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Subventricular Zone Microglia Transcriptional Networks

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Abstract

Microglia play an important role in inflammatory diseases of the central nervous system. There is evidence of microglial diversity with distinct phenotypes exhibiting either neuroprotection and repair or neurotoxicity. However the precise molecular mechanisms underlying this diversity are still unknown.

Using a model of experimental autoimmune encephalomyelitis (EAE) we performed transcriptional profiling of isolated subventricular zone microglia from the acute and chronic disease phases of EAE. We found that microglia exhibit disease phase specific gene expression signatures, that correspond to unique gene ontology functions and genomic networks. Our data demonstrate for the first time, distinct transcriptional networks of microglia activation in vivo, that suggests a role as mediators of injury or repair.

Introduction

The adult mammalian brain harbors neurogenic stem cells only within specialized compartments or niches (Doetsch, 2003), such as the subventricular zone (SVZ) of the lateral wall or the subgranular zone (SGZ) of the hippocampal dentate gyrus. These areas are considered repositories of stem cell activity and plasticity, engage in tissue homeostasis (Carleton et al., 2003; Kohwi et al., 2005; Kosaka et al., 1995), and promote repair after CNS injury (Corti et al., 2005; Imitola et al., 2004; Miller et al., 2005; Ohab et al., 2006; Robin et al., 2006).

During the course of experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis (MS), neural stem cells (NSCs) in the SVZ show increased cellular activation during the acute phase (Rasmussen et al., In Press) followed by significant decrease in proliferation and migration during the chronic phase of EAE (Pluchino et al., 2008a). Furthermore, alterations of the endogenous NSC compartment are not cell-autonomous but dependent on the SVZ microenvironment (Pluchino et al., 2008b; Rasmussen, 2010, in press).

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Conflict of Interest Statement

All Authors declare that there are no conflicts of interests.

The SVZ niche area is heterogeneous and in addition to stem/progenitor cells, includes endothelial cells, that are considered bona fide niche cells that secrete products enabling stem cells to survive and maintain their identity (Shen et al., 2004).

We have recently demonstrated that microglia populate the SVZ and proliferate during the acute phase of EAE showing marked activation, and remain activated during chronic disease (Rasmussen et al., 2007) suggesting that microglia can influence the SVZ microenvironment and impact the NSC compartment during EAE. We further showed that inactivating the microglia results in recovery of progenitor cell proliferation and enhanced repair (Rasmussen, 2010, in press). In order to investigate whether microglia cells play a beneficial or detrimental role in EAE and particularly on the neural stem cell microenvironment during the course of EAE, we analyzed gene expression of isolated, flow sorted SVZ resident microglia during acute and chronic EAE followed by Gene Ontology (GO) and system-level network analysis. We identified independent genomic signatures of microglia from acute and chronic EAE: microglia display different sets of signature genes that are associated with a distinct impact on the SVZ microenvironment.

Material and Methods

Animals and EAE Induction

Female SJL/J were purchased from Jackson Laboratories Inc. (Bar Harbor, ME). Mice were housed in conventional, pathogen-free facility at the New Research Building, Harvard Medical School (Boston, MA). For induction of EAE, SJL/J mice were immunized with 150µg of PLP139-151 (New England Peptide LLC, Gardener, MA, USA) as described previously (Rasmussen et al., 2007). Clinical disease was assessed according to the following score: 0, no disease; 1, loss of tail tone; 1.5, poor righting ability; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb paralysis and fore limb weakness; 5, moribund.

The mice were sacrificed at different time points according to the disease phase and clinical score. Acute EAE was defined as peak of disease (around 13 days post immunization, dpi) when mice reached a minimal clinical score of 2; EAE was considered chronic after the first relapse (between 50-60 dpi) when mice reached a minimal score of 1.5. Healthy control mice were sacrificed at least 10 days after immunization with CFA followed by PT.

All mice were housed according to National Institutes of Health guidelines and all experiments were done with the approval of the Animal Care Committee of Harvard University.

Microglia isolation of Subventricular Zone (SVZ) tissue

Mice were deeply anesthetized in a CO₂ chamber and transcardially perfused with 30ml PBS. Subventricular zone tissue was dissected from a 2mm block containing the lateral ventricle and the ventricular wall. The tissue was cut into pieces and gently digested using the papain containing Neural Tissue Dissociation Kit (Miltenyi Biotec) according to the manufacturer's instructions. Mononuclear cells were isolated by percoll gradient (70%/37%) centrifugation and removed from the interphase, washed and resuspended in PBS containing 2% fetal bovine serum (FBS).

