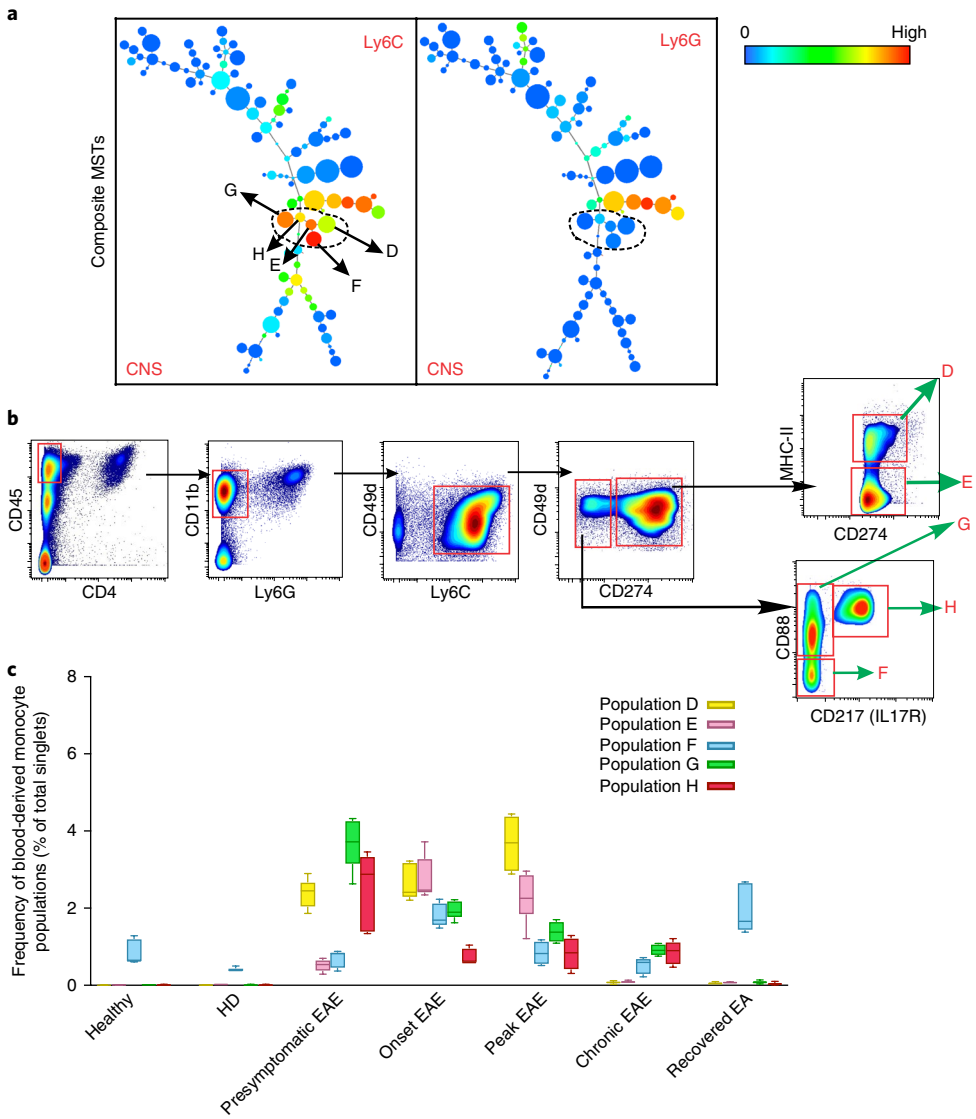

Fig. 4 | Kinetics of peripheral monocytes in CNS under inflammatory versus degenerative conditions. a, Composite MSTs of CNS samples ($n = 37$) reveals five distinct Ly6C$^+$Ly6G$^-$ myeloid populations (peripheral monocytes) in CNS. The color code (as in Fig. 2a) shows the expression levels of Ly6C and Ly6G. **b**, Manual gating based on markers and threshold defined by the X-shift DMT algorithm confirmed each population. This panel represents data from peak EAE, where 7 independent experiments confirmed a similar gate. **c**, Frequency analysis based on manual gating demonstrates that there is a minimum accumulation of peripheral monocytes in healthy and neurodegenerative conditions. In EAE disease, different peripheral monocyte populations accumulated depending on the disease state. Center line is average; boxes extend to 25th and 75th percentile; whiskers extend to 5th and 95th percentiles ($n = 5$ independent experiments for healthy, end-stage HD, presymptomatic EAE, chronic EAE; $n = 6$ independent experiments for onset EAE, peak EAE).

Discordance in expression of signaling molecules and cytokines in infiltrating versus resident myeloid cells. We next asked whether the five peripheral monocyte populations had distinctive signaling states in response to the same disease model conditions compared to the three CNS-resident myeloid cell populations. Several signaling proteins were indeed differentially expressed under the same disease conditions (Fig. 5a).

Expression of the transcription factor pSTAT3 was higher in several peripheral monocyte populations at the onset (populations D and E) and peak (populations D, E and H) of EAE compared to all three CNS-resident myeloid cell populations (Fig. 5a). An increase in pSTAT3 is recognized as an important mediator of inflammation in multiple sclerosis[33].

In contrast, pCREB expression was markedly higher in CNS-resident myeloid cells, particularly populations B and C, in relation to the peripheral monocyte populations (Fig. 5a), thus supporting a fundamental difference between infiltrating monocytes and CNS-resident myeloid cells. The proliferation of CNS-resident myeloid cells but not peripheral monocytes and the upregulation of proliferation-related genes such as *Fos* during the course of EAE in CNS-resident myeloid cells have recently been reported[34]. CREB is the main transcriptional regulator of the *Fos* gene[35]. The present results demonstrating pCREB expression are concordant with patterns of microglial proliferation and *Fos* expression, and suggest that CREB pathways promote proliferation of CNS-resident myeloid cells during EAE. NF-κB and C/EBPβ expression were also increased in CNS-resident myeloid cell populations but not in peripheral monocyte populations during EAE disease (Fig. 5a).

