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Abstract: Microglia act as sensors of inflammation in the central nervous system (CNS) and respond to many stimuli. Other key players in neuroinflammatory diseases are CD4⁺ T helper cell (Th) subsets that characteristically secrete IFN- γ (Th1) or IL-17 (Th17). However, the potential of a distinct cytokine milieu generated by these effector T cell subsets to modulate microglial phenotype and function is poorly understood. We therefore investigated the ability of factors secreted by Th1 and Th17 cells to induce microglial activation. In vitro experiments wherein microglia were cultured in the presence of supernatants derived from polarized Th1 or Th17 cultures, revealed that Th1-associated factors could directly activate and trigger a proinflammatory M1-type gene expression profile in microglia that was cell-cell contact independent, whereas Th17 cells or its associated factors did not have any direct influence on microglia. To assess the effects of the key Th17 effector cytokine IL-17A in vivo we used transgenic mice in which IL-17A is specifically expressed in astrocytes. Flow cytometric and histological analysis revealed only subtle changes in the phenotype of microglia suggesting only minimal effects of constitutively produced IL-17A on microglia in vivo. Neither IL-23 signaling nor addition of GM-CSF, a recently described effector molecule of Th17 cells, changed the incapacity of Th17 cells to activate microglia. These findings demonstrate a potent effect of Th1 cells on microglia, however, the mechanism of how Th17 cells achieve their effect in CNS inflammation remains unclear.

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K. Kelley
Editor-in-Chief
Brain, Behaviour and Immunity

Mein Zeichen:

Nachrichtlich:

23. Dec. 2013

Dear Prof. Kelley,

Thank you very much for your kind e-mail dated 26 Nov. 2013 and the reviewer comments on our manuscript **“Effector molecules released by Th1 but not Th17 cells drive a M1 response in microglia.”**

We have closely followed the reviewers recommendations and have revised the manuscript accordingly. Please find a detailed point-to-point list with our response to the reviewers comments. We have addressed each point and included new experiments. All changes in the manuscript are marked in blue.

We believe that we have addressed all reviewer comments comprehensively and hope that the manuscript is now acceptable for *Brain, Behaviour and Immunity*.

Thank you for consideration,
yours sincerely,
Prof. Dr. Martin Stangel

Reviewers' comments:

Reviewer #1: In this study, the authors investigated the ability of factors secreted by Th1 and Th17 cells to induce microglial activation. They claim Th1-associated factors can directly activate and trigger a proinflammatory M1-type gene expression profile in microglia, whereas Th17 cells or its associated factors do not have any direct influence on microglia. This work is interesting, and some results and concepts are novel and challenging.

Major concerns:

1) This large variance of IL-17A-producing cells ranging from 60% to 90% (line 25, page 12) in the culture system may have influences on the effects of Th17 cells observed in this study. For example, in figure 1A, IFN- γ /IL-17A-double negative cells account for almost 10% in Th17 cultures and perhaps, these IFN- γ /IL-17A-double negative cells play an opposite role to that of IL-17A-producing cells. Thus, the Th17-derived culture supernatants could not produce a significant influence on microglial activation, owing to the antagonistic action one another. This possibility should be considered and discussed by the authors.

Reply:

We share this concern raised by the reviewer. The logical solution for this problem would have been to purify the IL-17A⁺ IFN- γ ⁻ cells from the Th17 cultures. Still the major challenge is lack of a specific surface marker that would allow us to purify cells Th17 cells. However we ruled out the possibility that lack of activation of microglia is a result of inhibitory effects of contaminating non IL-17 producing cells for two reasons: Firstly, irrespective of purity of the Th17 cells (60% or 90%, the supernatants yielded similar result i.e. no activation of microglia. Secondly, to prove that no inhibitory factors are present in Th17 supernatants we mixed equal volumes of Th1 and Th17 supernatants and treated microglia with this mixture. In this case the Th17 supernatant was not able to inhibit the activation induced by Th1 factors. This is now mentioned in the manuscript and the data is provided as supplementary figure 1.

2) As stated by the authors (line 57 of page 12 and line 1 of page 13), the IL-21 and IL-22, which are described as Th17-related cytokines, were detected only in lower amounts in the Th17- compared to the Th1-derived culture supernatants. Therefore, how can they assess the purified and representative Th1 or Th17 cells?

Reply:

We defined the Th1 and Th17 cells primarily on their expression of IFN- γ and IL-17, respectively. We established the differentiation conditions such that neither of the cells were found as contaminants in either culture (as shown in fig. 1A). As mentioned above the solution would be to purify the cells and this was not possible for our experimental setup. The total amount of IL-22, although higher in Th1 cells compared to Th17 cells, was rather low (~30-60pg/ml). A recent report has demonstrated that IL-21 is also produced by a small percentage of in vitro differentiated Th1 cells. Nevertheless we could show that microglial activation was not mediated by IL-21, IL-22 or GM-CSF. Exogenous addition of recombinant murine IL-21 (20ng/ml), IL-22 (20ng/ml) and GM-CSF (10ng/ml) to the Th17 supernatants did not result in the activation of microglia. These results have now been discussed in the manuscript and data is shown in supplementary figure 2.

3) In this result, "Th1-derived factors trigger a pro-inflammatory (M1) gene expression profile in microglia", more than one of third of the M2-related genes in the microglia was upregulated (not downregulated) by the Th1-derived supernatants. These results were not discussed in this manuscript. Importantly, the same experiments were not performed for the Th17-derived supernatants. Therefore, this is insufficient in the evidence for showing influence of M1 response in microglia by Th1 but not by Th17 cells. My suggestion is to supplement measurement of some factors that are specific for M1-like and M2-like phenotypes of microglia, respectively, under the action of Th1 or Th17 culture supernatants.

Reply:

We would like to draw the attention of the reviewer that IL-10 was also detected in our Th1 supernatants. We also mentioned in our discussion that highly activated Th1 cells can produce IL-10 as a self control mechanism to minimize inflammatory damage. There is recent evidence in the literature that IL-10 plays a crucial role in the switch of macrophages from M1 to M2 phenotype. We believe that the M1 and M2 phenotype is not absolute and exclusive.

4) In the Th1 culture supernatants, GM-CSF is significantly increased. However, it does not affect microglial activation. So, what role does the increased GM-CSF play in the Th1-mediated microglial activation? Or which factors that are increased in the Th1 culture supernatants have the promoting effects on microglial activation? And also, they address that IFN- γ alone is less effective in activation of microglia (line 15 on page 20). These results are not easy to be understood. The authors should explain and discuss these issues.

Reply:

Complete activation of microglia into pro-inflammatory cells cannot be attributed to a single factor produced by Th1 cells. Although IFN- γ is one known factor we have evidence that the magnitude of microglial activation (measured in terms of CD40 and MHC class II expression) achieved by IFN- γ alone is much less compared to that achieved by adding Th1 supernatants. This again is a proof that several factors synergize to drive a proinflammatory phenotype of microglia. The statement "IFN- γ alone is less effective" appears to be misleading and has now been rephrased in the discussion.

At this moment the role of GM-CSF is not clear. We speculate that it synergizes with other factors to drive complete activation of microglia. In this regard we tested the effects of GM-CSF in the presence of IFN- γ and TNF- α but did not observe any synergistic increase in CD40 and MHC class II expression on microglia. To pin point that a particular factor(s) are involved in microglial activation requires a detailed analysis of the Th1 culture supernatants.

5) In figure 7, Iba-1 staining of brain sections indicates no obvious differences in the morphology of microglia between the GF/IL-17A transgenic and wild type animals, but the CD11b expression is significantly increased on the GF/IL-17 microglia. What are the reasons for the inconsistent results? In addition, the figure 7B is not so convincing, because it did not have statistical diagram. On the other hand, if the CD11b expression on the GF/IL-17 microglia is significantly increased, how do we understand the failed effects of Th17 on microglial activation?

Reply:

The confusion seems to arise from the usage of the phrase "no obvious difference" in our figure 7 legend instead saying minor change in morphology as indicated in our result section. We

intended to say that no drastic changes were observed in microglia and tried to be more precise in explaining the figure. This has been corrected in the figure 7 legend and is now stated in the same way as it is stated in the results section.