Mononuclear cells from HC, acute and chronic EAE SVZ tissue were labeled with FITC-conjugated anti-CD11b and APC -conjugated CD45 and sorted into a CD11b⁺/CD45^{lo} microglia population using a FACS Aria sorter (BD Bioscience). Microglia cells were lysed and total RNA samples were extracted employing TRIzol (Invitrogen) followed by RNeasy Mini Elute Cleanup (Qiagen).

Microarray analysis and statistical analysis

For microarray analysis, a total of 120 mice were sacrificed to isolate microglia. The SVZ area of 20 mice per group was pooled to sort microglia resulting in one RNA sample per group. Two independently isolated RNA samples per group were submitted for microarray analysis.

RNA integrity was confirmed using the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) and total RNA samples were then labeled and hybridized to Mouse Genome 430 2.0 Arrays (Affymetrix), employing standardized protocols and reagents from Affymetrix. Microarray data from biological replicates were combined and normalized using Bioconductor R. Student's t-test was used to test for significant differences between gene expression levels of acute EAE vs. control samples, chronic EAE vs. control samples and acute EAE vs. chronic EAE samples. A correction for the false discovery rate (FDR) was not used in this pair-wise comparison. Only genes that were significantly modulated (p value <0.05) and passed a fold change of $FC > 2.0$ were implemented in differential gene expression signatures and considered for further analysis.

Gene Ontology, canonical pathway and functional network analysis

Gene Ontology, canonical pathway and functional network analysis were executed using Ingenuity Pathways Analysis (IPA) tools (Ingenuity Systems, Mountain View, CA). IPA is a curated database of previously published findings on mammalian biology from the public literature. Reports on individual studies of genes in human, mouse or rat were first identified from peer-reviewed publications, and findings were then encoded into an ontology by content and modeling experts.

Each clone identifier was mapped to its corresponding gene in the Ingenuity pathway knowledge base. This knowledge base of pathway interactions is scientifically accurate, semantically consistent, contextually rich, broad in coverage, and up-to-date¹.

Location mapping and functional analysis were then applied on the so-called focus genes, to identify cellular location and biological functions and/or diseases that were algorithmically significant (Fischer's exact test) to the data set. For the generation of functional networks, focus genes were used as a starting point. A score was computed for each network, that reflects the negative logarithm of the P that indicated the likelihood of the focus genes in a network being found together due to random chance, where only networks of a score >10 were considered as biologically relevant.

Quantitative real time RT-PCR

Quantitative real-time reverse transcription (RT) PCR, total RNA samples were subjected to cDNA generation using the Applied Biosystems high capacity cDNA Reverse Transcription Kit. Samples were then subjected to real-time PCR analysis on an ABI 7500 system (Applied Biosystems) under fast PCR conditions. Genes analyzed were detected using commercially available assays (Applied Biosystems). Relative mRNA level was normalized against GAPDH. Gene expression levels resulting from RT-PCR were compared to gene expression levels from microarray to validate the microarray data, where the following genes were picked for validation according to their differential expression or biological relevance to the set: C3, il18bp, lgals1, thra1, insl6, jag1.

¹<http://www.ingenuity.com/products/PathwayKnowledge.pdf>.

Co-immunostaining and Confocal microscopy and Histology

At the appropriate time points, mice were deeply anesthetized in a CO₂ chamber and then transcardially perfused with cold PBS. Brains were removed and snap frozen in liquid Nitrogen. Tissues were then stored in -80°C until further use.

Tissues were cut into sections of 25µm thickness in a freezing microtome. For immunostaining, sections containing the SVZ area were fixed with 4% PFA for 10 min, washed with PBS for 15 min, blocked with 8% horse serum in PBS for 1 hours and then incubated over 2 nights with the following antibodies: rat anti CD11b (1:50, BD Biosciences), rabbit anti iba1 (1:200, abcam), goat anti Jag1 (1:50, Santa Cruz Biotechnology) rat anti C3 (1:100, abcam), mouse anti I18BP (1:100, Stressgen), rabbit anti Galectin-1 (1:100, kindly provided by Dr. GA Rabinovich), goat anti INSL6 (1:50, Santa Cruz Biotechnology) and rabbit anti Htra1 (1:50, abcam). Sections were rinsed and incubated for 1 h with the appropriate Alexa Flour 488 and 594 secondary antibodies (1:500, Molecular Probes) and mounted on coverslips. Negative control sections for each animal were treated identically, except that the primary antibodies were omitted. The SVZ region was analyzed with a confocal microscope (LSM 510 Laser Scanning Microscope and LSM 3D analysis software, Linuz, Ogdensburg, NY).

