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MicroRNA-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages via the C/EBP- α –PU.1 pathway

Eugene D Ponomarev¹, Tatyana Veremeyko¹, Natasha Barteneva², Anna M Krichevsky^{1,3}, and Howard L Weiner^{1,3}

¹Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA.

²The Immune Disease Institute, Harvard Medical School, Boston, Massachusetts, USA.

Abstract

MicroRNAs are a family of regulatory molecules involved in many physiological processes, including differentiation and activation of cells of the immune system. We found that brain-specific miR-124 is expressed in microglia but not in peripheral monocytes or macrophages. When overexpressed in macrophages, miR-124 directly inhibited the transcription factor CCAAT/enhancer-binding protein- α (C/EBP- α) and its downstream target PU.1, resulting in transformation of these cells from an activated phenotype into a quiescent CD45^{low}, major histocompatibility complex (MHC) class II^{low} phenotype resembling resting microglia. During experimental autoimmune encephalomyelitis (EAE), miR-124 was downregulated in activated microglia. Peripheral administration of miR-124 in EAE caused systemic deactivation of macrophages, reduced activation of myelin-specific T cells and marked suppression of disease. Conversely, knockdown of miR-124 in microglia and macrophages resulted in activation of these cells *in vitro* and *in vivo*. These findings identify miR-124 both as a key regulator of microglia quiescence in the central nervous system and as a previously unknown modulator of monocyte and macrophage activation.

MicroRNAs (miRNAs) belong to a family of small non-protein-coding RNAs that regulate expression of multiple target genes and are involved in many fundamental biological processes, such as embryonic development, cell proliferation, differentiation and apoptosis^{1–5}. miRNAs promote degradation of mRNA or prevent translation of the target genes, and they can be viewed as endogenous mediators of RNA interference (RNAi)¹. miRNAs have been identified as crucial regulators of differentiation of various cell types,

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Correspondence should be addressed to H.L.W. (hweiner@rics.bwh.harvard.edu) and A.M.K. (akrichevsky@rics.bwh.harvard.edu).

³These authors contributed equally to this work.

AUTHOR CONTRIBUTIONS

E.D.P. performed all flow-cytometry assays, EAE experiments, experiments with chimeric, knockout and transgenic mice, *in vivo* injections of oligonucleotides, cell isolations, cell cultures, coculture assays and immunohistochemistry; collected and analyzed the data; and wrote the manuscript. T.V. performed *in vitro* transfections, miRNA and mRNA expression assays and data analysis, western blots, luciferase target validation assay, immunohistochemistry and *in silico* target prediction analysis and helped to write the manuscript. N.B. performed imaging cytometry. E.D.P. and A.M.K. conceived the project. E.D.P., T.V. and A.M.K. developed the hypothesis and designed the experiments. A.M.K. and H.L.W. discussed the hypothesis, helped with data interpretation, coordinated and directed the project and wrote the manuscript.

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including neuronal cells in the central nervous system (CNS) and myeloid cells in the innate immune system^{2,6}. Furthermore, deregulated expression of specific miRNAs is associated with many pathological processes, including viral infection, heart disease and cancer^{7–10}.

Macrophages are traditionally subdivided into tissue-resident cells (such as microglia in the CNS), which are present in normal tissues, and activated inflammatory peripheral macrophages, which are known to migrate to the site of inflammation during processes such as EAE^{11–16}. EAE is a mouse model of the human disease multiple sclerosis, characterized by autoimmune inflammation of the CNS associated with microglia activation and infiltration of encephalitogenic T cells and leukocytes from the periphery. In contrast to other tissue-specific resident macrophages, microglia show a resting phenotype with low expression of both the pan-leukocyte marker CD45 and MHC class II (CD45^{low}MHC class II^{low}) in the normal CNS, but the mechanisms maintaining this unique phenotype remain unknown. Our goal was to identify miRNAs that are specifically expressed in microglia and contribute to their phenotypes.

We found that miR-124, the most abundant brain-specific miRNA that regulates neuronal differentiation during CNS development and adult neurogenesis^{17–20}, is also expressed in microglia. Here we show that miR-124 acts both as a key regulator of microglia quiescence in the CNS and as a new modulator of monocyte and macrophage activation in the periphery during EAE.

RESULTS

miR-124 is highly expressed by CNS-resident microglia

To identify miRNAs that are enriched in microglia, we selected 31 miRNAs known to be expressed in immune cells and in the CNS²¹ and analyzed their expression in sorted CD11b⁺F4/80⁺ macrophages from different organs of healthy adult mice by real-time quantitative RT-PCR (qRT-PCR; Supplementary Data and Supplementary Table 1). This analysis revealed that only resident macrophages from brain and spinal cord (microglia) expressed miR-124 (Fig. 1a), whereas myeloid-specific miR-223 (refs. ^{6,22}) was expressed in the macrophages in the periphery, but not in the microglia (Fig. 1b). The level of miR-124 expression in microglia was comparable to that in cultured cortical neurons (Supplementary Data and Supplementary Fig. 1), the cells expressing the highest miR-124 levels^{21,23,24}.