In this regard we would like to clarify that these minor changes in the phenotype of microglia were consistent. We now added the average fluorescence intensities value, the SEM and the statistical significance when appropriate in the results section.

Minor concerns:

1) In these histograms, the data in figure 1 are mean $\pm$ SEM, and others are mean $\pm$ SD. They should be uniformed.

Reply:

The data in figure 1 has now been represented as mean $\pm$ SD.

2) The asterisks in most figures were not indicated for their statistical significance.

Reply:

This information has been added to all figure legends.

3) The live CD11b+ cells are 94% in figure 2 but 72% in figure 4. This difference is large, why?

Reply:

In figure 2 the microglia were cultured in Th1 or Th17 derived supernatants, whereas in figure 4 microglia were co-cultured with T cells in an attempt to study cell-cell contact dependent activation. The CD11b-negative population found in figure 4 is residual T cells from the co-culture

4) The order of the figures is confused.

Reply:

We apologise for the confusion that arised due to the wrong numbering of figure legends. This has been corrected now.

5) There were no data to support this description, "Th17-derived factors only had minimal effect on the gene expression of microglia (line 1 of page 30)".

Reply:

Our Gene array analysis yielded 17 genes that were differentially regulated in Th17 treated microglia. These 17 genes that were regulated are presented in a supplementary table.

6) The two images in figure 7A seem to be not consistently magnified.

Reply:

This has now been corrected.

7) In the Materials and Methods, the authors described that isolated leukocytes were incubated with fluorochrome-conjugated antibodies (eBioscience) to detect CD11b (APC), CD11c (PE-

Cy7), CD45 (FITC), CD45 (eFluor 450), I-A/I-E (line 1 of page 11). However, in relative figure 7, only CD11b, MHCII and CD45 are displayed. Why?

Reply:

This was an error and has now been corrected. CD11c was deleted from the methods.

Reviewer #2: This is an interesting study investigating the contribution of Th1- vs. Th17-derived factors that regulate microglial activation and effector function, in light of the pre-clinical EAE data showing that both Th1- and Th17-cells promote/perpetuate the CNS autoimmunity. The premise of this study is warranted. To complement the authors work, I have included the comments, questions and/or suggestions below:

1) It's not clear whether the Th1 and Th17 supernatants being added to microglia still possess high levels of the cytokines and antibodies required to derive the different T cell subsets. If they do, then I have a problem with this study, since you are stratifying the conditions, dramatically and non-physiologically, based on these strong differential signals; ie. IL-12 in the absence of TGF-beta (for Th1 priming) and vice versa (for Th17 priming).

Reply:

The Th1 and Th17 supernatants used in our experiments were totally devoid of polarizing cytokines used for differentiation of naïve T cells into Th1 or Th17 cells. At the end of a 5-day culture in differentiation medium the cells were harvested and washed extensively before subjecting them to restimulation with plate bound anti-CD3 and CD28 antibodies. The supernatants collected post-restimulation were only used in our experiments. This information is now added to the methods

2) Please define GF/IL17 mice. It's not clear from the materials and methods without having to investigate the cited manuscript.

Reply:

This information is now included in the manuscript.

3) Related to question 1, from above, do the microarray analyses reflect the Th1 and Th17 subsets, exclusively, or do they reflect a combination of the distinct T cell subsets in exclusion of the T cell priming conditioning factors?

Reply:

An effect of polarizing cytokines can be excluded as the cells were extensively washed to remove the polarizing factors.

4) The data from Figure 7 is interesting, but lacks parallel construction. What happens when astrocytes express IFN-gamma? Is there a different effect than what is shown for constitutively expressed IL-17? Another issue related to this finding deals with timing. Most M.S. patients do not develop the disease until later in life. Do the authors believe that studying 6 week old mice reflects the adult form of M.S.? This question is particularly important given data showing that in aged mice, the CNS has a higher level of neuroinflammation? Please consider Karen Bulloch's work with regard to the natural evolution of inflammation in the CNS.

Reply:

We are thankful for these comments and extended the discussion accordingly. In Lin *et al* in 2004 has demonstrated that ectopic expression of IFN- γ in astrocytes resulted in activation of microglia. A similar observation was also made by Vass *et al.* and Corbin *et al.* where the former administered IFN- γ intrathecally and the later studied the response in mice that expressed IFN- γ under the MBP promoter.

The *in vivo* data should complement our *in vitro* studies and does clearly not reflect the situation in a human autoimmune disease like MS. We used young mice because we found it more suitable to complement the *in vitro* data describing an acute response to IL-17A. In particular we wanted to exclude the effects of aging in this study. Older transgenic GF-IL17A mice (9-month old) were characterized in our recent study describing the phenotype of these mice.

Karen Bullochs work is now discussed.

5) With regard to Figure 6 (microarray data), it's difficult to compare between the Th1 and Th17 conditions with respect to comparison of genes. Please use parallel construction so that one can directly compare the gene expression levels between the different experimental conditions.

Reply:

Firstly, we would like to apologize for the confusion with figure legends as they were wrongly numbered. In figure 4 our comparisons were always between resting microglia (medium treated) vs Th 1 treated or Th17 treated microglia. Microarray analysis revealed that only Th-1 factors were able to alter the gene expression profile (4368 genes altered > 2-fold) in microglia, whereas those treated with Th17-derived factors did not show any change in gene expression except for 17 genes (supple Table.1) Therefore for simplicity we limited our comparison to medium treated microglia vs Th-1 supernatant treated microglia. The confusion has now been eliminated by rephrasing the sentence in figure legend 4

Reviewer #3: NEW----The authors need to explain their data better. Many of the responses are quite low and it is unclear how certain conclusions can be derived, in particular Fig 1A. Also, Fig 7 presents data that may disagree with the failed effect on microglial activation of Th17. What is the role of GM-CSF? I would suggest that the results be presented in a manner that are reflective of the data shown. It is unclear as to whether more data would help.

Reply:

The reviewer did not specify which responses were low and inconclusive. We guess the reviewer was pointing out at low concentrations of certain factors (IL-1b, IL-22) in the culture supernatants in figure 1C (the differences shown in figure 1a is very clear). We would like to reiterate that Th1 and Th17 cells were primarily defined according to IFN- γ and IL-17 expression, respectively. The rationale behind the cytokine array was to precisely define the factors found in Th1 and Th17 culture supernatants. Nevertheless in the manuscript our results were interpreted considering only those factors that were detected in high amounts in culture supernatants (IFN γ , IL-17, GM-CSF etc).

In Fig 7 the *in vivo* experiments were aimed to complement our *in vitro* findings. Surprisingly we see no drastic differences except for minor changes in the phenotype (morphological and CD11b expression). We are sure the reviewer would agree with the fact that *in vivo* situation is quite complex and involves interaction with other cell types. Unlike *in vitro* condition there is constant

exposure of microglia to IL-17 for longer periods in GF/IL17 mice. These factors can be attributed to the changes observed in microglia.

Effector molecules released by Th1 but not Th17 cells drive an M1 response in microglia

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Abstract

Microglia act as sensors of inflammation in the central nervous system (CNS) and respond to many stimuli. Other key players in neuroinflammatory diseases are CD4⁺ T helper cell (Th) subsets that characteristically secrete IFN- γ (Th1) or IL-17 (Th17). However, the potential of a distinct cytokine milieu generated by these effector T cell subsets to modulate microglial phenotype and function is poorly understood. We therefore investigated the ability of factors secreted by Th1 and Th17 cells to induce microglial activation. *In vitro* experiments wherein microglia were cultured in the presence of supernatants derived from polarized Th1 or Th17 cultures, revealed that Th1-associated factors could directly activate and trigger a proinflammatory M1-type gene expression profile in microglia that was cell-cell contact independent, whereas Th17 cells or its associated factors did not have any direct influence on microglia. To assess the effects of the key Th17 effector cytokine IL-17A *in vivo* we used transgenic mice in which IL-17A is specifically expressed in astrocytes. Flow cytometric and histological analysis revealed only subtle changes in the phenotype of microglia suggesting only minimal effects of constitutively produced IL-17A on microglia *in vivo*. Neither IL-23 signaling nor addition of GM-CSF, a recently described effector molecule of Th17 cells, changed the incapacity of Th17 cells to activate microglia. These findings demonstrate a potent effect of Th1 cells on microglia, however, the mechanism of how Th17 cells achieve their effect in CNS inflammation remains unclear.

