RESEARCH ARTICLE

REVISED Myelin-specific T helper 17 cells promote adult hippocampal neurogenesis through indirect mechanisms [version 2; peer review: 3 approved]

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v2 **First published:** 24 Jul 2014, **3**:169
https://doi.org/10.12688/f1000research.4439.1
Latest published: 07 Apr 2017, **3**:169
https://doi.org/10.12688/f1000research.4439.2

Abstract

$CD4^+$ T cells provide a neuro-immunological link in the regulation of adult hippocampal neurogenesis, but the exact mechanisms underlying enhanced neural precursor cell proliferation and the relative contribution of different T helper (Th) cell subsets have remained unclear. Here, we explored the pro-proliferative potential of interleukin 17-producing T helper (Th17) cells, a developmentally and functionally distinct Th cell subset that is a key mediator of autoimmune neurodegeneration. We found that base-line proliferation of hippocampal precursor cells in a T cell-deficient mouse model of impaired hippocampal neurogenesis can be restored upon adoptive transfer with homogeneous Th17 populations enriched for myelin-reactive T cell receptors (TCR). In these experiments, enhanced proliferation was independent of direct interactions of infiltrating Th17 cells with precursor cells or neighboring cells in the hippocampal neurogenic niche. Complementary studies in immunocompetent mice identified several receptors for Th17 cell-derived cytokines with mRNA expression in hippocampal precursor cells and dentate gyrus tissue, suggesting that Th17 cell activity in peripheral lymphoid tissues might promote hippocampal neurogenesis through secreted cytokines.

Keywords

adult neurogenesis; hippocampus; stem cells; immune deficiency; regulatory T cells; cytokines; plasticity; learning and memory

Open Peer Review

Approval Status ✓ ✓ ✓

	1	2	3
version 2 (revision) 07 Apr 2017	✓ view		✓ view
version 1 24 Jul 2014	? view	✓ view	? view

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Competing interests: No relevant competing interests were disclosed.

Grant information: This work was supported by the CRTD, Center for Regenerative Therapies Dresden and Cluster of Excellence (Deutsche Forschungsgemeinschaft FZT 111) and a CRTD Seed Grant to K.K. and G.K.
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

How to cite this article: Niebling J, E. Rünker A, Schallenberg S *et al.* **Myelin-specific T helper 17 cells promote adult hippocampal neurogenesis through indirect mechanisms [version 2; peer review: 3 approved]** F1000Research 2017, **3**:169 https://doi.org/10.12688/f1000research.4439.2

First published: 24 Jul 2014, **3**:169 https://doi.org/10.12688/f1000research.4439.1

REVISED **Amendments from Version 1**

The revised version of our manuscript addresses the points brought up by our three referees and clarifies a few issues.

See referee reports

Background

In the hippocampus of the adult brain, neurogenesis originates from neural precursor cells residing in the subgranular zone of the dentate gyrus that proliferate and differentiate in response to intrinsic and extrinsic stimuli, allowing adaptation of the neuronal network to changing needs throughout life[1,2]. Besides innate immune mechanisms[3,4], $CD4^+$ T cells of the adaptive immune system promote adult hippocampal neurogenesis and convey functional benefits in reversal learning that have been related to adult neurogenesis[5–7]. However the molecular and cellular mechanisms underlying $CD4^+$ T cell-mediated enhancement of adult neurogenesis have largely remained unclear. For example, whether or not the infiltration of myelin-reactive T cells into the CNS is a prerequisite for the proneurogenic activity of $CD4^+$ T cells has been controversially discussed[5,6,8,9]. Additionally, the relative contribution of the activation or differentiation status of the $CD4^+$ T cells to their proneurogenic activity remains to be determined. This includes $CD4^+$ T cell-derived soluble factors that could either act directly on hippocampal precursor cells or promote precursor cell activity through indirect mechanisms, e.g. by acting on neighbouring cells within the neurogenic niche of the adult hippocampus.

Upon appropriate T cell and cytokine receptor signals, initially naïve $CD4^+$ T cells can differentiate into different T helper (Th) cell subsets with distinct cytokine profiles and effector functions[10–12]. This includes interleukin-17 (IL-17)-producing Th17 cells that additionally express the orphan nuclear receptor ROR-γt. Besides mediating anti-microbial immunity at epithelial barriers[13–17], ROR-γt^+ Th17 cells have been broadly linked to the pathogenesis of various autoimmune and chronic inflammatory conditions[18–23], most notably demyelinating inflammatory disorders of the CNS, such as multiple sclerosis in humans and experimental autoimmune encephalomyelitis (EAE) in rodents. In EAE, a local reactivation of myelin-reactive Th17 cells that have crossed the blood-brain barrier initiates a cascade of neuroinflammatory responses, ultimately leading to demyelination in the CNS and neurodegeneration. More recent evidence suggests that there are different subsets of Th17 cells comprising a wide spectrum of effector phenotypes. Among these are nonpathogenic Th17 cells with regulatory properties that restrict tissue destruction during inflammatory responses and promote tissue remodeling and repair[14,24–29]. This together with the broad expression of surface receptors for Th17-derived cytokines on both immune and non-immune cells[15,16,30], prompted us to assess the capacity of myelin-reactive Th17 cells to enhance precursor cell proliferation in an αβ T cell-deficient mouse model of impaired hippocampal neurogenesis. We concentrated on investigating proliferation as the most validated sub-process of adult hippocampal neurogenesis affected by T-cell alterations.

Methods

Mice

C57BL/6 mice were purchased from Janvier. $Nestin^{GFP}$[31], $TCR\alpha^{-/-}$[32], and 2D2 mice[33] expressing a transgenic TCR recognizing amino acids 35-55 of myelin oligodendrocyte glycoprotein (MOG_{35-55}), were on the C57BL/6 background. 2D2 mice additionally expressed a transgenic $Foxp3^{GFP}$ reporter (2D2 × $Foxp3^{GFP}$[34]). C57BL/6 and $TCR\alpha^{-/-}$ mice were intercrossed to obtain heterozygous $TCR\alpha^{+/-}$ F1 mice. All mice were housed at the Experimental Center of the Medizinisch-Theoretisches Zentrum (Technische Universität Dresden, Germany) under specific pathogen-free (SPF) conditions. They received food (standard mouse food „R/M-H“ from Ssniff Spezialitäten GmbH, Soest, Germany) and water ad libitum and lived on a light/dark cycle of 12 h/12 h with lights on at 8 am. Animal experiments were approved by the responsible regulatory authority at Regierungspräsidium Dresden (Approval numbers 24-9168.24-1/2008-5 and 24-9168.11-1/2008-12).

Flow cytometry and cell sorting

Single cell suspensions of pooled spleen and lymph nodes (mesenteric and subcutaneous) from four- to six-week-old 2D2 × $Foxp3^{GFP}$ mice were prepared using 70 µm cell strainers (BD). Monoclonal antibodies (mAbs) to CD4 (Monoclonal Rat IgG, GK1.5, BD Biosciences, Cat. No. 5532728), CD25 (Monoclonal Rat IgG, PC61, BD Biosciences, Cat. No. 551071) and CD62L (Monoclonal Rat IgG, MEL-14, eBioscience, Cat. No. 17-0621) and Pacific Blue-conjugated streptavidin were purchased from eBioscience or BD Biosciences. Before FACS, for some experiments, $CD4^+$ cells were enriched using biotinylated mAbs against CD4, streptavidin-conjugated microbeads and an AutoMACS (Miltenyi Biotec). Intracellular ROR-γt expression was analyzed using the Foxp3 staining buffer set (eBioscience) and an anti-ROR-γt mAb (Monoclonal Rat IgG, AFKJS-9, eBioscience, Cat. No. 12-6988). Intracellular cytokine staining was performed using the Cytofix/Cytoperm kit and mAbs to IL-17 (Monoclonal Rat IgG, TC11-18H10.1, eBioscience, Cat. No. 51-7172-80) and IFN-γ (Monoclonal Rat IgG, XMG1.2, BD Biosciences, Cat. No. 554412). Samples were analyzed on a FACSCalibur or sorted using a FACSAria II or III (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Inc.).