We next investigated expression of specific miRNAs, including miR-124, in microglia during EAE, a disease characterized by microglia activation and infiltration of peripheral macrophages into the CNS. As there are no markers to distinguish microglia from peripheral macrophages that migrate into the CNS during inflammation, we used chimeric mice to discriminate between these populations. In these chimeras, microglia and peripheral macrophages can be separated easily by FACS (Fig. 1c, Supplementary Data and Supplementary Fig. 2). In this study, we refer to cells expressing CD11b⁺F4/80⁺ as ‘macrophages’ even though they may include small numbers of CD11b⁺CD11c⁺F4/80⁺ myeloid dendritic cells. Peripheral macrophages may also be contaminated by small numbers of GFP⁺ microglia that enter the CNS during reconstitution¹⁴. We measured miR-124 expression in sorted populations of CD11b⁺F4/80⁺GFP[−] microglia and CD11b⁺F4/80⁺GFP⁺ CNS-infiltrating peripheral macrophages of healthy chimeras and chimeric mice with EAE at different stages of disease (Fig. 1d, Supplementary Data and Supplementary Table 1). In microglia, miR-124 expression decreased by ~70% during the course of the disease, compared to healthy chimeras (Fig. 1d). Peripheral macrophages, however, began to express low levels of miR-124 during onset and in the recovery phase (Fig. 1d, days 14 and 40). These results demonstrate that miR-124 is strongly expressed in microglia both in the normal CNS and during EAE; it is undetectable in macrophages in the

periphery but is slightly induced in CNS-infiltrating peripheral macrophages during the onset and recovery phases of the disease.

Activated microglia downregulate miR-124

In our model, microglia in healthy chimeric mice had a CD45^{low}MHC class II⁻ phenotype, whereas during the onset and peak of EAE, microglia upregulated CD45 and MHC class II (Fig. 2a). During recovery, the level of MHC class II in microglia returned to normal, but a subset of cells remained CD45^{hi}, suggesting some residual level of activation. Peripheral macrophages in the CNS of healthy mice showed an activated CD45^{hi}MHC class II^{hi} phenotype, which was consistent with previous studies²⁵. Most of the peripheral macrophages that infiltrated the CNS during EAE onset and at the peak of disease also showed an activated CD45^{hi}MHC class II⁺ phenotype (Fig. 2a). During onset of EAE, ~40% of peripheral macrophages showed a partially deactivated MHC class II⁻ phenotype, whereas during recovery, ~40% of peripheral macrophages became CD45^{low}MHC class II⁻ (Fig. 2a). This suggests further deactivation, which is in agreement with recent studies²⁶. The miR-124 expression pattern (downregulation in microglia at EAE onset when the cells become activated, and upregulation in peripheral macrophages at EAE onset and recovery when the subset of cells deactivates) suggests a link between miR-124 and the activation state of microglia and macrophages (Figs. 1 and 2).

We therefore further compared the levels of miR-124 expression in the following four populations sorted from the CNS of mice with EAE (onset of disease): resting CD45^{low}MHC class II⁻ microglia, activated CD45^{int-hi} MHC class II⁺ microglia (MHC class II⁺ microglia have an intermediate or high level of CD45 expression), activated CD45^{hi}MHC class II⁺ peripheral macrophages, and deactivated CD45 MHC class II⁻ peripheral macrophages (MHC class II⁻ peripheral macrophages have an intermediate or high level of CD45 expression). The highest level of miR-124 expression was observed in resting CD45^{low}MHC class II⁻ microglia, whereas activated MHC class II⁺ microglia expressed a 60% lower level of miR-124 (Fig. 2b). Activated MHC class II⁺ peripheral macrophages expressed very low levels of miR-124, whereas deactivated MHC class II⁻ macrophages upregulated miR-124 by 4.2-fold (Fig. 2b). To investigate whether activated microglia downregulate miR-124 *in vitro*, we used microglia isolated from healthy adult mice, culturing them for 6 h in media alone, with the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon- γ (IFN- γ), or with liposaccharide (LPS) and IFN- γ ; these three agents are known to activate microglia. Microglia cultured with GM-CSF and IFN- γ or with LPS and IFN- γ downregulated miR-124 compared to *ex vivo*-isolated cells or cells cultured in media alone (Fig. 2c). During mouse prenatal and postnatal development, microglia showed an activated phenotype with a low level of miR-124 expression (Supplementary Data and Supplementary Fig. 3). Collectively, these data demonstrate that miR-124 expression correlates inversely with the activation state of microglia and macrophages in the CNS.

miR-124 deactivates bone marrow-derived macrophages *in vitro*

In the next series of experiments, we asked whether transfection of peripheral macrophages with miR-124 resulted in downregulation of activation markers. We transfected bone marrow-derived macrophages (BMDMs) with either miR-124 or negative-control RNA (control miRNA). BMDMs transfected with negative control showed an activated phenotype, with high protein expression of surface markers CD45, CD11b, F4/80, MHC class II and CD86. Transfection of BMDMs with miR-124 resulted in downregulation of CD45, CD11b, F4/80, MHC class II and CD86 (Fig. 3a,b).

