Generalized Lévy walks and the role of chemokines in migration of effector CD8⁺ T cells

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Chemokines have a central role in regulating processes essential to the immune function of T cells, such as their migration within lymphoid tissues and targeting of pathogens in sites of inflammation. Here we track T cells using multi-photon microscopy to demonstrate that the chemokine CXCL10 enhances the ability of CD8⁺ T cells to control the pathogen *Toxoplasma gondii* in the brains of chronically infected mice. This chemokine boosts T-cell function in two different ways: it maintains the effector T-cell population in the brain and speeds up the average migration speed without changing the nature of the walk statistics. Notably, these statistics are not Brownian; rather, CD8⁺ T-cell motility in the brain is well described by a generalized Lévy walk. According to our model, this unexpected feature enables T cells to find rare targets with more than an order of magnitude more efficiency than Brownian random walkers. Thus, CD8⁺ T-cell behaviour is similar to Lévy strategies reported in organisms ranging from mussels to marine predators and monkeys, and CXCL10 aids T cells in shortening the average time taken to find rare targets.

*T. gondii* is an opportunistic pathogen that causes encephalitis in patients with acquired defects in T-cell function. Several studies have established that resistance to this parasite in the central nervous system (CNS) relies on T-cell production of interferon (IFN)-γ and cytotoxic T cells, but little is known about the factors that regulate the behaviour of effector T cells at this site. To understand the role of chemokines in directing T cells to regions of infection during toxoplastic encephalitis, real-time PCR (rtPCR) was performed to assess changes in chemokine-receptor expression in the brains of infected mice (Supplementary Fig. 1a).

Notably, messenger RNA transcripts for CXCR3, a receptor expressed by activated and memory T cells and associated with T-helper 1 (Th1)-type responses and its ligands, CXCL9 and CXCL10, were highly expressed during toxoplastic encephalitis (Fig. 1a). Previous studies have demonstrated extensive production of *Cxcr3* mRNA by activated astrocytes during toxoplastic encephalitis. Analysis of lymphocytes isolated from the brains of mice infected with ovalbumin (OVA)-expressing Prugniaud-strain (*PruOVA*) tachyzoites showed that CD8⁺ T cells, including those specific for ovalbumin, express CXCR3 (Fig. 1b) and migrate towards CXCL10 *ex vivo* (Fig. 1c). Thus, parasite-specific CD8⁺ T cells present in the CNS during toxoplastic encephalitis are responsive to CXCR3 ligands.

Although CXCL10 is required for resistance to acute *T. gondii* infection, little is known about how this molecule affects T-cell responses during chronic toxoplastic encephalitis. Therefore, we treated chronically infected mice with anti-CXCL10 antibodies. One week later, mononuclear cells from the brain were isolated, and T cells were quantified by flow cytometry. Anti-CXCL10 treatment led to a 40% decrease in the number of CD8⁺ T cells (Fig. 2a, *P* = 0.04) and an increase in parasite burden (Fig. 2b, *P* = 0.04). Immunohistochemical staining for *T. gondii* showed latent cyst forms in control mice (Fig. 2c), whereas regions of active parasite replication were observed in the brains of anti-CXCL10-treated mice (Fig. 2d). To address the role of CXCL10 in the recruitment and maintenance of antigen-specific T cells in the CNS, we used an adoptive-transfer system. *In vitro*-activated OVA-specific OT-I cells were transferred to mice chronically infected with Pru⁩OVA⁩, resulting in the migration and accumulation of these cells within the CNS. When OT-I T cells were transferred to chronically infected wild-type C57BL/6 or *Cxcl10*-deficient mice, knockout mice had 60% fewer transferred cells in the brain in comparison to wild-type mice, whereas equivalent numbers were recovered from the spleen and lymph node in both groups (Supplementary Fig. 1b, c). Similar results were obtained when *Cxcr3*⁻/⁻ and wild-type OT-I cells were transferred to wild-type mice chronically infected with Pru⁩OVA⁩ (Supplementary Fig. 1d, e).