T cell culture and adoptive T cell transfer

T cells were cultured in IMDM medium, supplemented with 10% FCS (v/v), 1 mM sodium pyruvate, 1 mM HEPES, 2 mM Glutamax, 100 U/ml Penicillin-Streptomycin, 0.1 mg/ml Gentamicin, 0.1 mM non-essential amino acids, and 0.55 mM β-mercaptoethanol (all Invitrogen), at 37°C and 5% CO_2. For Th17 differentiation *in vitro*, FACS-purified naïve $CD4^+$ T cells ($CD4^+CD62L^{high}CD25^-Foxp3^{GFP-}$) were cultured for one week in 24-well plates (0,5 × 10^6 cells/ml) together with 20 Gy irradiated T cell-depleted C57BL/6 splenocytes at a 1:5 ratio, in the presence of soluble anti-CD3ε (2 µg/ml, Monoclonal Armenian Hamster IgG, 145-2C11, BD Biosciences, Cat. No. 550275), recombinant human TGF-β1 (1 ng/ml), murine IL-6 (50 ng/ml) (PeproTech), and neutralizing mAbs to IL-4 (10 µg/ml, Monoclonal Rat IgG, 11B11, eBioscience, Cat. No. 14-7041) and IFN-γ (10 µg/ml, Monoclonal Rat IgG, XMG1.2, eBioscience, Cat. No. 16-7311). After 2–3 days, cultures were supplemented with fresh cytokines. Murine IL-23

(10 ng/ml; R&D Systems) was added on day 4. Prior to flow cytometry of cytokine expression, Th17 differentiation cultures were briefly (4 h) restimulated on day 7 with 50 ng/ml Phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 200 ng/ml Ionomycin (Iono; Calbiochem), in the presence of 10 µg/ml brefeldin A (BFA; Sigma-Aldrich). On day 7 of Th17 differentiation cultures, 4×10^6 cells/200 µl PBS were injected i.v. into six-week-old TCR$\alpha^{-/-}$ recipients. Control mice received PBS only. Adoptively transferred CD4$^+$ T cells were tracked by flow cytometry after 2 weeks in the peripheral blood of recipients, as indicated.

BrdU administration and immunohistochemistry

Eight-week-old mice received 3 consecutive i.p. injections of BrdU (50 mg/kg body weight in 100 µl NaCl; Sigma-Aldrich) at intervals of 6 hours. Twenty-four hours after the first injection, mice were killed with an overdose of anesthetics and perfused transcardially, first with ice-cold saline and then with 4% paraformaldehyde (Sigma-Aldrich). The brains were removed from the skull, postfixed overnight, washed with PBS and cryoprotected for ≥ 3 days in a 30% sucrose solution. Free-floating, 40 µm coronal sections were obtained on a freezing microtome (Leica SM2010R) and stored at 4°C. Immunohistochemistry was performed on 1-in-6 series of free-floating sections of each brain as previously described[35]. To visualize the immune reaction we used the peroxidase method (ABC-Elite; Vector Laboratories) with biotinylated anti-rat and anti-rabbit antibodies (Jackson ImmunoResearch) and nickel-intensified diaminobenzidine (DAB; Sigma-Aldrich) as chromogen. Primary antibodies were rat anti-BrdU (Monoclonal Rat IgG, BU1/75 (ICR1), AbD Serotec, Cat. No. MCA2060) or polyclonal rabbit anti-CD3 (Abcam, Anti-CD3 antibody, ab 5690). Sections were mounted on gelatine-coated slides, air-dried, incubated in Neoclear (Merck) for 90 min and coverslipped. BrdU$^+$ cells in the granule cell layers and within two cell diameters below in the subgranular zone of the dentate gyrus on both sides were counted exhaustively throughout the rostro-caudal extension of the dentate gyrus by an observer blind to the treatment conditions on a light microscope (Leica DM750, 40x objective). Numbers of BrdU$^+$ cells in the selected coronal sections of each brain were multiplied by 6 as an estimate of total BrdU$^+$ cell numbers in both dentate gyri.

Dentate gyrus microdissection and neural precursor cell culture

For RNA isolation, dentate gyri of seven- to eight-week-old Nestin^{GFP} mice were dissected as described before[36]. For hippocampal precursor cell cultures from microdissected dentate gyri of adult seven- to eight-week-old C57BL/6 mice, tissue dissection, digestion and cell enrichment were performed as previously described[37,38]. After enrichment, 1×10^4 cells/cm^2 were cultured in poly-D-lysine- and laminin-coated (Sigma-Aldrich and Roche, respectively) T25 cell culture flasks (TPP) in proliferation medium, consisting of Neurobasal Medium supplemented with B27, Glutamax and 50 U/ml Penicillin-Streptomycin (all Invitrogen), as well as 20 ng/ml human Fibroblast Growth Factor-basic (FGF-2) and 20 ng/ml human Epidermal Growth Factor (EGF; both PeproTech). Every other day, 75% of the medium was replaced by fresh medium. Cells were passaged, when 80% of confluence was reached.

RNA isolation and RT-PCR

For mRNA expression analysis of cells from microdissected dentate gyri, the tissue was passed several times through a 25-gauge needle in RLT buffer (QIAGEN) supplemented with 1% β-Mercaptoethanol (Bio-Rad). For mRNA expression analysis of isolated neural precursor cells, cultured cells were detached from the flask surface with Accutase (PAA) and washed with PBS prior to lysis in RLT buffer. Total RNA was extracted using the RNeasy Mini kit according to the manufacturer's protocol (Qiagen), including on-column DNase I digestion to minimize genomic DNA contaminations. For real-time RT-PCR, cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). cDNA was analyzed in duplicates using a Mastercycler ep realplex thermal cycler (Eppendorf), the QuantiFast SYBR Green PCR kit (Qiagen), and primers listed in Table 1. With the exception of GAPDH[39], primers were designed using NCBIPrimer-BLAST (http://www.ncbi.nlm.nih.gov/tools/ primer-blast/). Relative mRNA expression was calculated using the ΔCt method and GAPDH as housekeeping gene. Only mRNAs with an expression below ΔCt = 15 were considered to be expressed. PCR specificity was confirmed employing melting curve analysis and gel-electrophoresis of PCR products.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 5 software and the GraphPad web calculator (http://www.graphpad.com/quickcalcs/). A two-tailed unpaired Student's t-test was used for analysis of the experiments shown in Figure 2. Data from the experiments shown in Figure 1 were analyzed with ANOVA followed by Dunnett's Multiple Comparison Test. Differences were considered statistically significant at $p < 0.05$.

Results

Dataset 1. Hippocampal neurogenesis data from T helper 17 cell-deficient and control mice

http://dx.doi.org/10.5256/f1000research.4439.d157588

Dataset 1a. Baseline proliferation levels of hippocampal precursor cells in TCR$\alpha^{-/-}$ and TCR$\alpha^{+/-}$ mice compared to C57BL/6 controls. The values shown are the numbers of BrdU$^+$ cells in the bilateral dentate gyrus of each single animal in the three different groups. The data refer to the results presented in Figure 1.

Dataset 1b. Proliferation levels of hippocampal precursor cells in TCR$\alpha^{-/-}$ mice after adoptive Th17 cell transfer compared to sham-injected TCR$\alpha^{-/-}$ controls. The values shown are the numbers of BrdU$^+$ cells in the bilateral dentate gyrus of each single animal in the two different groups. The numbers in brackets represent the percentages of total CD4$^+$ T cells/Vα3.2$^+$ T cells among gated CD4$^+$ T cell populations from the peripheral blood of recipient mice two weeks after adoptive Th17 cell transfer. The data refer to the results presented in Figure 2. The last entry in the left-hand column was excluded from statistical analysis due to determination as significant outlier by Grubbs' test.

Dataset 1c. Quantification of expression levels of cytokine receptor genes in cells of the neurogenic niche of the hippocampus and hippocampal precursor cells from cell culture by real-time RT-PCR. Shown are mean values for ΔCt and the relative expression levels ± range of duplicate samples, using the ΔCt-method and GAPDH for normalization. The data refer to the results presented in Figure 3.

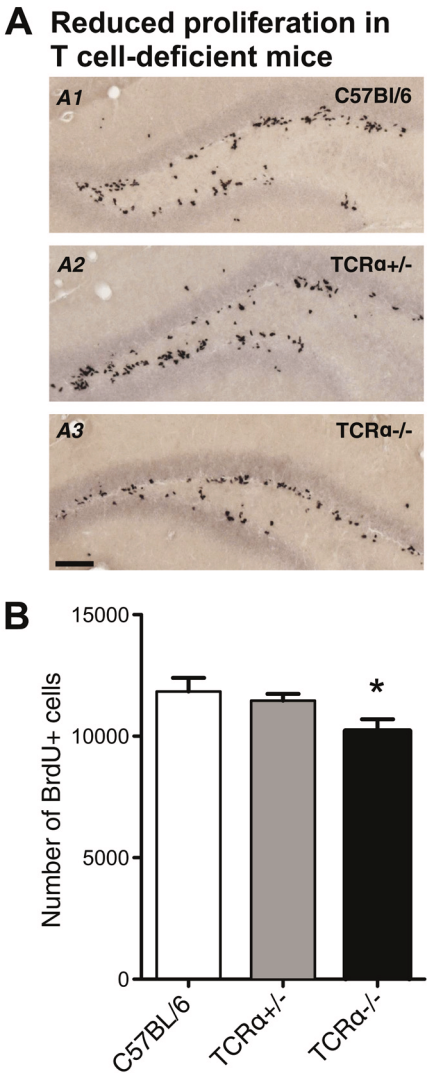

Figure 1. Impaired base-line proliferation in the hippocampus in the absence of αβ T cells. (**A**) Representative BrdU immunohistochemistry of the hippocampal dentate gyrus from eight week-old wild-type (A1), TCRα$^{+/-}$ (A2) and TCRα$^{-/-}$ (A3) mice, 24 hours after the first of 3 consecutive BrdU injections. Scale bar, 100 µm. (**B**) Quantification of BrdU^{+} cells in the dentate gyrus of wild-type (n = 6), TCRα$^{+/-}$ (n = 7) and TCRα$^{-/-}$ (n = 8) mice. All numbers are mean ± SEM. ANOVA, * $p < 0.05$.