Introduction

Neuroinflammation is a consequence of a crosstalk between resident immune cells of the CNS and cells infiltrating from the periphery (Ransohoff and Brown, 2012; Ransohoff and Engelhardt, 2012). Microglia are crucial in maintaining the integrity of the CNS, as they constantly sample the microenvironment and act as sensors of pathological conditions. The activation state of microglia strikes a balance between tissue damage and repair. In steady state, the resting microglia express low levels of major histocompatibility complex (MHC) class II molecules and co-stimulatory molecules on their surface and are mostly involved in maintenance of homeostasis in CNS (Aguzzi et al., 2013; Goldmann and Prinz, 2013; Tambuyzer et al., 2009). However, an inflammatory insult leads to activation of microglia and triggers a proinflammatory cascade that can cause tissue damage. Reports suggest that microglial activation is a key event in most of the neuroinflammatory and neurodegenerative disorders (Bhasin et al., 2007; Heppner et al., 2005).

It is well understood that IFN- γ -producing Th1 cells and IL-17-producing Th17 cells play a crucial role in the pathology of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE) and similar neurodegenerative disorders (Goverman, 2009; Pierson et al., 2012). The individual contribution of Th1 and Th17 cells in CNS pathologies is highly debatable but a consensus arises from the fact that both subsets can mediate EAE although with varying degree of severity when adoptively transferred into mice (Domingues et al., 2010; Gocke et al., 2007; Lee et al., 2012; Yang et al., 2009). Characteristically, these subsets have distinct cytokine secretory profiles as their differentiation is controlled by mutually exclusive signaling molecules and transcription factors (Sallusto et al., 2012; Stockinger and Veldhoen, 2007; Wilson et al., 2009). Further evidence suggests that both Th1 and Th17 cells can interact with microglia and activate them (McQuillan et al., 2010; Murphy et al., 2010). However, the effectiveness of the effector molecules secreted by Th1 and Th17 cells in

1 activating and inducing a robust inflammatory response in microglia is only poorly
2 understood.
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4 Several methods have been published describing the polarizing conditions used to generate
5 Th1 and Th17 cells from naïve CD4⁺ T cells (Carlson et al., 2009; Nurieva et al., 2009). In
6 this study, we employed the supernatants generated from *in vitro* differentiated highly pure
7 Th1 and Th17 cells and compared their effects on microglia harvested from mixed glial
8 cultures. Our findings reveal that Th1-, but not Th17-derived supernatants were effective in
9 microglial activation as they significantly upregulated the expression of co-stimulatory
10 molecules on microglia and bestow them with a pro-inflammatory phenotype. Interestingly,
11 key Th-17 effector molecules, IL-17A and GM-CSF, had no direct influence on microglia *in*
12 *vitro*. Even constitutive long-term expression of IL-17A in the CNS of transgenic mice led to
13 only subtle changes in microglia *in vivo*.
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Materials and Methods

Mice

C57BL/6 male mice were obtained from Charles River (Sulzfeld, Germany) or from Janvier and housed under specific-pathogen-free conditions in the central animal facility of Helmholtz Centre for Infection Research (HZI), Braunschweig, Germany, Hannover Medical School (MHH), Germany and at Institute of pathology, University of Veterinary Medicine (TiHo), Hannover, Germany. Generation of the GF/IL17 transgenic mice where IL17 is specifically expressed under GFAP promoter has been previously described. (Zimmermann et al., 2013)

For generation of GF/IL17 mice, *Il17a* cDNA fragment was cloned into a GFAP expression vector containing a human growth hormone polyadenylation signal sequence downstream of the insert. The resulting fusion gene construct was microinjected into the germline of (C57BL/6×C3H/HeN) F1 mice. Genotyping of the animals was accomplished by PCR analysis of genomic tail DNA using primers targeted at the human growth hormone sequence and the *Il17a* sequence included in the transgene construct. Hemizygote transgenic founder mice were backcrossed to the C57BL/6 background for at least 8 generations before experiments were performed. Transgene negative mice served as wild-type littermate controls

GF/IL17 transgenic mice were maintained under specific-pathogen-free conditions in the closed breeding colony of the University Hospital of Muenster, Germany. All research and animal care procedures were approved by the Review Board of the care for Animals Subjects of the district government (Lower Saxony, Germany) and performed according to international guidelines on the use of laboratory animals (Nicklas et al., 2002).

Antibodies and reagents

Antibodies specific for mouse, anti-CD3 PerCp Cy5.5 (clone: 145-2C11), anti-CD4 FITC (clone: RM 4.5), anti-CD25 APC (clone: PC61), and anti-CD40 PE and APC (clone: 1C10) were purchased from eBioscience, Frankfurt, Germany. Antibodies to anti-IFN- γ PE (clone:

1 XMG1.2), anti-IL-17A APC (clone: TC11-18H10.1), anti-CD11b PE and PerCp (clone:
2 M1/70), anti-I-A/I-E FITC (clone: M5/114.15.2) and anti-CD86 FITC (clone: GL-1) were
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4 purchased from Biolegend, San Diego, CA. Rat anti-mouse IgG2a κ PE, and FITC (clone:
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6 eBR2A) and IgG2b κ FITC (clone: eB149/10H5) were purchased from eBioscience,
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8 Frankfurt, Germany. Unconjugated anti-CD3 (clone: 145-2C11), anti-CD28 (clone: 37.51),
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10 anti-IFN- γ (clone: XMG1.2) and anti-IL4 (clone: 11B11) were purchased from eBioscience,
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12 Frankfurt, Germany. Unconjugated anti-IL-2 (clone JES6-1A12) were obtained from
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14 Biolegend, San Diego, CA. Recombinant murine cytokines TNF- α , IL-1 β , GM-CSF and IL-
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16 23 were all purchased from R&D systems, Wiesbaden-Nordenstadt, Germany whereas
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18 recombinant murine IL-6 was bought from Peprotech, Hamburg, Germany.
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26 *Preparation of primary mouse mixed glial cells*

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28 Primary cultures of mixed glial cells were prepared from brains of postnatal 1-3 day old
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30 C57BL/6 mice. Briefly, the brains were freed from meninges, and digested enzymatically
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32 with 0.1% trypsin (Sigma-Aldrich) and 0.25% DNase (Roche, Mannheim, Germany). Single
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34 cell suspensions obtained from the digested brains were seeded into poly-L-lysine-coated
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36 T75mm² culture flasks in medium consisting of DMEM + L-Glutamine + 4.5g/L D-Glucose
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38 (Gibco®, Darmstadt, Germany) supplemented with 10% fetal calf serum (FCS), 50 U/ml
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40 penicillin, and 50 μ g/ml streptomycin (all Biochrom AG, Berlin, Germany). After 24 hours all
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42 media containing cell debris was removed and fresh media was added. Medium was changed
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44 every fourth day and the microglia were harvested at day 9-11 by shaking the flask at 37°C
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46 and 180 rpm for 30 min on an orbital shaker.
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54 *In vitro differentiation of Th1 and Th17 cells*

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56 Naive CD4⁺ (CD25⁻CD62L^{hi}) from spleen and lymph nodes of C57BL/6J mice were sort
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58 purified using autoMACSPro (Miltenyi Biotec Bergisch Gladbach Germany). Cells (5.0 $\times$
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10⁵/ml) were stimulated with plate-bound anti-CD3 (2 µg/ml) and anti-CD28 (2 µg/ml) in 12-well plates (Corning Life Science, Acton, MA) in complete IMDM (IMDM, 10% FCS, 1mM Sodiumpyruvate 50uM b-mercaptoethanol, 25mM HEPES and non-essential amino acids) supplemented with either Th1-polarizing factors IL-12 (20ng/ml), anti-IL4 (10ug/ml) or with Th17-polarizing factors TGF-β1 (2 ng/ml), IL-6 (30 ng/ml), TNF-α (20 ng/ml), IL-1β (10 ng/ml), anti-IL-2 (10 µg/ml), and anti-IFN-γ (10 µg/ml). After five and six days culture of Th1 and Th17 cells, respectively, the cells were harvested [at the end of the culture period](#), [washed extensively](#) and restimulated in 12-well plates coated with anti-CD3 and anti-CD28 antibodies for 6h. In some experiments the restimulation was carried out in the presence of mouse recombinant IL-23 (20 ng/ml). Supernatants were collected and stored at -80°C until further use. For determining the purity of Th1 and Th17 cultures, cells were harvested and restimulated in the presence of Phorbol 12-myristate 13-acetate (PMA; 10 ng/ml; Sigma) and ionomycin (500 ng/ml) before staining for intracellular cytokines.