For histological assessment, 20µm thick frozen brain sections were stained with hematoxylin and eosin (H&E).

Results

Genomic signature of microglia in the acute and chronically inflamed SVZ

SJL/J mice immunized with PLP 130-151 develop relapsing-remitting EAE with a peak of disease at around day 14 post immunization (dpi) followed by a remission and then a relapse (Rasmussen et al., 2007). Mice enter the phase of chronic inflammation at around 50-55 days post immunization, characterized by only minor changes in clinical disease severity (Fig. 1) Our group has reported that microglia activation persists through the chronic phase of EAE (Rasmussen et al., 2007), and that microglia activation in the subventricular zone (SVZ) correlates with alterations in endogenous stem cell repair mechanism and niche activity (Rasmussen, 2010, in press).

In order to investigate how microglia influence the subventricular zone (SVZ) niche during the course of EAE, we dissected the subventricular zone of mice from acute (n=40) and chronic EAE (n=40) as well as healthy control mice (HC; n=40) that were immunized with Freud's Complete Adjuvant (CFA). We isolated microglia, defined as CD11b⁺/CD45^{lo} expressing cells, by flow cytometric sorting. Microarray profiling was then performed to investigate patterns of gene expression. (Fig. 1)

In the primary analysis, we compared gene expression between CD11b⁺/CD45^{lo} microglia isolated from HC mice and acute or chronic EAE. We found 1414 transcripts that are differentially regulated in EAE SVZ microglia. Among these, several genes were shared between acute and chronic EAE but were not expressed among control SVZ microglia (fold change (FC) of acute vs. HC >2 or chronic vs. HC >2). Despite being expressed both in acute and chronic EAE many genes were significantly enriched in one disease phase more than the other (FC acute vs. chronic >2 or FC chronic vs. acute >2). Genes sharing similar expression pattern between acute and chronic EAE, but different than HC are likely important for microglia functions throughout the whole disease course, whereas genes that are enriched in either acute or chronic EAE are more likely to reflect functions that are disease phase specific. Thus the differentially regulated transcripts converged into a pattern of 1171 transcripts with significant enrichment in acute EAE microglia, 41 transcripts were

upregulated in chronic microglia and 201 transcripts were co-expressed and co-regulated in both acute and chronic EAE (common EAE) (**Venn diagram**, Fig. 2a,b).

The known microglia genes HLA-DQ A1, CD40, Vcam1, IL1b, Cx3cr1, CCL2 and P2×7 were highly abundant, whereas neuronal, astroglial, oligodendroglial and precursor genes were not expressed in the sorted microglia population in HC or EAE, confirming the high purity of the CD11b⁺/CD45^{lo} microglia population. (Suppl. Figure 1). The SVZ did not contain foci of inflammation (Suppl. Fig 1), making it unlikely that we had contaminating peripheral immune cells (Rasmussen et al., 2007)

Gene Ontology analysis

Bioinformatic analysis was performed by uploading the previously identified differentially expressed gene patterns (**Venn Diagramm**, Fig. 2) into the Ingenuity pathway knowledge base (IPA). Transcripts were clustered according to the reported subcellular location of the transcripts' gene products (Fig. 3a). We were interested in how microglia influence the stem cell niche, so we analyzed transcripts of molecules that are secreted or expressed on the surface of microglia. We found a large number of upregulated transcripts of secreted molecules (86 transcripts in acute EAE, and 11 shared by acute and chronic EAE), and plasma membrane expressed molecules (202 transcripts in acute EAE, and 29 shared between acute and chronic EAE). In contrast, only a small number of such transcripts were downregulated: for secreted molecules 6 transcripts in acute EAE, and 4 shared by acute and chronic EAE were downregulated and for surface expressed molecules 14 transcripts in acute EAE, and 16 shared by acute and chronic EAE were downregulated. In chronic EAE we found only 3 up-regulated and no down-regulated transcripts for secreted gene products, and for membrane expressed products 2 up-regulated transcripts and 2 down-regulated transcripts. Furthermore, we observed a large number of differentially regulated genes whose products have a nuclear (134 up-regulated molecules, 29 down-regulated molecules) and cytoplasmic location (209 up-regulated molecules, 33 down-regulated molecules) to be specific for the acute phase of EAE. These findings suggest that microglia respond to the inflammatory microenvironment especially during acute disease, but many of these genes revert to normal expression during the chronic phase (Fig. 3a).