Impaired base-line proliferation of hippocampal precursor cells in adult TCRα$^{-/-}$ mice

We first assessed steady-state levels of cell proliferation in the hippocampal dentate gyrus of adult, 8-week-old TCRα$^{-/-}$ mice, which are characterized by a complete lack of αβ T cells (both CD4^{+} and CD8^{+}) due to targeted deletion of the gene encoding the TCRα subunit (TCRα$^{-/-}$). For *in vivo* labeling of dividing cells, TCRα$^{-/-}$ mice received three consecutive i.p. injections of the thymidine analog bromodeoxyuridine (BrdU) at intervals of six hours. In these experiments, age-matched cohorts of fully immunocompetent TCRα$^{+/-}$ and C57BL/6 wild-type mice were included for comparison. Twenty-four hours after the first BrdU injection, experimental mice were subjected to immunohistochemical quantification of BrdU^{+} cells in the dentate gyrus (Figure 1A). Consistent with a published study in TCRα$^{-/-}$ mice based on endogenous Ki67 expression as proliferation marker[7], we found that TCRα$^{-/-}$ mice (10205 ± 492 BrdU^{+} cells, n = 8) exhibited significantly reduced levels of proliferation compared to C57BL/6 wild-type mice (11843 ± 556, n = 6; ANOVA, $F(2, 18) = 3.698$, $p < 0.05$; Figure 1B). TCRα$^{+/-}$ mice (11469 ± 273, n = 7) ranged between controls and knockouts. Overall, these results are in agreement with our previous observation that CD4^{+} T cells provide a neuroimmunological link in the base-line regulation of hippocampal precursor cell activity[6].

Th17 cell-mediated restoration of proliferation in adult TCRα$^{-/-}$ mice

To assess the impact of myelin-reactive Th17 cells on proliferation *in vivo*, we employed adoptive T cell transfers using six-week-old mice and quantified BrdU^{+} cells in the hippocampus of TCRα$^{-/-}$ recipients two weeks later. For the generation of Th17 cells, naïve CD4^{+} T cells (CD4^{+}CD62L^{high}CD25^{-}Foxp3^{GFP-}) carrying the MOG_{35-55}-specific 2D2 TCR as a transgene were FACS-purified from peripheral lymphoid tissues of four- to six-week-old 2D2 × Foxp3^{GFP} mice (Figure 2A) and cultured under T cell stimulatory conditions that promote efficient differentiation into Th17 cells with a ROR-γt^{+}IL-17^{+} phenotype (Figure 2B and C). As expected based on previous observations with differentiated Th17 cells *in vitro*, these cultures exhibited limited IFN-γ production (Figure 2C).

On day 7 of Th17 differentiation cultures, 4×10^{6} total cells were injected i.v. into adult, age-matched cohorts of TCRα$^{-/-}$ mice. TCRα$^{-/-}$ mice that received PBS only were included as controls. Two weeks later, small populations of adoptively transferred CD4^{+} T cells could be detected in the peripheral blood of recipient mice (Figure 2D). In these experiments, significant proportions of CD4^{+} Th17 cells expressed the MOG_{35-55}-specific 2D2 TCR transgene (ranging from 15.6 % to 56.1%), as judged by flow cytometry of the TCRα subunit of the transgenic 2D2 TCR, employing anti-Vα3.2 mAbs (Figure 2D). Importantly, throughout the observation period, TCRα$^{-/-}$ recipients of *in vitro* generated Th17 cells appeared phenotypically healthy and exhibited no clinical symptoms of EAE. Consistently, immunohistochemistry for the pan-T cell marker CD3 revealed that infiltrating Th17 cells in the dentate gyrus were below the detection level in all mice analyzed (Figure 2E and F), with the exception of an individual recipient with immune cell infiltrates, including some CD3^{+} T cells (Figure 2E2).

Two weeks after adoptive T cell transfer, cell proliferation in the dentate gyrus of TCRα$^{-/-}$ recipients was assessed by immunohistochemical quantification of BrdU^{+} cells, as described above. After applying Grubbs' outlier test, one animal in the control group with exceptionally high numbers of BrdU-positive cells was excluded from further analysis. The results showed that TCRα$^{-/-}$ recipients of Th17 cells exhibit significantly increased numbers of BrdU^{+} cells in the hippocampal dentate gyrus (11758 ± 347, n =7), as compared to control-injected TCRα$^{-/-}$ mice (10602 ± 214, n = 7; t-test,