On the basis of these results, we hypothesized that different signaling properties of CNS-resident myeloid cells and peripheral monocytes should be reflected in distinct cytokine expression profiles during EAE pathology. Therefore, we next assessed the cytokine production capacity of each of the five peripheral monocyte populations. Peripheral monocyte and CNS-resident myeloid cell populations had similar cytokine expression profiles, predominantly producing TNF-α, followed by IL-6, GM-CSF, IL-10 and TGF-β (Supplementary Fig. 12).

We next analyzed the profile of multiple cytokines produced by single-cell populations using the X-shift clustering algorithm. A comparative analysis of the five peripheral monocyte populations and the three CNS-resident myeloid cell populations revealed that, in addition to the seven distinct populations of cytokine-producing cells that were identified in CNS-resident myeloid cell populations (Fig. 3e–g), some of the peripheral monocyte populations have three more multiple-cytokine-producing subsets in EAE (Fig. 5b). These three new multifunctional subsets consisted of triple-cytokine-producing TNF-α$^+$GM-CSF$^+$IL-6$^+$ and TNF-α$^+$IL-6$^+$IL-10$^+$ cells and quadruple-cytokine-producing TNF-α$^+$GM-CSF$^+$IL-6$^+$IL-10$^+$ cells (Fig. 5b), whereas multifunctional subsets in CNS-resident myeloid populations did not go beyond double positive (Fig. 3e–g). These three additional subsets were only present at the onset and peak of EAE and had a noticeably higher frequency at the peak of the disease compared to the onset (Fig. 5b).

Differential expression of cell surface phenotype on infiltrating versus resident myeloid cells reveals therapeutic targets. Microglia and peripherally derived myeloid cells have distinct developmental origins[36], distinct renewal mechanisms[29] and, as shown here, different signaling and cytokine profiles under the same disease condition. Next we explored these different cell types in reference to phenotypic surface proteins.

When comparing the cell surface markers in the CNS-resident myeloid cell populations (A, B and C) with the peripheral monocyte populations (D, E, F, G and H), we observed that adhesion molecules CD49d (α4 integrin) and CD49e (α5 integrin) were expressed only on blood-derived myeloid populations and not on CNS-resident myeloid cell populations (Fig. 6a). CD49a, CD49b and CD49c were not present in any population in our pilot screen. While CD49d was also expressed by lymphocytes and dendritic cells, CD49e was only expressed by Ly6C$^+$ subpopulations (Fig. 6a). CD49e binds fibronectin[37] and is found in multiple sclerosis lesions, particularly around blood vessels[38]. The expression of CD49e on monocytes suggests that a CD49e–fibronectin interaction is critical to migration of these cells into the CNS parenchyma.

To investigate whether blocking the entry of monocytes into the CNS would affect the course of EAE disease, we treated EAE mice with either the MFR5 antibody specific to CD49e or its isotype antibody control. We used two treatment regimens to test the effect of anti-CD49e antibody treatment on EAE.

In the first regimen, we started the treatment on day 1, the day on which we induced EAE in C57BL/6 mice, and continued treatment every day. The onset of the disease in mice treated with anti-CD49e antibody in this prophylactic regimen was significantly delayed compared with the control group (Fig. 6b). Notably, antibody treatment reduced the severity of the disease, and the animals never reached a severe paralytic stage (Fig. 6b). Fibronectin and α5β1 integrin have been reported to be upregulated in the endothelium at the height of vascular remodeling during presymptomatic EAE[39]. Therefore, starting anti-CD49e antibody treatment at the time of disease induction could function by both blocking monocyte trafficking into the CNS and inhibiting brain endothelial cell proliferation and angiogenic remodeling.

In the second treatment regimen, we started administration of anti-CD49e antibody once all mice exhibited clinical signs of EAE (day 15). Anti-CD49e antibody treatment also attenuated established EAE (Fig. 6c). Analyzing the cellular profile of mice treated with anti-CD49e antibody versus the isotype control group with CyTOF revealed a notable reduction in the size of the nodes representing each of the five monocyte populations following anti-CD49e antibody treatment (Fig. 6d,e). Treatment of EAE by blocking CD49e (α5-integrin) recruitment of peripheral myeloid cells to the CNS thus suggests that these infiltrating cells are essential for pathogenesis.

Discussion

For the last few decades, the concept of inflammation in response to brain pathologies has been under constant debate[1,8,40,41]. The results of our study challenge a view whereby any cellular and molecular activation of glial cells across various neuropathological conditions is simply labeled 'neuroinflammation'. We used CyTOF coupled with an unbiased[18] bioinformatics approach to characterize the myeloid cell compartment at two opposite poles of CNS pathology in an mouse model of neuroinflammatory disease, EAE, versus mouse models of neurodegenerative diseases such as HD and ALS. We initially identified two CNS-resident myeloid populations (A and B) in healthy brains. Notably, in the EAE, HD and ALS models, these two populations increased in total frequency in parallel with the expansion of a third, disease-specific population, population C. These observations provide a basis for the contention that different CNS diseases involving microglia have similarities. However, delving further into the phenotypes of these populations allowed us to demonstrate that the three CNS-resident myeloid populations have nuanced differences when comparing EAE with the R6/2 model of HD and the mSOD1 model of ALS.