To understand the functional significance of the gene expression data, we applied gene ontology (GO) analysis onto data sets of transcripts with subcellular location in plasma membrane and extracellular space in acute, chronic and common EAE signatures using IPA. (Calvano et al., 2005). Biological functions were assigned to each data set by using the knowledge base as a reference set. Using the web-based entry tool IPA, biological functions are assigned to each data set and Fisher's exact tests were then performed to calculate a p-value determining the probability that genes of interest participate in a given biological function, relative to the total number of occurrences of these genes in all functions stored in IPA.

IPA organizes GO terms into three categories ("Molecular Functions", "Physiological Functions" and "Diseases and Disorders") and ranks them according to their p value. The functional profiles of acute EAE, chronic EAE and common microglia gene expression are shown in pie charts in Fig. 3b. Under the category of "Molecular and cellular functions" it can be clearly seen that genes related to "cellular growth and proliferation", "cellular movement" and "cell-to-cell signaling" are predominantly regulated during acute EAE with a fraction of these being regulated during both phases of disease, but very few genes with these functions are differentially regulated during the chronic phase (Suppl. Table 2). This is consistent with the hypothesis that microglia proliferation, migration and interactions occur predominantly during acute disease and with our observation that the largest increase in number of microglia in the SVZ occurs during acute EAE (Rasmussen et al., 2007). More

intriguing, under the categories of “physiological system development” and “diseases and disorders”, one can see that genes related to tissue development and cancer are predominantly regulated in acute EAE (Suppl. Table 1). Brain tumors and neural stem cells share several genes and recently a hypothesis that tumor-derived stem cells originate from a population of endogenous neural stem cells has been proposed (Germano et al., 2010). So the fact that we observed regulation of genes in these categories during the acute and not the chronic phase of EAE, supports our hypothesis that microglia produce niche promoting molecules only during acute disease. Some of the genes listed in Suppl Table 1, such as neurite outgrowth, tubulation of endothelial cells, adhesion of tumor cells, assembly of extracellular matrix, and development of blood vessels were unique to the acute EAE signature and suggest an active role of acute microglia in supporting the SVZ niche.

On the other hand, genes related to inflammation “immunological disease”, “connective tissue disease” and “inflammatory response” are regulated during both phases of EAE (Suppl. Table 3) consistent with the observation that microglia remain activated throughout the disease course (Rasmussen et al., 2007).

Interestingly, signature genes were associated with canonical pathways such as antigen presentation pathway, allograft rejection pathway and graft-versus-host disease signaling in acute and chronic EAE; leukocyte extravasation signaling, atherosclerosis signaling and complement system for genes in acute EAE and reelin signaling, ephrin receptor signaling and phospholipase C signaling in chronic EAE. The latter three pathways are associated with cytoskeleton reorganization. Taken together these results predict that microglia cells display specific canonical pathway phenotypes in EAE with additional distinct phenotypes in acute versus chronic EAE.

Functional Network analysis

To understand how genes associated with different EAE signatures are related, we performed network analysis using Ingenuity Pathway Analysis (IPA) (ingenuity.com). IPA generated networks of 35 genes that are stored in the knowledge base as having protein-protein interactions with the given set of focus genes. Furthermore IPA computed a score, reflecting the relevance of the network to the empirical data sets, which allowed identification of networks with highest importance to the data set. Based on the computed score (score above 10, with a maximum score of 50 and a minimal score of 0) we identified 16 networks in the signature set of acute EAE, 1 in chronic and 2 networks in common EAE to be significant. Additionally, biological functions were calculated and assigned to each network, based on their significance and relevance to the network ($p < 0.05$).

Given its overrepresentation of up- and down-regulated genes and its unique GO functional profile, we focused on acute EAE signature genes.

