Integrative single cell and spatial transcriptomic analysis reveal reciprocal microglia-plasma cell crosstalk in the mouse brain during chronic *Trypanosoma brucei* infection

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Running title: Spatial transcriptomics analysis of the murine forebrain during chronic *T. brucei* infection
Abstract

Human African trypanosomiasis, or sleeping sickness, is caused by the protozoan parasite *Trypanosoma brucei* and induces profound reactivity of glial cells and neuroinflammation when the parasites colonise the central nervous system. However, the transcriptional and functional responses of the brain to chronic *T. brucei* infection remain poorly understood. By integrating single cell and spatial transcriptomics of the mouse brain, we identified that glial responses triggered by infection are readily detected in the proximity to the circumventricular organs, including the lateral and 3rd ventricle. This coincides with the spatial localisation of both slender and stumpy forms of *T. brucei*. Furthermore, *in silico* predictions and functional validations led us to identify a previously unknown crosstalk between homeostatic Cx3cr1+ microglia and Cd138+ plasma cells mediated by IL-10 and B cell activating factor (BAFF) signalling. This study provides important insights and resources to improve understanding of the molecular and cellular responses in the brain during infection with African trypanosomes.
Introduction

Chronic infection with *Trypanosoma brucei*, the causative agent of Human African trypanosomiasis (HAT) or sleeping sickness, is associated with extensive and debilitating neuroinflammation (Barrett et al., 2008; Fèvre et al., 2008; Lundkvist et al., 2004; Maclean et al., 2012). Widespread glial cell activation in the CNS, measured by ionized calcium-binding adapter molecule 1 (IBA1) and glial fibrillary acidic protein (GFAP) reactivity, has also been reported in both human brain biopsies and in murine models of infection (Barrett et al., 2008; Etet et al., 2012; Laperchia et al., 2016; Maclean et al., 2012). There is also extensive infiltration of adaptive immune cells that are thought to be critical mediators of the neuroinflammation induced when the parasites colonise the CNS (Laperchia et al., 2016; Olivera et al., 2021). However, an in-depth characterisation of the transcriptional responses to infection, in particular that of innate immune cells in the CNS, is lacking.

The application of single cell RNA sequencing (scRNAseq) has been transformative to understanding brain pathologies such as Alzheimer’s disease and has also been recently applied to understand immunological responses to viral infections (Jiang et al., 2020; Mathys et al., 2019; Stephenson et al., 2021; Wang et al., 2021a). Nevertheless, a major limitation of scRNAseq is that it cannot preserve the spatial distribution in the tissue of origin. The integration of scRNAseq with spatial transcriptomics enable us to characterise cellular and tissue responses to infections on regional and global scales. This has been successfully applied to characterise local immune responses to *Mycobacterium tuberculosis* (Carow et al., 2019) and *M. leprae* (Ma et al., 2021) and in the heart during viral myocarditis (Mantri et al., 2021). However, to our knowledge, similar approaches have not been implemented to study tissue responses to protozoan parasites. Here, we present a spatially resolved single cell atlas of the murine CNS in response to *T. brucei*. This integrative approach led us to identify that glia responses triggered by infection are not limited to the hypothalamus but can also be readily detected in close proximity to the circumventricular organs (CVOs), coinciding with the localisation of slender and stumpy forms of *T. brucei*. Furthermore, we identified a previously unknown interaction between homeostatic Cx3cr1+ microglia and Cd138+ plasma cells mediated by IL-10 and B cell activating factor (BAFF) signalling. Our spatiotemporal atlas offers novel insights into the interaction between the innate and adaptive immunity during chronic CNS infections and represents a resource to improve our understanding of the molecular and cellular responses triggered in the brain upon infection.
**Materials and methods**

**Ethical statement.** All animal experiments were approved by the University of Glasgow Ethical Review Committee and performed in accordance with the home office guidelines, UK Animals (Scientific Procedures) Act, 1986 and EU directive 2010/63/EU. All experiments were conducted under SAPO regulations and UK Home Office project licence number PC8C3B25C to Dr. Jean Rodger. The *in vivo* work related to the single cell and spatial transcriptomic experiments were conducted at 25- and 45-days post-infection (dpi) and correlated with increased clinical scores and procedural severity. Subsequent *in vivo* experiments for experimental validation (flow cytometry and imaging) were terminated earlier in line with ethical recommendations from the veterinary team at the University of Glasgow.

**Cell lines and in vitro culture.** Murine microglia cell line BV2 (kindly gifted by Dr. Marieke Pingen, University of Glasgow) were cultured in DMEM medium (Sigma) supplemented with 10% foetal bovine serum (FBS) (Sigma) and 1000 IU/mL penicillin, and 100 mg/ml streptomycin. Cells were maintained at 37°C and 5% CO₂. All of the experiments presented in this study were conducted with cells between passages 3 to 6. We challenged BV2 cells with *Escherichia coli* B55:O5 LPS (Sigma) for a period of 2 hours to trigger an initial pro-inflammatory response, and then incubated these cells with either untreated B cell supernatant, or B cell supernatant pre-treated with a recombinant antibody to deplete IL-10 (IgG2b, clone JES5-16E3, Biolegend). As controls, BV2 cells were left untreated or were incubated with *E. coli* LPS.

**Gene expression analysis by qRT-PCR.** Total RNA from BV2 murine microglia cell lines was extracted using RNeasy Kit (Qiagen), eluted in 30 µl of nuclease-free water (Qiagen), and quantified using Qubit broad range RNA assay (Invitrogen). qRT-PCR analysis was carried out using the Luna Universal One-Step RT-qPCR kit (NEB) using 100 ng RNA as input according to the manufacturer’s protocol, using the primers listed below. For each sample, two technical replicates were included, as well as a nuclease-free water sample as a “no template sample” control to determine background signal. The relative expression was calculated using the 2^ΔΔCt formula, where ΔΔCt represents the normalized Ct value of the target RNA relative to the 18S rRNA and compared to naïve controls. Statistical analysis was conducted using the Mann-Whitney test and *p* values <0.05 were considered statistically significant.
Murine infections with *Trypanosoma brucei*. Six to eight week old female C57Black/6J mice (JAX, stock 000664) were inoculated by intra-peritoneal injection with ~2 x 10^3 parasites of strain *T. brucei brucei* Antat 1.1E (Le Ray et al., 1977). Parasitaemia was monitored by regular sampling from tail venesection and examined using phase microscopy and the rapid “matching” method (Herbert and Lumsden, 1976). Uninfected mice of the same strain, sex and age served as uninfected controls. Mice were fed *ad libitum* and kept on a 12 h light–dark cycle. All the experiments were conducted between 8h and 12h. For sample collection, we focussed on the onset of the CNS stage (25 days post-infection) and the onset of neurological symptoms (>30 days post-infection), defined in this study as altered gait, reduced co-ordination of hind limbs, and flaccid and/or intermittent paralysis in at least one hind limb.

**Brain slice preparation for hypothalamus single-cell RNA sequencing**

**Tissue processing and preparation of single cell suspension.** Single-cell dissociations for scRNAseq experiments were performed as follow. Animals were infected for 25 and 45 days (*n* = 2 mice / time point), after which hypothalami were harvested for preparation of single cell suspensions. Uninfected animals were also included as naive controls (*n* = 2 mice). Briefly, all mice were killed by rapid decapitation following isoflurane anaesthesia, within the same time (between 8:00 and 10:00 AM). Using a rodent brain slicer matrix (Zivic Instrument), we generated ~150 μm coronal brain sections around the hypothalamic area (bregma −1.34 mm to −1.82 mm, including anterior and posterior hypothalamic structures). The hypothalami were then rapidly excised under a dissection microscope, and the excised hypothalami were then enzyme-treated for ~30 min at 37 °C using protease XXIII (2.5 mg/ml; Sigma) and DNAse I (1 mg/ml; Sigma) in Hank’s Balanced Salt Solution (HSBB) (Invitrogen). Slices were washed three times with cold dissociation solution then transferred to a trypsin inhibitor/bovine serum albumin (BSA) solution (1 mg/ml; Sigma) in cold HBSS...
Single-cell suspensions were passed through 70 μm nylon mesh filters to remove any cell aggregates, diluted to ~1,000 cells/μl (in 1X phosphate buffered saline (PBS) supplemented with 0.04% BSA), and kept on ice until single-cell capture using. In parallel, a fraction of these samples was analysed by flow cytometry to estimate the relative proportion of various glial cell types in the single cell suspensions (S1A Figure).

The single cell suspensions were loaded onto independent single channels of a Chromium Controller (10X Genomics) single-cell platform. Briefly, ~20,000 single cells were loaded for capture using 10X Chromium NextGEM Single cell 3 Reagent kit v3.1 (10X Genomics). Following capture and lysis, complementary DNA was synthesized and amplified (12 cycles) as per the manufacturer’s protocol (10X Genomics). The final library preparation was carried out as recommended by the manufacturer with a total of 14 cycles of amplification. The amplified cDNA was used as input to construct an Illumina sequencing library and sequenced on Nextseq 500 and Novaseq 6000 sequencers by Glasgow polyomics.