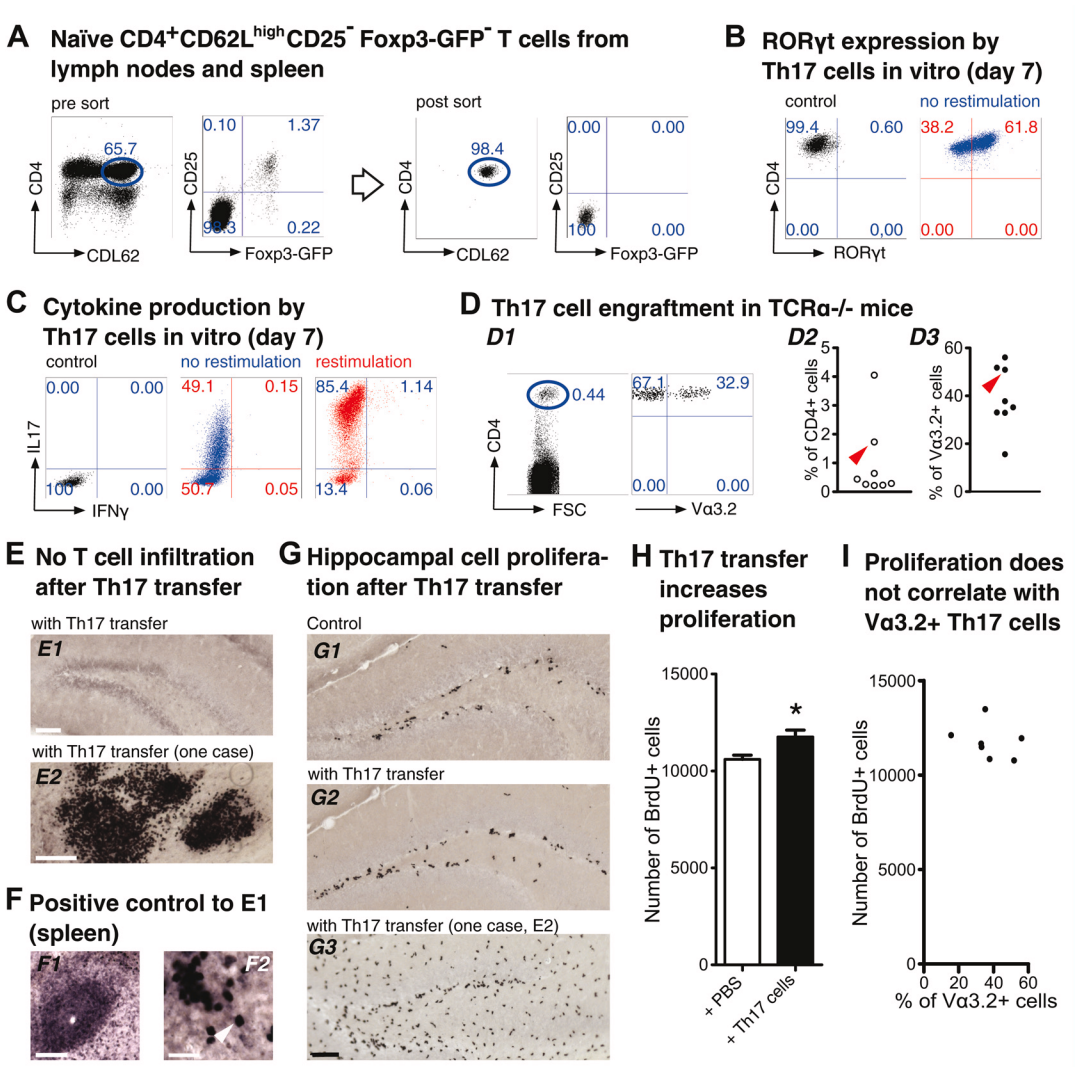

Figure 2. Th17-polarized CD4⁺ T cells are sufficient to promote hippocampal precursor cell proliferation in adult TCRα$^{-/-}$ mice. (**A**–**C**) Th17 polarization *in vitro*. (**A**) Flow cytometry of encephalitogenic CD4⁺ T cells. Dot plots show pre-sort (left) and post-sort (right) analysis of naïve T cells (CD4⁺CD62L^{high}CD25⁻Foxp3^{GFP-}) from pooled spleen and lymph nodes of 2D2 x Foxp3^{GFP} mice. FACS-purified T cell populations were cultured under Th17-polarizing conditions, as described in the Methods section. On day 7, efficiency of Th17 cell differentiation was confirmed by intracellular flow cytometry of (**B**) the Th17 transcription factor ROR-γt and (**C**) the Th17 and Th1 signature cytokines IL-17 and IFN-γ, respectively. (**D**–**I**) Impact of adoptive Th17 cell transfer on hippocampal precursor cell proliferation in TCRα$^{-/-}$ mice. (**D**) On day 7, 4 × 10^6 total cells from Th17 polarization cultures were injected i.v. into adult TCRα$^{-/-}$ mice. (D1) Dot plots show representative flow cytometry of CD4⁺ T cells in peripheral blood of recipient mice that express the Vα3.2 subunit of the transgenic 2D2 TCR, two weeks after adoptive transfer. (D2) and (D3) Graphs show composite percentages of total CD4⁺ T cells (D2) and MOG_{35-55}-reactive Vα3.2⁺ T cells among gated CD4⁺ T cell populations (D3) from peripheral blood of recipient mice. The arrowheads in (D2) and (D3) highlight an individual mouse that exhibited immune cell infiltrations in the brain (see below). Numbers in dot plots in (**A**–**D**) indicate the percentage of cells in the respective quadrant or gate. (**E**,**F**) Anti-CD3 immunohistochemistry. (**E**) Immunohistochemistry of the dentate gyrus of TCRα$^{-/-}$ recipient mice for the pan-T cell marker CD3, two weeks after adoptive Th17 cell transfer. Infiltrating CD3⁺ T cells were found to be below the level of detection in all mice analyzed (E1, scale bar, 100 μm), with the exception of an individual recipient mouse that exhibited CD3⁺ T cell and other immune cell infiltrations in some brain areas, including the hippocampus (E2, scale bar, 100 μm). (**F**) Anti-CD3 immunohistochemistry of the spleen from wild-type C57BL/6 mice was included as a positive control (F1, scale bar, 100 μm; F2, scale bar, 25 μm). The arrowhead in (F2) indicates an individual CD3⁺ T cell. (**G**,**H**) Quantification of hippocampal cell proliferation. (**G**) BrdU immunohistochemistry of the dentate gyrus of TCRα$^{-/-}$ mice, which had been injected with either (G1) PBS or (G2) Th17 cells two weeks earlier, was performed 24 hours after the first of 3 consecutive BrdU injections. (G3) depicts the dentate gyrus of the mouse exhibiting immune cell infiltrations (see E2). Scale bar, 100 μm. (**H**) Quantification of BrdU⁺ cells in the dentate gyrus of TCRα$^{-/-}$ mice injected with either PBS (n = 7) or Th17 cells (n = 7). All numbers are mean ± SEM. t-test, * $p < 0.05$. (**I**) Scatter diagram to visualize a possible relationship between cell proliferation in the dentate gyrus and the percentage of MOG_{35-55}-reactive Vα3.2⁺ T cells among CD4⁺ T cells in the peripheral blood of recipient mice two weeks after adoptive transfer. No statistically significant correlation was found.

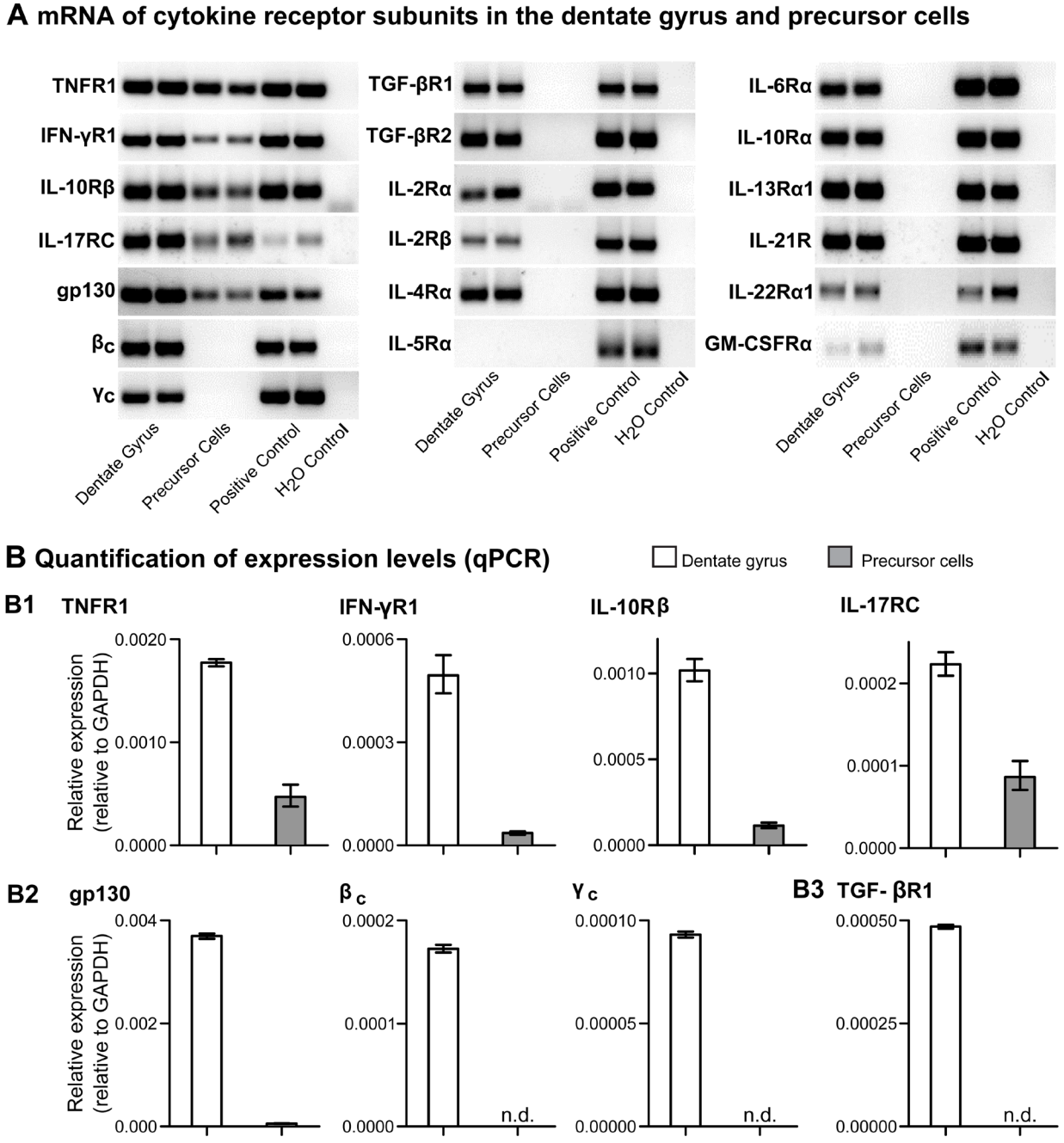

Figure 3. Cytokine receptor expression in the neurogenic niche of the hippocampal dentate gyrus. Freshly microdissected tissue and cultured neural precursor cells from the dentate gyrus of adult Nestin^{GFP} and C57BL/6 wild-type mice were subjected to mRNA expression analysis of T cell-relevant cytokine receptor genes by real-time RT-PCR, as indicated. (**A**) Gel electrophoresis of RT-PCR duplicate samples. For details on indicated cytokine receptor genes, see below. Total cells from spleen and pooled lymph nodes were included as positive control. (**B**) Quantification by real-time RT-PCR. Relative mRNA expression values of indicated genes encoding cytokine receptor subunits, as revealed by quantitative RT-PCR using GAPDH for normalization. Only mRNAs with an expression below ΔCt = 15 were considered to be expressed (n.d., not detected). Shown are mean values ± range of duplicate samples. (B1) Receptor subunits for essential T cell effector cytokines, including Th17 cell-derived cytokines, were expressed in the dentate gyrus as well as isolated precursor cells. TNFR1, IFN-γR1, IL-10Rβ and IL-17RC are components of the receptors for TNF-α, IFN-γ (Th1), IL-10 (Treg/Th2) as well as IL-22, IL-17A and IL-17F (Th17). (B2) Shared receptor subunits of class I cytokine receptors (glycoprotein 130, gp130; common beta subunit, β_c; common gamma subunit, γ_c) were expressed in the dentate gyrus but, with the exception of gp130, not by the isolated precursor cells. Similar results were obtained for TGF-βR1, TGF-βR2, IL-2Rα (ΔCt = 17,86), IL-2Rβ (ΔCt = 16.75), IL-4Rα, IL-6Rα, IL-10Rα, IL-13Rα1, IL-21R, IL-22Rα1 (ΔCt = 18.78) and GM-CSFRα (ΔCt = 19.22). As a representative example, relative mRNA expression values for TGF-βR1 are shown (B3). Expression of the cytokine receptor mRNA for IL-5Rα could be detected neither in microdissected tissue nor in cultured precursor cells from the dentate gyrus.