Transfection of macrophages with miR-124 also inhibited expression of TNF- α (Fig. 3c) and inducible nitric oxide synthase (Fig. 3d) by these cells, and upregulated both the anti-inflammatory cytokine TGF- β 1 (Fig. 3d,e) and markers of alternatively activated (M2) macrophages arginase I and FIZZ1 (Fig. 3d). Transfection with miR-124 also resulted in a reduced number of proliferating cells and altered cell morphology (Supplementary Data and Supplementary Fig. 4). Further analysis of the extent and mechanisms of macrophage transfection is described in **Supplementary Data** (Supplementary Figs. 5 and 6).

miR-124 downregulates C/EBP- α and PU.1

Having found that miR-124 deactivated macrophages (Fig. 3), we investigated the mechanism underlying this effect. Using the TargetScan algorithm²⁷, we performed *in silico* analysis of mRNA targets predicted for miR-124. C/EBP- α , a master transcription factor involved in differentiation of myeloid cells^{28,29}, was predicted as a putative target, with three conserved miR-124 binding sites within its 3' untranslated region (UTR; Fig. 4a). C/EBP- α expression was reduced in miR-124-transfected BMDMs by 50–70% on the protein level (Fig. 4b) and by 50% on the mRNA level (Fig. 3d). Two-color flow cytometry further confirmed that transfection of macrophages with miR-124 resulted in downregulation of both CD45 and C/EBP- α (Fig. 4c), and indicated that the C/EBP- α [−] phenotype corresponded to the CD45^{low} phenotype of macrophages. Expression of the transcription factor PU.1, which is regulated by C/EBP- α and is also required for differentiation of monocytic-lineage cells^{29–31}, was downregulated by miR-124 as well (Fig. 4c). Quantification of the four experiments shows that miR-124 overexpression decreased the proportion of CD45^{hi} C/EBP- α ⁺ and CD45^{hi}PU.1⁺ cells by 70% and 80%, respectively (Fig. 4d).

Next we sought to confirm, using a luciferase reporter system, that miR-124 directly binds the mRNA encoding C/EBP- α and downregulates expression of this protein. The mouse neuroblastoma cell line NIE115, which does not express C/EBP- α and has a low level of endogenous miR-124 expression, was transfected with a construct containing the full-length C/EBP- α 3' UTR sequence downstream of firefly luciferase. The transfected cells showed luciferase activity, which the cotransfection with miR-124 inhibited by 72% (Fig. 4e). Mutations of this reporter construct within two predicted miR-124 binding sites (C/EBP- α mut1–2) substantially reduced the luciferase responsiveness to miR-124, and mutations within all three predicted sites (C/EBP- α mut1–3) nearly abolished it (Fig. 4e). These data confirm that miR-124 directly targets the mRNA encoding C/EBP- α and reduces expression of this protein.

Our experiments showed that miR-124 overexpression causes downregulation of transcription factor PU.1 (Fig. 4c) and activation markers CD45, MHC class II and CD86 (Fig. 3a,b). According to the target-prediction analysis *in silico*, miR-124 does not directly regulate either PU.1 mRNA or mRNAs for activation markers CD45, CD11b, F4/80, MHC class II and CD86. As C/EBP- α binds the promoter region of PU.1 and induces its transcription^{30,31}, we hypothesized that miR-124 downregulates PU.1 indirectly through the inhibition of C/EBP- α . To test this hypothesis, we used mice with a conditional knockout of the *Cebpa* gene. This conditional knockout reduced levels of C/EBP- α protein, which resulted in reduced expression of PU.1, CD11b, MHC class II and CD86 (Supplementary Data and Supplementary Fig. 7). These data suggest that miR-124 controls multiple markers of macrophage activation by direct inhibition of C/EBP- α and its downstream transcription factor PU.1.

miR-124 inhibits EAE and reduces CNS inflammation

As miR-124 expression regulated macrophage activation, we investigated whether systemic administration of miR-124 *in vivo* affects the course of EAE. Administration of miR-124 during the preclinical stage of EAE (beginning on day 7 after disease induction) completely prevented disease symptoms (Fig. 5a). Treatment of mice with miR-124 at the onset of EAE (starting day 13 after EAE induction) also substantially ameliorated clinical symptoms and enhanced recovery (Fig. 5b). These studies were carried out in C57BL/6 mice. We then investigated whether miR-124 suppressed EAE in other strains. Disease symptoms were also substantially ameliorated in both EAE-prone SJL mice as well as in *Ifng*^{-/-} mice, which have a more severe form of EAE (Supplementary Data and Supplementary Fig. 8a,b).