Intracellular cytokine staining

In vitro differentiated Th1 and Th17 cells were restimulated in 96-well plates in the presence of PMA (10 ng/ml; Sigma), ionomycin (500 ng/ml; Sigma- Aldrich) and Brefeldin A (5µg/ml; Sigma- Aldrich). The surface markers were stained with flurochrome conjugated anti-CD3 and anti-CD4 antibodies. To eliminate dead cells from the analysis Live/Dead staining was performed using Live/Dead fixable blue dead cell stain kit. Cells were washed thoroughly, fixed and permeabilized using the Foxp3 staining buffer set (eBioscience, Frankfurt, Germany) according to the manufacturer's recommendations. Appropriate dilutions of antibodies for intracellular cytokines anti-IFN-γ and anti-IL-17A were added and incubated for 30 min. Cells were washed and recorded immediately on an LSRII or Fortessa (BD Biosciences, Heidelberg, Germany). The data was analyzed by using the FlowJo software (Tree Star, USA).

Stimulation of microglia with Th1- and Th17-derived supernatants

After harvesting, 2.5×10^5 microglia were plated in 6-well or 12-well plates and incubated for 24h to regain their resting phenotype. After aspirating the existing medium, 1 ml of Th1- or Th17-derived culture supernatants diluted with equal volume of DMEM containing 10 % FCS was added to the plated microglia. In some experiments, microglia were treated with Th17-derived supernatants supplemented with recombinant mouse GM-CSF (5ng/ml). For flow cytometry the cells were released by adding pre-warmed 1x trypsin-EDTA solution. After blocking the Fc-receptors with anti-CD16/32, cells were stained with fluorochrome conjugated anti-mouse anti-CD11b, anti-CD40, anti-I-A/I-E and anti-CD86 antibodies. Rat anti-mouse IgG2a κ and rat anti-mouse IgG2b κ were used to stain the isotype controls. The cells were immediately analyzed on FACScaliburTM (BD Biosciences Heidelberg, Germany).

Co-culture of microglia and T cells

Microglia harvested from the mixed glial cultures were plated in 12-well plates at 2.5×10^5 cells/well. Polarized Th1 or Th17 cells were restimulated with plate bound anti-CD3 and anti-CD28 antibodies for 6 hrs and added to microglia at 5×10^5 cells/well. After 16 hrs of co-culture the T cells were collected separately by gently pipetting the medium and microglia were harvested for flow cytometry by adding pre-warmed 1x trypsin EDTA.

Nitrite measurement

NO production was measured indirectly by measuring the stable end product nitrite in the cell culture media. An aliquot of the cell culture media (100 μ l) was mixed with an equal volume of Griess reagent and incubated at room temperature for 10 mins. The absorbance was measured at 490 nm. The concentration was calculated by linear regression to a standard curve.

MCP-1/CCL-2 ELISA

Supernatants of microglia cultured under different conditions were collected from several experiments and were stored in aliquots at -80°C until further use. CCL-2 in culture supernatants was measured using an enzyme-linked immunosorbent assay (ELISA) for mouse MCP-1/CCL-2 (R&D Systems,) and carried out according to the manufacturer's instructions. A standard curve was generated in the concentration range 0-250 pg/ml using the MCP-1 standard provided in the kit. The standard curve was calculated by a computer-generated four-parameter log (4-PL) fit curve.

Gene expression analysis.

Microglia were harvested from the 6-well plates by mild trypsinization and washed thoroughly in PBS. RNA was isolated from the cell pellet using the RNeasy Micro kit (Qiagen) according to the manufacturer's instructions. Quality and integrity of the total RNA was controlled on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany). 500ng of total RNA were applied for Cy3-labelling reaction using the one color Quick Amp Labeling protocol (Agilent Technologies; Waldbronn, Germany). Labeled cRNA was hybridized to Agilent's murine 4x44k microarrays for 16h at 68°C and scanned using the Agilent DNA Microarray Scanner. Expression signal values were calculated by the software package Feature Extraction 10.5.1.1 (Agilent Technologies; Waldbronn, Germany). For further statistical analysis raw intensities were log2 transformed and expressed as mean centralized relative gene intensity. Statistical analysis of the expression data was performed using the Gene Spring Software package (Agilent Technologies; Waldbronn, Germany). A background subtraction method eliminates all the genes whose detection flag was negative in any of the three conditions. Statistical T-Test were applied to identify differentially expressed gene sets using a *p*-value less than 0.05 and showing a fold change of more than two-fold. The entire microarray data set is available

under Gene Expression Omnibus (GEO) accession number: GSE45384
(<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE45384>)

Th1/ Th17 Cytokine profiling (Luminex assay)

Cytokine profiling was done using the mouse Th17 magnetic bead panel (Millipore) designed to simultaneously detect Th1 and Th17 cytokines (GM-CSF, TNF- α , IFN- γ , IL-1 β , IL-2, IL-10, IL-17A, IL-17F, IL-21 and IL-22). Aliquots of cell culture supernatants were analysed according to the manufacturer's instructions. Briefly, after removing cellular debris by centrifugation, 25 μ l supernatant was mixed with 25 μ l magnetic beads covered with catch antibodies against the cytokines listed above. After overnight incubation at 4°C and washing, the magnetic beads were resuspended in 25 μ l detection antibody solution. After one-hour incubation at RT 25 μ l Streptavidin-Phycoerythrin was added to each sample and incubated for 30 minutes at RT. Finally, the beads were washed twice and resuspended in 150 μ l sheath fluid. Beads incubated with standard proteins, control proteins and supernatants were measured on a MAGPIX (Millipore, Billerica, MA) and analyzed with the xPONENT 4.2 software (Luminex, Austin, Texas). The determination of the cytokine concentration was based on the standard curve (5- or 4- parameter logistic method).

CNS leukocyte isolation and flow cytometry

CNS microglia were isolated from whole brain homogenates as described previously (de Haas et al., 2008) with modifications. In brief, mice were perfused transcardially with ice cold PBS until flow through was completely clear to remove intravascular leukocytes. After dissection brains were grinded in Hank's Balanced Salt Solution (HBSS, Gibco, Eggenstein) using a tissue homogenizer (glass Potter, Braun, Melsungen) followed by a needle (0.6 $\times$ 25) and a syringe (5 ml) before passing through a 70 μ m cell strainer (BD biosciences, Heidelberg).