$p < 0.05$; Figure 2G and H). Thus, populations of Th17 cells enriched for myelin-reactive TCRs appear sufficient to restore impaired base-line proliferation of hippocampal precursor cells in adult TCR$\alpha^{-/-}$ recipient mice, in the absence of T cell infiltration and direct interaction with neural precursor cells or cellular components of the hippocampal neurogenic niche such as microglia. Consistently, the enhanced proliferative activity of precursor cells did not correlate with the proportion of Vα3.2$^+$ Th17 cells that accumulated in recipient mice (Pearson's $r = -0.28$, $p = 0.538$, 95% CI –0.85 to 0.60, $n = 7$; Figure 2I).

Cytokine receptor expression in the neurogenic niche of immunocompetent mice

Besides the signature cytokines IL-17A and IL-17F, ROR-γt^+ Th17 cells have been reported to produce a variety of cytokines such as TNF-α, IFN-γ, IL-9, IL-10, IL-21 and IL-22. In a first attempt to provide insight into possible mechanisms underlying the enhancement of hippocampal precursor cell proliferation by Th17 cell-secreted cytokines, we assessed expression levels of mRNAs encoding relevant cytokine receptors in the neurogenic niche. To this end, we performed quantitative RT-PCR analysis of freshly microdissected dentate gyrus as well as isolated precursor cells from the dentate gyrus of adult, immunocompetent C57BL/6 mice (Figure 3).

This approach identified several subunits of receptors for Th17-derived cytokines with detectable mRNA expression levels in both total dentate gyrus tissue and isolated precursor cells (Figure 3A and B), namely IL-17 receptor C (IL-17RC), tumor necrosis factor R1 (TNFR1), interferon-gamma R1 (IFN-γR1) as well as IL-10R beta (IL-10Rβ), a common subunit involved in the formation of the receptors for IL-10 and IL-22[40]. Next, we extended our analysis to the type I cytokine receptor family (glycoprotein 130: gp130, CD130; common γ subunit: γc, CD132; common β subunit: βc, CD131), which is involved in the formation of more than 20 different cytokine receptors. In these experiments, mRNA expression of all three receptor family members (gp130, βc, γc) could be detected in total dentate gyrus tissue (Figure 3A and B). Furthermore, mRNA encoding gp130, a subunit shared between the receptors for cytokines such as IL-6, leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF), was also expressed in isolated neural precursor cells. Interestingly, IL-6, LIF and CNTF directly affect the differentiation of adult hippocampal precursor cells *in vitro*[41–44].

Cytokine receptor subunits with detectable mRNA expression levels in microdissected total tissue but not in isolated precursor cells from the dentate gyrus included transforming growth factor beta receptor 1 and 2 (TGF-βR1 and TGF-βR2), IL-2R alpha and beta (IL-2Rα and IL-2Rβ), IL-4Rα, IL-10Rα, IL-13Rα1, IL-21R, IL-22Rα1 and granulocyte-macrophage colony stimulating factor receptor alpha (GM-CSFRα). Among these the ΔCt-values for IL-2Rα (ΔCt = 17,86), IL-2Rβ (ΔCt = 16.75), IL-22Rα1 (ΔCt = 18.78) and GM-CSFRα (ΔCt = 19.22) were found to be below the chosen cut-off (ΔCt < 15). Nevertheless, in all of these cases a specific product could be detected by gel electrophoresis of RT-PCR samples (Figure 3A). Additionally, and in contrast to previous reports on IL-6Rα mRNA expression in dentate gyrus-derived precursor cells[42], we found IL-6Rα mRNA to be expressed in whole dentate gyrus but not in isolated neural precursor cells. The underlying reason for this apparent discrepancy between studies remains to be determined, but may include methodological differences in the preparation and/or purity of isolated precursor cells. Lastly, among the cytokine receptor subunits whose expression was analyzed in the present study, we failed to detect mRNA expression for IL-5R alpha (IL-5Rα) in both microdissected tissue and isolated precursor cells (Figure 3A).

Discussion

Previous studies on mice with transgenic expression of a myelin-specific TCR on CD4$^+$ T cells[5] and a non-TCR transgenic mouse model of MOG-inducible EAE[9] have provided the first evidence that encephalitogenic CD4$^+$ T cell activity can promote hippocampal precursor cell proliferation and adult neurogenesis. Here, we have extended these observations and show that, in the absence of autoimmune neuroinflammation, small numbers of myelin-reactive CD4$^+$ T cells with a ROR-γt^+IL-17$^+$ phenotype are sufficient to restore base-line proliferation of hippocampal precursor cells in TCR$\alpha^{-/-}$ mice that lack endogenous $\alpha\beta$ T cells.

Mechanistically, and consistent with the proneurogenic activity of non-infiltrating CD4$^+$ T cells with a polyclonal TCR repertoire[6,7], the overall absence of immune cell infiltrations in our Th17 adoptive transfer model emphasizes that direct cell-cell interaction is not a prerequisite of enhanced Th17-mediated hippocampal precursor cell proliferation. Alternatively, Th17 cells residing in peripheral lymphoid tissues outside the brain may secrete cytokines that are actively transported across the blood-brain barrier[45,46] and act on the hippocampal neurogenic niche to promote precursor cell proliferation. Indeed, it is becoming increasingly clear that the impact of inflammatory cytokines on hippocampal neurogenesis appears much more context-dependent than anticipated based on previous studies highlighting overall detrimental effects[3,4,47–49]. Factors that influence the impact of inflammatory cytokines on neurogenesis include the administration route and local cytokine concentrations, the strength and duration of enhanced cytokine receptor signalling as well as the target cell within the neurogenic niche. While the present study suggests that several receptors for Th17 cell-derived cytokines (TNF-α, IFN-γ, IL-17, IL-22) are expressed on hippocampal precursor cells as well as neighbouring cells in the dentate gyrus, it will be important to investigate whether the pattern of expressed cytokine receptors observed in mice under physiological baseline conditions is subject to differential regulation in response to intrinsic or extrinsic stimuli. Another important, unresolved question is whether the proneurogenic effect of Th17 cells can be attributed to an individual inflammatory cytokine or is rather mediated by the combined action of different Th17-cell derived factors.

At present, cytokines with reported proneurogenic potential in hippocampal neurogenesis include IFN-γ[50,51], TNF-α[52,53], TGF-β[54], CNTF[44] as well as IL-1β and IL-6[42,55]. Interestingly, the Th17 signature cytokine IL-17 has recently been found to increase neurite outgrowth from adult postganglionic sympathetic neurons, a process that required NFkB activation[28]. Importantly, the NFkB pathway, which is shared between many cytokine receptor signaling pathways, has previously been implicated in the regulation of neural precursor cell proliferation and differentiation[56,57]. Clearly, future studies are warranted to directly address a putative role of IL-17 and the NFkB pathway in hippocampal proliferation and neurogenesis.

In summary, the present study exemplifies that the TCR$\alpha^{-/-}$ mouse represents a suitable experimental model to assess the proneurogenic potential of homogeneous Th cell populations that had been generated under well-defined *in vitro* conditions. This is likely to facilitate mechanistic studies on the relative contribution of various CD4$^+$ Th cell subsets (Th1, Th2, Th17 etc.) to the regulation of adult hippocampal neurogenesis.

Data availability

F1000Research: Dataset 1. **Hippocampal neurogenesis data in T helper 17 cell-deficient and control mice**, http://dx.doi.org/10.5256/f1000research.4439.d157588[58]

Author contributions

JN performed and analyzed the experiments and contributed to the data interpretation and wrote the manuscript. AR and SS performed experiments, contributed to the research design and the analysis and interpretation of data. KK and GK conceived the research, guided its design, analysis and interpretation, and wrote the manuscript.

Competing interests

No relevant competing interests were disclosed.

Grant information

This work was supported by the CRTD, Center for Regenerative Therapies Dresden and Cluster of Excellence (Deutsche Forschungsgemeinschaft FZT 111) and a CRTD Seed Grant to K.K. and G.K.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

The authors are indebted to Marie Boernert, Tina Koenig, Carmen Friebel (Kretschmer Group) and Daniela Lasse (Kempermann Group) for excellent technical assistance. We would like to thank Marcin Dembinski, Pei-Yun Tsai, Cathleen Petzold (Kretschmer Group) and Rupert Overall (Kempermann Group) for help with some experiments and fruitful discussions.