Single-cell analysis of several signaling markers revealed that in EAE two of the CNS-resident myeloid populations (B and C) developed a closely coordinated series of signaling events with pCREB and pMAPKAPK2 as their signature. This occurred during the presymptomatic stage of disease, before clinical paralysis, and at the peak of disease, when paralysis is manifest, whereas both NF-κB and C/EBPβ signaling pathways characterized the chronic disease state. By contrast, these two populations in the HD model did not exhibit any marked expression of these same signaling pathways,

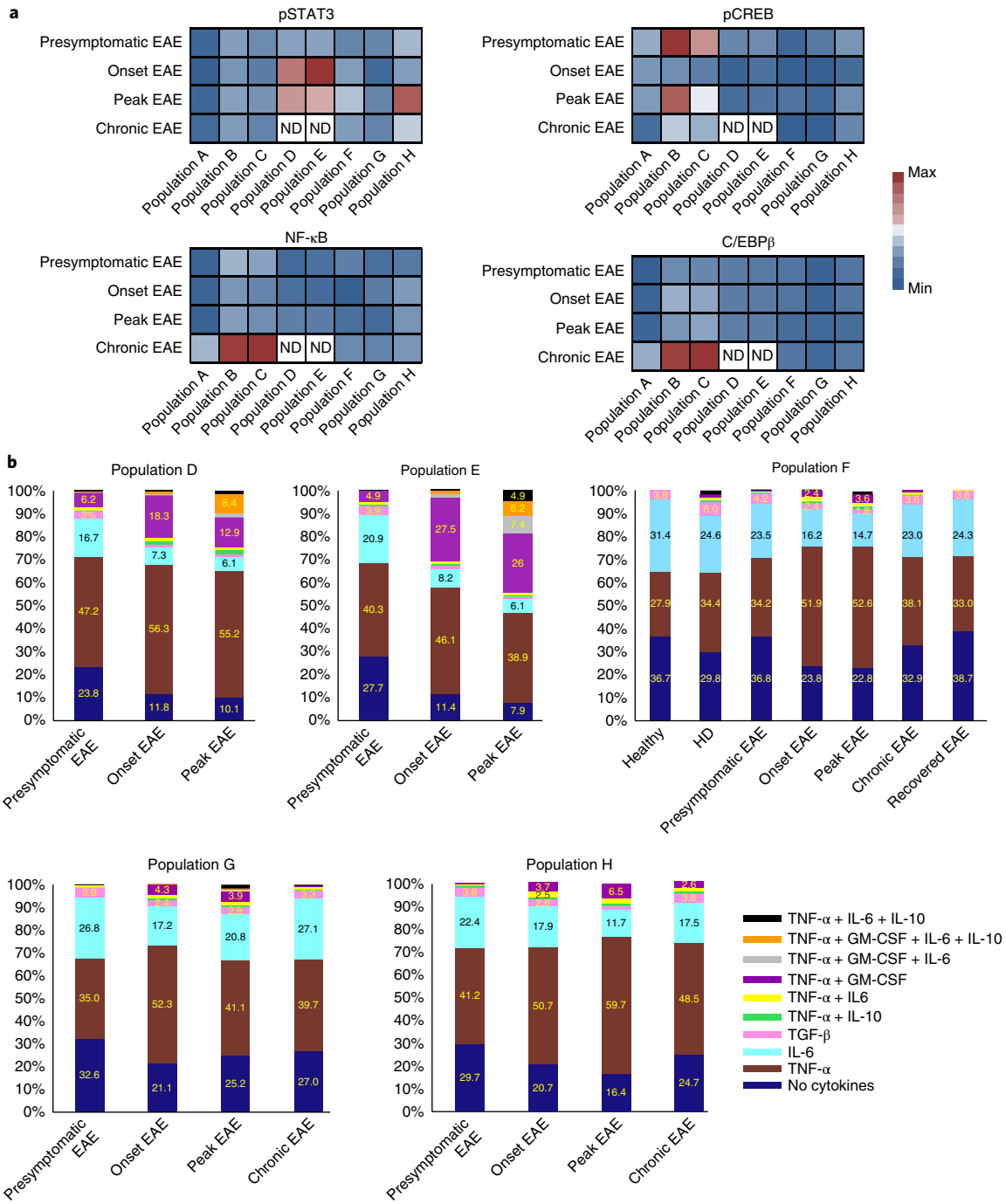

Fig. 5 | Single-cell analysis of signaling molecules and cytokine production in different peripheral monocyte populations in response to different disease conditions. a, Heat map representing the comparison of median raw CyTOF signal intensity for each signaling molecule between CNS-resident myeloid populations and peripheral monocyte populations in presymptomatic, onset and peak EAE, when all five peripheral monocyte populations are present. The color representing the signaling molecule expression ranges from blue (undetectable) to white (intermediate) to red (maximum); $n = 5$ independent experiments for healthy, end-stage HD, presymptomatic EAE, chronic EAE; $n = 6$ independent experiments for onset EAE, peak EAE. ND, not distinguishable. **b**, Single-cell analysis of cytokine production by different peripheral monocyte populations in response to different disease conditions. X-shift analysis of the coexpression of cytokines in peripheral monocyte populations suggests that each population contains heterogeneous subsets depending on each disease conditions. Percentages of single cells expressing zero, one, two, three or four cytokines are represented in a stacked bar graph ($n = 3$ independent experiments).

contrary to previous reports[42]. The lack of similarity in signaling activity of CNS-resident myeloid cells between the HD model and chronic stage EAE is notable, considering that mice in both models developed permanent functional neurologic impairment. Chronic EAE has been often described as the neurodegenerative phase of progressive multiple sclerosis[43,44]. In our results, NF-κB and C/EBP signaling in CNS-resident myeloid cells in chronic EAE contrasted with the lack of any such signaling activity in an HD model.