1 After pelleting, homogenates were resuspended in 75 % isotonic Percoll (GE-healthcare,
2 Uppsala, Sweden) at 4°C. A discontinuous Percoll density gradient was layered as follows: 75
3 %, 25 % and 0% isotonic Percoll. The gradient was centrifuged for 25 min, 800 g at 4°C.
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5 Microglia, leukocytes, and astrocytes were collected from the 25 % / 75 % interface. For
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7 surface marker staining the collected cells were directly washed in PBS, and blocked with
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9 CD16/CD32 (Fc block; eBioscience, Frankfurt/Main, Germany) antibody. Isolated leukocytes
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11 were incubated with fluorochrome-conjugated antibodies (eBioscience) to detect CD11b
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13 (APC), CD45 (FITC), CD45 (eFluor 450), I-A/I-E. After washing, bound Ab was detected
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15 using a BD FACSCanto II (BD Biosciences), and the acquired data were analyzed using the
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17 flow cytometry software, FlowJo (TreeStar, San Carlos, CA).
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26 *Statistical analysis*

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28 All statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software). All
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30 data are expressed as group mean $\pm$ SD unless otherwise stated. All experiments were
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32 performed multiple times and the data obtained was analyzed using one-way ANOVA with
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34 Tukey's post test. Results were considered statistically significant at $p < 0.05$.
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Results

In vitro differentiation and characterization of Th1 and Th17 cells

In order to study the effectiveness of effector molecules secreted by Th1 and Th17 cells in activating and inducing a robust inflammatory response in microglia our initial efforts were to establish *in vitro* cultures that yielded high percentage of cells producing IFN- γ and IL-17, respectively. Naïve CD4⁺ T cells were sorted from the spleen and lymph nodes of mice and were cultured under Th1- or Th17-polarizing conditions. After five (Th1) or six (Th17) days of culture, the cells were harvested, restimulated and their phenotype was assessed by intracellular staining for IFN- γ and IL-17A. We consistently achieved > 99 % of IFN- γ -producing Th1 cells after five days of culture, whereas the yield of Th17 cells was slightly variable ranging from 60-90 % of IL-17A-producing cells at day 6 of the culture. Importantly, Th1 cells did hardly express IL-17A and Th17 cells were largely devoid of IFN- γ -producing cells (< 0.5 %) (Fig. 1a & 1b).

We intended to use the culture supernatants harvested following restimulation of Th1 and Th17 cells to treat microglia in further experiments. Hence, it was essential to comprehensively characterize the cytokine secretion profile of these cells. A multiplex assay designed to measure Th1- and Th17-specific cytokines was used to profile the culture supernatants. Confirming our above findings high amounts of IFN- γ were detected only in Th1-derived supernatants and IL-17A and IL-17F were present in large amounts in Th17-derived culture supernatants (Fig. 1c). In addition to IFN- γ , Th1 cells produced large amounts of IL-2 and TNF- α , and these cytokines were detected in lower quantities in Th17-derived supernatants. Interestingly, the pro-inflammatory cytokine GM-CSF and anti-inflammatory cytokine IL-10 were also detected in surplus amounts in Th1- but not in Th17-derived supernatants. Unexpectedly, IL-21 and IL-22, which are described as Th17-related cytokines

(Dong, 2008), were detected only in lower amounts in the Th17- compared to the Th1-derived culture supernatants (Fig. 1c).

Th1- but not Th17-derived factors activate microglia

Few studies have demonstrated that both pathogenic Th1 and Th17 cells are capable of activating and inducing a proinflammatory response in microglia (McQuillan et al., 2010; Murphy et al., 2010). However, the contribution of effector molecules secreted by these T cell subsets during the activation of microglia is not well understood. To study the effect of factors secreted by Th1 and Th17 cells on microglial activation, culture supernatants collected after restimulation of polarised Th1 and Th17 cells were added to microglia. The majority of purified microglia were CD11b⁺ (Fig. 2a). After 16h culture in the respective supernatants we examined the expression of MHC class II and co-stimulatory molecules CD86 and CD40 on the surface of microglia (Fig. 2b). We observed that microglia cultured in Th1-derived supernatants significantly upregulated MHC class II, CD86, and particularly CD40 on their surface whereas microglia cultured in Th17-derived supernatants had an unaltered expression of these surface markers comparable to control cells cultured in non-conditioned medium (Fig. 2b & 2c). Further attempts were made to investigate if any inhibitory factors released by a varying percentage of contaminating IL17⁺ IFN- γ ⁺ cells found in the Th17 cultures were responsible for the observed inefficiency of Th17 derived supernatants to activate microglia. For this purpose, we mixed equal volumes of Th1- and Th17-derived supernatants and the mixture was added to microglia to see if Th17-derived supernatants inhibited or reduced the efficacy of Th-1 derived supernatants to activate microglia. The observed microglial activation induced by Th1-Th17 mixture was similar to that observed in microglia treated with Th1-derived supernatants alone, thereby suggesting that no inhibitory factors are present in Th17 derived supernatants (Supplementary fig. 1).

1 Microglia respond to inflammation by secreting reactive oxygen species and are the major
2 source of toxic nitric oxide (NO) in the CNS (Boje and Arora, 1992). Additionally, microglia
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4 release certain chemokines such as monocyte chemoattractant protein (MCP-1/CCL2) that
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6 attract other cells including macrophages and T cells to the sites of inflammation (Conductier
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8 et al., 2010). To assess if microglia respond to Th1- and Th17-derived factors in a similar
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10 manner, we measured the levels of nitrite (as the stable end product of NO secretion) and
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12 CCL2 in the culture supernatants of microglia that were cultured in the presence of Th1- and
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14 Th17-derived supernatants. Further strengthening our above findings, we detected high levels
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16 of nitrite and CCL2 in culture supernatants of microglia that were exposed to Th1-derived
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18 factors, whereas Th17-derived supernatants only had a minimal impact (Fig. 3).
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26 *Th1-derived factors trigger a pro-inflammatory (M1) gene expression profile in microglia*

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28 In order to study the response of microglia in an unbiased manner, we performed gene
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30 expression profiling of microglia that were cultured with Th1- and Th17-derived supernatants,
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32 respectively. Microglia treated with Th1-derived supernatants showed a highly altered gene
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34 expression profile compared to medium-treated control cells. A total of 4368 genes were
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36 differentially regulated more than 2-fold in microglia in response to Th1-derived factors.
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38 Surprisingly, Th17-derived supernatants did not affect the gene expression profile in
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40 microglia and resulted in the regulation of only 16 genes. The genes regulated by Th17-
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42 derived factors are listed in the supplementary Table 1.
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48 Macrophages and similarly microglia can be classified into pro-inflammatory M1-like or anti
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50 inflammatory M2-like cells based on their function in promoting or suppressing
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52 inflammation, respectively (David and Kroner, 2011; Kigerl et al., 2009). Although there is
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54 no clear demarcation with respect to the M1 and M2 phenotype, it is known that several genes
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56 are more specifically expressed in M1-like cells while few others are predominantly
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58 expressed in M2-like cells. In our further attempts, we screened for differentially regulated
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1 M1/M2 genes in microglia treated with Th1-derived supernatant in terms of their
2 inflammatory profile. From the existing literature we selected a list of 84 genes that were
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4 reported to be differentially expressed in M1 or M2 macrophage/microglia and classified
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6 them into M1- (39 genes; Fig. 4a), M2- (28 genes; Fig. 4b) and M1+M2- (seven genes;
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8 Fig. 4c) specific genes (David and Kroner, 2011; Durafour et al., 2012; Kigerl et al., 2009;
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10 Martinez et al., 2006). The results indicated that pro-inflammatory M1-like genes were
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12 predominantly up-regulated in microglia that was exposed to Th1-derived factors and at the
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14 same time many of the M2-related genes were downregulated (Fig. 4). Altogether these
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16 results suggest that Th1-derived factors might augment neuroinflammation by conferring a
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18 strong pro-inflammatory M1-like phenotype to microglia whereas Th17-derived factors were
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20 least effective in activating and inducing a pro-inflammatory response in microglia.
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29 *Cell-cell contact cannot restore the inability of Th17 cells to activate microglia*

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31 From the above experiments it is understood that Th1 cells can mediate their effects
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33 independent of being in close contact with microglia. Nevertheless, the inability of Th17-
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35 derived supernatants to activate microglia was puzzling and led us to speculate if there was a
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37 need of cell-cell contact for Th17 cells to mediate their effects on microglia. After
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39 restimulation, polarized Th1 and Th17 cells were harvested and added to microglia at a ratio
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41 of 2:1 and co-cultured for another 16h. Again, the activation was assessed based on the
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43 expression levels of co-stimulatory molecules CD40 and CD86 on microglia. In accordance
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45 with the above findings, we did not observe any upregulation of CD40 and CD86 on
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47 microglia when co-cultured with Th17 cells (Fig. 5). In contrast, co-culture of microglia with
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49 Th1 cells yielded similar results as observed with Th1-derived supernatants. Taken together,
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51 these results indicate that Th1 cells can activate microglia whereas Th17 cells have no effect
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53 on this cell type even at direct cell-cell contact.
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Role of IL-23 signaling and GM-CSF in mediating Th17 effector functions