To assess changes in inflammatory responses in mice with EAE treated with miR-124, we sacrificed groups of four or five mice at day 21 (when the control group had peak EAE) and isolated mono-nuclear cells from the CNS and spleens. We detected CD11b⁺CD45^{hi} peripheral macrophages and activated microglia (Fig. 5c, control miRNA, region R2), and CD11b⁺CD45^{hi} leukocytes (Fig. 5c, control miRNA, region R3), in the CNS of mice from the control group. In contrast, the mice treated with miR-124 had primarily CD11b⁺CD45^{low} resting microglia (Fig. 5c, miR-124, region R1) and no signs of microglia activation or leukocyte infiltration in the CNS (Fig. 5c, miR-124, regions R2 and R3). This suggests that peripheral administration of miR-124 suppressed EAE symptoms and leukocyte infiltration in the CNS (Supplementary Data and Supplementary Table 2). Figure 5d shows how the treatment with miR-124 decreased the absolute numbers of lymphocytes, CD4 T cells and macrophages and activated CD45^{hi} microglia in the CNS. Inflammatory lesions containing nucleated and CD11b⁺ cells in the white matter of the lumbar spinal cord were evident in the control group but undetectable in the miR-124-treated mice (Fig. 5e). Thus, treatment of mice with miR-124 ameliorated both EAE symptoms and inflammation in the CNS.

We next assessed whether amelioration of EAE symptoms was due to the reduced level of C/EBP- α expression. Intravenously injected small interfering RNA (siRNA) specific for C/EBP- α had similar effects on disease symptoms, whereas mutant miR-124 lacking the C/EBP- α mRNA binding sequence had no effect on the disease score (Supplementary Data and Supplementary Fig. 9a). Finally, we found that conditional knockout of the *Cebpa* gene also ameliorated EAE (Supplementary Data and Supplementary Fig. 9b). Subsequent experiments showed that peripheral administration of miR-124 during EAE directly affected macrophages and deactivated them, and indirectly reduced the activation of autoimmune CD4 T cells (Supplementary Data, Supplementary Figs. 10–15 and Supplementary Table 3).

Effect of miR-124 inhibitors on microglia and macrophages

To further investigate our hypothesis that expression of miR-124 is required for microglia to maintain the quiescent CD45^{low}MHC class II^{low} phenotype in the normal CNS, we performed miR-124 knockdown experiments *in vivo* and *in vitro*. To knock down miR-124 *in vivo*, we injected a miR-124 antisense oligonucleotide inhibitor (anti-miR-124) intracranially (Supplementary Fig. 16a). When injected into C57BL/6→*Cx3cr1*^{GFP/+} chimeric mice that express GFP in microglia but not in peripheral macrophages, anti-miR-124 induced the activation of CD11b⁺GFP⁺ microglia and altered their morphology (Supplementary Data and Supplementary Fig. 16b–e).

As *ex vivo*-isolated adult microglia are not durable in long-term cultures, *ex vivo* cultures are unsuitable for studying relatively long-term effects of the miRNA inhibitors. We therefore chose a different approach. We hypothesized that macrophages upregulate miR-124 under the influence of the CNS microenvironment by receiving specific signals from CNS stromal cells such as astrocytes and neurons, and thus these macrophages could be used for miR-124

inhibition experiments. To test this hypothesis, we cocultured GFP⁺ BMDMs with either an astroglial or neuronal cell line. In these cocultures, the macrophages acquired a deactivated microglia-like phenotype, as indicated by downregulation of both CD45 and MHC class II (Fig. 6a) and upregulation of miR-124 (Fig. 6b). This coculture system allowed us to investigate whether inhibition of miR-124 affected the expression of MHC class II and CD45 in macrophages. Using the coculture system, we found that transfection of macrophages with anti-miR-124 attenuated the downregulation of MHC class II and CD45 (Fig. 6c–e). It also inhibited the development of the ramified microglia-like morphology in GFP⁺ macrophages cocultured with astroglial and neuronal lines (data not shown). These data demonstrate that miR-124 has a role in maintaining the quiescent phenotype of microglia in the normal CNS, and this phenotype is driven by the paracrine interaction of microglia with local stromal cells.