We identified 2 networks that were most interesting, according to their gene content and assigned GO functions (Fig. 4). The top-scoring network (Fig. 4a, score=45) was associated with the GO functions of “cell-to-cell signaling and interaction”, “tissue development” and “tissue morphology”. Furthermore, it included genes that are known to be associated with the proliferation of oligodendroglial precursors (vitronectin; VTN, (Baron et al., 2002)), neurogenesis (jagged 1; Jag1 (Katoh, 2006)), increase of neurite outgrowth (discoidin domain receptor tyrosine kinase 1 (DDR1) (Bhatt et al., 2000); syndecan 1 (SDC1) (Kiryushko et al., 2006); tissue plasminogen activator (PLAT) (Pardridge, 2007); gap junction protein alpha 1 (GJA1) (Belliveau et al., 2006)) neuritogenesis (tissue plasminogen activator (PLAT) (Jacovina et al., 2001)), axonal growth (SPACR-like 1 (SPARCL1) (Dunbar et al., 2006)) neuroprotection (tissue plasminogen activator (PLAT) (Lu et al., 2002)) and tubulogenesis of endothelial cells (thrombospondin 1 (THBS1) (Sternlicht and

Werb, 2001)). Furthermore, we identified a network that was enriched in complement genes highly over-expressed in acute EAE, such as complement component 3 (C3) (FC=48.85), CD40 (FC=7.66), and vascular endothelial growth factor α (VEGFA) (FC=6.199) and (Fig. 4b). The complement component C3 was among the highly interconnected nodes within this pathway, supporting an important role of microglia in the SVZ niche in acute EAE, since C3a is reported to increase neurogenesis in addition to its role in inflammation (Rahpeymai et al., 2006a). These results suggest that microglia play a neuroprotective and neuroregenerative role during the acute phase by up-regulating non-classical niche supporting factors.

Target gene validation by real-time reverse transcription PCR

Several target genes seemed biologically interesting for their differential expression in acute and chronic EAE as well as their ontological association with cancer or stem cell functions. Furthermore, the products of the identified target genes are secreted in the extracellular space or located in the plasma membrane; *Jag1* (jagged1) promotes proliferation of cancer cell lines (Purow et al., 2005) and tumor cells (Jundt et al., 2002), the complement component 3 (*c3*), which is primarily known for its immune function, has been shown to be a potent inducer of neurogenesis (Rahpeymai et al., 2006b). The gene for IL18bp (Interleukin 18 binding protein) is of interest since IL18bp neutralizes IL18 (Martin and Wesche, 2002) that acts as an inhibitor of neuronal differentiation (Liu et al., 2005). *lgals1* (*Galectin-1*) was shown to promote neural progenitor proliferation in the dentate gyrus (Kajitani et al., 2009), whereas *htral* (HtrA serine peptidase 1) and *insl6* (insulin-like 6) are members of the insulin-like growth factor 2 pathway (IGF-2) that plays a role in stem cell recruitment to the site of injury (Mauney et al.) and in differentiation potential of stem cells (Shao et al., 2008).

In order to confirm the results of the cDNA microarray, real-time RT-PCR analysis was used for validation on the gene and immunostaining of brain sections facilitated validation on the protein level. For real-time RT-PCR, samples of SVZ microglia isolated from healthy control, acute EAE and chronic EAE were used for validation. Several target genes were chosen based on their differential expression in acute and chronic EAE. Figure 5 demonstrates the correlation between expression ratios (Log^2) of PCR samples and microarray data (acute EAE vs HC; chronic EAE vs HC) with a correlation coefficient of 0.78 and a slope of 1.18. For validation on the protein level, frozen brain sections of CFA control, acute EAE and chronic EAE mice were co-immunostained for CD11b as a microglia marker and the proteins of interest (Suppl. Figure 3). As expected we detected co-localization of either CD11b or Iba1 as a microglia marker and Jag1, C3, IL18BP, Galectin-1 and INSL6 in the SVZ area microglia during the acute phase of EAE. Colocalization of Iba1 with IL18BP or C3 was also detected in chronic EAE, but to a lower extend when compared to acute EAE microglia. No colocalization of expression of IL18BP or C3 was detected in CFA control microglia. No colocalization of CD11b and Galectin-1 or Insl6 was detected in chronic or control microglia. Furthermore no Htra1 positive cells were found in the SVZ area of healthy or EAE mice. Overall this result confirms the validity of the microarray study on the gene and protein level.