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These studies show that CXCL10 and CXC3R1 are required for optimal recruitment and/or retention of antigen-specific CD8+ T cells in the CNS during toxoplasmic encephalitis. To determine whether CXCL10 and chemokine signals also affect the migration of CD8+ T cells once they enter the CNS, we used multi-photon imaging to track green fluorescent protein (GFP)-expressing OT-I T cells (OT-I) in the CNS during toxoplasmic encephalitis. To determine whether parasites. On day 7 after transfer, brains from mice that received

PBS (control), 300 μg anti-CXCL10, or 8 μg PTX intraperitoneally (i.p.) were imaged in three dimensions over 10 min. e–g, Representative cells tracks from control e, anti-CXCL10-treated (f), and PTX-treated (g) mice are shown. Scale bar, 100 μm. h, Velocity software was used to calculate the average track velocity (the average over all cells of the total displacement divided by the total observation time). Ctrl, control. 1–k, Cell motility was visualized by plotting individual cell tracks from the origin from control (i), anti-CXCL10-treated (j) and PTX-treated (k) mice. ***P < 0.001 by one-way analysis of variance. Cell track data were obtained from three independent experiments with two mice per group. Control, 12 movies, n = 307 cells; anti-CXCL10, 10 movies, n = 280 cells; and PTX, 7 movies, n = 192 cells.

To determine the type of random walk that best describes the migration data, we focused not only on the behaviour of the m.s.d., but also on the shape of the tracks; the probability distribution P(r(t)) of cell displacements, r(t), as a function of the time interval, t, and the decay of normalized displacement correlations, \( K(t, t') = \langle r(0, t) r(t, t + t') \rangle / \langle r(0, 0) \rangle \), as a function of \( t \), where \( r(t, t + t') \) is the displacement between times \( t \) and \( t + t' \). Together, these properties provide a more complete description of the walk statistics than the m.s.d. alone, and therefore provide far more constraints that must be satisfied by a candidate random-walk model. First, by analysing statistics of the cell-trajectory shapes, we established that CD8+ T cells do not exhibit directional migration on the time and length scales relevant to this experiment (see Supplementary Fig. 3 and Supplementary Discussion). To analyse the displacement distribution, we introduced a time-dependent variable, \( \zeta(t) \), to scale the cell displacements. For Brownian walks, the distribution, \( P(\rho) \), of scaled displacements, \( \rho(t) = r(t)/\zeta(t) \), should be Gaussian, \( P(\rho) = \frac{1}{\sqrt{2\pi}} e^{-\rho^2/2} \), and the scale factor, \( \zeta(t) \), should be the root m.s.d. (r.m.s.d.). However, for the migrating CD8+ T cells, the distribution \( P(\rho) \) is not Gaussian (Fig. 3b, inset); the probability of large displacements is much larger than expected at all times studied. Notably, \( P(\rho) \) has the same shape at all times, indicating that the tracks are also not well described by persistent random walks. Moreover, the scale factor obeys \( \zeta(t) \sim t' \), with \( \gamma = 0.63 \), and not \( \gamma = 1/2 \), as expected for Brownian walks (Fig. 3c), and clearly differs from the r.m.s.d. (Supplementary Fig. 4) at all times studied. Finally, the displacement correlations do not decay exponentially in time, as for Brownian walks (Fig. 3c, inset). Thus,
Brownian walks do not describe effector T-cell migration during toxoplasmonic encephalitis.