Read mapping, data processing, and integration. For FASTQ generation and alignments, Illumina basecall files (*.bcl) were converted to FASTQs using bcl2fastq. Gene counts were generated using Cellranger v.6.0.0 pipeline against a combined Mus musculus (mm10) and Trypanosoma brucei (TREU927) transcriptome reference. After alignment, reads were grouped based on barcode sequences and demultiplexed using the Unique Molecular Identifiers (UMIs). The mouse-specific digital expression matrices (DEM) from all six samples were processed using the R (v4.1.0) package Seurat v4.1.0 (Stuart et al., 2019). Additional packages used for scRNAseq analysis included dplyr v1.0.7 (Wickham et al., 2022), RColorBrewer v1.1.2 (http://colorbrewer.org ), ggplot v3.3.5 (Wickham, 2016), and sctransform v0.3.3 (Choudhary and Satija, 2022). We initially captured 25,852 cells mapping specifically against the M. musculus genome across all conditions and biological replicates, with an average of 37,324 reads/cell and a median of ~587 genes/cell (Table S2A). The number of UMIs was then counted for each gene in each cell to generate the digital expression matrix (DEM) (Figure S1B). Low quality cells were identified according to the following criteria and filtered out: i) nFeature < 200 or >1,500, ii) nCounts < 200 or >5,000, iii) > 10% reads mapping to mitochondrial genes, and iv) > 40% reads mapping to ribosomal genes, v) genes detected < 3 cells. After applying this cut-off, we obtained a total of 13,195 high quality mouse-specific cells with an average of 12,162 reads/cells and a median of 565 genes/cell (S2A Table).
We noted that the overall number of UMIs was significantly higher in samples from 25 and 45dpi compared to naïve controls (Figure S1B). A closer examination of the number of genes/UMIs per cell type enabled us to determine that the overall increase in infected samples derived mostly from microglia and Oligodendrocytes/B cells (Figure S1B) and may be indicative of a “transcriptional burst” associated with cell activation. Based on these observations, we considered this differential feature and gene counts when scaling the data (see below). The gene counts for each cell were divided by the total gene counts for the cell and multiplied by the scale factor 10,000. Then, natural-log transformation was applied to the counts. To identify gene signatures that represent outliers we employed two independent but complementary approaches: i) The Seurat FindVariableFeatures function with default parameters, using vst as selection method, and ii) The plotHighestExprs in Scater package (McCarthy et al., 2017) with default parameters, which led us to identify additional highly variable genes such as pseudogenes and long non-coding RNAs such as Malat1. High-quality cells were then normalised using the SCTransform function, regressing out for total UMI and genes counts, cell cycle genes, and highly variable genes identified by both Seurat and Scater packages, followed by data integration using IntegrateData and FindIntegrationAnchors. For this, the number of principal components were chosen using the elbow point in a plot ranking principal components and the percentage of variance explained (10 dimensions) using a total of 5,000 genes, and SCT as normalisation method. In parallel, given the gene/UMIs discrepancies between experimental groups, we analysed the integrated dataset using the STACAS workflow (Andreatta and Carmona, 2021) with default parameters (10 dimensions) to determine if the clusters identified with the Seurat package can be reproduced by an independent method. Overall, we detected the same marker genes and cell types identified by the IntegrateData and FindIntegrationAnchors function in Seurat (Table S2D). We applied the same approach with the myeloid subset with similar results, suggesting that the differential gene/UMI counts between experimental groups (and accounted for when scaling the data) does not cofound downstream detection of marker genes or cell types.

**Cluster analysis, marker gene identification, and subclustering.** The integrated dataset was then analysed using RunUMAP (10 dimensions), followed by FindNeighbors (10 dimensions, reduction = “pca”) and FindClusters (resolution = 0.4). With this approach, we identified a total of 11 cell clusters. The cluster markers were then found using the FindAllMarkers function (logfc.threshold = 0.25, assay = “RNA”). To identify cell identity confidently, we employed a hierarchical approach, combining unsupervised and supervised
cell identity methods. For the unsupervised approach, we implemented two complementary
R packages, scCATCH (Shao et al., 2020) and SingleR (Aran et al., 2019), using
hypothalamic datasets built-in as references with default parameters (Table S2C). These
packages map a query dataset against selected reference atlases, scoring the level of
confidence for cell annotation. Cell identities were assigned based on confidence scores
and/or independent cell identity assignment by the two packages. Confidence scores >85%
were assumed to be reliable and the cell annotations were kept. When the two packages
failed to detect cell identity confidently (confidence scores <85% by at least one package),
we employed a supervised approach. This required the manual inspection of the marker
gene list followed by and assignment of cell identity based on the expression of putative
marker genes expressed in the unidentified clusters. This was particularly relevant for
immune cells detected in our dataset that were not found in the reference atlases used for
mapping. A cluster name denoted by a single marker gene indicates that the chosen
candidate gene is selectively and robustly expressed by a single cell cluster and is sufficient
to define that cluster (e.g., Cd79a, Cd8a, C1qa, Cldn5, among others). The addition of a
second marker was used to indicate a secondary identifier that is also strongly expressed
in the cluster but shared by two or more subclusters (e.g., Apoe, Gfap).

When manually inspecting the gene markers for the final cell types identified in our
dataset, we noted the co-occurrence of genes that could discriminate two or more cell types
(e.g., macrophages from microglia). To increase the resolution of our clusters to help
resolve potential mixed cell populations embedded within a single cluster and, we subset
vascular associated cells (endothelial cells, pericytes, tanycytes, and ependymocytes),
microglia, T cells, and oligodendrocytes (the latter also containing a distinctive B cell cluster)
and analysed them individually using the same functions described above. In all cases, upon
subsetting, the resulting objects were reprocessed using the functions
FindVariableFeatures, RunUMAP, FindNeighbors, and FindClusters with default
parameters. The number of dimensions used in each case varied depending on the cell
type being analysed but ranged between 5 and 10 dimensions. Cell type-level differential
expression analysis between experimental conditions was conducted using the
FindMarkers function (min.pct = 0.25, test.use = Wilcox) and (DefaultAssay = “SCT”). Where
indicated, the AddModuleScore function was used to assign scores to groups of genes of
interest (Ctrl = 100, seed = NULL, pool =NULL), and the scores were then represented in
violin plots. This tool measures the average expression levels of a set of genes, subtracted
by the average expression of randomly selected control genes. Statistical tests using the non-parametric Wilcoxon test comparing mean of normalised gene expression (basemean) was conducted in R. Cell-cell interaction analysis mediated by ligand-receptor expression level was conducted using NicheNet (Browaeys et al., 2020) with default parameters using “mouse” as a reference organism, comparing differentially expressed genes between experimental conditions (condition_oi = “Infected”, condition_reference = “Uninfected”). Pathways analysis for mouse genes were conducted using STRING (Szklarczyk et al., 2019) with default parameters. Raw data and scripts used for data analysis will be made publicly available after peer review.

10X Visium spatial sequencing library preparation and analysis

**Tissue processing and library preparation.** Coronal brain sections (bregma −1.34 mm to −1.82 mm) were frozen in optimal cutting temperature medium (OCT) and stored at −80 °C until sectioning. Optimization of tissue permeabilization was performed on 10-μm-thick sections using the Visium Spatial Tissue Optimization Reagents Kit (10X Genomics), which established an optimal permeabilization time of 18 min. Samples were mounted onto a Gene Expression slide (10X Genomics) and stored at −80 °C until haematoxylin and eosin (H&E) staining. To prepare for staining, the slide was placed on a thermocycler adaptor set at 37 °C for 5 min followed by fixation in ice-cold methanol for 30 min. Methanol was displaced with isopropanol and the samples were air-dried for 5-10 min before sequential staining with Mayer’s haematoxylin Solution (Sigma-Aldrich), Bluing Buffer (Dako) and 1:10 dilution of Eosin Y solution (Sigma-Aldrich) in 0.45 M of Tris-acetic acid buffer, pH 6.0, with thorough washing in ultrapure water between each step. Stained slides were scanned under a microscope (EVOS M5000, Thermo). Tissue permeabilization was performed to release the poly-A mRNA for capture by the poly(dT) primers that were precoated on the slide, including a spatial barcode and a Unique Molecular Identifiers (UMIs). The Visium Spatial Gene Expression Reagent Kit (10X Genomics) was used for reverse transcription and second strand synthesis, followed by denaturation, to allow the transfer of the cDNA from the slide to a collection tube. These cDNA fragments were then used to construct spatially barcoded Illumina-compatible libraries using the dual Index Kit TT Set A (10x Genomics) was used to add unique i7 and i5 sample indexes, enabling the spatial and UMI barcoding. The final Illumina-compatible sequencing library was sequenced on a single lane (2x150) of a NextSeq instrument (Illumina) by Glasgow Polyomics.

After sequencing, the FASTQ files were aligned to a merged reference transcriptome combining the *Mus musculus* genome (mm10) genome and the *Trypanosome brucei*
After alignment, reads were grouped based on spatial barcode sequences and demultiplexed using the UMIs, using the SpaceRanger pipeline version 1.2.2 (10X Genomics). Downstream analyses of the expression matrices were conducted using the Seurat pipeline for spatial RNA integration (Hao et al., 2021b; Stuart et al., 2019) (Table S3A). Specifically, the data was scaled using the SCTransform function with default parameters. We then proceeded with dimensionality reduction and clustering analysis using RunPCA (assay = “SCT”), FindNeighbours and FindClusters functions with default settings and a total of 30 dimensions. We then applied the FindSpatiallyVariables function to identify spatially variable genes, using the top 1,000 most variable genes and “markvariogram” as selection method. We optimised the parameters to obtain clustering of distinct spatially variable gene sets that broadly coincide with several brain regions, including cortex, hippocampus, 3rd and lateral ventricles, thalamus, hypothalamus, striatum, and amygdala (Materials and Methods, Figure S3), confirming the robustness, reproducibility, and reliability of our data. For the analysis of the T. brucei genes detected in the spatial transcriptomics dataset, we used the SpatialFeaturePlot function (alpha = 0.01, 0.1, min.cutoff = 0.1). The genes detected in the spatial transcriptomics dataset at 45dpi where further analysed using the gene ontology server built in the TriTrypDB website (Aslett et al., 2009) with default settings.

To integrate our hypothalamic scRNAseq with the 10X Visium dataset, we used the FindTransferAnchors function with default parameters, using SCT as normalization method. Then, the TransferData function (weight.reduction = “pca”, 30 dimensions) was used to annotate brain regions based on transferred anchors from the scRNAseq reference datasets. To predict the cell-cell communication mediated by ligand-receptor co-expression patterns in the spatial context, we employed NICHES v0.0.2 (Raredon et al., 2022). Upon dimensionality reduction and data normalisation, NICHES was run using fanton5 as ligand-receptor database with default parameters. The resulting object was then scaled using the functions ScaleData, FindVariableFeatures (selection.method = “disp”), RunUMAP with default settings and a total of 15 dimensions. Spatially resolved expression of ligand-receptor pairs was then identified using the FindAllMarkers function (min.pct = 0.25, test.use = “roc”). For visualisation, we used the SpatialFeaturePlot function with default parameters and min.cutoff = “q1”. Raw data and scripts used for data analysis will be made publicly available after peer review.