Discussion

Adult stem cells are viewed as repositories for the repair potential of tissues. Endogenous neural stem cells have the potential of participating in regeneration in neurological diseases. In MS, an inflammatory neurodegenerative disease of the CNS, the clinical disease course usually starts with reversible episodes of neurologic disability (Relapsing Remitting Multiple Sclerosis, RRMS), which later may become progressive with irreversible neurological decline (Trapp and Nave, 2008). In the acute phase of MS and EAE the CNS

may compensate for tissue damage through local repair mechanisms (Trapp et al., 1999), whereas such endogenous repair mechanisms are lost in the chronic phase (Rasmussen et al., In Press) resulting in progression of clinical disability (Li et al., 1998; Trapp and Nave, 2008; Trapp et al., 1999).

Recent studies have revealed that the NSC compartment of the SVZ is highly activated during acute EAE, and loses its capacity for endogenous repair during the chronic phase (Rasmussen, 2010, in press). We found that, the loss of stem cell activity during the chronic phase is reversible when the stem cells are cultured ex-vivo (Pluchino et al., 2008a), suggesting that the niche microenvironment is responsible for the stem cell dysfunction. The microglia are closely associated with the SVZ stem cells in the niche, so we hypothesized that microglia are able to influence the SVZ microenvironment.

There are conflicting reports on the effect of microglia on NSC function with some reports suggesting a deleterious role (Monje et al., 2003) while others suggest a beneficial role (Butovsky et al., 2006; Thored et al., 2009). Our data suggest that microglia have both beneficial and deleterious roles at different phases of disease, and that manipulation of microglia activation may impact the repair potential in the CNS (Rasmussen, 2010, In Press).

In this manuscript we performed a detailed analysis of microglia transcriptome by direct isolation of CD11b⁺/CD45^{lo} microglia from the subventricular zone. This analysis provides, for the first time, direct molecular information about the activation of microglia in the stem cell niche during the different phases of disease. The transcriptional profile of microglia showed no contamination with neurons, astroglia, oligodendroglia or progenitors cells (Suppl. Figure 2). However it is possible that the CD11b⁺/CD45^{lo} expressing cells contain microglia as well as peripheral macrophages. We validated some of the regulated genes by quantitative real time PCR.

Our results show that during the acute phase of EAE there are a larger number of up-regulated transcripts than during the chronic phase. Microglia remain activated during the chronic phase as evidenced by sharing inflammation related transcripts with the acute phase, but they lose the expression of genes that are niche supportive. Our data suggest that microglia may actively influence the SVZ microenvironment by secreting molecules in the extracellular space and expressing molecules on the plasma-membrane. During the acute phase of EAE microglia exhibits a dual phenotype: on the one hand microglia support and orchestrate the inflammatory process, while on the other hand they secrete molecules that actively support neural stem cell functions such as self-renewal, differentiation and recruitment to the site of injury. However, chronically activated microglia have predominantly a detrimental role since niche supporting functions do not remain upregulated.

Microglia secreted molecules may lead to the recruitment, adhesion and development of other immune cells, such as monocytes and T cells, but may also affect the neural stem cell niche through molecules that are known to influence adhesion, development and growth of tumor cells and tumor cell lines. Tumor cells and stem cell share many unique functions (Bonnet and Dick, 1997; Germano et al.; Hemmati et al., 2003; Ignatova et al., 2002). Furthermore, upregulation of genes that support cardiovascular functions such as the development of blood vessels and angiogenesis solely during acute EAE suggests a structural impact on the stem cell niche. Functions related to cancer and angiogenesis are thought to influence as self-renewal, differentiation and migration capacity of stem cells (Germano et al.; Hemmati et al., 2003; Ignatova et al., 2002).

We identified genes such as Jag1, C3 or vascular endothelial growth factor alpha (VEGF α) that affect stem cell functions. The transmembrane type notch ligand, Jag1 induces self-renewal in neural progenitors through the notch signaling pathway (Artavanis-Tsakonas et al., 1999; Chojnacki et al., 2003; Katoh, 2006; Nagao et al., 2007). In addition to its immunological functions, the complement component C3 is known to induce neurogenesis and migration of neural progenitors in vivo and in vitro (Rahpeymai et al., 2006b; Shinjyo et al., 2009), while VEGF α induces proliferation of neural (Daniel and Abrahamson, 2000; Guo et al.; Jin et al., 2002; Serini and Bussolino, 2004) and glial progenitors (Kim et al., 2009) as well as neurogenesis (Kim et al., 2006).

These findings are of great interest since they open the door for indentifying new therapeutic targets for chronically activated microglia, which are associated with neuronal and progenitor dysfunction (Rasmussen et al., In Press; Rasmussen et al., 2007; Stark et al., 2007).