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Version 2

Reviewer Report 18 April 2017

https://doi.org/10.5256/f1000research.12255.r21904

✔ **Francis G. Szele**
Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK

I have read the revised paper and approve it.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 10 April 2017

https://doi.org/10.5256/f1000research.12255.r21677

✔ **Stefano Pluchino**
Department of Clinical Neurosciences, John Van Geest Centre for Brain Repair, University of Cambridge, Cambridge, UK

The authors have now addressed in full my previous criticisms and this revised version of the paper is of an acceptable standard.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 26 August 2014

https://doi.org/10.5256/f1000research.4750.r5570

?

Francis G. Szele
Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK

The link between the immune system and neurogenesis has become increasingly interesting with some papers showing positive and others showing negative effects in health and disease. It is likely that different subsets of immune cells, and different states of activation are in part responsible for these discrepancies. This short paper begins to address the need for detailed examination of these immune cell subsets by showing that systemic adoptive transfer of myelin reactive T17 cells into a mouse model with T cell deficiency rescues proliferation in the hippocampal dentate gyrus. This appears to occur without the need for direct cell contact since the majority of mice did not have significant numbers of transplanted T cells in the hippocampus.

1. The decrease in TCRa$^{-/-}$ DG proliferation, though statistically significant, is rather small and one wonders whether these differences would translate into functional differences. Is there also increased neurogenesis? The paper would benefit from additional experiments showing neurogenic changes are paralleled by behavioral changes.

2. Could it be that T17 cells migrated into and then left the DG within the first week after adoptive transfer? What are the kinetics of T cell immigration and emigration into the DG?

3. Did you exclude the animal (Fig. 2E2) with significant T cell infiltration from your analysis?

4. The authors present a hypothesis that T cell derived cytokines affect DG neurogenesis. It would be interesting to examine cytokine receptor levels in TCRa$^{-/-}$ mice.

Minor Points:

- Please spell out TCR the first time it is used.
- Scull should be "skull".
- Fig. 3 title *"mRNA of cytokine receptor chains in the dentate gyrus and precursor cells"*; I recommend substituting "chain" with "subunit".

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response ( ) 03 Apr 2017

Gerd Kempermann, German Center for Neurodegenerative Diseases (DZNE) Dresden, Technische Universität Dresden, Germany

1. The decrease in TCRa-/- DG proliferation, though statistically significant, is rather small and one wonders whether these differences would translate into functional differences. Is there also increased neurogenesis? The paper would benefit from additional experiments showing neurogenic changes are paralleled by behavioral changes.
    - Impaired precursor cell proliferation has been observed in different animal models of adaptive immune deficiency. While this effect appeared to be rather discrete using TCRα$^{-/-}$mice in the present study and the work by Huang and colleagues[7], more pronounced results could be obtained in severe combined immune deficiency (SCID) and RAG1$^{-/-}$mice, respectively[5,6]. Since the relative decrease in proliferation in RAG1$^{-/-}$ and TCRα$^{-/-}$mice was fairly comparable using Ki67 as a marker of proliferation[7], it is possible that the different methodologies in addition to the genetic background of the animals caused the discrepancies between the mentioned studies.

The question on whether our observed increased proliferation after adoptive transfer results in more new-born neurons together with the rational to restrict our analysis to proliferation as one component of adult neurogenesis has been discussed above (our response to the 1. main comment of the first Reviewer, S. Pluchino). In brief, our aim was to follow up on previous studies showing increased proliferation and, likely as a consequence, more neurons after CD4^{+} T-cell reconstitution[5,6,7] and further define the relevant T-cell subtypes.

The functional relevance of T cells as a positive regulation factor of hippocampal neurogenesis is still a matter of debate. Due to resource limitations, we were not able to extend our observations on hippocampus-dependent learning processes, which can be objectified in the Morris water maze (MWM). In this regard, we can only refer to previous work done by our group, in which CD4-depleted mice had shown an impaired performance at least in the reversal learning phase of the MWM[6].

2. Could it be that T17 cells migrated into and then left the DG within the first week after adoptive transfer? What are the kinetics of T cell immigration and emigration into the DG?
    - Clearly we cannot exclude a transient infiltration of Th17 cells into the CNS during the two weeks between adoptive transfer and analysis. The absence of clinical symptoms during the observation period, however, argues against a neuroinflammatory process. As already stated in the manuscript, it is per se unlikely that a relevant amount of immune cells infiltrate into the brain parenchyma across an intact BBB under physiological conditions (Engelhardt & Ransohoff, 2005; Prendergast & Anderton, 2009). In murine EAE the first signs of clinical disease can be observed within a period of four up to >100 days post induction depending on the genetic background of the host animals and the mode of disease induction (Miller et al.. 2010). Demyelination and inflammation in the CNS thereby largely reflect the clinical expression of the disease (Pachner, 2011). Using intravital imaging in a rat model of passive EAE, an infiltration of transferred T cells into the brain parenchyma could be seen as early as on day three after transfer (Bartholomäus et al. 2009). By the adoptive transfer of polyclonal as well as non-activated myelin-specific T cell populations into immunodeficient host animals and subsequent CD3 immunohistochemistry, our group could, however, exclude an early immigration of T

cells into the brain within the first four days following injection, at least under the chosen experimental conditions (Wolf et al., 2009b; unpublished data).

3. Did you exclude the animal (Fig. 2E2) with significant T cell infiltration from your analysis?
   - Although the cell infiltrations found in the CNS of this specific mouse were limited to only a few brain regions, they affected some parts of the hippocampus as well. As it can be seen in Figure 2G3, this would have certainly prevented a correct and unbiased quantification of cellular proliferation, which is why the animal concerned was excluded from our analysis.
4. The authors present a hypothesis that T cell derived cytokines affect DG neurogenesis. It would be interesting to examine cytokine receptor levels in TCRa-/- mice.
   - Even though several other cell types within the brain, such as astrocytes and microglia, were found to produce a plethora of different effector cytokines (Sawada et al., 1995; Gonzalez-Perez et al., 2012), there seems to be no doubt that an absence of T cells, as a major source of cytokines in the organism, could potentially lead to differential expression of cytokine receptors in the putative target cells of the neurogenic niche. Quantitative RT-PCR could be adopted to verify differences in the mRNA expression levels of cytokine receptor genes between TCR$\alpha^{-/-}$and immunocompetent control mice. Still, although this kind of experimental approach might eventually contribute to a better understanding of the general picture of a T cell-mediated regulation of adult hippocampal neurogenesis, it would probably only allow very imprecise interpretation of the underlying molecular signalling, which is why we do not consider it to be a prerequisite for the conclusions drawn in the current manuscript.

Minor Points:

- Please spell out TCR the first time it is used.
- Scull should be "skull".
- Fig. 3 title "*mRNA of cytokine receptor chains in the dentate gyrus and precursor cells*"; I recommend substituting "chain" with "subunit".
  - These points have been addressed.

Competing Interests: No competing interests were disclosed.

Reviewer Report 05 August 2014

https://doi.org/10.5256/f1000research.4750.r5572

Carlos P. Fitzsimons
Center for Neuroscience, Swammerdam Institute for Life Sciences,, University of Amsterdam, Amsterdam, The Netherlands

In this paper, Niebling *et al.*, study the regulation of hippocampal neurogenesis by interleukin 17-producing T helper (Th17) cells. Using a T-cell deficient mouse model with impaired base-line levels of hippocampal neurogenesis, they found that the neurogenesis impairment present in these animals can be restored after transfer of homogenous populations of Th17 cells, in a way that was independent of direct interaction between the transferred Th17 cells and cells of the dentate gyrus. From these results, they conclude that Th17 cells may promote hippocampal neurogenesis via secreted cytokines.

The manuscript is well written and easy to read, the title is descriptive of the content and the abstract provides a clear summary of the article.

The experiments are clearly designed and information on how the data has been analyzed is provided.

The main conclusions made by the authors are supported by the data presented in the manuscript's figures.

Main Comments:

1. Some details in the description of the methods are unclear. Specifically, the age of the animals used in the experiments is not clearly stated in the corresponding methods section. Although eight weeks old is mentioned in legend to figure 1 this should be clarified in the methods section because age is a strong determinant of base-line hippocampal neurogenesis. Were animals transcardially perfused until complete elimination of blood cells before fixation? The authors should clarify this point to facilitate the reader's interpretation of the data presented in figure 3, discarding blood cell contamination in dentate gyrus microdissected tissue samples, which could be an alternative interpretation for the differences in cytokine receptor expression in tissue vs. cultured precursor cells (last paragraph of the results section).