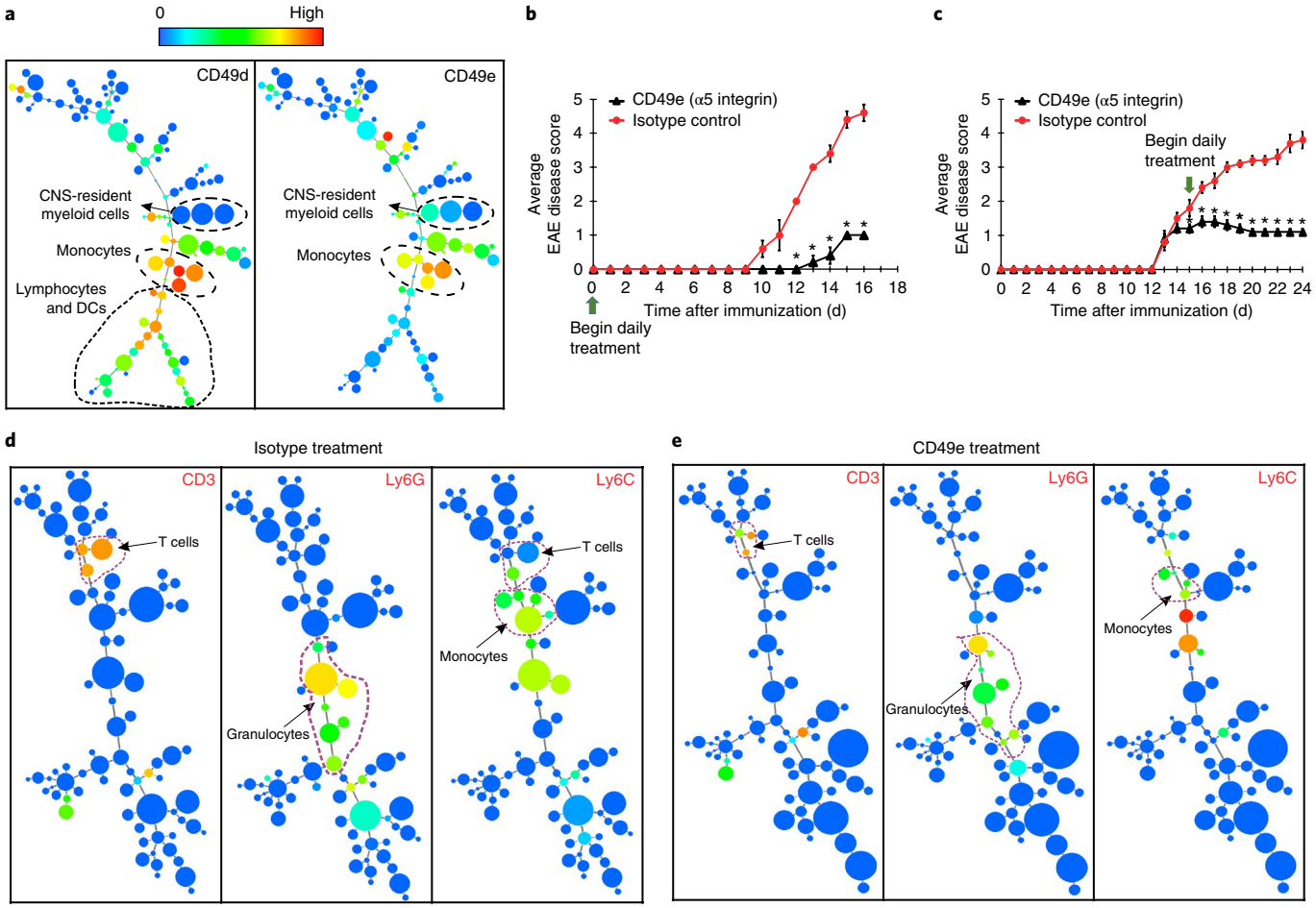

Fig. 6 | CyTOF analysis reveals a therapeutic target on infiltrating myeloid cells in inflammatory conditions. a, Cell surface phenotype analysis reveals high expression of CD49d (α4 integrin) and CD49e (α5 integrin) only on infiltrating myeloid cells compared to CNS-resident myeloid cells. CD49e is expressed only on myeloid cells whereas CD49d is also expressed on T cells and dendritic cells. **b**, Average clinical score for EAE mice treated daily with an anti-CD49e antibody compared to an isotype control antibody. In this prophylactic regimen, treatment started at the time of EAE induction. Mice treated with anti-CD49e antibody exhibit a delay in disease onset and have significantly reduced overall disease severity. The experiment was ended because of the high morbidity of control mice. Arrow indicates when the treatment was started. Each point represents the mean clinical disease score ± s.e.m. *$P < 0.05$ by Mann–Whitney one-tailed test comparing between the two groups ($n = 5$ mice). **c**, Average clinical score for EAE mice treated daily with anti-CD49e antibody compared to an isotype control starting when EAE disease is already established. Mice treated with anti-CD49e antibody exhibit reduced overall disease severity. Arrow indicates when the treatment started. Each point represents the mean ± s.e.m. *$P < 0.05$ by Mann-Whitney one-tailed test comparing between the two groups ($n = 10$ mice). **d**,**e**. CyTOF analysis from CNS tissue of EAE mice treated with either isotype control antibody or anti-CD49e antibody (experiment in **c**).

The difference in the functional properties of CNS-resident myeloid cells was also reflected in their respective profiles of cytokine secretion in HD and ALS compared to EAE. While from an analysis of the total population these three populations in healthy and disease conditions demonstrated the ability to generate similar cytokines, albeit with different frequencies, analysis at the single-cell level confirmed that each population itself in fact contains different subsets based on cytokine production profiles. The striking difference between EAE versus HD and ALS was the surge of cells that secrete multiple cytokines, such as the combination of TNF-α and GM-CSF, in EAE. Such dual secretors constituted a substantial portion of the total cytokine producing cells at the onset and peak of EAE disease. In both HD and ALS models, most cells were not multifunctional cytokine producers. Our data suggest that the analyses of cytokine levels as a marker of immune response should be interpreted in the context of whether single or multiple cytokines are produced within a cell population[25].

Of note, we observed that a substantial fraction of cells within populations A, B and C upon the transition from presymptomatic to mild symptomatic HD (up to 10 weeks of age) expressed IL-10, which is often associated with anti-inflammatory properties[26]. IL-10 production was notably absent at the late stage of the R6/2 HD model (13–14 weeks). Altered levels of IL-10 have been observed post-mortem in the striatum in HD[45]. This observation is interesting in the context of the protective (anti-inflammatory) and trophic support delivered directly by IL-10 to neurons[46]. These results emphasize the limitations of defining myeloid cells along single polarities such as M1 and M2 because, as we see from these studies, cytokine profiles are highly nuanced[47].