Recently, several independent studies have demonstrated the importance of IL-23 signaling in the induction of effector Th17 cells (Codarri et al., 2011; El-Behi et al., 2011; Lee et al., 2012). They have shown that IL-23 signaling induces GM-CSF production by Th17 cells, which is considered as a key molecule capable of mediating the effector function of Th17 cells in the CNS (Codarri et al., 2011; El-Behi et al., 2011; Lee et al., 2012). Incidentally, our multiplex data revealed that Th17 cells generated in the absence of IL-23 did not produce GM-CSF. However, we found large amounts of GM-CSF in Th1-derived supernatants, which had been demonstrated to be effective in activating microglia. Therefore, we next tested if GM-CSF was the key effector molecule responsible for activation of microglia. For this, Th17-derived supernatants generated as described above were supplemented with recombinant GM-CSF and added to the microglia. As depicted in Figure 6a, expression of MHC class II and CD40 was unaffected on microglia that were cultured in medium containing GM-CSF alone or in Th17-derived supernatants that were supplemented with GM-CSF, suggesting that GM-CSF had no effect on microglial activation. Similarly, we could confirm that the inability of Th17-derived supernatants to activate microglia was not due to minimal expression of key Th17 cytokines, IL-21 and IL-22. Exogenous addition of recombinant murine IL-21 and IL-22 to the Th17-derived supernatants did not lead to activate microglia (Supplementary fig.2)

Next we asked if IL-23 signaling is required to generate Th17 effector cells being capable of activating microglia. To address this, we differentiated Th17 cells from naïve CD4⁺ T cells as mentioned above and restimulated them in the presence of recombinant IL-23. The culture supernatants collected after restimulation were added to microglia and the activation was assessed by measuring CD40 and MHC class II expression on microglia. Strikingly, the addition of IL-23 during the restimulation did not rescue the inability of the Th17-derived supernatants to activate microglia as similar expression levels of CD40 or MHC class II were

observed under all (Th17) conditions tested (Fig. 6b). Altogether, we conclude that Th17 cells have a minimal or no effect on microglia and IL-23 has no influence in this process.

Constitutive expression of IL-17A has only minimal activating effects on microglia in vivo

So far the results have confirmed that effector molecules secreted by Th17 cells have no effect on microglial activation. Our next attempt was to study the effect of IL-17A, a key effector molecule of Th17 cells, on microglia *in vivo*. We have recently reported transgenic mice (GF/IL17), which produce IL-17A specifically and constitutively in astrocytes (Zimmermann et al., 2013). Immunofluorescence of the hippocampus of 6-week old GF/IL17 mice stained with Iba-1 did show minimal signs of microglial activation by morphological criteria (large cell body with distinct processes) compared to wild-type (wt) littermate control mice (Fig. 7a). Furthermore, surface expression of CD45, CD11b and MHC class II molecules was compared among the microglia harvested from the brain of GF/IL17 and wt mice. CD11b expression was slightly but significantly higher on microglia harvested from GF/IL17 mice compared to wt mice (mean fluorescence intensity 3377 ± 53 vs. 3070 ± 61 ; $p < 0.01$) (Fig. 7b). However, no significant difference in the surface expression of CD45 (mean fluorescence intensity 975 ± 33 vs. 925 ± 33) and MHC class II (mean fluorescence intensity 525 ± 12 vs. 542 ± 21) was observed among wt and GF/IL17 microglia (Fig. 7b). These results demonstrate that *in vivo* exposure to IL-17A by transgenic expression over weeks has only minimal effects on microglia. We believe, these effects might even be secondary and mostly due to microvascular changes observed in GF/IL17 mice (Zimmermann et al., 2013).

Discussion

It is known that IFN- γ -producing Th1 cells and IL-17-producing Th17 cells combine with CNS-resident microglia to promote certain neuroinflammatory disorders (Ransohoff and Brown, 2012; Ransohoff and Engelhardt, 2012). In the present study, we demonstrate that only factors secreted by Th1 cells had the potential to directly activate microglia and confer them an inflammatory, M1-like phenotype, whereas factors secreted by Th17 cells only had minimal effects. CD4⁺ T helper cell (Th) subsets are classified into distinct subsets based on their cytokine secretion profile, and the effector molecules secreted by Th cell subsets modulate the phenotype and functions of other cells. Th1 and Th17 cells can be obtained from naïve CD4⁺ T *in vitro* by culturing them under defined polarizing conditions (Carlson et al., 2009; Janke et al., 2010). In order to investigate these functions, it is a major challenge to select a protocol that yields pure cultures without contamination from either population. This is a crucial factor in studies aimed to assess the individual effects of Th1 and Th17 cells, as our experience suggests that even a minor contamination (< 10 %) with IFN- γ producing cells in the Th17 population yielded contrasting results (data not shown). Hence, in this study we standardized our conditions to obtain Th1 and Th17 cells largely free of contaminating IL-17⁺ and IFN- γ ⁺ cells, respectively. To the best of our knowledge this high yield for pure Th17 cells after six days of culture under polarizing conditions has not been reported in any other study.

Screening of the culture supernatants for Th1- and Th17-specific cytokines further confirmed the signature cytokine secretion profile in these cells. The cytokines IFN- γ , TNF- α and IL-2 were abundant in Th1-derived supernatants and high amounts of IL-17A and IL-17F were detected in Th17-derived culture supernatants. Another key cytokine found in the Th1 culture supernatants was IL-10 which is known to have anti-inflammatory properties. Several studies investigating the role of Th1 responses in controlling infections have demonstrated the production of IL-10 by the same IFN- γ producing cell as a mechanism to self-limit tissue

1 damage (Jankovic et al., 2007; O'Garra and Vieira, 2007). Additionally, higher amounts of IL-
2 21 which is regarded as Th-17 related cytokine was detected in Th1-derived supernatants.
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4 However, it has been shown by Nakayamada et al. that at the end of a 5-day culture period *in*
5 *vitro* generated Th1 cells showed a small percentage of cells that are capable of producing
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7 both IL-21 and IFN- γ . They describe this as transition phenotype that resembles follicular
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9 helper T (Tfh) cells. Tfh cells are a known source of IL-21 (Nakayamada et al., 2011).
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11 Interestingly, we also found high amounts of GM-CSF in Th1-, but not in Th17-derived
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13 supernatants, which is in contrast to recent reports highlighting that Th17 cells are the major
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15 source of GM-CSF in the CNS (Codarri et al., 2011; El-Behi et al., 2011). Although GM-CSF
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17 is emerging as a key Th17 effector cytokine, it is long known that other T cell subsets can also
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19 produce GM-CSF (Ponomarev et al., 2007). In this line, a study published by Domingues et
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21 al. also demonstrated that *in vitro* generated Th1 but not Th17 cells produced GM-CSF
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23 (Domingues et al., 2010). It should be noted that Th17 development is a complex process
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25 with the participation of distinct signals at different stages of development (Korn et al., 2009;
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27 Yosef et al., 2013). We believe that missing signals could be the reason for the slight
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29 variations observed among the *in vitro* differentiated and *in vivo* induced Th17 cells.
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32 Microglia play a significant role in regulating neuroinflammation, and microglial activation is
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34 considered to be indispensable in this process (Bhasin et al., 2007; Heppner et al., 2005). It is
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36 known that microglia have divergent effects in the CNS depending on their phenotype.
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38 Classically activated microglia have a damaging proinflammatory phenotype and are often
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40 regarded as M1-like cells whereas alternatively activated microglia have a more protective
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42 role and participate in anti-inflammatory M2-like responses (Kigerl et al., 2009). Few studies
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44 have demonstrated that both Th1 and Th17 cells can interact with and activate microglia
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46 (McQuillan et al., 2010; Murphy et al., 2010). Consistent with these studies, we show that
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48 Th1-derived supernatants were able to induce microglial activation. However, the effect of
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50 Th17 derived supernatants on microglia was indifferent. McQuillan et al. observed microglial
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activation when mixed glial cells were co-cultured with amyloid-beta (A β)-specific Th17 cell lines in the presence of A β antigen. This we believe could be an antigen specific response and it is less clear if the effects are directly mediated on microglia as the mixed glial cells have higher percentage of astroglial cells. On the other hand Murphy et al. demonstrate microglial activation only when the mixed glial cells were co-cultured with IL-17⁺ IFN γ ⁺ Th1/Th17 cells and failed to show the effect of IL-17⁺ Th17 cells for the reason that these cells were less stable and attained double phenotype. In this regard our findings are highly significant for two reasons. Firstly, Th1 and Th17 supernatants were obtained from highly pure cultures and secondly their effects were studied on purified microglia harvested from mixed glial cultures. The key role of Th cells is to provide factors that influence the function of other cells. IFN- γ produced by Th1 cells has long been known for its immunomodulatory effects on cells of both innate and adaptive immunity. IFN- γ alone induced only mild changes in the surface expression of CD40 and MHC class II molecules on microglia compared to profound upregulation of these molecules induced by Th1-derived supernatant. Furthermore the morphology of IFN- γ treated microglia showed more ramifications whereas the Th-1 activated microglia displayed amoeboid morphology, a typical characteristic of completely activated microglia (data not shown). Induction of a full pro-inflammatory microglial phenotype generally requires several other factors and involves more than one signaling pathway (Merson et al., 2010). *In vitro* differentiated Th1 cells used in this study were a rich source of several key inflammatory cytokines and hence were efficient in directly activating microglia. Furthermore, they also triggered a gene expression pattern, wherein several proinflammatory genes (M1 specific) were upregulated and simultaneously other anti-inflammatory genes (M2 specific) were downregulated. It is noteworthy that a significant proportion of M2 specific genes were also upregulated in microglia treated with Th1-derived supernatants. We believe this effect is due to the presence of IL-10 in the Th-1 derived