2. The authors suggest that one of the limitations of their study is the lack of direct characterization of the specific factor/cytokine(s) responsible for the cell-cell interaction-independent promotion of hippocampal neurogenesis they observe. However, it could have been very interesting to see some experimental effort made to support the hypothesis that the effects of Th17 cells are mediated by specific cytokines produced by these cells. This could have been done in vitro, using their cultured precursor cells, providing a stronger first proof-of-principle for the important concept they propose.

3. In the adoptive T cell transfer experiments, control mice were injected with PBS only. Although this experimental approach provides a suitable negative control, one wonders whether the injection of other non-IL17 producing T cells (e.g. naive CD4+ differentiated into Th1 or Th2 cells) could have been a more sensible negative control for these experiments, given the specific immune functions of these different T cell sets, which are highlighted by the authors in the last sentence of their discussion. Perhaps the authors could comment on the limitations of their experimental approach in this respect?

4. The discussion could benefit from a short paragraph including a more detailed discussion of possible pathological implications of the observations described in the paper. For example, other authors have recently found that hippocampal neurogenesis is enhanced in an

arthritis model in rat (Leuchtweis *et al.*, 2014). Perhaps another interesting point of discussion could be the possible implications for depression, a disease commonly linked to alterations in hippocampal neurogenesis and possibly linked to immune function (Eyre and Baune, 2012).

5. The data presented in dataset 1 seems compatible with the figures presented with the article. However, the data could be presented in a format more easily understandable for the reader. i.e. Dataset 1a is labelled "C57BL/6,TCR?+/?,TCR??/?", while is should presumably be "C57BL/6, TCRα$^{+/-}$, TCRα$^{-/-}$" and the data in rows 8 and 9 seems to be in an odd format compared to the other data rows?

Minor comments:

- In "*BrdU administration and immunohistochemistry*" "*scull*" should be "*skull*".
- Abbreviations should be defined the first time they are used in the text.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response ( ) 03 Apr 2017

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1.Some details in the description of the methods are unclear. Specifically, the age of the animals used in the experiments is not clearly stated in the corresponding methods section. Although eight weeks old is mentioned in legend to figure 1 this should be clarified in the methods section because age is a strong determinant of base-line hippocampal neurogenesis.

Were animals transcardially perfused until complete elimination of blood cells before fixation? The authors should clarify this point to facilitate the reader's interpretation of the data presented in figure 3, discarding blood cell contamination in dentate gyrus microdissected tissue samples, which could be an alternative interpretation for the differences in cytokine receptor expression in tissue vs. cultured precursor cells (last paragraph of the results section).

- Microdissection of DGs was done without prior perfusion of Nestin^{GFP} mice (according to Ref.[36]). Even though the dissected tissue was thoroughly washed before RNA extraction, we cannot exclude the possibility that residues of blood cells might have contributed to our quantitative RT-PCR results in whole DG samples. Still, perfusion before microdissection cannot be regarded as a guaranteed way to exclude blood cell contamination, but rather as another measure to reduce its likelihood. Apart from that, we believe that the differences in cytokine receptor expression in tissue vs. cultured precursor cells can be very well explained by the absence of virtually all bystander cells in samples of neural precursor cell (NPC) cultures.

As mentioned in the manuscript, neighbouring cells together with systemic factors and

components of the extracellular matrix comprise a neurogenic niche, which provides a supportive microenvironment for the activity of the residing precursor cells whilst at the same time it regulates their expansion, differentiation and migration (Li & Xie, 2005). In this regard, several studies particularly emphasized the role of microglia as important mediators of immune-based regulatory mechanisms of adult hippocampal neurogenesis[3,4,5] (also: Cacci et al, 2005; Butovsky et al., 2006). The expression of several cytokine receptor molecules assessed in our study has been repeatedly demonstrated previously for cells that contribute to the neurogenic niche such as microglia, astrocytes, brain endothelial cells and mature neurons (Sawada et al., 1993; Rock et al., 2004; Mathieu et al., 2010; Szelényi, 2001). Taken together, we consider the differences in cytokine receptor expression between cultured NPCs and microdissected DG tissue to be mainly caused by the heterogeneous cellular composition of the DG and not by blood cell contamination, which rather has to be regarded as potential confounding factor, that could only be partially prevented by prior perfusion of the experimental animals.

2. The authors suggest that one of the limitations of their study is the lack of direct characterization of the specific factor/cytokine(s) responsible for the cell-cell interaction-independent promotion of hippocampal neurogenesis they observe. However, it could have been very interesting to see some experimental effort made to support the hypothesis that the effects of Th17 cells are mediated by specific cytokines produced by these cells. This could have been done in vitro, using their cultured precursor cells, providing a stronger first proof-of-principle for the important concept they propose.

- One possible next approach is indeed to test whether cultured NPCs are in principle responsive to the candidate cytokines (TNFa, IFNg, IL-10, IL-17, IL-6). However, a possible NPC response to particular cytokines in culture will have limited value as supporting evidence of an influence of Th17 cell-secreted cytokines on hippocampal NPCs in vivo. In fact, in the brain, activated microglia are the main source of several cytokines including TNFa, IL-6, IL-10, or INFg. Activated microglia is mostly known as a suppressor, but might also act as enhancer of adult neurogenesis dependent on their phenotype or mode of activation (i.e. inflammation associated activation[3,4,52] versus Th cell associated IL-4 or INFg activation[5] (also: Butovsky et al., 2006)). In addition some cytokines such as TNFa, IFNg, and IL-6 are expressed by astrocytes[49] or adult hippocampal NPCs themselves (Klassen et al., 2003). Finally, the impact of Th17 cell-derived cytokines on adult neurogenesis might be indirect, i.e. mediated by cells of the neurogenic niche, such as astrocytes, microglia or endothelial cells. In summary, Th17 influences on hippocampal neurogenesis are presumably complex and highly context-dependent, and are thus ideally addressed in vivo.

3. In the adoptive T cell transfer experiments, control mice were injected with PBS only. Although this experimental approach provides a suitable negative control, one wonders whether the injection of other non-IL17 producing T cells (e.g. naive CD4+ differentiated into Th1 or Th2 cells) could have been a more sensible negative control for these experiments, given the specific immune functions of these different T cell sets, which are highlighted by the authors in the last sentence of their discussion. Perhaps the authors could comment on the limitations of their experimental approach in this respect?

- Our observation of an increased hippocampal precursor cell proliferation by Th17 cell-mediated indirect mechanisms provides the first evidence of a distinct $CD4^+$ T helper subset affecting adult hippocampal neurogenesis . Certainly more work will have to be done in order to present a cohesive picture of the particular contribution

of different Th cell subtypes to the pro-proliferative effect on adult NPCs. In fact, our original objective was to expand our adoptive transfer model in TCRα$^{-/-}$ mice towards Th1 and Th2 lineages as well. Actually, we consider the Th1 subtype to be an obvious and promising candidate. The receptor for IFN-γ, the signature cytokine of Th1 cells, is also expressed on adult neural precursor cells and several studies demonstrated that IFN-γ could influence their proliferation both and [50,51] (also: Butovsky et al. 2006). Moreover, Th1 cells together with Th17 cells are key factors in the pathogenesis of EAE, during which modulations of adult hippocampal neurogenesis have been observed[9]. Unfortunately the transfer experiments involved higher effort and expenses as initially assumed. Especially the generation of naïve T cells into effector cells with a ROR-γt^{+} IL-17^{+} phenotype, due to the small yields, could only be achieved by including high numbers of mice. Therefore, we could not follow up on our initial goals in the scope of the present paper.

4. The discussion could benefit from a short paragraph including a more detailed discussion of possible pathological implications of the observations described in the paper. For example, other authors have recently found that hippocampal neurogenesis is enhanced in an arthritis model in rat (Leuchtweis *et al.*, 2014). Perhaps another interesting point of discussion could be the possible implications for depression, a disease commonly linked to alterations in hippocampal neurogenesis and possibly linked to immune function (Eyre and Baune, 2012).
    - In a previous study, our group could already establish a relationship between adaptive peripheral immune responses and enhanced neurogenesis in the adult hippocampus (Wolf et al. 2009b). Similar to the work by Leuchtweis and colleagues, a T cell-dominated adaptive immune response was generated either by intraperitoneal injection of staphylococcus enterotoxin B or the induction of adjuvant-induced rheumatoid arthritis in C57BL/6 mice. Under both conditions a transient increase in hippocampal precursor cell proliferation and neurogenesis could be observed, whereas the intraperitoneal administration of lipopolysaccharide (LPS), an extremely potent activator of innate immune responses, had the opposite effect. Reduced levels of hippocampal precursor cell proliferation in different animal models of adaptive immunodeficiency and its restoration upon adoptive T cell transfers, observed in previous studies[5,6,7] and in the present paper, underline once again the potential role of T cell-mediated immune responses in brain homeostasis and neurogenesis.