DISCUSSION

Our investigation provides new insights into the role of CNS-specific miR-124 in the function of normal microglia and the control of macrophage activation during inflammation. We have demonstrated that miR-124 directly inhibits expression of the C/EBP- α transcription factor, which results in downregulation of PU.1, another master regulator of monocytic cell differentiation and decreases expression of the macrophage activation markers CD45, CD11b, F4/80, MHC class II and CD86. It has previously been shown that C/EBP- α directly activates transcription of PU.1 in monocytes³⁰, which, in turn, can induce expression of CD45, CD11b, F4/80 and MHC class II^{32,33}. Moreover, PU.1 controls proliferation of macrophages by upregulating the receptor for M-CSF³⁴. We also found that miR-124 inhibited expression of TNF- α in macrophages. It has previously been shown that the TNF receptor (TNFR1) is a direct downstream target for C/EBP- α and C/EBP- β ³⁵, and that TNF- α and TNFR1 may enhance each other's expression by an autocrine-paracrine mechanism^{36,37}. Normal adult microglia do not express C/EBP- α , but this transcription factor can be induced during inflammation³⁸. In our experiments, activated microglia downregulated miR-124, and high levels of C/EBP- α expression were associated with the activated CD45^{hi} phenotype. As BMDMs with an inactivated *Cebpa* gene closely mimicked the phenotype of BMDMs overexpressing miR-124, we assume that C/EBP- α is the main miR-124 target in the monocytic-lineage cells.

miR-124 is known to be expressed in the CNS by neuronal cells^{23,39–41}. It promotes differentiation of neuronal progenitors to mature neurons by targeting small C-terminal domain phosphatase-1 and polypyrimidine tract-binding protein-1 that repress expression of neuronal genes in non-neuronal cells^{18,20,40}. It has been shown with human HeLa cells and synoviocytes that miR-124 can regulate expression of multiple target genes, including those encoding CDKs and MCP-1 (refs. ^{17,42}). In addition, miR-124 inhibits proliferation and induces differentiation of medulloblastoma and glioblastoma cancer cells, which originate from immature neural cells^{43,44}. We found that miR-124 is also expressed in microglia of the normal CNS, where it has a role in maintaining the cells in a quiescent state, as shown in our *in vitro* and *in vivo* knockdown experiments. Similarly to its function in neural cells, miR-124 may restrict proliferation of microglia and promote differentiation of monocytes and macrophages into adult microglia. Therefore, miR-124 may be viewed as a master regulator of differentiation of various cell types in the CNS during development. In the case of pathological events such as brain cancer or inflammation, when miR-124 is downregulated, treatment with miR-124 may have a beneficial effect by restricting proliferation, inducing differentiation of the immature CNS cells into mature phenotypes and by deactivating macrophages. Indeed, we found that deactivation of macrophages by intravenous injection of miR-124 led to suppression of EAE in three different mouse models of the disease.

Transfection of macrophages with miR-124 resulted in down-regulation of markers and cytokines associated with the phenotype of the classically activated (M1) proinflammatory macrophages CD86, MHC class II, TNF- α and inhibitor of nitric oxide synthase, whereas cytokines and markers associated with the phenotype of alternatively activated (M2) regulatory macrophages—TGF- β 1, arginase I and FIZZ1—were upregulated. These data suggest that miR-124 not only deactivates macrophages but also skews their polarization from an M1 toward an M2 phenotype. These results are consistent with our observations that quiescent microglia show properties of M2 macrophages and that M2 macrophages are crucial for the suppression of EAE⁴⁵. Our extensive analysis showed that *in vivo* administration of miR-124 directly affected macrophages, and this, in turn, resulted in deficient activation of encephalitogenic T cells (Supplementary Discussion).

In summary, our findings demonstrate that miR-124 has a key role in inhibiting macrophage activation and promoting microglial differentiation under physiological conditions in the CNS microenvironment. Furthermore, *in vivo* administration of miR-124 suppresses EAE by affecting macrophages, and this treatment could also be applied to other inflammatory diseases associated with macrophage activation, such as rheumatoid arthritis, type 1 diabetes and atherosclerosis.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemedicine/>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ONLINE METHODS

Mice

C57BL/6, SJL/J, *Ifng*^{-/-} (B6.129S7-*Ifng*^{tm1Ts}/J), CX3CR1-GFP (B6.129P-*Cx3cr1*^{tm1Litt}/J), *Cebpa*^{F/F} (B6.Cg-*Cebpa*^{tm1Dgt} Tg(Mx1-cre)1Cgn/J) and ACTB-GFP transgenic (C57BL/6-Tg(ACTB-EGFP)131Osb/LeySopJ) mice were purchased from Jackson Laboratories. MOG-TCR transgenic 2D2 mice were maintained in our colony. All animal protocols were approved by the Institutional Animal Care and Use Committee of Harvard Medical School.