supernatants and it has been recently demonstrated that IL-10 plays a central role in regulating the switch of muscle macrophages from M1 to M2 like phenotype (Deng et al., 2012).

The most prominent effect of Th1-derived factors was observed in the upregulation of CD40 on microglia. CD40 interacts with CD40 ligand (CD154) expressed on activated T cells, and this interaction is reported to be involved in the development of EAE (Gerritse et al., 1996; Howard et al., 1999). Another key observation was that Th1-derived supernatants also strongly induced production of NO and CCL2 by microglia. Reactive oxygen species such as NO are toxic when present in high amounts in the CNS and can cause neuronal damage (Calabrese et al., 2007). Furthermore, chemokines such as CCL-2 are known for their ability to attract monocytes, T lymphocytes, basophils, natural killer cells, and astrocytes to the inflammatory foci in the CNS (Semple et al., 2010). All these findings strongly hint that Th1-derived factors orchestrate neuroinflammation by activating microglia directly.

Ever since the discovery of Th17 cells the concept of Th1 cells being the major cause of autoimmunity has taken backseat. Th17 cells are believed to mediate their encephalitogenicity via their effector molecules such as IL-17 and GM-CSF. In this context, our findings are surprising that Th17 cells and their secreted factors had no influence on microglia *in vitro*. Recent studies have demonstrated the importance of IL-23 signaling in triggering highly pathogenic Th17 effectors cells (Codarri et al., 2011; El-Behi et al., 2011; Lee et al., 2012). We speculated the observed ineffectiveness of Th17 cells in our initial experiments might be due to the fact that they were generated in the absence of IL-23. We were aware that naïve CD4⁺ T cells do not express IL-23 receptor and are unresponsive to IL-23 (Bettelli et al., 2006; Veldhoen et al., 2006). However, IL-23 receptor is upregulated in polarized Th17 cells differentiated in the presence of TGF- β and IL-6 (Ivanov et al., 2006) and hence we included IL-23 during the restimulation of *in vitro* polarized Th17 cells. Surprisingly, supernatants collected from IL-23-primed Th17 cells also did not alter the phenotype of microglia.

Recent reports highlighted the ability of GM-CSF as an effector molecule of Th17 cells that can induce encephalitogenicity in the CNS (Codarri et al., 2011; El-Behi et al., 2011; Ponomarev et al., 2007). GM-CSF was largely present in the culture supernatants of Th1 cells and was negligible in Th17-derived supernatants. However, it is unlikely that GM-CSF is the mediator of microglia activation [at least as a single factor](#) as we show that GM-CSF alone or when added along with Th17-derived supernatants, did not promote the expression of co-stimulatory molecules on the surface of microglia. The concentration of GM-CSF (5 ng/ml) was chosen on the basis of the amount detected in Th1-derived supernatants. We also tested higher amounts of GM-CSF (50-100 ng/ml), but observed no effects on microglia (data not shown). [We could speculate that GM-CSF might synergize with other factors released by Th1 and hence a greater effect was observed when Th1 derived supernatants were added to microglia.](#) Although Codarri *et al.* recently demonstrated that CNS-resident glial cells show less activation in GM-CSF-deficient mice, it is not clear from this study if GM-CSF has a direct effect on glial cells (Codarri et al., 2011). As a support to our findings Fischer *et al.* and Esen *et al.* earlier reported that microglia exposed to GM-CSF did not alter the surface expression of MHC class II and co-stimulatory molecules but they attain dendritic cell like properties (Esen and Kielian, 2007; Fischer et al., 1993).

IL-17A and IL-17F are predominantly found in the Th17 culture supernatants, which were for many years regarded as key effector molecules produced by Th17 cells capable of mediating CNS disease. The ability of IL-17A to act on a broad spectrum of immune cells and propagate autoimmune response has been clearly demonstrated for peripheral organs (Kolls and Linden, 2004). However, the role of IL-17A in CNS pathology is poorly understood and highly controversial. Elevated levels of IL-17 paralleled with the severity of MS or EAE. In order to dissect the potential role of IL-17A in neuropathology, we recently characterized transgenic mice (GF/IL17 mice) in which IL-17A is specifically expressed in astrocytes (Zimmermann et al., 2013). There we have shown that despite the continuous presence of IL17A in the CNS of

GF/IL17 mice, comparable to IL-17A CNS levels in neuroinflammatory diseases like EAE, these mice did not develop spontaneous leukocyte infiltration or major tissue destruction (Zimmermann et al., 2013). It has been reported that aging can influence the phenotype and function of microglia and render them more sensitive to inflammatory stimuli (Sierra et al., 2007). In order to exclude aging related effects we used young (6 week old) mice and screened for the phenotype and activation status of microglia and found only very minor signs of microglial activation in GF/IL17 mice. This might either be an indirect effect mediated via astrocytes, as IL-17 is known to activate astrocytes and cause the release of proinflammatory mediators such as IL-6 and IL-1 β (Trajkovic et al., 2001) or due to microvascular changes observed in GF/IL17 mice (Zimmermann et al., 2013). These results are underlined by the findings that in contrast to IL-17 an overexpression of IFN- γ in the CNS or intrathecal administration of recombinant cytokine induced pronounced activation of microglia (Corbin et al., 1996; Lin et al., 2004; Vass et al., 1992). Therefore we believe that one of the targets of Th1 cells and its effectors in the CNS are microglia whereas Th17 cells and its effectors show no direct effects on microglia.

Dissecting the individual contribution of Th1 and Th17 cell in propagating neuroinflammation is a daunting task as it involves a complex interaction of cells in the CNS. Here we provide strong evidence that Th1 cells known to infiltrate into the CNS are thoroughly fitted with the arsenal to directly activate microglia. Potentially, these proinflammatory microglia on one hand can cause tissue damage and on other hand with increased MHC class II and co-stimulatory molecules on their surface can act as efficient antigen-presenting cells in reactivation of infiltrating Th17 cells. Furthermore, our data confirm that Th17 cells and their effector molecules lack the ability to directly modulate microglia.