Several immune mediators have been proposed in the pathogenesis of depression and the associated changes in adult hippocampal neurogenesis. Among them the proinflammatory cytokine IL-1β, which is known to be a key molecular mediator of innate immune responses, has repeatedly been shown to be partly responsible for the anti-neurogenic effects and depression-like behaviour in mice exposed to acute or chronic stress[48] (also: Goshen et al. 2008). Regarding the opposite effects of innate versus adaptive immune responses on adult hippocampal neurogenesis, these observations could eventually be the basis for new therapeutic approaches. By steering peripheral immune responses towards an adaptive, more T cell-dominated phenotype, it might be possible to modulate the cytokine milieu in the adult brain in a way that supports neurogenesis and reduces anhedonic behaviour in patients suffering from depression.

We believe, however, that such forward-looking statements would potentially oversimplify the underlying mechanisms of CD4^{+} T cell-mediated neuro-immunoregulation, which still remains largely unclear. To this end, our manuscript has to be considered only as a starting

point for future research. We would therefore prefer to keep the previous paragraphs out of the manuscript in order not to overstrain the current data situation.
5. The data presented in dataset 1 seems compatible with the figures presented with the article. However, the data could be presented in a format more easily understandable for the reader. i.e. Dataset 1a is labelled “C57BL/6,TCR?+/?,TCR??/?”, while is should presumably be “C57BL/6, TCRα+/-, TCRα−/−” and the data in rows 8 and 9 seems to be in an odd format compared to the other data rows?
    - It appears that there have been problems with the formatting. The datasets will therefore be converted into PDF for easier accessibility.

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- In"*BrdU administration and immunohistochemistry*" “*scull*” should be “*skull”.*
- Abbreviations should be defined the first time they are used in the text.
    - These points will be addressed.

Competing Interests: No competing interests were disclosed.

Reviewer Report 30 July 2014

https://doi.org/10.5256/f1000research.4750.r5569

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Study design, methods are adequate. Results are generally sound. A few claims are only in part justified by the proposed approach and controls. The importance or relevance of the myelin-specificity/reactivity in the observed promotion of endogenous hippocampal neurogenesis by adoptively transferred $CD4^{+}CD62L^{high}CD25^{-}FoxP3^{GFP-}$ Th17 cells is not fully addressed in this paper. It is in fact quite clear that adoptive transfer of Th17 cells increases BrdU incorporation (and hence proliferation) at the level of the DG of the hippocampus. What is instead much less clear, and only speculated, is that higher proliferation leads to more neurons, and that reactivity to MOG_{35-55} (via the expression of the Vα3.2 chain of the transgenic 2D2 TCR) is indispensable for promoting adult hippocampal neurogenesis.

Main comments

1. Newly generated neurons should be quantified.

2. Experiments in Figure 2 and 3 should include also mice adoptively transferred with non-myelin reactive Th17 cells. This is a key aspect of this paper, especially in the perspective of proposing a mechanism of brain homeostasis that is promoted by a specific subset of

immune cells acting in periphery (e.g. peripheral lymphoid tissues; as anticipated in the abstract). As such, also experiments in Figure 2B should include the very same positive controls as those in Figure 2A.

3. Ideally, one would like to see at least what is the contribution of the Th17 cell adoptive transfer to the cytokine milieu of the host, both at the level of the DG as well as of peripheral lymphoid tissues.

Minor comments

1. Wondering whether it would read better if the characterization of the pattern of expression for cytokine receptors is showed prior to the transfer experiments.

2. Nice to briefly show or comment on the clinical phenotype of that individual mouse showing remarkable accumulation of adoptively transferred Th17 cells in the brain (did it develop EAE-like signs?).

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response ( ) 03 Apr 2017

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1. Newly generated neurons should be quantified.
    - It is correct that we quantified neural precursor cell proliferation as one component of adult hippocampal neurogenesis. Indeed, the term neurogenesis is inclusive of the entire process from cell division to full maturity of new-born neurons after about 4 weeks (Kempermann et al., 1997). Beside proliferation, quiescence, self-renewal vs. cell fate decision (neuron vs. astrocyte, microglia; mostly few days after birth) and survival of new-born cells (30 – 70% die mainly between their first and second week) are further determinants of neurogenesis. Neurogenesis can be regarded as affected once any of the mentioned sub-processes is altered. Changes in individual sub-processes will inevitably affect the outcome, i.e. the number of new mature neurons ("net neurogenesis"), unless additional, counteracting changes occur in additional sub-processes. We, as many authors, use the term neurogenesis in this "inclusive" sense, for example in the title.

Of note, assessing new-born cells or neurons at a later stage of neurogenesis allows a better estimate of the resulting number of newly generated mature neurons (most precise at 4 weeks), but give a "collapsed" view of preceding sub-processes, i.e. assessed differences can not be assigned to a specific underlying cellular mechanism. Obviously, the reverse argumentation has to be considered as well.

Others and we have previously shown that lack of ($CD4^+$) T cells (depletion or deficiency) negatively impacts neurogenesis by lowering baseline proliferation and result in fewer

mature neurons. Changes in further sub-processes, such as cell fate decision/differentiation (7d after division) or survival (up to 4 weeks) have not consistently been reported[5,6,7]. In line with this, reconstitution of T-cell deficiency with (preparations that contain) $CD4^+$ T-cells led to increased proliferation of neural precursor cells ($BrdU^+$) and/or neuronally committed cells ($BrdU^+$ after 7d and Dcx^+)[5,6,7].
One aim of our study was to follow up on these studies and further define whether $CD4^+$ T-cells with a specific status of activation or differentiation are responsible for the reported pro-proliferative effect. Therefore, we concentrated on the investigation of proliferation as the most validated affected measure of neurogenesis. We state this now in manuscript (Background, last sentence). We agree that this does not allow a direct conclusion on the number of newly generated neurons and we did not intend to give this impression.
2. Experiments in Figure 2 and 3 should include also mice adoptively transferred with non-myelin reactive Th17 cells. This is a key aspect of this paper, especially in the perspective of proposing a mechanism of brain homeostasis that is promoted by a specific subset of immune cells acting in periphery (e.g. peripheral lymphoid tissues; as anticipated in the abstract). As such, also experiments in Figure 2B should include the very same positive controls as those in Figure 2A.

- Myelin-specific T cells seem to possess a pronounced ability to promote the proliferation of neural precursor cells in the adult hippocampus[5]. The authors of this initial report therefore suspected an underlying mechanism based on the infiltration of these self-reactive T cells into the CNS in order to directly interact with cellular components of the neurogenic niche of the dentate gyrus. In our manuscript, we report that adoptively transferred myelin-specific Th17 cells promote hippocampal cell proliferation in $TCR\alpha^{-/-}$ mice evidently without entering the brain in significant numbers. Therefore, we concluded that peripheral effector functions must be responsible for this Th17 cell-mediated effect on hippocampal precursor cells. We do not, however, claim that specificity for a CNS-antigen would be a prerequisite for this effect. In fact, we do believe that Th17 cell populations with a polyclonal TCR repertoire would potentially have a similar effect on the proliferative activity of hippocampal precursor cells. We decided to use generated Th17 cells with a transgenic TCR recognizing a CNS-antigen, since we wanted to point out that under physiological conditions T cell subpopulations can affect the neurogenic region of the adult hippocampus and despite their specificity do so without infiltrating the CNS. The crucial point may simply be that the T cells become activated in the peripheral lymphoid tissues upon encounter of their cognate antigen. This should be the fact for both self-reactive as well as polyreactive T cell populations.

3. Ideally, one would like to see at least what is the contribution of the Th17 cell adoptive transfer to the cytokine milieu of the host, both at the level of the DG as well as of peripheral lymphoid tissues.

- Analyzing the cytokine milieu after adoptive T cell transfer could indeed help deciphering underlying clues of a Th17 cell-mediated regulatory mechanism of hippocampal cell proliferation. By additionally using neutralizing antibodies against certain Th17 cell-derived cytokines, one could potentially obtain even more precise results in this regard. However, the transfer experiments turned out to involve high costs and considerable expenditure of time and effort. An assay, using our cultured precursor cells, might probably be a more cost efficient alternative in the first instance (see below). We consider such experiments beyond the scope of the present

manuscript.

Minor comments

1. Wondering whether it would read better if the characterization of the pattern of expression for cytokine receptors is showed prior to the transfer experiments.
    - Our intention was to use our main finding of an increased hippocampal cell proliferation after adoptive Th17 cell transfer as a starting point and subsequently descent to the molecular level in order to find first clues for potential underlying mechanisms and provide a basis for further experimentation.
2. Nice to briefly show or comment on the clinical phenotype of that individual mouse showing remarkable accumulation of adoptively transferred Th17 cells in the brain (did it develop EAE-like signs?).
    - The affected animal did not develop clinical signs of EAE during the observation period. The cellular infiltrations found in the brain of this animal, while thwarting a quantification of hippocampal cell proliferation, were found to be limited rather than widespread, which is probably why they did not result in a clinical phenotype.

Competing Interests: No competing interests were disclosed.