Antibodies, cytokines and peptides

The fluorochrome-conjugated antibodies specific for CD3, CD4, CD11b, CD11c, CD19, CD25, CD62L, CD69, CD86, IL-6, TNF- α , TGF- β 1 and IFN- γ were purchased from BD Biosciences; CD44, CD205, MHC class II and F4/80 were purchased from eBioscience; CD45R (B220) and CD45 were purchased from BioLegend. Antibodies to C/EBP- α were purchased from Epithomics and antibodies to PU.1 from Cell Signaling Technology. MOG_{35–55} peptide was purchased from Sigma. All cytokines were purchased from R&D Systems.

Macrophage cell culture

Bone marrow was isolated from B6, *Cebpa*^{F/F} or ACTB-GFP Tg mice 4–6-weeks-old; after lysis of erythrocytes, mononuclear cells were incubated with M-CSF (10 ng ml⁻¹) in DMEM medium (American Type Culture Collection) supplemented with 10% (vol/vol) FBS for 5 d. The medium was changed every 2–3 d.

Isolation of mononuclear cells

Mice were perfused intracardially with PBS before the dissection of the brain, spinal cord or liver, which were homogenized; mononuclear cells were isolated using 40%/70% Percoll gradients. Peritoneal macrophages were isolated by peritoneal lavage of intact mice 5 d after a single injection of 2 ml 4% (wt/vol) thioglycolate broth media.

Transfections and injections of miR-124, anti-miR-124 and small interfering RNA

We performed transfections and injections of oligonucleotides as described in Supplementary Methods.

Analysis of miRNA and mRNA expression

For analysis of RNA expression, we carried out real-time qRT-PCR using TaqMan (Applied Biosystems), as described in detail in Supplementary Methods.

Flow cytometry and cell sorting

We performed one- to five-color flow cytometry analysis in the Flow Cytometry sorting core facility of Harvard Medical School according to standard procedures. See Supplementary Methods for more details.

Immunohistochemistry

We assessed the extent of inflammation and demyelination in the CNS on 10- μ m coronal sections of spinal cord that were stained for myelin (Luxol fast blue) or CD11b.

Induction of experimental autoimmune encephalomyelitis

We induced EAE by subcutaneous immunization of 8- to 12-week-old C57BL/6, *Cebpa*^{F/F}, chimeric, SJL or *Ifng*^{-/-} mice with 150 μ g myelin oligodendrocyte glycoprotein (MOG) or proteolipid protein (PLP) in 4 mg ml⁻¹ complete Freund's adjuvant. Pertussis toxin was given intraperitoneally (150 ng per mouse) on days 0 and 2 after immunization. To conditionally knock out the *Cebpa* gene in *Cebpa*^{F/F} mice, animals were injected intraperitoneally with 100 μ g per mouse poly(I:C) (Sigma) at indicated time points. Mice were watched for signs of disease starting on day 5 after transfer, and disease severity was scored on a numerical scale from 0–5 as follows: 0, no disease; 1, weak tail or wobbly walk; 2, hind limb paresis; 3, hind limb paralysis; 4, hind and forelimb paralysis; 5, death or euthanasia for humane reasons.

Irradiation of bone marrow chimeras

C57BL/6 or CX3CR1^{GFP/+} mice (8-weeks-old) were lethally irradiated (950 rads) and reconstituted with 7×10^6 to 10×10^6 total mononuclear BM cells from the CX3CR1^{GFP/+}

or C57BL/6 mice, respectively (CX3CR1^{GFP/+}→C57BL/6 or C57BL/6→CX3CR1^{GFP/+} chimeras). They were allowed to reconstitute for 8 weeks. We determined the percentage of chimerism 8 weeks after transplant by examining cells from the spleen, as described¹⁴. We found that 95–97% of F4/80⁺CD11b⁺ cells in the spleen were GFP⁺ (data not shown). EAE was induced 8–9 weeks after bone marrow transplantation.

Western blot analysis

The cells were lysed, and total cell lysates were resolved on SDS electrophoresis gels by standard procedures. Immunoblotting was performed with mouse primary antibodies to C/EBP- α (Epitomics) and actin (Abcam).

Luciferase reporter assay for target validation

Briefly, mouse neuroblastoma NIE115 cells were transfected with psiCHECK-2 vectors containing wild-type or mutated C/EBP- α 3'-UTR variants and with 50 nM of either miR-124 or negative-control miRNA. The cells were lysed and luciferase activity was measured 48 h after transfection. See Supplementary Methods for more details.

Coculture assay

We obtained BMDMs from ACTB-GFP transgenic mice as described above and cocultured them for 6 d with astrocyte type I C8-D1A cell line or neuroblastoma NIE115 cells (both from American Type Culture Collection) in DMEM media supplemented with 10% (vol/vol) FBS and 10 ng ml⁻¹ macrophage colony-stimulating factor (M-CSF). Neuroblastoma cells were induced to differentiate to neuronal-like cells by treatment with retinoic acid (10 μ M, Sigma) in DMEM media with 10% (vol/vol) FBS for 3 d before coculture. After treatment, media was replaced two or three times to remove retinoic acid before addition of macrophages for coculture.