On the basis of these walk statistics, we considered several variations of Lévy walks (see Supplementary Table 1, Supplementary Fig. 5 and Supplementary Discussion). We find that, consistent with early observations of runs and pauses in lymphocytes, T-cell migration is well described by the following model of a generalized Lévy walk. Walkers make straight runs at fixed velocity in random directions over distances chosen randomly from a Lévy distribution, \( L_n(t) \approx t^{-\frac{2.15}{ \alpha} \cdot (t + \mu_{\text{run}})} \), with \( \mu_{\text{run}} = 2.15 \). After each run, a walker pauses for a duration of time that is drawn from a Lévy distribution with \( \mu_{\text{pause}} = 1.7 \). The values of the exponents \( \mu_{\text{run}} \) and \( \mu_{\text{pause}} \) were determined from a maximum likelihood analysis (see Supplementary Discussion). The model captures quantitatively the observed displacement distributions at different times (Fig. 3b), the time evolution of the m.s.d. and scale factor (Fig. 3a and c, respectively), the decay of displacement correlations (Fig. 3c, inset), and qualitative features of cell tracks (Supplementary Fig. 6).

In the absence of CXCL10 or signals through G\(x\)-coupled receptors, the migration statistics for CD8\(^+\) T cells are well described by the same generalized Lévy walk model, characterized by \( \mu_{\text{run}} = 2.15 \) and \( \mu_{\text{pause}} = 1.7 \) (Supplementary Figs 6 and 8), as for control cells, with either a reduced instantaneous speed during runs or longer pauses. Therefore, the chemokine CXCL10 and signals through G\(x\)-coupled receptors speed up migration without otherwise changing the walk statistics. This result, together with the fact that we find no evidence of directed migration over the timescales investigated (see Supplementary Discussion), suggests a chemokinetic role for CXCL10 during toxoplasmonic encephalitis.

Previous studies have demonstrated that neutrophil or CD8\(^+\) T-cell control of bacteria or tumour cells, respectively, can be understood by a rate equation in which the killing of targets is modelled as a collision-based process. We incorporated the generalized Lévy walk statistics into a similar model to predict the time required to find rare target cells. In our model, we placed N generalized Lévy walkers randomly in a sphere of volume \( V \) with a target of radius \( a \) at the origin (Supplementary Fig. 9a). We find that cells migrating by generalized Lévy walks are considerably more efficient in finding target cells than those performing Brownian walks (Fig. 4 and Supplementary Fig. 9b, c). Here, the efficiency is the inverse of the sum of the displacements of all the walkers at the instant when the first walker reaches the target. In the absence of CXCL10 or signals through G\(x\)-coupled receptors, our model predicts that for estimated values of \( a \), \( V \) and \( N \), the capture time for a CD8\(^+\) T cell to reach the target is increased by factors of 1.9 or 3.0, respectively, in comparison to the control setting (see Supplementary Fig. 9d–f and Supplementary Discussion). These results suggest that the ability of CD8\(^+\) T cells to find and control \( T. gondii \)-infected targets in the CNS is aided by a generalized Lévy walk search strategy, and the capture time is shortened by CXCL10, and probably by other chemokines as well. We emphasize that the generalized Lévy walk is not necessarily an optimal search strategy, and a model with \( \mu_{\text{run}} = 2.0 \) would be more efficient according to this definition. Moreover, the efficiency is highly dependent on details of the environment and search/capture process that are not presently known, so determination of the optimal search strategy remains an open question.

Lévy search strategies may be used by diverse species, including microzooplankton, fruitflies, honeybees, mussels, predatory fish, sea turtles, penguins and spider monkeys. Our results show that a
generalization of this search strategy seems to be relevant, at the single-cell level, to the ability of effector cells to find rare targets. In addition, our findings provide a new insight into the role of CXCL10 as a chemokine that specifically influences the capture time for CD8+ T cells to find infected targets during toxoplasma encephalitis.

Altogether, our findings raise several fundamental questions as to whether lymphocytes execute generalized Lévy walks in other environments, how activation status affects walk statistics, and whether the pauses suggested by our model arise from factors internal to the cell or from interactions of the cells with their external environment.