We next analyzed blood-derived myeloid cells. Myelomonocytic cells ($Ly6C^+Ly6G^-$) differentiate into five peripheral monocyte populations. Blood-derived myeloid cells do not contribute to myeloid population in the brain under neurodegenerative conditions such as ALS[29]. Similarly, we confirmed that the recruitment of myelomonocytic cells to the brain was absent in the HD model as well. By contrast,

these five peripheral monocyte populations were present in all different clinical stages of EAE, but at varied frequencies.

We investigated the functional differences between CNS-resident myeloid cells versus recruited blood-derived myeloid cells in the pathogenesis of different CNS disease models. These two cell types had different signaling phenotypes under the same disease conditions. In addition, we were able to demonstrate that inflammatory signaling, such as pSTAT3 upregulation, occurred in peripheral monocytes during the active stages of EAE. Later, in the chronic disease stage, the inflammatory signaling switched to CNS-resident myeloid cells that express NF-κB.

The implications of the shift in inflammatory signaling from peripheral myeloid cells to brain-specific myeloid cells, with different signaling programs, may have broad implications for designing new drugs targeting the NF-κB pathway beyond the blood–brain barrier. The progressive stage of diseases such as multiple sclerosis may be largely generated from cells that lie within the CNS and thus beyond the blood–brain barrier.

An emerging theme from these data, in concert with our previous findings and those of others[28,34], is that the substantial recruitment of monocytes is a transient event largely driven by classical inflammation. Once inflammation is substantially diminished or disappears, monocytes also largely vanish. The image of monocytes as the key cellular player that triggers the progression of EAE disease to the paralytic stage, a concept put forward by our own previous studies and others[28,48], now becomes even more complex and nuanced given the present discovery of the considerable heterogeneity of this monocyte population.

Blocking the entry of leukocytes to the brain has been explored as a therapeutic strategy[49]. We found that blocking the homing of T lymphocytes and monocytes to the CNS with an antibody specific for CD49d (α4 integrin) suppressed EAE, and recent clinical studies confirmed reduced relapse rates in multiple sclerosis patients, leading to the approved therapeutic natalizumab[50]. Unfortunately, this approved treatment for relapsing-remitting multiple sclerosis that blocks T cells from entering the brain may result in the reactivation of the JC virus, leading to the sometimes fatal infection known as progressive multifocal leukoencephalopathy[49].

We also previously reported that by preventing the infiltration of blood-derived myeloid cells to the CNS, the activation of CNS-resident myeloid cells is required for the initiation of EAE and precedes the entry of blood-derived cells[28]. Furthermore, the progression of EAE is due to the entrance of blood-derived myeloid cells into the CNS[28]. Here we identified CD49e (α5 integrin) expression on only the peripheral monocyte populations and not on CNS-resident myeloid cell populations. Treatment with anti-CD49e antibody significantly reduced EAE disease severity and now provides a strong rationale for a new therapeutic approach that specifically targets and inhibits monocyte trafficking into the CNS. Such a strategy might even have fewer deleterious side effects than existing multiple sclerosis therapies.

These studies illustrate the power of mass cytometry for understanding previously undefined populations of CNS myeloid cells. The varieties of molecular programs in myeloid cells between inflammatory and degenerative conditions in these models of HD, ALS and multiple sclerosis, may allow us to further distinguish between neuroinflammation and neurodegeneration. Extrapolation of these studies to humans is one of the next steps. As we have shown here, unexpected therapeutic targets, such as α5 integrin, have emerged by application of CyTOF to the analysis of neuropathology.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41593-018-0100-x.

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Author contributions

B.A. conceived the study; designed, directed and performed all experiments; analyzed and interpreted the data; and wrote the manuscript. N.S. developed the analysis algorithms and carried out analysis. P.W. and Z.B. designed experiments, performed data analysis and interpretation. P.P.H. designed experiments, performed data analysis and interpretation, and wrote the manuscript. A.C. provided advice on data analysis and interpretation and wrote the manuscript. M.P., W.J.F. and G.P.N. provided important advice on experimental design and data analysis and interpretation. L.S. conceived the study, directed the project and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

Mice. C57BL/6J female mice were purchased from the Jackson Laboratory (Sacramento, CA) at 7 weeks. Animals were rested at Stanford University's research animal facility for 2 weeks and were induced with EAE at 9 weeks of age.

R6/2 female mice were purchased from the Jackson Laboratory. CNS tissues were collected at the ages of 4, 7 and 10 weeks, before development of severe symptoms, and at the age of 13–14 weeks (late stage disease), when they had developed severe tremor, irregular gait, abnormal movements and seizures and decreased survival[15]

Female mice overexpressing the G93A mutant human transgene for *SOD1* were purchased from Jackson Laboratory. CNS tissues were collected at an age of 95 d, when a substantial decline in motor function of mSOD1 mice compared to wild-type controls begins[14], and 140 d of age, with disease late stage being defined as the time when mice were completely paralyzed.

Animal experiments were approved by the Institutional Animal Care and Use Committee at Stanford University and performed in compliance with National Institute of Health guidelines. All animals were housed under a 12-h light cycle. The maximum number of animals housed per cage was 5 mice. Animals were randomly selected for use in this study.