Immunofluorescence and single molecule fluorescence in situ hybridisation (smFISH) using RNAscope. Formalin-fixed paraffin embedded coronal brain sections were
section on a microtome (Thermo) and fixed in 4% PFA for 10 min at room temperature. Sections were blocked with blocking buffer (1X PBS supplemented with 5% foetal calf serum and 0.2% Tween 20) and incubated with primary antibodies at 4°C overnight, followed by incubation with fluorescently conjugated secondary antibodies for 1 hour at room temperature. All the antibodies were diluted in blocking buffer. Slides were mounted with Vectashield mounting medium containing DAPI for nuclear labelling (Vector Laboratories) and were visualized using an Axio Imager 2 (Zeiss). The list of antibodies for immunofluorescence and RNAscope probes used in this study is presented in the table below.

smFISH experiments were conducted as follow. Briefly, to prepare tissue sections for smFISH, infected animals and naïve controls were anesthetized with isoflurane, decapitated and brains were dissected out into ice-cold 1X HBSS. Coronal brain sections were prepared as described above and embedded in paraffin. Cryopreserved coronal brain sections (5 μm) were prepared placed on a SuperFrost Plus microscope slides. Sections were fixed with 4% paraformaldehyde (PFA) at 4 °C for 15 min, and then dehydrated in 50, 70 and 100% ethanol. RNAscope 2.5 Assay (Advanced Cell Diagnostics) was used for all smFISH experiments according to the manufacturer’s protocols. All RNAscope smFISH probes were designed and validated by Advanced Cell Diagnostics. For image acquisition, 16-bit laser scanning confocal images were acquired with a 63x/1.4 plan-apochromat objective using an LSM 710 confocal microscope fitted with a 32-channel spectral detector (Carl Zeiss). Lasers of 405nm, 488nm and 633 nm excited all fluorophores simultaneously with corresponding beam splitters of 405nm and 488/561/633nm in the light path. 9.7nm binned images with a pixel size of 0.07um x 0.07um were captured using the 32-channel spectral array in Lambda mode. Single fluorophore reference images were acquired for each fluorophore and the reference spectra were employed to unmix the multiplex images using the Zeiss online fingerprinting mode. smFISH images were acquired with minor contrast adjustments as needed, and converted to grayscale, to maintain image consistency. The resulting images were processed and analysed using QuPath (Bankhead et al., 2017), and the values plotted using Prism v8.0. The in situ hybridisation images were acquired from the publicly available resource the Allen Mouse Brain Atlas (www.mouse.brain-map.org/) and used in Figure S3B.
**List of RNAscope probes used for smFISH**

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<th>Sequence</th>
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<td>314221-C2</td>
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<td>Mm-Il10ra</td>
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**List of antibodies used for immunofluorescence**

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<td>RE-Affinity CD68-PE</td>
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**Flow cytometry analysis and ex vivo stimulation of brain-dwelling B cells.** To discriminate circulating versus brain-resident immune cells, we performed intravascular staining of peripheral CD45^+ immune cells as previously reported (Anderson et al., 2014). Briefly, a total of 2 μg of anti-CD45-PE antibody (in 100 μl of 1X PBS) was injected intravenously 3 minutes prior culling. Mice were euthanised as described above and transcardially perfused with ice-cold 0.025% (wt/vol) EDTA in 1X PBS. Whole brain samples were collected and placed on ice-cold 1X HBSS (Invitrogen) and processed as recently described (Guldner et al., 2021). Whole brain specimens were minced and digested using the Adult Brain dissociation kit (Miltenyi) for 30 min at 37 °C, following manufacturer’s recommendations. The digested tissue was gently pressed through 70 μm nylon mesh cell strainers to obtain a single cell suspension. The cell suspension was cleaned up and separated from myelin debris using a Percoll gradient, as previously reported (Guldner et al., 2021). The resulting fraction was then gently harvested and used as input for glia profiling or for B cell purification using the B cell isolation kit II (negative selection approach) using MACS sorting (Miltenyi). Cells from spleens were used as positive controls. The
resulting cell fraction was diluted to a final density of ~1x10^6 cells/ml. The resulting suspension enriched in B cells were seeded on a 96 well plate and stimulated with 1X cell Stimulation cocktail containing phorbol 12-myristate 13-acetate (PMA), Ionomycin, and Brefeldin A (eBioSciences™) for 5 hours at 37°C and 5% CO₂, as previously reported (Radomir et al., 2021). Upon stimulation, the resulting supernatant was harvested and used to quantify IL-10 by ELISA (Biolegend), or to test its capacity to block BV2 polarisation in the presence of E. coli LPS. As control, anti-mouse IL-10 antibody (IgG2b, clone JES5-16E3. Biolegend) was applied to the B cell-derived supernatant for 30 min at room temperature to sequester and block IL-10 signalling in vitro.

For flow cytometry analysis, single cell suspensions were resuspended in ice-cold FACS buffer (2 mM EDTA, 5 U/ml DNAse I, 25 mM HEPES and 2.5% Foetal calf serum (FCS) in 1× PBS) and stained for extracellular markers at 1:400 dilution. The list of flow cytometry antibodies used in this study were obtained from Biolegend and are presented in the table below. Samples were run on a flow cytometer LSRFortessa (BD Biosciences) and analysed using FlowJo software version 10 (Treestar). For intracellular staining, single-cell isolates from brain or draining lymph nodes were stimulated as above in Iscove’s modified Dulbecco’s media (supplemented with 1× non-essential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μM β-mercaptoethanol, 1 mM sodium pyruvate and 10% FBS; Gibco). Cells were then permeabilized with a Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and stained for 30 min at 4°C. All antibodies used were diluted at 1:250.

<table>
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Results

Single cell transcriptomic analysis of the mouse hypothalamus over the course of T. brucei infection

To resolve the complexity of the different cell types and transcriptional pathways involved in the CNS response to T. brucei infection with as much singularity and spatial resolution as possible, we employed a combined single cell (scRNAseq) and spatial transcriptomic approach (Figure 1A), from samples harvested during the onset of the CNS stage (25dpi) and appearance of neurological symptoms (45dpi) (Figure 1B and C). The overall inflammation in the brain neuroparenchyma and the meningeal space was confirmed at these time points by histological examination (Table S1). To further refine our scRNAseq dataset, we focused on the hypothalamus, given its critical role in controlling circadian behaviour (Rijo-Ferreira et al., 2018; Tesoriero et al., 2018). We obtained a total of 13,195 cells with an average of 500 genes/cell and 1,500 transcripts/cell (Materials and Methods, Figure S1). Overall, we identified 11 clusters spanning 8 cell types, including microglia (clusters 0, 1, 6, and 9), oligodendrocytes/B cells (cluster 7), astrocytes (clusters 2 and 5), T cells (cluster 3), and vascular-associated cells including endothelium (cluster 4), pericytes (cluster 8), and ependymocytes (cluster 10) (Figure 1D and Table S2B and S2C). The microglia subclusters were dominated by the expression of putative markers including C1qa, Lyz2, Aif1, and Cx3cr1 (Prinz et al., 2021), whereas the astrocyte cluster was characterised by the expression of bona fide markers of mature astrocytes, including Gfap and Agt (Figure 1E, Figure S2, and Table S2B) (Batiuk et al., 2020). The vascular-associated cells were further divided into three Cldn5+ endothelial cell subclusters, two clusters representing Pdgfrb+ pericytes/tanyocytes, one cluster representing Acta2+ pericytes, and one Ccdd153+ ependymocyte cluster (Figure 1E, Figure S3, and S2E Table). These data are in agreement with the diversity of the glial cell types previously reported in healthy mouse hypothalamus (Chen et al., 2017; Mickelsen et al., 2019).

Disease state analysis revealed differential distribution of cells within the microglia, B cell, and T cell clusters in infected samples compared to naïve controls (Figure 1F). Furthermore, by computing an in silico gene module score to assess the global expression level of inflammatory mediators (e.g., cytokines and chemokines), we identified that responses to chronic T. brucei infection (at both 25 and 45dpi) were largely observed in the microglia subclusters (in particular microglia 1 and 2) and, to a lesser extent in endothelial cells, T cells, and adaptive immune cells and were significantly higher than naïve controls (ANOVA, p < 2.2^-16) (Figure 1G). Taken together, our data demonstrate that T. brucei
infection in the CNS induces an inflammatory response predominantly in microglia, as well as T and B cells, and to a lesser extent in endothelial cells. Notably, we did not detect *T. brucei* enough reads in our scRNAseq dataset for downstream analysis, perhaps owing to the low parasite burden in the brain parenchyma; however, we were able to detect them in our spatial transcriptomics dataset (see below).

**Spatial transcriptomics reveals both *T. brucei* long slender and stumpy forms predominantly in the circumventricular organs**

When analysing our spatial transcriptomics datasets, we noted the expression of multiple *T. brucei*-specific genes in infected samples, especially at 45dpi ([Figure 2A and S3B Table]). The majority of the *T. brucei*-specific genes were distributed in discrete locations in the mouse forebrain. For example, *Tb927.6.4280* (GAPDH), typically associated with slender forms (Briggs et al., 2021; Creek et al., 2015; Kovářová et al., 2018), was highly expressed in spatial clusters 0, 1, 4, 10, 14, and 17, that define the anatomical regions corresponding to cerebral caudoputamen or corpus striatum, thalamus, hippocampus, cerebral cortex, hypothalamus, and the circumventricular organs (CVOs; including the lateral ventricle and the 3rd ventricle) ([Figure 2A, 2B, and S3B Table]). Similarly, *Tb927.7.5940* (*PAD2*) was restricted to cluster 17 ([Figure 2A, 2B, and S3B Table]). The localisation of parasites in these brain regions at 45dpi coincided, at least partly, with an increase in the expression of inflammatory mediators in several brain regions, including around cluster 4 and 17 (both CVOs-related clusters) ([Figure 2C]). The spatial distribution of the different developmental stages of *T. brucei* was further confirmed using smFISH against parasite-specific marker genes associated with slender (*GAPDH* and *PYK1*) or stumpy (*PAD2* and *EP1*) life cycle stages (Briggs et al., 2021; Dean et al., 2009) ([Figure 2D]) and by independent histological scoring ([Table S3B]). These observations confirm that in addition to passage through the blood-brain barrier, African trypanosomes also exploit the CVOs as points of entry into the CNS (Bentivoglio et al., 2018; Kristensson et al., 2010).