Statistical analysis

We used Student's *t*-test to validate the significance of the observed differences. *P* < 0.05 was considered statistically significant.

Additional methods

Detailed methodology is described in the Supplementary Methods.

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Figure 1.

Analysis of expression of miR-124 in CNS-resident microglia compared to peripheral macrophages. **(a,b)** Real-time qRT-PCR analysis of miR-124 **(a)** and miR-223 **(b)** expression in F4/80⁺CD11b⁺ mononuclear cells isolated from different tissues of healthy adult C57BL/6 mice. Mean $\pm$ s.d. of triplicate wells is shown. The data are representative of three different experiments. BM, bone marrow; Perit., peritoneal cavity; TGM, thioglycolate-elicited inflammatory macrophages. **(c,d)** Analysis of expression of miR-124 in populations of microglia and peripheral macrophages isolated from the CNS of chimeric mice during EAE. **(c)** GFP fluorescence (*x* axis) and staining for CD11b (*y* axis) are shown for mononuclear cells isolated from the CNS of chimeric mice at the peak of EAE (day 21). In **d**, miR-124 expression in populations of F4/80⁺CD11b⁺GFP⁻ microglia **(c, left rectangle)** and F4/80⁺CD11b⁺GFP⁺ peripheral macrophages **(c, right rectangle)** sorted from CNS of healthy chimeric mice (no disease) or chimeras with EAE at the disease onset (day 14), peak (day 21) and recovery phase (day 40). One representative of four experiments is shown.

Figure 2.

Quantitative analysis of expression of miR-124 in activated microglia. **(a)** Analysis of expression of activation markers CD45 and MHC class II in populations of gated F4/80⁺CD11b⁺GFP⁻ microglia (upper row) and F4/80⁺CD11b⁺GFP⁺ peripheral macrophages (lower row) isolated from CNS of healthy chimeric mice (no disease) or chimeric mice with EAE at the disease onset (day 14), peak (day 21) or recovery (day 40). Four to five mice per group were used. The isotype controls are shown in upper left quadrants of contour plots. Percentages of CD45⁺ cells (right quadrants) and MHC class II⁺ cells (upper quadrants) are shown. **(b)** Comparison of miR-124 expression in populations of resting CD45^{low}MHC class II⁻GFP⁻ and of activated CD45^{int-hi}MHC class II⁺GFP⁻ microglia, as well as populations of activated CD45^{hi}MHC class II⁺GFP⁺ and deactivated CD45^{int-hi}MHC class II⁻GFP⁺ peripheral macrophages. The cells were sorted by FACS from the CNS of chimeric mice with EAE at day 14, and miR-124 expression was assessed by real-time qRT-PCR. Representative results from two independent experiments are shown, with mean $\pm$ s.d. of quadruplicate wells plotted; ** $P < 0.01$. **(c)** Analysis of expression of miR-124 in microglia activated *in vitro* by GM-CSF and IFN- γ or LPS and IFN- γ . Microglia cells were isolated from healthy mice and incubated in medium alone, with GM-CSF and IFN- γ , or with LPS and IFN- γ for 6 h, and miR-124 expression was assessed by real-time qRT-PCR. Mean $\pm$ s.d. of triplicate experiments is shown; * $P < 0.05$ compared to *ex vivo*-isolated microglia.

Figure 3.

Analysis of expression of activation markers, pro- and anti-inflammatory cytokines and markers for M2 macrophages in BMDMs with ectopic overexpression of miR-124. **(a,b)** Flow cytometry analysis of the expression of CD45 (*x* axes) and either CD11b, F4/80, MHC class II or CD86 (*y* axes) in BMDMs transfected with miR-124 or control miRNA. Percentages of CD45^{hi} cells (right quadrants) and activation marker-positive cells (upper quadrants) are shown. A representative experiment is shown in **a**, and mean $\pm$ s.e.m. of four independent experiments is shown in **b**. MFI, mean fluorescence intensity. * $P < 0.05$. **(c)** Flow cytometry analysis of TNF- α production by BMDMs transfected with miR-124 or control miRNA. **(d)** Real-time qRT-pPCR analysis of the mRNA expression of C/EBP- α , inducible form of nitric oxide synthase (iNOS), IL-4, IL-10, TGF- β 1, arginase I and FIZZ1 in BMDMs transfected with miR-124 or control miRNA. **(e)** Analysis of TGF- β 1 protein expression. The cells were stained for intracellular TGF- β 1 or for isotype control and analyzed by FACS. Open histograms show TGF- β 1 staining; filled histograms show staining for isotype control. Percentages of TGF- β 1⁺ cells are shown.

Figure 4.