Induction of EAE in mice by immunization with MOG and adjuvant. EAE was induced in female C57BL/6J mice (the Jackson Laboratory) at 9 weeks of age by subcutaneous immunization in the flank with an emulsion containing 200 µg myelin oligodendrocyte glycoprotein$_{35–55}$ (MOG$_{35–55}$; MEVGWYRSPFSRVVHLYRNGK) in saline and an equal volume of complete Freund's adjuvant containing 4 µg/ml *Mycobacterium tuberculosis* H37RA (Difco Laboratories Inc., Detroit, MI). All mice were given 400 ng of pertussis toxin (PTX; List Biological Laboratories, Inc., Campbell, CA) intraperitoneally 0 and 48 h after immunization. Neurological impairment was scored as follows: presymptomatic; 10 d after EAE induction with no clinical disease; onset: loss of tail tone and hindlimb weakness, peak; complete hindlimb paralysis, recovered; recovery from hindlimb paralysis and persistence of the improvement, chronic; development of permanent functional impairment after 3–6 months and lack of recovery.

Although the inclusion of PTX for EAE induction was originally thought to facilitate entry of immune cells into the CNS by increasing the permeability of the blood–brain barrier[51], more recent work has shown that this is a byproduct of PTX. PTX has been reported to act as an adjuvant, acting on dendritic cells to drive the development of T_H1 cells[52,53] and suppressing the number and function of T regulatory cells[54]. Repeated PTX administration actually prevents EAE[55].

Cx3cr1^{CreER} Rosa26-YFP mice. *Cx3cr1^{CreER} Rosa26-YFP* mice were generated in Marco Prinz's laboratory in Freiburg, Germany, by crossing *Cx3cr1^{CreER}* (*B6.129P2(C)-Cx3cr1tm2.1(cre/ERT2)Jung/J*) mice with *Rosa26-YFP* (*B6.129×1-Gt(ROSA)26Sortm1(EYFP)Cos/J*) mice, both available from Jackson Laboratory, and housed under SPF conditions in the local animal facility. *Cx3cr1^{CreER}* mice were backcrossed for over nine generations onto the C57Bl/6 background[21,56]. *Cx3cr1^{CreER}* mice were genotyped by PCR using the forward primer 5′-CCT CTA AGA CTC ACG TGG ACC TG-3′, the reverse primer 5′-GAC TTC CGA GTT GCG GAG CAC-3′ and a specific primer GCC GCC CAC GAC CGG CAA AC, which amplify a 750-bp fragment from wild type or a 304-bp fragment from the transgenic *Cx3cr1* locus. For induction of Cre recombinase, 5- to 7-week-old *Cx3cr1^{CreER}* female mice were treated with 4 mg tamoxifen (Sigma) dissolved in 200 µl corn oil (Sigma) injected subcutaneously at two time points 48 h apart. The brain, spinal cord and blood of these mice were collected at 15 weeks of age for CyTOF analysis. Anti-GFP antibody (Supplementary Table 2a) conjugated to a metal was used to detect the YFP expression. The lack of YFP expression in blood cells was confirmed by CyTOF using the same antibody panel (Supplementary Fig. 13a).

Antibodies. A high-throughput flow cytometric screen of 255 monoclonal antibodies against mouse cell surface markers was performed using the LEGENDScreen Mouse PE Kit (Biolegend cat # 700005) (Supplementary Table 1). CNS tissues from healthy mice, EAE mice and HD mice were used for the initial screen. The CyTOF panel was assembled on the basis of the high-throughput screen results, as well as including proteins known to regulate myeloid cell functions (such as MerTK and Axl), transcription factors and signaling molecules known to be relevant to neuroinflammation.

A summary of antibodies used for each panel can be found in Supplementary Tables 2a–c, including their primary manufacturer, clone, corresponding metal conjugate and final operating concentration. Antibodies were prepared in amounts varying from 100 to 500 µg at a time using the MaxPAR antibody conjugation kit (Fluidigm, Markham, ON, Canada) following the manufacturer's protocol. After being labeled with their corresponding metal conjugate, the percentage yield was determined by measuring their absorbance at 280 nm using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). Antibodies were diluted using Candor phosphate-buffered saline (PBS) antibody stabilization solution (Candor Bioscience GmbH, Wangen, Germany) to 0.3 mg/mL and then stored at 4 °C. Each antibody was titrated for optimal staining concentrations using primary murine samples and cell cultures.

Single-cell isolation. Mice were deeply anesthetized and monitored. Upon the loss of nociceptive reflexes, animals were perfused transcardially with ice-cold PBS. Brains and spinal cords were removed and gently homogenized in cold HBSS (Life Technologies, 14175-095) on ice. Mononuclear cells were separated with 30%–70% Percoll (GE Healthcare, Marlborough, MA) gradient centrifugation according to a previously reported protocol[57].

Cell suspensions were washed in PBS with 2% FCS and 2 mM EDTA two times and were immediately fixed for 10 min at RT using a 1:1.4 proteomic stabilizer (to preserve the expression of signaling molecules) according to the manufacturer's instructions (Smart Tube Inc., Palo Alto, CA) and frozen at –80 °C.

Peripheral blood was collected retro-orbitally before perfusion of the animal and transferred to sodium heparin-coated vacuum tubes at 1:1 dilution in RMPI 1640, fixed immediately for 10 min at room temperature using 1:1.4 proteomic stabilizer according to the manufacturer's instructions (Smart Tube Inc., Palo Alto, CA) and frozen at –80 °C. Sample preparation for cytokine analysis were performed as described previously[24]. Protein transporter inhibitor (eBioscience, 00-4980-93) was used to avoid the secretion of cytokines.

In each experiment, 10–12 mice were pooled to provide enough cells. Each experiment was repeated 7 to 10 times with separate immunization and cohorts of mice.

Mass-tag cell barcoding. Samples from each condition were mass-tag cell barcoded (MCB). In each sample a unique combination of 6 palladium isotopes was used to encode 20 unique mass-tag barcodes as previously described[58,59]. This technique allows all the samples to be pooled and stained in a single tube, eliminating tube-to-tube variability in antibody staining and minimizing the effect of variable instrument sensitivity. For each sample, 1.5×10^6 cells from each condition were barcoded. Methanol-permeabilized cells were washed once with cell staining medium (CSM, PBS with 0.5% BSA, 0.02% NaN_3) and then once with PBS. Different combinatorial mixtures of palladium-containing MCB reagents in DMSO were then added to the individual samples at 1:100 DMSO with vortexing and then incubated at room temperature for 15 min, followed by three washes with CSM. The individual samples were then pooled for antibody staining and mass cytometry analysis. After data collection, the conditions were deconvoluted using a mass cytometry de-barcoding algorithm[58].