METHODS SUMMARY

T. gondii infection was established by intraperitoneal injection of PruWA. rPCR was performed for chemokine receptor expression and T. gondii DNA quantification. Brain mononuclear cells were stained with fluorescently conjugated antibodies for flow-cytometric analysis. OT-I cells were activated in vitro and transferred to recipient mice. Mice were treated with four doses of 100 μg anti-CXCL10 for week-long depletion studies or 300 μg 18 h before imaging studies. PTX was administered at 400 μg kg⁻¹ h⁻¹ before imaging. For multi-photon microscopy, explant brain was imaged by two-photon microscopy in lymphoid organs.

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Figure 4 | Generalized Lévy walks find targets more efficiently than random walks. We determined efficiency, η, for generalized Lévy walkers (black circles) and Brownian walkers (open red squares) as a function of the target radius, a. The generalized Lévy search is considerably more efficient, especially when the targets are small. Error bars denote s.e.m. Small inset shows an example trajectory for Brownian walks and the large inset shows the generalized Lévy walk model.
METHODS

Mice, parasites and antibodies. C57BL/6, CXCL10-deficient, C57BL/6 Thy1.1, OT-I transgenic, and mice expressing DsRed under the actin promoter were purchased from The Jackson Laboratory. Mice expressing GFP in all T cells (TgOVA) were originally obtained from U. von Andrian and crossed with OT-I mice. Ccr3<sup>-/-</sup> mice were originally obtained from C. Gerard and crossed to OT-I mice. DsRed P14-transgenic mice were a gift from S. Reiner. All procedures were performed in accordance to the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee. Ptd<sup>-</sup>parasites were generated and maintained as previously described<sup>3,12</sup>. Female mice were infected with 10<sup>5</sup> tachyzoites in 200 μl PBS i.p. Hamster anti-CXCL10 antibodies (clone 1F11, generated as previously described<sup>9</sup>) and normal hamster IgG (Jackson Immunoresearch) were administered i.p. in PBS. PTX (400 μg kg<sup>-1</sup>; Sigma) was administered i.p. for 6 h before imaging experiments.

rtPCR. For the analysis of gene expression, brain tissue was placed in Trizol (Invitrogen) and mRNA was extracted as instructed by the manufacturer. Purified RNA was treated with DNase I to eliminate any contamination with genomic DNA (Promega). Complementary DNA was generated using Superscript II reverse transcriptase (Invitrogen). rtPCR was performed using Quantitect primers (Qiagen) specific for Ccr1, Ccr3, Ccr5–6, Cxcr1, Cxcl9 and Cxcl10 or primers for Ccr2 (forward 5’–CACACCCTGTGTTTGCTGTA-3’ and reverse 5’–TGATGGCCCTGTTCAAGTG-3’) and were normalized to Hprt (Qiagen). To measure the amount of parasite DNA in the brain, rtPCR was used as previously described<sup>12</sup>. PCR was performed using Power SYBR® Green PCR Master Mix and a 7500 Fast Real-Time PCR System (Applied Biosystems).

Flow cytometry. Single-cell suspensions were generated from spleen and lymph nodes by macerating the tissues through a 70-μm nylon mesh filter (BD Falcon). Spleen samples were subjected to hypotonic red blood cell lysis. Brain mononuclear cells were isolated as previously described<sup>11</sup>. In brief, perfused brains were homogenized and digested with collagenase/disperse and DNase (Roche). After the digestion, the cells were purified using a percoll gradient. For flow cytometry, 1 × 10<sup>7</sup>–2 × 10<sup>8</sup> cells were incubated with 0.1 μl μl<sup>-1</sup> 24G2 antibody before surface staining with phycoerythrin (PE)-conjugated-R<sup>-</sup>K<sup>-</sup>SIFNEKL tetramer reagent (Benton-Dickinson), CXCR3–APC (R&D Systems), Thy1.2–PECy7, CD4–FITC, CD8–PerCPCy5.5, CD8–eFlour780, CD45–APC and CD45.1–PerCPCy5.5 (eBioscience). All flow cytometry was performed on a FACsCanto using FACS DIVA software (BD Biosciences). Analysis was performed using FlowJo software (Treestar Inc.).