To provide insights into the potential diversity of brain-dwelling trypanosomes, including the presence of various developmental stages, we performed gene ontology and pathway analysis on the most abundant *T. brucei* transcripts based on their relative spatial distribution ([Figure 2E and S3C Table]). Overall, we observed an overrepresentation of genes typically associated with metabolically active parasites in the CVOs, such as protein translation (*p adj = 3.35^{-57}* ) and biosynthetic processes (*p adj = 1.05^{-24}* ), irrespective of their spatial distribution ([Figure 2E and S3C Table]) (Briggs et al., 2021; Creek et al., 2015; Kovářová et al., 2018). Additionally, the transcriptome of the parasites in the CVOs was
dominated by genes pathways broadly associated with translation (\(p\) adj = 9.14\(^{-22}\)), control of gene expression (\(p\) adj = 2.23\(^{-04}\)), and biosynthetic processes (\(p\) adj = 3.46\(^{-08}\)), indicating that the CVO-dwelling parasites are metabolically active. Notably, the parasites in the CVOs also expressed genes considered critical regulators of parasite differentiation, such as RBP7A and RBP7B (encoded by \(Tb927.10.12090\) and \(Tb927.10.12100\), respectively), PAD1 (encoded by \(Tb927.7.5930\)), and PAD2 (encoded by \(Tb927.7.5940\)) (S3C Table) (Dean et al., 2009; Mony et al., 2014; Rojas et al., 2019). Together, these results provide an overview of the spatial distribution of African trypanosomes in the mouse forebrain and support the hypothesis that the majority of brain-dwelling trypanosomes display features of replicative slender forms, protein translation and control of gene expression, together with differentiation commitments in the CVOs.

**Infection-associated microglia and border-associated macrophages occupy spatial niches in proximity to the ventricular spaces**

Having established that microglia display a high inflammatory score upon infection, and *T. brucei* slender and stumpy forms are found in or surrounding CVOs, we next asked whether the spatial distribution of different microglia cell clusters correlates to the distribution of parasites in the forebrain. After subclustering, we identified six discrete myeloid subclusters that displayed a marked differential gene expression signature (Figure 3A). For example, cluster 1 and cluster 2 express high levels of putative homeostatic microglia marker genes including \(P2ry12\), \(Trem1\), and \(Cx3cr1\) and may correspond to different homeostatic subsets. Cells in cluster 1 express high levels of \(Tgfbr1\), \(Ifngr1\), and \(Il6ra\), whereas cluster 2 expresses high levels of \(Iil10ra\) (Figure 3B and S2F Table), suggesting a potential divergent response to cytokine signalling (e.g., interferon gamma (IFN\(_{\gamma}\)) in cluster one versus IL-10 in cluster 2). Cluster 3 is characterised by the expression of monocyte-specific lineage markers, including \(Cd14\), \(Ccr2\), \(Fcgr2b\), and several MHC-II associated molecules (\(H2-Aa1\), \(H2-Ab1\)) (Figure 3B and S2F Table). Cluster 4 and 5 express high levels of \(Aif1\), as well as canonical pro-inflammatory chemokines and mediators of innate immunity (\(Ccl5\), \(Mif\), \(Cxc13\)), components of the complement cascade (\(C1qa\), \(C1qb\)), antigen processing and presentation genes (\(H2-Ab1\), \(H2-Eb1\)), several interferon-stimulated genes (\(Ifitm3\), \(Ifih1\)), and markers of disease-associated microglia (\(Apoe\), \(Itgax\), \(Trem2\), \(Cst7\)) (Fitz et al., 2021; Krasemann et al., 2017; Shi and Holtzman, 2018), but decreased expression of microglia homeostatic markers (Figure 3B and S2F Table). Lastly, cluster 6 expresses putative marker genes associated with border-associated macrophages such as
Lyz2, Ms4a7, Ms4a6c, Tgfb1, H2-Ab1, and Lyz2 (Van Hove et al., 2019; Jordão et al., 2019), as well as gene sets characteristic of anti-inflammatory responses, such as Chil3, Arg1, and Vegfa (Figure 3B and S2F Table), indicative of an anti-inflammatory phenotype. Based on these results we catalogued clusters 1 to 6 as follow: Cx3cr1+ 1 (29.5%), Cx3cr1+ 2, (30.4%), Cd14+ Monocytes (27.6%), Arg1+ border-associated macrophages (Arg1+ BAMs; 12.6%), Aif1+ 1 (10.5%), Aif1+ 2 (4.85%) (Figure 3C and D). Notably, Cx3cr1+ microglia 1 and 2, and Cd14+ monocytes accounted for ~75% of all the microglia detected under homeostatic conditions, but Aif1+ microglia 1 and 2, and Arg1+ BAMs subclusters progressively increased in frequency over the course of infection, suggesting an adoption of an infection-associated phenotype (Figure 3C and 3D). In the spatial context, we found that the gene expression of Aif1, Adgre1, specific marker genes for the Aif1+ 1 and Aif1+ 2 subclusters, were highly expressed around the hippocampus, CVOs, and caudoputamen at 25dpi and 45dpi compared to naïve controls (Figure 3F). Similarly, Arg1 and Chil3, putative marker genes for Arg1+ BAMs, were predominantly located in the lateral ventricle and the dorsal 3rd ventricle in the infected brain (Figure 3E), further corroborated by immunofluorescence analysis on independent brain sections (Figure 3F). Together, our combined analyses demonstrate that infection-associated myeloid subsets (Aif1+ 1, Aif1+ 2, and Arg1+ BAMs) are detected in regions proximal to the CVOs, coinciding with the spatial distribution of trypanosomes and suggesting a functional compartmentalisation of the myeloid subsets in responses to infection.

Microglial responses to T. brucei infection share common transcriptional features with neurodegeneration diseases

To gain a more comprehensive understanding of microglia responses to infection at the molecular level, we analysed the differentially expressed genes (DEGs) of microglia subtypes in response to T. brucei infection, defined as genes with a Log2 fold change >0.25 or < -0.25 and an adjusted p value < 0.05. Most of the upregulated DEGs were detected in the Cx3cr1+ 1, Cx3cr1+ 2, and Cd14+ monocyte subclusters (Figure 3G) and were associated with an upregulation of MHC-II-mediated antigen presentation (iH2-Aa1, H2-Ab1), Neutrophil chemotaxis (Ccl2, Ccl4), adaptive immune responses (Cd274, Mif, Tnfsf13b), and responses to IFNγ (Ifitm3, Aif1) (Figure 3H top and S2G Table). As the infection progresses, we noted an enrichment for gene pathways associated with neurodegenerative disorders, including Amyotrophic lateral sclerosis, Huntington disease, Parkinson disease, and Alzheimer’s disease, including Apoe, Trem2, Psen2, and Cd22.
(Figure 3H bottom and S2G Table). These cells also downregulate homeostatic processes associated with organ development (Tgfbr1, Mertk, Fos), neurone homeostasis (Cx3cr1, Itgam), and responses to cAMP (Fosb, Junb) (Figure 3H, S2G and S2H Table). Overall, our data demonstrates a dynamic response of the microglia during T. brucei infection; during the onset of the CNS stage (25dpi), homeostatic Cx3cr1⁺ microglia upregulate transcriptional programmes associated with antigen processing and presentation and development of adaptive immune responses, whilst downregulating genes associated with homeostasis. As the infection progresses (45dpi), the microglia signatures share many commonalities to those identified in neurodegenerative disorders (e.g., Apoe, Trem2, Cd22) (Fitz et al., 2021; Krasemann et al., 2017; Pluvinage et al., 2019; Shi and Holtzman, 2018), coinciding with the development of clinical symptoms in these animals. These data suggest a common transcriptional response to inflammatory processes in the CNS. Other myeloid cell types such as Cd14⁺ monocytes and Arg1⁺ BAMs constitute additional responders to the infection, albeit with opposing effects; Cd14⁺ monocytes and Arg1⁺ BAMs display pro- and anti-inflammatory phenotypes, respectively.

Chronic T. brucei infection recruits follicular-like regulatory Cd4⁺ T cells and cytotoxic Cd8⁺ T cells into the CNS

We next sought to characterise the population of adaptive immune cells identified in our dataset. We identified three T cell subclusters based on the expression of putative T cell marker genes such as Trac and Cd3g (Figure 4A to 4C, and S2I Table). Cluster 0 (44%) was discarded owing to the lack of identifiable marker genes. Cluster 1 (18%) and 2 (18.5%) express marker genes associated with cytotoxic T cells such as Cd8a and Gzmb (Figure 4A to 4C, and S2I Table). Cells within cluster 2 also express high levels of genes associated with cytotoxic T cell activation and effector function (Ccl5, Klrδ1), a gamma TCR receptor subunit (Trgv2), interferon-stimulated genes (Ilf3m1), and high levels of Cd52 which is involved in T cell effector function (S2I Table) (von Kutzleben et al., 2017). This suggests that cells in cluster 2 potentially represent a specialised cytotoxic T cell subset. Lastly, in addition to Cd4, cells in cluster 3 (18.5%) express high levels of genes associated with regulatory CD4⁺ T cells including surface markers (Cd5, Ctlα4, Icos, Cd274), transcription factors (Mxd1, Izkf2), and effector molecules (Il10, Areg, Il21) (Figure 4A to 4C, and S2I Table). Notably, these regulatory Cd4⁺ T cells also express high levels of marker genes typically associated with follicular helper T cells such as Maf and Slamf5 (Figure 4A to 4C, and S2I Table) (Bauquet et al., 2009; Cannons et al., 2010; Nurieva and Chung, 2010). These follicular-like regulatory CD4⁺ T cell subsets have been postulated as critical
regulators of adaptive responses in lymphoid organs (Hao et al., 2021a; Miles and Connick, 2018; Vaeth et al., 2019, 2019; Wollenberg et al., 2011), but so far have not been reported in the brain during infections. These populations seemed dynamic over the course of infection, with chronic stages associated with a two-fold increase in the abundance of $\text{Cd4}^+$ T cells compared to other subclusters (8.2%, 19.3%, and 20.85% in naïve, 25dpi, and 45dpi, respectively) (Figure 4B and 4D), consistent with previous reports (Laperchia et al., 2016; Lyck et al., 2003). Of note, the subcluster identified as cluster 2 $\text{Cd8}^+$ T cells ($\text{Cd8}^+$ 2 T cells) was only detected in infected samples but not in naïve controls (23% and 20% at 25 and 45dpi, respectively) (Figure 4B), indicating a disease-associated T cell subset in the brain. When compared across the brain in the spatial transcriptomics data set, the $\text{Cd4}^+$ T cell subcluster was mostly detected in the lateral ventricle, external capsule, and the caudoputamen in both naïve and infected samples, whereas the $\text{Cd8}^+$ T cell subsets showed a more widespread distribution in the brain parenchyma (Figure 4E).