Antibody staining. Barcoded cells were resuspended in PBS with 0.5% BSA and 0.02% NaN_3, and antibodies against CD16/32 were added at 20 µg/ml for 10 min at room temperature on a shaker to block Fc receptors. Cells were mixed with a cocktail of metal-conjugated surface marker antibodies (Supplementary Table 1), yielding 500-µL final reaction volumes, and stained at room temperature for 30 min on a shaker. Following staining, cells were washed twice with PBS with 0.5% BSA and 0.02% NaN_3. Next, cells were permeabilized with 4 °C methanol for 10 min at 4 °C. Cells were then washed twice in PBS with 0.5% BSA and 0.02% NaN_3 to remove remaining methanol. They were stained with intracellular antibodies (Supplementary Table 2b for signaling experiments and Supplementary Table 2c for cytokine experiments) in 500 µL for 30 min at room temperature on a shaker. Samples were then washed twice in PBS with 0.5% BSA and 0.02% NaN_3. Cells were incubated overnight at 4 °C with 1 mL of 1:4,000 $^{191/193}$Ir DNA intercalator (DVS Sciences/Fluidigm, Markham, ON) diluted in PBS with 1.6% PFA overnight. The following day, cells were washed once with PBS with 0.5% BSA and 0.02% NaN_3 and then two times with double-deionized water (ddH_2O).

Mass cytometry measurement. Prior to analysis, the stained and intercalated cell pellet was resuspended in ddH_2O containing polystyrene normalization beads containing lanthanum-139, praseodymium-141, terbium-159, thulium-169 and lutetium-175 as described previously[59]. Stained cells were analyzed on a CyTOF 2 (Fluidigm, Markham, ON) outfitted with a Super Sampler sample introduction system (Victorian Airship & Scientific Apparatus, Alamo, CA) at an event rate of 200 to 300 cells per second. All mass cytometry files were normalized together using the mass cytometry data normalization algorithm freely available for download from https://github.com/nolanlab/bead-normalization.

Analysis. Samples are labeled with iridium nucleic acid ($^{191/193}$Ir DNA intercalator) (DVS Sciences/Fluidigm, Markham, ON). For each event, many features are recorded, including signal duration (called event length) and iridium intensity. Single events have lower iridium intensity (since they have less DNA) and lower event length values than aggregates. These characteristics enable gating of single cells (Supplementary Fig. 13b). Live cells are identified on the basis of lack of c-PARP (cleaved poly-(ADP)-ribose polymerase (PARP)) binding as described before[60] (Supplementary Fig. 13b).

Clustering. Minimum Spanning Tree (MST)[61] creates a two-dimensional layout. Differences in cell frequency of each subpopulation across different conditions are visualized by varying the size of each node proportionally to the frequency of the respective cluster in a given condition. Differences in marker expression levels across populations are visualized by coloring the nodes according to condition-specific marker expression levels (blue, no expression; green, yellow and red, increasing levels of expression).

The raw CyTOF data was subject to arcsinh(x/5) transformation[18]. We selected cells from each sample, which were then pooled for clustering, generating a dataset with a total of 1,800,183 cells for the signaling dataset and 1,967,893 cells for the cytokine dataset. These datasets were clustered with a density-based clustering method known as X-shift. X-shift was developed to compute large multidimensional datasets and automatically determine the optimal number of clusters. In short, X-shift uses weighted K-nearest neighbor density estimation to find the local maxima of data-point (cell event) density in the multidimensional marker space. X-shift computes the density estimate for each data point and then searches for the local density maxima in a nearest-neighbor graph, which become cluster centroids. All remaining data points are then connected to the centroids via density-ascending paths in the graph, thus forming clusters. Finally, the algorithm checks for the presence of density minima on a straight line segment between the neighboring centroids, merging closely aligned clusters as necessary. In summary, cells were assigned to different populations on the basis of the local gradient of cell event density in the marker expression space. Two cell populations counted as separate if cell density in any point on a straight line between centers of populations was lower than the density in the population centers. In other words, the peaks of cell event density that represent two populations must be separated by a cleft. Furthermore, clusters separated by a Mahalanobis distance of less than 2.0 were merged together. The optimal nearest neighbor parameter K was chosen to be 70 in a data-driven manner, by finding the elbow point of the plot of the number of clusters over K. All data processing was performed with the VorteX clustering environment (https://github.com/nolanlab/vortex).

Divisive marker tree (DMT) for gating. To facilitate back-gating of X-shift clustered populations, we organized the clusters into a divisive marker tree (DMT)[18]. The DMT algorithm constructs a binary decision tree that starts with a root node encompassing all clusters; this set of clusters is then subject to iterative binary division. This process results in a hierarchical binary classification of cell types that resembles manual gating hierarchies. By tracing the sequence of marker divisions from the root, we were able to infer a concise marker-based signature for each cell population that differentiates it from other populations.

CD49e (α5 integrin) treatment. *Prophylactic administration of anti-CD49e antibody.* For Fig. 6b, EAE mice (n = 5 per group) were treated daily with 200 µg of CD49e (α5 integrin) antibody (Clone = 5H10-27(MFR5)) or rat IgG2a, κ isotype control (low endotoxin, azide-free antibody, custom-made by Biolegend for this experiment). Treatment with antibody and isotype was initiated on day 0 after immunization via intraperitoneal injection.

EAE treatment with anti-CD49e antibody. For Fig. 6c, EAE mice (n = 10 per group) were treated daily with 200 µg of CD49e (α5 integrin) antibody (clone 5H10-27 (MFR5)) or rat IgG2a, κ isotype control (low endotoxin, azide-free antibody, custom-made by Biolegend for this experiment). Treatment with antibody and isotype was initiated once immunized mice developed paralysis (representing clinical EAE) on day 15.