Validation of downstream target genes for miR-124. **(a)** Alignment of three predicted miR-124 binding sites to C/EBP- α 3' UTR is shown for different species (*Mus musculus*, *Homo sapiens*, *Pan troglodytes*, *Macaca mulatta*, *Rattus norvegicus* and *Oryctolagus cuniculus*). **(b)** Western blot analysis of C/EBP- α expression in BMDMs transfected with miR-124 or control miRNA **(c)** Flow cytometry analysis of the expression levels of C/EBP- α and CD45, or PU.1 and CD45 in BMDMs transfected with miR-124 or control miRNA. Percentages of CD45^{hi} C/EBP- α ⁺ and CD45^{hi} PU.1⁺ cells are shown in upper right quadrants. Populations of miR-124-transfected CD45^{low} cells were negative for C/EBP- α and PU.1 expression, as shown in double staining for cell-surface CD45 and intracellular C/EBP- α or PU.1 (lower left quadrants). Staining for CD45 (x axes) and either C/EBP- α , PU.1 or corresponding isotype controls (y axes) are shown. **(d)** The mean $\pm$ s.e.m. of percentages of CD45^{hi} C/EBP- α ⁺ and CD45^{hi} PU.1⁺ cells from four independent experiments. $**P < 0.01$. **(e)** Luciferase activity in NIE115 cells transfected with reporter constructs containing either intact or mutated C/EBP- α 3' UTR. The NIE115 cell line was co-transfected with the indicated constructs and either miR-124 or control miRNA, and normalized levels of luciferase activity are shown.

Figure 5.

Flow cytometry and histology analysis of extent of inflammation and demyelination in the CNS of mice treated with miR-124 or control miRNA. **(a,b)** EAE disease course (scored as described in Online Methods) in mice treated with miR-124. Mice with EAE were injected intravenously (i.v.) with miR-124 or control miRNA on days 7, 11, 15 and 18 **(a)** or days 13, 16, 18, 20 and 22 **(b)** after EAE induction, as indicated by arrows. The data represent average disease scores from three experiments with four or five mice per group. **(c)** Flow cytometry analysis of immune cell infiltrate in the CNS of mice with EAE treated with miR-124 or control miRNA. Mononuclear cells were isolated from the CNS on day 21 after induction of EAE, stained for CD11b and CD45 and analyzed by FACS. Percentages of resting CD11b⁺CD45^{low} microglia (region R1), CD11b⁺CD45^{hi} activated microglia and peripheral macrophages (R2) and CD11b⁻CD45^{hi} lymphocytes (R3) are shown. **(d)** Quantification of absolute number of activated microglia and macrophages (CD11b⁺CD45^{hi}), lymphocytes and CD4 T cells in the CNS of mice treated with either miR-124 or control miRNA. Mean $\pm$ s.e.m. of three independent experiments is shown. **(e)** Histology analysis of extent of inflammation and demyelination in the spinal cords of mice with EAE treated with miR-124 or control miRNA. Spinal cords were harvested on day 21 after induction of EAE, and sections of spinal cord were stained for myelin or CD11b. Each panel shows a representative histopathology image (scale bar, 200 μ m); three mice were analyzed. Myelin sheath is stained light blue and nucleated cells are stained dark blue (left images). The cells colored dark gray are positive for CD11b (right images).

Figure 6.

Effect of miR-124 inhibitor on phenotype of macrophages cocultured with neural and astroglial cells. **(a)** Flow cytometry analysis of the expression of CD45 (*x* axis) and MHC class II (*y* axis) in populations of CD11b⁺GFP⁺ gated BMDMs cultured alone (left contour plot) or cocultured with an astroglial (middle contour plot) or neuronal (right contour plot) cell line. Percentages of CD45^{hi}MHC class II⁺ cells are shown in upper right quadrants. BMDMs were isolated from ACTB-GFP transgenic mice that ubiquitously express GFP under the actin promoter. The cells were then analyzed for the expression of GFP, CD11b, MHC class II and CD45 using four-color flow cytometry. **(b)** qRT-PCR analysis of miR-124 expression in microglia, astroglial and neuronal lines, in BMDMs cultured alone or in CD11b⁺GFP⁺ BMDMs sorted from the cocultures. **(c–e)** BMDMs isolated from ACTB-GFP transgenic mice were cocultured with either an astroglial (**c**) or neuronal (**d**) cell line in the presence of anti-miR-124 or a control antagonist. **(c,d)** CD11b⁺GFP⁺ gated cells were analyzed for expression of CD45 (*x* axis) and MHC class II (*y* axis). Percentages of CD45^{hi}MHC class II⁺ cells are shown in upper right quadrants. **(e)** Data from three independent experiments are summarized, which show mean $\pm$ s.e.m. of percentages of CD45^{hi}MHC class II⁺ macrophages. **P* < 0.05; ***P* < 0.01.