Cell-cell interaction analysis using NicheNet (Browaeys et al., 2020) revealed a network of molecular communication between T cells, stromal cells, and the vasculature, in infected samples compared to naïve controls (Figure 4F and 4G). For instance, ependymocytes and endothelia cells express high levels of Cxcl10 and Cxcl12, respectively, which are critical mediators of lymphocytic recruitment into the brain parenchyma during neuroinflammation (Christensen et al., 2006; Fife et al., 2001; Klein et al., 2005). Microglia also express additional subsets of chemokines (Ccl2, Ccl3, Ccl4) with no overlap to those detected in vascular-associated cells (Figure 4F and 4G), potentially indicating non-redundant mechanisms of T cell recruitment into the brain parenchyma during infection. Furthermore, the endothelial cells and microglia expressed high levels of cell adhesion markers including Icam1, Icam2, and Pecam1 (Figure 4F and 4G), which mediate immune cell transendothelial and extravascular tissue migration (Abadier et al., 2015; Dias et al., 2021; Lyck et al., 2003). We also detected additional mediators of T cell activation, including endothelial cell-derived Il15 and astrocyte-derived Il18, which are involved in T cell activation and enhancement of IFNγ production (Alves et al., 2003; Pien et al., 2000; Sareneva et al., 1998; Strengell et al., 2003; Takeda et al., 1998) (Figure 4F and 4G).

Together, our data provide an overview of the T cell diversity in the CNS during chronic T. brucei infection, including regulatory $\text{Cd4}^+$ T cells that accumulate in the brain over the course of T. brucei infection. Moreover, ligand-receptor mediated cell-cell communication suggests that microglia, ependymocytes, endothelial cells, and astrocytes are involved in
the recruitment and activation of T cells into the brain during chronic *T. brucei*, albeit through divergent signalling molecules.

**Cd138**⁺ plasma cells are detected in the mouse brain during chronic *T. brucei* infection

Next, we characterised the cells contributing to the genes expressed in cluster 7 (Figure 1D and S2J Table). This appeared to represent a heterogeneous grouping of cells expressing high levels of oligodendrocyte markers (*Olig1, Sox10*, and *S100b*) and bona fide B cell markers (*Cd79a, Cd79b, Ighm*) (Figure 1D and S2J Table). Dimensional reduction analysis after subsetting cluster 7 led us to identify five clusters identified as follow: clusters 1 (34.7%) and 2 (11.41%) expressed high levels of *Olig1* and *Pdgfra* and corresponded to oligodendrocytes, cluster 3 (8.48%) corresponds to *Epcam*⁺ neuroepithelium, and cluster 4 (6.02%) composed of Map2⁺ neurons (Figure 5A and S2J Table). Lastly, in addition to *Cd79a* and *Cd79b* (which encode for the B cell receptor), cluster 0 (39.3%) was also characterised by high expression levels of putative markers associated with plasma cells, including surface markers (*Sdc1* or *Cd138, Slamf7*) and plasma cell-specific transcription factors (*Prdm1, Xbp1, Irf4*) (Figure 5A, 5B, and S2J Table). These cells also express genes associated with regulatory function, including *Il10* and *Cd274* (Figure 5A, 5B, and S2J Table), and was thus labelled as *Cd138*⁺ plasma cells. Furthermore, the *Cd138*⁺ plasma cells were detected at low levels in naïve controls (~8% of the cells in this cluster) but increased over the course of infection at 25dpi (61.6%) and 45dpi (88%) (Figure 5C and 5D). The enrichment of *Cd138*⁺ plasma cells during chronic infections was further confirmed by flow cytometry on independent *in vivo* experiments, mirroring the proportions detected by scRNAseq (Figure 5E and 5F). Notably, *Cd138*⁺ plasma occupied discrete niches in the naïve brain around the CVOs (dorsal 3rd ventricle) and subthalamic regions (Figure 5G), but were preferentially detected in the external capsule, corpus callosum, and lateral ventricle at 25dpi, or in the leptomeninges, cingulate cortex, lateral ventricle, and dorsal 3rd ventricle at 45dpi (Figure 5G). Taken together with the flow cytometry findings, these data suggest a potential expansion of this population in the CVOs and proximal regions. Furthermore, the predicted expression of *Il10*, an anti-inflammatory cytokine shown to be expressed in B cells with a regulatory phenotype (Brioschi et al., 2021; Radomir et al., 2021; Rosser and Mauri, 2015), was tested and confirmed by ELISA of *ex vivo* stimulation brain-dwelling B cells from infected mice (Figure 5H), corroborating the *in silico* data and indicating a regulatory phenotype. Together, these data show the presence of *Cd138*⁺
plasma cells with a regulatory phenotype in the CVOs and leptomeninges in the murine brain during chronic *T. brucei* infection.

**Cd138**⁺ plasma cell supernatant suppresses microglia polarisation towards an inflammatory phenotype

Our scRNAseq data indicates that microglia in the forebrain of *T. brucei*-infected mice express both *Il10ra* and *Il10rb* (which together form the functional IL-10 receptor (Burmeister and Marriott, 2018; Holly A. Swartz, Jessica C. Levenson, 2012; Lobo-Silva et al., 2016) (**Figure 5I**), and that brain-dwelling *Cd138**⁺ plasma cells produce IL-10 when stimulated *ex vivo* (**Figure 5H**). Thus, we hypothesised that plasma cell-derived IL-10 may play a role in modulating pro-inflammatory responses in microglia. *In silico* spatial ligand-receptor interaction analysis around the CVOs identified several significant ligand-receptor interactions upregulated during infection, including Clec1b-Klrb1c, involved in regulating NK cell-mediated cytolytic activity (Hao et al., 2006), and Lpl-Lrp2, which are involved in ApoE-mediated cholesterol intake in neurons (Spuch et al., 2012) (**Figure S5**). Additionally, we also identified a robust co-expression of *Il10* and *Il10ra* in the CVOs and leptomeninges (**Figure 5J and Figure S5**), coinciding with the predicted localisation of *Cd138**⁺ plasma cells at 45dpi, which was independently validated using single molecule fluorescence in situ hybridisation (smFISH) (**Figure 5K**). This confirmed that expression of *Il10* in brain dwelling *Cd138**⁺ plasma cells and *Il10ra* in homeostatic Cx3cr1⁺ microglia. Next, we hypothesised that the supernatant from stimulated *Cd138**⁺ plasma cells might also block microglia polarisation towards a pro-inflammatory state. As expected, BV2 microglia-like cells exposed to *E. coli* LPS for 24h expressed high levels of the pro-inflammatory cytokines *Il1β* and *Tnfα* (**Figure 5L**), which was abrogated when BV2 cells were exposed to supernatant from *Cd138**⁺ plasma cells (**Figure 5L**). Moreover, pre-treatment of the *Cd138**⁺ plasma cells supernatant with a blocking antibody against IL-10 restores the expression of *Il1β* and *Tnfα* in BV2 microglia, strongly indicating that IL-10 is a key plasma cell-derived anti-inflammatory modulator. Taken together, these data suggest a functional interaction between *Cd138**⁺ plasma cells and microglia mediated, mediated at least partially, by IL-10 signalling.

**Cx3cr1**⁺ microglia express the B cell pro-survival factor B cell activation factor (BAFF) signalling

Having established that microglia, T cells and plasma cells are associated with chronic *T. brucei* infection, we next decided to evaluate relevant cell-cell interactions based on the expression level of canonical ligand-receptor pairs. Of these, we observed a network
of complex molecular communication between plasma cells and microglia. A group of ligands were redundantly detected in microglia from Cx3cr1+ 1, Cx3cr1+ 2, and Arg1+ BAMs (Vcam1, Spp1, and Agt) (Figure 6A), whereas a second subset of ligands displayed a more cell-restricted expression profile. For example, the pro-survival factor Tnfsf13b (encoding for the B cell survival factor, BAFF) was abundantly expressed upregulated by homeostatic Cx3cr1+ microglia upon infection, whereas the expression of its cognate receptor Tnfrsf17 (or B cell maturation antigen, BCMA) was highly expressed in Cd138+ plasma cells from infected animals (Figure 6B and 6C). Furthermore, the expression of Tnfsf13b was higher in microglia from infected mice (Figure 6D), suggesting that this B cell pro-survival factor is induced upon infection. Spatial ligand-receptor interaction analysis based on co-expression revealed that the co-expression of both the gene for the pro-survival factor, Tnfsf13b and the gene for its receptor, Tnfrsf17, was restricted to the CVOs and leptomeninges (Figure 6E and Figure S5), as identified for the Il10-Il10ra ligand-receptor pair. The expression pattern for these two genes was independently confirmed by smFISH analysis and showed that plasma cells expressing Tnfrsf17 were in close proximity to microglia expressing Tnfsf13b, particularly in the vicinity of the lateral ventricle in the brains of T. brucei-infected mice (Figure 6F and Figure S5). The expression of BAFF in microglia upon infection was further analysed by flow cytometry experiments (Figure 6G), corroborating the in silico predictions. Together, our data suggest that crosstalk between microglia and Cd138+ plasma cells; In this context, Cx3cr1+ microglia promote Cd138+ plasma cell survival via BAFF, and in turn Cd138+ plasma produce IL-10 to dampen down inflammatory responses in microglia during T. brucei infection.
Discussion

To address fundamental questions regarding the innate and adaptive immune responses of the CNS to unresolved, chronic *T. brucei* infection, this study had three main goals: *i*) to characterise the temporal transcriptional responses of glial and recruited immune cells to the CNS using single cell transcriptomics, *ii*) to understand the spatial distribution of candidate cell types from the scRNAseq dataset using 10X Visium spatial transcriptomics, and *iii*) to model cell-cell interactions taking place in the CNS during chronic infections based on putative ligand-receptor interactions at both single cell and spatial level. Our combined atlas provides novel and important insights for future analyses of the innate and adaptive immune response to chronic CNS infection by *T. brucei*.