EAE scores were assessed daily for clinical signs of EAE in a blinded fashion, without knowing which mouse was receiving treatments. Mice were assessed daily and scored as follows: 0, no clinical disease; 1, tail weakness; 2, hindlimb weakness; 3, complete hindlimb paralysis; 4, hindlimb paralysis and some forelimb weakness; 5, moribund or dead. The experiment was ended because of high morbidity of control mice.

Statistics. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications[62]. Mice were randomized such that both treatment groups had the same average disease score. We did not exclude any animals for data point from the analysis. Data collection were randomized. Data distribution was assumed to be normal, but this was not formally tested. Data collection was not performed blind to the conditions of the experiments. Analysis was performed blind to the conditions of the experiments.

A Mann–Whitney one-tailed test was used for the CD49e treatment studies of EAE, comparing isotype antibody control and CD49e antibody treatments. Cell population frequency data were compiled with Prism GraphPad7 and shown as box-and-whisker plots where the center line is the mean, boxes extend to 25th and 75th percentiles, and whiskers extend to 5th and 95th percentiles.

Life Sciences Reporting Summary. Further information on experimental design is available in the Life Sciences Reporting Summary.

Code availability. All data processing performed with the VorteX clustering environment called X-shift algorithm can be found at https://github.com/nolanlab/vortex.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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☐ Initial submission ☐ Revised version ☒ Final submission

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Experimental design

1. Sample size

Describe how sample size was determined.

In each condition, 10-12 mice were pooled in order to provide enough cell number (each experiment used 1 million cells per condition to assure that we are able to collect "enough cells" after barcoding / staining and CyToF run).
In data analysis, the algorithm chose a total of 1,800,183 cells from the signaling dataset and 1,967,893 cells from the cytokine dataset for analysis. These cell numbers give us enough confidence for the accuracy of the experiment and are the maximum number of cells the algorithm can handle before crashing.

This has been reported in figure 1 legend and material and methods under the "single cell isolation" section.

2. Data exclusions

Describe any data exclusions.

We did not exclude any data point.

3. Replication

Describe whether the experimental findings were reliably reproduced.

Each experiment repeated 7 to 10 times from separate immunization and cohort of mice in order to make sure the same data is produced. All attempts at replication were successful.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

1) Healthy, HD and mSOD1 mice were taken randomly from different cages.
In EAE experiments, different conditions were taken from random cages. Not all the cages provided the same condition for each experiment. 2) In alpha 5 treatment experiment: mice from several cages were randomly assigned to treatment and control.
This has been reported in Material and Methods, "mice" section.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

EAE scores were assessed daily for clinical signs of EAE in a blinded fashion without knowing which mouse was receiving treatments. Analysis was performed blind to the conditions of the experiments.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ A statement indicating how many times each experiment was replicated
☐	☒ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
☐	☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☐	☒ The test results (e.g. *P* values) given as exact values whenever possible and with confidence intervals noted
☐	☒ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☐	☒ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

An analysis algorithm called x-shift was used to analyze the data in an unbiased data-driven approach. It was used to identify different populations. Based on the gating criteria that was identified by the software, each cluster was confirmed by traditional manual gating. The algorithm is published in a manuscript : Samusik et al 2016. Description of the algorithm and the link to the software has been provided in the Materials and Methods "analysis" and "code availability" sections.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

There is no restriction on availability of materials.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

The information regarding the antibodies are provided in supplementary Tables 2a-c .
We have titrated all the CyToF antibodies. The antibodies were titrated on primary mouse cells that express each antibody and the proper negative control was chosen.
The following concentrations of antibody were used (0, 0.5 ug, 1 ug, 2 ug, 4 ug, 6 ug, 8 ug, 12 ug). The signaling and cytokine antibodies were titrated according to literature using proper stimulation.
For each antibody, the best signal-to-noise ratio was calculated and the titration curve was plotted and optimal dilution determined.
The optimal concentrations have been reported in the supplementary tables. All the validation data are available from authors.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

No eukaryotic cell line was used in this paper.

b. Describe the method of cell line authentication used.

Describe the authentication procedures for each cell line used OR declare that none of the cell lines used have been authenticated OR state that no eukaryotic cell lines were used.

c. Report whether the cell lines were tested for mycoplasma contamination.

Confirm that all cell lines tested negative for mycoplasma contamination OR describe the results of the testing for mycoplasma contamination OR declare that the cell lines were not tested for mycoplasma contamination OR state that no eukaryotic cell lines were used.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

Provide a rationale for the use of commonly misidentified cell lines OR state that no commonly misidentified cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

C57BL/6J female mice were purchased from the Jackson Laboratory (Sacramento, CA) at 7 weeks. Animals were rested at Stanford University's research animal facility for 2 weeks and were induced with EAE at 9 weeks of age.
R6/2 female mice were purchased from the Jackson Laboratory. The CNS tissues were harvested at the age of 4, 7 and 10 weeks old prior to development of severe symptoms, and at the age of 13- 14 weeks of age when they developed severe tremor, irregular gait, abnormal movements and seizures, and decreased survival.
Female mice overexpressing the G93A mutant human transgene for SOD1 were purchased from Jackson Laboratory. CNS tissues were harvested at the age of 95 days of age when a significant decline in motor function of mSOD
mice compared to wt controls begins and 140 days disease end-stage being defined as the time when mice were completely paralyzed. 5- to 7-week-old Cx3cr1CreER female mice were treated with 4 mg tamoxifen injected subcutaneously at two time points 48 h apart and were a gift from Professor Marco Prinz in Freiburg, Germany. The brain, spinal cord and blood of these mice were harvested at 15 weeks of age.
All animals were housed under a 12-hour light cycle. The maximum number of animals housed per cage was 5 mice. Animals were randomly selected and used in this study.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

Human research participants were not used in this study.