Our results demonstrate the delicate interactions between T cell subsets and microglia in the CNS. While Th1 cells directly activate microglia and drive them into an M1-like phenotype, Th17 cells have no or only little direct effects. Although these findings contribute to a better

1 understanding of the role of Th1 cells in CNS diseases, the mechanism of how Th17 cells
2 achieve their effect in the CNS still remains to be clarified.
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19 **Conflict of interest:** The other authors declare that they have no conflict of interest.
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Figure legends:

Figure 1: *In vitro* differentiation of Th1 and Th17 cells. Th1 and Th17 cells were generated from purified naïve CD4⁺CD25⁻CD62L^{high} T cells under specific polarizing conditions for 5 and 6 days, respectively. (A) Dot plot showing the expression of IFN- γ and IL-17 among differentiated Th1 and Th17 cells. (B) Percentage of IFN γ ⁺ IL-17⁻ (black bars) and IFN γ ⁻ IL-17⁺ (grey bars) cells pooled from six independent Th1 and Th17 cultures. Results are represented as mean $\pm$ SD. (C) Aliquots of culture supernatants from the Th1 and Th17 cultures were tested for the of various Th1/Th17 cytokines in a multiplex assay. Results shown are represented as mean $\pm$ SD from four independent cultures. ** p-value < 0.01, *** p-value < 0.001

Figure 2: *Th1- but not Th17-derived supernatants upregulate MHC class II and co-stimulatory molecules on microglia.* (A) The purity of microglia harvested from mixed glial cultures was estimated by the staining for CD11b. Live CD11b⁺ cells were used for further analysis. (B) Microglia treated with medium (dotted lines), Th1- (solid line) or Th17-derived (dashed line) supernatants were stained for the expression of MHC class II and co-stimulatory molecules CD40 and CD86. The histograms represent the expression levels of these molecules on the microglial surface. Isotype controls (filled) were included for each staining. The data are representative of five independent experiments. (C) The mean fluorescence intensities (MFI) obtained were normalized to medium controls and the fold change in MFI in microglia treated with Th1- and Th17-derived supernatants is depicted. Mean $\pm$ SD of five independent experiments. ** p-value < 0.01, *** p-value < 0.001

Figure 3: *Th1-, but not Th17-derived induce a pro-inflammatory response in microglia.* The supernatants were collected from microglia that were cultured in the presence or absence of Th1- and Th17-derived supernatants and the levels of nitrite (as an indirect measure of

nitric oxide, NO) and the chemokine CCL-2 were measured by using the Greiss assay and CCL-2 ELISA, respectively. Results are represented as mean $\pm$ SD from four independent experiments. * p-value < 0.05, *** p-value < 0.001

Figure 4: *Pro-inflammatory M1-specific genes upregulated in microglia in response to Th1-derived factors.* RNA isolated from microglia cultured in medium or with Th1- or Th17 derived supernatants was probed on Agilent mouse gene array chips. Altogether expression of only 17 genes were altered in Th17 treated microglia and none of those genes were M1 or M2 specific genes. Hence for simplicity here we only show comparison of medium vs Th1 treated microglia. We selected the genes that were reported to be specifically expressed in M1- (A) and M2- (B) or in both M1- and M2-like (C) microglia and compared their expression in microglia cultured in medium or in Th1-derived supernatants. Absolute fold change in gene expression compared to medium control is shown here. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001. The results are from four independent experiments.

Figure 5: *Co-culture of in vitro differentiated Th17 cells with microglia does not alter the phenotype of microglia.* Microglia were co-cultured with *in vitro* differentiated Th1 or Th17 cells at a ratio of 1:2 and surface expression of CD40 and CD86 was analysed on CD11b⁺ gated events. Histograms of CD40 and CD86 on microglia cultured in medium (dotted line) or co-cultured with Th1 (solid line) or Th17 cells (dashed line). Isotype controls (filled) were included for each staining. A representative of two independent experiments is shown here.

Figure 6: *IL-23 signaling on Th17 cells and GM-CSF had minimal effects on activation of microglia.* (A) The effects of GM-CSF on microglial activation was tested by culturing microglia in the presence or absence of Th1-derived supernatant, Th17-derived supernatant, or Th17-derived supernatant supplemented with GM-CSF (5 ng/ml) or GM-CSF alone.

Expression of MHC class II and CD40 was measured on microglia by flow cytometry. Fold change in MFI was calculated from the normalized medium controls and presented here. Mean $\pm$ SD of four independent experiments *** p-value < 0.001 (B) Microglia were cultured in the presence or absence of Th17-derived supernatants collected after restimulation in the presence or absence of recombinant IL-23 (20 ng/ml). Microglia cultured in medium and Th1-derived supernatants served as controls. Expression of MHC class II and CD40 was measured on microglia by flow cytometry. Fold change in MFI from three independent experiments are shown here. Data is presented as mean $\pm$ SD.

Figure 7: *CNS expression of IL-17A induces activation of microglia.* (A) Iba-1 (red) staining of frozen brain sections of 6-week old GF/IL17A transgenic animals (right panel) and wild type (WT) littermate controls (left panel) revealed **only a minor increase of microglial activation** in GF/IL17A transgenic mice. Representative of six animals analyzed per group. (B) Flow cytometric analysis of surface marker expression of cells harvested from the brain of GF/IL17A (red line) and WT mice (green line). Isotype control is shown in black line. Small but significant increase in the surface expression of CD11b (left panel) observed on GF/IL17 microglia. No significant changes in the surface expression of MHC class II (middle panel) and CD45 (right panel) was observed between GF/IL17 and WT microglia. Representative of 6 animals analyzed per group.

Supplementary table 1: *Gene regulated in microglia in response to Th17-derived factors.* This table shows the genes that are differentially regulated in microglia exposed to Th17-derived culture supernatants compared to medium controls.

Supplementary figure 1: *There are no inhibitory factors present in Th17 supernatants.* To test if IL-17⁺ IFN- γ ⁺ cell present in Th-17 cultures released inhibitory factors that prevented microglial activation by Th17 supernatants we treated microglia with medium (black line), Th-1 derived supernatants (red line) and Th1 supernatants mixed with equal volume of Th17 derived supernatants (green line). The surface expression of MHC class II (left histogram) and CD40 (right histogram) was analyzed by flow cytometry. The FACS histogram represents the overlays depicting differential expression of these molecules on microglia. The data is representative one out of three independent experiments.

Supplementary figure 2. *IL-21 and IL-22 are not the effectors that activate the microglia.* Exogenous addition of IL-21 (20ng/ml), IL-22 (20ng/ml) and GM-CSF (10ng/ml) to Th17 supernatants did not result in microglial activation. The surface expression of MHC class II (left histogram) and CD40 (right histogram) was analyzed on microglia treated with medium, (black line) Th17 derived supernatants (red line) and Th17 derived supernatants supplemented with above mentioned cytokines (green line). The histogram overlay is a representative of one out of three independent experiments.

Figure(s)
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Gene Symbol	Gene Name	Fold Change [Th17] Vs [IMDM]	Regulation	p -value
Chka	choline kinase alpha	2.0251074	↑	0.01449766
Atrnl1	attractin like 1	2.7631235	↑	0.00237678
BC055004	cDNA sequence BC055004	5.882539	↑	1.54E-04
Cd38	CD38 antigen	2.352381	↑	0.00752265
Mex3b	mex3 homolog B (C. elegans)	3.5809052	↓	0.03828006
Marco	macrophage receptor with collagenous structure	2.2470522	↑	0.04091039
Gbp11	guanylate binding protein 11	2.342218	↑	0.02371074
LOC100041550	hypothetical protein LOC100041550	3.7419481	↓	0.03470071
Ifi203	interferon activated gene 203	6.0953736	↑	0.00448672
Zfp783	zinc finger protein 783	2.8574996	↓	0.04947
Socs3	suppressor of cytokine signaling 3	2.5982282	↑	0.02323626
Ctse	cathepsin E	2.21074	↓	0.001792
Tmprss4	transmembrane protease, serine 4	2.652192	↑	0.03463934
Gbp8	guanylate-binding protein 8	3.98757	↑	0.01600175
Wdr96	WD repeat domain 96	2.0562932	↑	0.02830346