Our data describe critical and previously unappreciated cell types and cell-cell interactions associated with chronic CNS infections. We demonstrate that microglia drive inflammatory and anti-parasitic responses in the CNS, and also provide insights into the transcriptional features border associated macrophages (BAMs). These responses are heterogeneous, with microglia and *Cd14*+ monocytes displaying strong pro-inflammatory signatures. Their transcriptional programme is consistent with pro-inflammatory responses expected to be triggered in response to pathogenic challenges during the onset, including the production of cytokines (i.e., *Il1b*, *Tnf*), chemokines (i.e., *Ccl5*, *Cxcl10*), and an upregulation of molecules associated with antigen processing and presentation. Based on the differential gene expression analysis over the course of infection, we propose a model in which homeostatic *Cx3cr1*+ microglia undergo extensive transcriptional remodelling during infection, leading to the acquisition of an infection associated phenotype, which coincides with the onset of clinical symptoms. This includes the upregulation of gene programmes involved in other neurodegenerative disorders, such as *Apoe*, *Trem2*, and *Cd22* at the point of infection in which clinical symptoms are detected (Fitz et al., 2021; Krasemann et al., 2017; Pluvinage et al., 2019; Shi and Holtzman, 2018). The transcriptional signatures identified in the IAMs state are reminiscent of those previously reported in neurodegenerative disorders, thus it is tempting to speculate that this represents a core “pathological” transcriptional module that is triggered in microglia in response to insults, irrespective of the nature of such insults (e.g., parasites versus protein aggregates). These similarities provide new insights into the mechanisms behind chronic brain inflammation.

Additionally, our datasets suggest that *Arg1*+ BAMs acquire an anti-inflammatory state in the chronically infected brain, which might counterbalance the inflammatory responses of infection-associated microglia (IAM). This is consistent with the responses
observed in other organs in which macrophages with an anti-inflammatory phenotype act to limit inflammatory damage by promoting tissue repair (Mosser et al., 2021; Nobs and Kopf, 2021; Watanabe et al., 2019; Wynn and Vannella, 2016). To our knowledge, this is the first report describing the responses of the BAMs to infection by protozoan pathogens. Given their seemingly important role in promoting anti-inflammatory responses, further work is required to explore whether these BAMs consists of ependymal (epiplexus or supraependymal macrophages) or stromal choroid plexus macrophages, as recently discussed (Munro et al., 2022).

This study also improves our understanding of the components of the adaptive immune response that are recruited into the hypothalamic and brain parenchyma during chronic infection. These include T cells, consistent with previous findings that these cells have a prominent role in modulating CNS responses to T. brucei infection (Laperchia et al., 2016; Masocha et al., 2004; Olivera et al., 2021). In addition to conventional cytotoxic Cd8+ T cells, we have identified a Cd4+ T cell subset that shares many features with T follicular helper cells, including the expression of immunomodulatory genes and effector molecules. We also identified and validated, for the first time, a population of Cd138+ plasma cells that display a marked regulatory phenotype, characterised by the expression of Il10, Lgals1, and Cd274. These plasma cells are exclusively detected in chronic infections but not in healthy controls or during the onset of the CNS stage, suggesting a positive correlation between CNS invasion and plasma cell recruitment. Using an in vitro approach, we also show that these cells dampen pro-inflammatory responses in microglia mediated by IL-10 signalling, consistent with previous studies (Laffer et al., 2019; Shemer et al., 2020; Sun et al., 2019), although additional factors (e.g., Lgals1) might also play a role in this process. In silico predictions suggest that microglia, in particular homeostatic microglia, are able to promote plasma cell survival via Tnfsf13, encoding the B cell survival factor BAFF (Mackay and Browning, 2002; Mackay et al., 2003; Ng et al., 2004; Smulski and Eibel, 2018). We validated this using smFISH, confirming that both Tnfsf13 and its cognate receptor Tnfrsf17 (encoding for the B cell maturation antigen, BCMA) are upregulated in microglia and B cells, respectively, upon infection.

Based on these results, we propose a two-phase model to explain the behaviour of microglia and adaptive immune cells over the course of CNS infection by T. brucei, centrally coordinated at the CVOs. The first phase, or “priming phase” takes place during the onset of the CNS stage and is associated with an upregulation of genes involved in antigen presentation, cell migration and response to chemokine signalling, potentially derived from...
The second stage, or the “pathology phase”, and is characterised by the upregulation of a core transcriptional programme previously reported in neurodegenerative disorders, including Apoe and MHC-II (Bohlen et al., 2019; Grubman et al., 2021; Sala Frigerio et al., 2019; Shi and Holtzman, 2018; Wang, 2021). This coincides with the worsening of the clinical scoring and the appearance of severe neurological symptoms in this model of infection. In this context, the activation of homeostatic microglia leads to the recruitment and survival of plasma cells mediated by BAFF-BCMA signalling. In turn, these regulatory plasma cells alleviate inflammation by dampening microglia activation via IL-10 signalling, limiting pathology, providing novel insights into the mechanisms of B cell-microglia interactions in the brain during infection. Our model is in line with previous work demonstrating that systemic IL-10 administration ameliorates neuroinflammation during chronic T. brucei infection (Rodgers et al., 2015), thus highlighting a prominent role of IL-10, derived from either Cd138+ plasma cells or follicular-like Cd4+ T cells, in limiting brain pathology. Future work is required to understand the origin of the Cd138+ plasma cells recruited into the brain parenchyma upon CNS colonisation, but the meningeal space and the lymphopoietic niche at the CNS border is a plausible candidate (Brioschi et al., 2021; Wang et al., 2021b). Our results also demonstrate that the CNS invasion by African trypanosomes is orchestrated and fine-tuned by a myriad of cellular interactions between resident stromal cells and recruited peripheral immune cells in and around the CVOs, suggesting a previously unappreciated role for the CVOs in the pathogenicity of Sleeping Sickness. Although the formation of tertiary lymphoid structures in the CNS upon T. brucei infection has not been observed before, we speculate that this population of follicular-like T cells, together with stromal cells that might function to support T-B cell interactions (e.g., ependymal cells) might be involved in supporting primary humoral responses. We suggest that the Cd138+ plasma cells identified in our study facilitate this response, especially around the CVOs. Further work is required to understand how these various signals translate into changes in sleep/wake patterns and circadian behaviour in sleeping sickness.

**Limitations of this study**

In this paper, we have provided for the first time a detailed, spatially resolved, single cell atlas of the murine CNS in response to chronic infections with African trypanosomes. Although our work represents a valuable gene expression resource of the murine CNS in response to infection, validated by complementary approaches, further work on examining the expression of other key molecular markers may offer additional information in the field.
Similarly, we have also defined several key cell-cell communications taking place in the infected brain using in silico ligand-receptor interaction analysis, but detailed functional experiments are required to validate their role in vitro and in vivo. For instance, the origin of the brain-resident plasma cells, observed in the dorsal 3rd ventricle under homeostatic conditions, remains to be evaluated. Additionally, the clonality and antibody repertoire of brain-dwelling plasma cells, expanded after the onset of the CNS stage, merits further investigation. From the parasite perspective, although we did not capture enough parasites in our single cell transcriptomics datasets to make statistical inferences, we have resolved the spatial distribution of slender and stumpy developmental stages and have provided insights into the transcriptional signatures of these developmental stages in different parts of the murine forebrain, which has remained elusive. Future sorting strategies to purify tissue-dwelling parasites will be greatly beneficial to overcome these challenges. We envision that integration of our work with future scRNAseq, and spatial transcriptomic datasets will address some of the questions arising from this study.
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Contributions

Methodology: JFQ, AML, NAM, LDL. In vivo work and sample collection: JFQ, MCS. Bioinformatic data analysis (single cell transcriptomics): JFQ, PC, EMB, TDO. Bioinformatic data analysis (spatial transcriptomics): JFQ. Flow cytometry: JFQ, MCS. Imaging: JFQ, PC, RH, CAB, CL. The single-cell atlas was created by TDO. All authors participated in discussions related to this work. JFQ wrote the manuscript. All authors reviewed and approved the manuscript.
**Figure legend**

**Figure 1. Diversity of hypothalamic glial cells during chronic T. brucei infection.**

A) Overview of the experimental approach applied in this work. Upon infection, the levels of parasitaemia (in log_{10} scale; B) and clinical scoring (C) were measured in infected animals. D) Combined UMAP plot for 13,195 high-quality cells coloured by cell type across all the biological replicates. E) Heatmap representing the expression level of the top 25 marker genes for each of the cell clusters identified in D. F) Frequency of the different cell types detected in the murine hypothalamus at the three experimental groups analysed in this study. G) Inflammatory gene module score of genes typically associated with inflammation across all the cell types detected in (D), split and colour-coded by time point of infection. Statistical analysis using analysis of variance (ANOVA) for multiple comparison testing (p = 2.2^{-16}) is also reported.