Taken together our result demonstrate for the first time that during inflammatory CNS disease, microglia display distinct genomic signatures for different disease phases and may provide strong stem cell support during the acute phase of EAE that does not persist during the chronic phase of EAE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science*. 1999; 284:770–776. [PubMed: 10221902]
- Baron W, Shattil SJ, French-Constant C. The oligodendrocyte precursor mitogen PDGF stimulates proliferation by activation of $\alpha(v)\beta_3$ integrins. *EMBO J*. 2002; 21:1957–1966. [PubMed: 11953315]
- Belliveau DJ, Bani-Yaghoob M, McGirr B, Naus CC, Rushlow WJ. Enhanced neurite outgrowth in PC12 cells mediated by connexin hemichannels and ATP. *J Biol Chem*. 2006; 281:20920–20931. [PubMed: 16731531]
- Bhatt RS, Tomoda T, Fang Y, Hatten ME. Discoidin domain receptor 1 functions in axon extension of cerebellar granule neurons. *Genes Dev*. 2000; 14:2216–2228. [PubMed: 10970885]
- Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med*. 1997; 3:730–737. [PubMed: 9212098]
- Butovsky O, Landa G, Kunis G, Ziv Y, Avidan H, Greenberg N, Schwartz A, Smirnov I, Pollack A, Jung S, Schwartz M. Induction and blockage of oligodendrogenesis by differently activated microglia in an animal model of multiple sclerosis. *The Journal of clinical investigation*. 2006; 116:905–915. [PubMed: 16557302]
- Calvano SE, Xiao W, Richards DR, Felciano RM, Baker HV, Cho RJ, Chen RO, Brownstein BH, Cobb JP, Tschoeke SK, Miller-Graziano C, Moldawer LL, Mindrinos MN, Davis RW, Tompkins RG, Lowry SF. A network-based analysis of systemic inflammation in humans. *Nature*. 2005; 437:1032–1037. [PubMed: 16136080]