Ex vivo chemotaxis assay. Purified splenocytes and brain mononuclear cells were rested for 1 h at in complete RPMI before the chemotaxis assay. Cells were re-suspended in chemotaxis assay medium (RPMI, 0.5% BSA, 25 mM HEPES). Cells (2 × 10<sup>6</sup>) were placed in the upper chamber of a 6.5-mm transwell insert with a 5-μm pore-size membrane (Corning), CXCL9, CXCL10 or CXCL11 (R&D Systems) was present in the lower chamber at various concentrations (0–300 nM). After 90 min, CD45<sup>+</sup> cells that migrated through the filter were enumerated using fluorescent beads (Polysciences) and phenotyped by flow cytometry.

In vitro activation of T cells. OT-I cells were expanded from the spleen and lymph nodes of OT-I transgenic mice. Lymphocytes were cultured with 500 μg ml<sup>-1</sup> chicken ovalbumin protein ( Worthington) for 24 h. The cells were washed and rested for 2 days and received 200 U ml<sup>-1</sup> interleukin (IL)-2 on days 4 and 6 of culture. On day 7 of culture, cells were washed in PBS and enumerated. A total of 2 × 10<sup>5</sup>–5 × 10<sup>6</sup> cells were transferred intravenously (i.v.). For polyclonal expansion of T cells, CD8<sup>+</sup> T cells were enriched from the lymph nodes and spleens of C57BL/6, DsRed, DsRed P14 or GFP OT-I mice by magnetic separation (Miltenyi Biotech). T cells were plated in the presence of platebound anti-CD3 (1 μg ml<sup>-1</sup>; eBioscience) and anti-CD28 (3 μg ml<sup>-1</sup>; eBioscience), 200 U ml<sup>-1</sup> IL-2, and anti-IL-4 (1 μg ml<sup>-1</sup>; NCI Preclinical Repository). The cells were split and supplemented with 200 U ml<sup>-1</sup> IL-2 on day 2 of culture. On day 4 of culture, cells were washed and 10 × 10<sup>4</sup> T cells were transferred i.v.

Immunohistochemistry. For immunohistochemistry, organs were embedded in OCT and flash frozen. Anti-CD8 (5 μg ml<sup>-1</sup>; eBioscience), anti-Me49 (gift from F. Araujo), anti-rabbit Alexa 488 (Invitrogen) and anti-rat Cy3 (Jackson Immunoresearch) were used for fluorescence staining. DAPI (Invitrogen) was used to visualize nuclei. Images were captured using standard fluorescence microscopy using a Nikon Eclipse E600 microscope (Melville) equipped with a Photometrics Cool Snap EZ CCD camera (Tucson). Nikon NIS Elements software was used to capture and overlay images.

Multi-photon imaging. Mice were euthanized by CO<sub>2</sub> asphyxiation and the brains were removed immediately, with minimal mechanical disruption, and placed in heated chamber where specimens were constantly perfused with warmed (37°C), oxygenated media (phenol-red free RPMI 1640 supplemented with 10% FBS; Gibco). The temperature in the imaging chamber was maintained at 37°C using heating elements and monitored using a temperature-control probe. For the imaging of live mice, mice were anaesthetized and cells were imaged through thinned skull. The core temperature of the mice was monitored and maintained at 37°C. Imaging was done using a Leica SP5 2-photon microscope system (Leica Microsystems) equipped with a picosecond or femtosecond laser (Coherent). GFP was excited using laser light of 920 nm. Images were obtained using a ×20 water-dipping lens. Four-dimensional imaging data were collected by obtaining images from the x-, y-, and z-planes, with a z thickness of 28 μm and step size of 4 μm to allow for the capture of a complete z-series every 22 s. This was carried out for approximately 10 min, which was the typical time elapsed before an appreciable number of cells had left the field of view. The resulting images were analysed with Velocity software to obtain individual cell track data (PerkinElmer).

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