**Figure 2. Spatially resolved transcriptomics of brain-dwelling Trypanosoma brucei.**

A) Spatial expression of Tb927.6.4280 (GAPDH) and Tb927.7.5940 (PAD2) and in the spatial transcriptomics from naïve (top row), 25dpi (middle), and 45dpi (bottom) coronal brain datasets. The circumventricular organs (CVOs)-specific marker Transthyretin (Ttr) is included for anatomical reference. B) Top: spatial feature plot depicting 18 different transcriptional clusters at in the murine forebrain from infected (45dpi) samples. Bottom: Violin plot depicting the relative expression of Tb927.6.4280 (Gapdh, marker for slender forms) and Tb927.7.5940 (Pad2, marker for stumpy forms) in the different spatially resolved transcriptional clusters in the infected murine brain. Ttr (mouse-specific gene) is also included to depict that cluster 17 corresponds to CVOs. Additional regions are also indicated, including caudoputamen or corpus striatum (clusters 0 and 8), hippocampus (cluster 4), Amygdala (cluster 10), and CVOs (cluster 17). The full list of T. brucei genes detected can be found in **S3B Table.** C) Inflammatory gene module score of genes typically associated with inflammation across the different spatially resolved transcriptional clusters at 45dpi. Non-parametric Wilcoxon test for multiple comparisons was applied and identified significant differences using the normalised gene expression (basemean) as reference. Asterisk denotes significant differences of <0.05. D) Representative smFISH probing putative slender markers (GAPDH and PYK1; middle panel) and stumpy marker genes (PAD2 and EP1; right panels), around the lateral ventricle (LV) and choroid plexus (ChP) in naïve animals (left panels) and infected brain samples (right panels). Scale bar = 25 µm. E) Top 10 GO terms that characterise brain-dwelling African trypanosomes. The GO terms...
were chosen using significant genes (-0.25 < Log$_2$ fold change > 0.25; adjusted $p$ value < 0.05). Ctx, cerebral cortex; CPu, caudoputamen; Hip, Hippocampus; Am, Amygdala; CVOs, circumventricular organs, including the lateral ventricle (LV) and the dorsal 3$^{rd}$ ventricle (d3V); Th, thalamus.

**Figure 3. Diversity and spatial distribution microglia, monocytes, and border-associated macrophages during chronic *T. brucei* infection.**

A) UMAP plot depicting the six subclusters identified as microglia, including the total number of cells in this plot. The dotted line represents the clusters preferentially detected in infected samples compared to naïve controls. B) Heatmap representing the expression level of putative microglia marker genes for each of the microglia and myeloid subclusters. The cell origin within each cluster (Naïve in teal, infected in orange) is also indicated at the bottom of the heatmap. C) As in (A) but depicting the identified different time points. The dotted line represents the clusters preferentially detected in infected samples compared to naïve controls. D) Cell type proportion of the various microglia subclusters detected in Figure 1E over the course of infection. E) *In silico* projection of top marker genes for the infection-associated clusters, including *Aif1*, *Adgre1*, *Arg1*, and *Chil3* from naïve (top), 25dpi (middle), and 45dpi (bottom) coronal mouse brain sections. Specific brain regions are also indicated. F) Imaging analysis of Arg1$^+$ BAMs in proximity to the lateral ventricle of naïve and infected mice using immunofluorescence staining for the detection of CD68 (pan-microglia marker) and ARG1 (BAM specific marker). DAPI was included as nuclear staining, and GFAP as a marker for astrocyte reactivity. Scale = 25 $\mu$m. G) Bar plot indicating the total number of differentially regulated genes (DEGs) at 25dpi (left) and 45dpi (right) compared to naïve controls. Upregulated genes are indicated in red, and downregulated genes are indicated in blue. These genes were defined as having a -2 < Log$_2$ Fold change < 2, and an adjusted $p$ value of < 0.05. I) KEGG gene pathways overrepresented in cluster 2 at 25 and 45dpi.

**Figure 4. Chronic *T. brucei* infection leads to an expansion of resident follicular-like Cd4$^+$ T cells in the CNS.**

A) UMAP plot depicting the three main T cell subclusters identified in figure 1, including the total number of cells in this group. B) As in (A) but depicting the identified different time points including in this study. C) Heatmap representing the expression level of putative microglia marker genes for each T cell subcluster. The cell origin within each cluster (Naïve in teal, infected in orange) is also indicated. D) Proportion of the main T cell subclusters identified in Figure 1E over the course of infection with *T. brucei*. E) Spatial feature plot
depicting the expression of Cd4 and Cd8a, putative marker genes for Cd4+ and Cd8+ T cells, respectively, from samples harvested from naïve (top) and 25dpi (middle), and 45dpi (bottom) coronal mouse brain sections. F) Circos plot representing significant cell-cell interactions mediated by ligand-receptor communication between T cells (CD4+ and CD8+ T cells) and microglia (dark blue), astrocytes (dark green), T cells (dark pink), endothelial cells (light blue), and ependymocytes (light pink). Ligand-receptor interactions that were redundantly observed in more than one cell type are shown in light green. G) Heatmap representing the most significant ligand-receptor interactions between T cells and stromal cells detected in the scRNAseq datasets. The ligand-receptor interaction probability is scored based on the prior interaction potential scale. Abbreviations: Ctx, cerebral cortex; CPu, caudoputamen; Hip, Hippocampus; Lateral ventricle (LV); Dorsal 3rd ventricle (d3V).

Figure 5. Chronic T. brucei infection is associated with the expansion of resident regulatory-like Cd138+ plasma cells

A) UMAP plot depicting the oligodendrocyte/B cell subclusters, including the total number of cells. B) Heatmap of the top 50 most abundant genes in the B cell / oligodendrocyte subcluster, including a second heatmap depicting selected marker genes of plasma cell. C) As in (A) but depicting the identified different time points. The dotted line represents the clusters preferentially detected in infected samples compared to naïve controls. D) Proportion of the main B cells and oligodendrocytes subclusters identified in Figure 1E over the course of infection with T. brucei. E) Representative quantification of CD138+ (encoded by Sdc1) plasma cells and CD19+ B cells during the first peak of infection (7dpi) and after the onset of the CNS stage (30 dpi) using flow cytometry. F) Total number of cells detected by flow cytometry (n = 3-4 mice). A p value of < 0.05 is considered significant. G) In silico prediction of Cd138+ plasma cells distribution onto the spatial transcriptomics slides from naïve (left), 25dpi (middle), and 45dpi (right) coronal mouse brain sections. Sdc1 or Cd138, which is a plasma cell-specific marker, is used to depict cell distribution. The relative expression level of is colour coded. H) IL-10 production by ex vivo brain-dwelling B cells measured by ELISA. An adjusted p value of < 0.05 is considered significant. I) Dot Plot representing the expression level of Il10ra and Il10rb as well as Il10 in the various cell types identified in naïve and infected mouse hypothalami. J) Predicted spatial ligand-receptor interaction analysis for Il10-Iil10ra in the mouse brain at 45dpi. The relative expression level is indicated, and colour coded. K) Representative smFISH imaging targeting Il10 (red), Il10ra (orange), Cd79a (green), and Cx3cr1 (purple) around the lateral ventricle in the infected mouse brain, including an inlet section, highlighting the co-expression of the
predicted ligand-receptor pairs. Abbreviations: d3V, dorsal 3rd ventricle; Th, Thalamus; Ctx, Cerebral cortex; CPu, Caudoputamen; LV, Lateral ventricle.

Figure 6. Crosstalk between Cd138+ plasma cells and Cx3cr1+ microglia in the brain of chronically infected animals.

A) Circos plot of significant ligand-receptor interactions between Cd138+ plasma cells and Cx3cr1+ microglia (light blue), Cx3cr1+ microglia (dark blue), and Arg1+ BAMs (purple). Redundant interactions (shared by >1 cell type) are shown in light green. B) Heatmap of the most significant ligand-receptor interactions between Cd138+ plasma cells and microglia. C) Dot Plot representing the expression level of Tnfsf13b (BAFF) and its cognate receptor Tnfrsf17 (BCMA). D) Dot Plot representing the expression level of Tnfsf13b (BAFF) in the various microglia subsets identified in the mouse hypothalamus during T. brucei infection. E) Predicted spatial ligand-receptor interaction analysis for Tnfsf13b-Tnfrsf17 in the mouse brain at 45dpi. The relative expression level is indicated, and colour coded. F) Chronic T. brucei infection induces the expression of Tnfsf13b and Tnrsf17 in Cx3cr1+ microglia and B cells, respectively. Representative smFISH probe targeting Cx3cr1 (purple), Cd79a (green), Tnfsf13b (red), and Tnfrsf17 (orange) around the lateral ventricle (LV) in an infected mouse brain coronal section. Scale, 25 µm. G) Representative flow cytometry analysis and quantification of BAFF+ microglia in naïve and infected animals (30 dpi) using flow cytometry (n = 3-4 mice). ** p < 0.005 is considered significant.
Supplementary figures

Figure S1. Quality control of the hypothalamic scRNAseq datasets over the course of infection with *T. brucei*.

**A)** Representative flow cytometry analysis from naïve (Top panel) and infected (bottom panel) animals showing the relative proportion of immune cells (Cd45^{high}), microglia (CD11b^{High} CD45^{low}), oligodendrocytes (O4^+), and astrocytes (ACSA2^+). **B)** Average number of genes (top) and transcripts (bottom) per cell in the hypothalamic scRNAseq after filtering low quality cells, split by biological replicate. For normalisation, we accounted for differential gene and UMI counts using two independent approaches (SCT and STACAS). Both packages broadly identified the same cell populations. **C)** Gating strategy for the identification of resident immune cells, related to the quantification of CD138^+ plasma cells, as well as the expression of *Tnfsf13b* and *Tnfrsf17* in microglia and B cells, respectively.

Figure S2. Transcriptional landscape of hypothalamic astrocytes and vasculature during chronic *T. brucei* infection.

**A)** UMAP plot depicting the various vascular-associated cell types identified in the hypothalamic scRNAseq dataset. **B)** Spatial feature plot of putative marker genes for the various vasculature-associated cells in the 10X Visium dataset from naïve (top), 25dpi (middle), and 45dpi (bottom) coronal mouse brain sections. The relative expression level is indicated, and colour coded.

Figure S3. Quality control of 10X Visium datasets from the mouse forebrain over the course of infection with *T. brucei*


Figure S4. Characterisation of CNS myeloid responses to *T. brucei* infection.
A) Imaging analysis of Arg1+ BAMs in coronal brain sections of naïve and 45 day-infected mice using immunofluorescence staining for the detection of CD68 (pan-microglia marker) and ARG1 (BAM specific marker). DAPI was included as nuclear staining, and GFAP as a marker for astrocyte reactivity. An IgG isotype control is also included. Scale bar = 25 µm.

B) Examples of downregulated gene pathways overrepresented in Cx3cr1+ microglia at 25 and 45dpi.

Figure S5. Ligand-receptor interaction network in the mouse brain at 45 days post-infection.

A) Spatially resolved cluster genes for the 45dpi 10X Visium spatial transcriptomic dataset.

B) Uniform Manifold Approximation and Projection (UMAP) plot depicting the predicted cluster-cluster interactions in the spatial context. Clusters are grouped based on their predicted ligand-receptor interaction. C) Heatmap representing the top 5 most significantly enriched ligand-receptor pairs in each of the spatially resolved transcriptional units in (A) and (B), clustered based on their relative expression.

Figure S6. Microglia – plasma cell communication mediated by Il10 and Tnfsf13b.

A) Chronic T. brucei infection induces the expression of Il10 and Il10ra in B cells and Cx3cr1+ microglia, respectively. Representative smFISH probe targeting Cx3cr1 (purple), Cd79a (green), Il10 (red), and Il10ra (orange) around the lateral ventricle (LV) in naïve (left) and infected (right) mouse brain coronal section. Scale bar, 25 µm. B) Top panel: Insets taken from the infected sample in (A), representing B cells expressing Il10 (left) and Cx3cr1+ microglia expressing Il10ra (right). Bottom panel: Quantification of fluorescence intensity of the smFISH probes across four biological replicates per group and from >1,000 cells/replicate. Non-parametric T test p value < 0.01 is considered significant. C) Chronic T. brucei infection induces the expression of Tnfsf13b and Tnfrsf17 in Cx3cr1+ microglia and plasma cells, respectively. Representative smFISH probe targeting Cx3cr1 (purple), Cd79a (green), Tnfsf13b (red), and Tnfrsf17 (orange) around the lateral ventricle (LV) in naïve (left) and infected (right) mouse brain coronal section. Scale bar, 25 µm. D) Top panel: Insets taken from the infected sample in (A), representing plasma cells expressing Tnfrsf17 (left) and Cx3cr1+ microglia expressing Tnfsf13b (right). Bottom panel: Quantification of fluorescence intensity of the smFISH probes across four biological replicates per group and from >1,000 cells/replicate. P value < 0.01 (non-parametric T test) is considered significant. G) Proposed microglia-plasma cell crosstalk mediated by Il10 and Tnfsf13b. In this context, microglia promote plasma cell maintenance and survival via Tnfsf13b (BAFF) signalling,
whereas plasma cells dampen pro-inflammatory responses in microglia via Il10 signalling.

E) Proposed model to explain the reciprocal cell-cell communication between plasma cells and homeostatic microglia mediated by IL-10 and BAFF signalling. In this context, plasma cells limit inflammatory processes triggered in homeostatic microglia via IL-10 signalling (that undergo transcriptional changes towards an infection-associated phenotype). In turn, microglia promote plasma cell activation and survival via BAFF signalling.
Table legend

Table S1. Characterisation of intra- and extra-ventricular trypanosomes in the mouse forebrain. Histopathology analysis from C57BL/6 female mice infected with *T. brucei brucei* Antat 1.1E were harvested at 25- and 45- days post-infection (*n* = 3-4 mice/group), fixed in 10% PFA and counterstained with the *T. brucei*-specific antibody TbHSP70. Uninfected animals (*n* = 3) were included as naïve controls. Clinical scoring on coronal sections were scored using a double-blinded approach. In addition to relative parasite localisation, H&E staining was also included to assess meningeal and parenchymal inflammation. The classification used for the scoring system is included in the table.

Table S2. Overview of the mouse hypothalamic single cell transcriptomics during chronic *T. brucei* infection. S2A) Quality control including mean reads per cell and median genes per cell before and after filtering out low quality cell types. S2B) Overview of the major cell types detected in the single cell dataset at a resolution of 0.4. The total number of cells per cluster, percentages, and marker genes are also included. S2C) Overview of the outputs generated by scCATCH and SingleR. The predicted cell identity, as well as the prediction score (where applicable), are included. S2D) Marker genes detected with the integration workflow STACAS for the cells detected in the hypothalamic dataset, and the microglia subset. S2E) Overview of the vascular-associated cells detected in our hypothalamic dataset at a resolution of 0.3. The marker genes for these clusters, as well as representative UMAP plots are also included. S2F) As in S2E but for the microglia/macrophage subclusters at a resolution of 0.3. S2G) List of differentially expressed genes in all the myeloid subsets at 25dpi, defined having a -0.25 < Log$_2$Fold change >0.25 and an adjusted *p* value < 0.05. S2H) List of differentially expressed genes in all the myeloid subsets at 45dpi, defined having a -0.25 < Log$_2$Fold change >0.25 and an adjusted *p* value < 0.05. S2I) As in S2E but for T cells at a resolution of 0.3. S2J) Overview of the oligodendrocytes and B cells detected in our hypothalamic dataset at a resolution of 0.3. The marker genes for these clusters, cell counts, as well as representative UMAP plots are also included.

Table S3. Overview of the spatial transcriptomics of the mouse forebrain during chronic *T. brucei* infection. S3A) Overview of the spatial transcriptomics project, including total number of reads sequenced per biological replicate, the median number of genes per spot and the percentage of mappable reads to the mouse (mm10) or *T. brucei* (TREU927) genomes. S3B) Mouse and *T. brucei* marker genes identified in the 10X Visium spatial transcriptomics datasets. S3C) Gene Ontology analysis (Biological processes) generated
using TriTrypDB and default settings. Significantly enriched pathways are defined as having an adjusted $p$ value < 0.05.
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Figure 1

A) C57BL/6 mice infected with Trypanosoma brucei

B) Parasitemia over time

C) Clinical score over time

D) UMAP representation of cell clusters

E) Heatmap of gene expression

F) Cell frequency across conditions

G) ANOVA analysis of inflammatory module score
Figure 2

(A) Slender forms
T927.6.4280 (GAPDH)

Stumpy forms
Tb927.7.5940 (PAD2)

Circumventricular organs
(Ttr)

Expression level
Low

Expression level

B) 45dpi

Tb927.6.4280 (GAPDH)

Tb927.7.5940 (PAD2)

Expression level

Ttr (CVOs)

D) Naive

Infected

LV

GAPDH

PYK1

PAD2

EP1

Thalamus (43 genes)

Hippocampus (83 genes)

Cc (32 genes)

CVOs (1067 genes)

Adjusted p value

gene expression

cellular process

biosynthetic process

cellular protein metabolic process

translation

peptide biosynthetic process
Figure 4

A) T cells - subsets

B) T cells - time point

C) Cd8+ 1
Cd8+ 2
Cd4+

D) Cell proportions

E) Cd4
Cd8a

F) Cd4+ T cells (receivers)

G) T cells (receptors)
Figure 5

A) Clusters of cells in the brain:
- Cluster 3 (Neuroepithelium)
- Cluster 2 (Olig 2)
- Cluster 5 (B cells)
- Cluster 4 (Neurons)
- Cluster 1 (Olig 1)

648 cells

B) Heatmap of gene expression levels across different clusters.

C) Gating on CD45+ resident lymphocytes:
- Naive
- 7dpi
- 30dpi

D) Cell proportion chart:
- B cells
- Neuroepi.
- Olig 2
- Olig 1
- Neurons


F) Plasma cells (x10):
- Naive
- 7dpi
- 30dpi

G) Images of d3V and Th regions:
- Naive
- 25dpi
- 45dpi

H) IL-10 concentration (pg/ml):
- Resting
- Stimulated

I) Expression level and percent expressed for Il10ra, Il10rb, Il10.

J) Spatial LR: Il10-Il10ra

K) Immunofluorescence images of LV,
- Cd79a
- Il10
- Cx3cr1

L) Fold change for Il1/β and Tnfa:
- Untreated
- LPS
- LPS + Bup
- LPS + Bup + α-IL10
- Bup alone

**P < 0.01
*P < 0.05
Figure S1

(A) Lymphocytes, Single cells, Live/dead, Myeloid cells (Cd45⁺/Cd11b⁺), Oligodendrocytes (O₂⁻), Astrocytes (ACSA²⁺)

(B) Average No. genes/cell, Average No. transcripts/cell

(C) Lymphocytes, Singlets, Live cells, Resident vs circulating CD45⁺ cells
Figure S2

A) 2,235 cells

B) Expression level

Ependymocytes
Endothelial cells 1
Endothelial cells 2
Pericytes
Tanyocytes

Cldn5
Pdgfrb
Acta2
Ccdc153

Figure 2
Figure S4

(A) Immunofluorescence images of DNA, GFAP, ARG1, CD68, and merged images for Isotype control, Naive, and Infected conditions.

(B) KEGG pathways downregulated genes for 25dpi and 45dpi conditions.
Figure S5

A) 45dpi

B) 10X Visium 45dpi - Cellular microenvironment

C) Gene expression heatmap with clusters and expression levels.
Figure S6

(A) Naive vs. Infected

(B) Il10* plasma cells vs. Il10ra* microglia

(C) Naive vs. Infected

(D) Tnfrsf17* plasma cells vs. Tnfsf13b* microglia

(E) Schematic of interaction between Il10 and Tnfrsf17+ plasma cells, and Il10ra+ microglia.