- Carleton A, Petreanu LT, Lansford R, Alvarez-Buylla A, Lledo PM. Becoming a new neuron in the adult olfactory bulb. *Nat Neurosci.* 2003; 6:507–518. [PubMed: 12704391]
- Chojnacki A, Shimazaki T, Gregg C, Weinmaster G, Weiss S. Glycoprotein 130 signaling regulates Notch1 expression and activation in the self-renewal of mammalian forebrain neural stem cells. *J Neurosci.* 2003; 23:1730–1741. [PubMed: 12629177]
- Corti S, Locatelli F, Papadimitriou D, Donadoni C, Del Bo R, Fortunato F, Strazzer S, Salani S, Bresolin N, Comi GP. Multipotentiality, homing properties, and pyramidal neurogenesis of CNS-derived LeX(ssea-1)+/CXCR4+ stem cells. *FASEB J.* 2005; 19:1860–1862. [PubMed: 16150803]
- Daniel TO, Abrahamson D. Endothelial signal integration in vascular assembly. *Annu Rev Physiol.* 2000; 62:649–671. [PubMed: 10845106]
- Doetsch F. The glial identity of neural stem cells. *Nat Neurosci.* 2003; 6:1127–1134. [PubMed: 14583753]
- Dunbar GL, Sandstrom MI, Rossignol J, Lescaudron L. Neurotrophic enhancers as therapy for behavioral deficits in rodent models of Huntington's disease: use of gangliosides, substituted pyrimidines, and mesenchymal stem cells. *Behav Cogn Neurosci Rev.* 2006; 5:63–79. [PubMed: 16801683]
- Germano I, Swiss V, Casaccia P. Primary brain tumors, neural stem cell, and brain tumor cancer cells: where is the link? *Neuropharmacology.* 58:903–910. [PubMed: 20045420]
- Germano I, Swiss V, Casaccia P. Primary brain tumors, neural stem cell, and brain tumor cancer cells: where is the link? *Neuropharmacology.* 2010; 58:903–910. [PubMed: 20045420]
- Guo Y, Shi D, Li W, Liang C, Wang H, Ye Z, Hu L, Li Y. Effects of cerebral microvascular endothelial cells and vascular endothelial growth factor on the proliferation and differentiation of NSCs: a comparative study. *Br J Neurosurg.* 24:62–68. [PubMed: 20158355]
- Hemmati HD, Nakano I, Lazareff JA, Masterman-Smith M, Geschwind DH, Bronner-Fraser M, Kornblum HI. Cancerous stem cells can arise from pediatric brain tumors. *Proc Natl Acad Sci U S A.* 2003; 100:15178–15183. [PubMed: 14645703]
- Ignatova TN, Kukekov VG, Laywell ED, Suslov ON, Vrionis FD, Steindler DA. Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. *Glia.* 2002; 39:193–206. [PubMed: 12203386]
- Imitola J, Raddassi K, Park KI, Mueller FJ, Nieto M, Teng YD, Frenkel D, Li J, Sidman RL, Walsh CA, Snyder EY, Khoury SJ. Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1 α /CXC chemokine receptor 4 pathway. *Proc Natl Acad Sci U S A.* 2004; 101:18117–18122. [PubMed: 15608062]
- Jacovina AT, Zhong F, Khazanova E, Lev E, Deora AB, Hajjar KA. Neuritegenesis and the nerve growth factor-induced differentiation of PC-12 cells requires annexin II-mediated plasmin generation. *J Biol Chem.* 2001; 276:49350–49358. [PubMed: 11679580]
- Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA. Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. *Proc Natl Acad Sci U S A.* 2002; 99:11946–11950. [PubMed: 12181492]
- Jundt F, Anagnostopoulos I, Forster R, Mathas S, Stein H, Dorken B. Activated Notch1 signaling promotes tumor cell proliferation and survival in Hodgkin and anaplastic large cell lymphoma. *Blood.* 2002; 99:3398–3403. [PubMed: 11964309]
- Kajitani K, Nomaru H, Ifuku M, Yutsudo N, Dan Y, Miura T, Tsuchimoto D, Sakumi K, Kadoya T, Horie H, Poirier F, Noda M, Nakabeppu Y. Galectin-1 promotes basal and kainate-induced proliferation of neural progenitors in the dentate gyrus of adult mouse hippocampus. *Cell Death Differ.* 2009; 16:417–427. [PubMed: 19008923]
- Katoh M. Notch ligand, JAG1, is evolutionarily conserved target of canonical WNT signaling pathway in progenitor cells. *Int J Mol Med.* 2006; 17:681–685. [PubMed: 16525728]
- Kim BK, Kim SE, Shim JH, Woo DH, Gil JE, Kim SK, Kim JH. Neurogenic effect of vascular endothelial growth factor during germ layer formation of human embryonic stem cells. *FEBS Lett.* 2006; 580:5869–5874. [PubMed: 17027979]
- Kim HM, Hwang DH, Lee JE, Kim SU, Kim BG. Ex vivo VEGF delivery by neural stem cells enhances proliferation of glial progenitors, angiogenesis, and tissue sparing after spinal cord injury. *PLoS One.* 2009; 4:e4987. [PubMed: 19319198]

- Kiryushko D, Novitskaya V, Soroka V, Klingelhofer J, Lukanidin E, Berezin V, Bock E. Molecular mechanisms of Ca(2+) signaling in neurons induced by the S100A4 protein. *Mol Cell Biol*. 2006; 26:3625–3638. [PubMed: 16612001]
- Kohwi M, Osumi N, Rubenstein JL, Alvarez-Buylla A. Pax6 is required for making specific subpopulations of granule and periglomerular neurons in the olfactory bulb. *J Neurosci*. 2005; 25:6997–7003. [PubMed: 16049175]
- Kosaka K, Aika Y, Toida K, Heizmann CW, Hunziker W, Jacobowitz DM, Nagatsu I, Streit P, Visser TJ, Kosaka T. Chemically defined neuron groups and their subpopulations in the glomerular layer of the rat main olfactory bulb. *Neurosci Res*. 1995; 23:73–88. [PubMed: 7501303]
- Li C, Trapp B, Ludwin S, Peterson A, Roder J. Myelin associated glycoprotein modulates glia-axon contact in vivo. *J Neurosci Res*. 1998; 51:210–217. [PubMed: 9469574]
- Liu YP, Lin HI, Tzeng SF. Tumor necrosis factor-alpha and interleukin-18 modulate neuronal cell fate in embryonic neural progenitor culture. *Brain Res*. 2005; 1054:152–158. [PubMed: 16054598]
- Lu W, Bhasin M, Tsirka SE. Involvement of tissue plasminogen activator in onset and effector phases of experimental allergic encephalomyelitis. *J Neurosci*. 2002; 22:10781–10789. [PubMed: 12486171]
- Martin MU, Wesche H. Summary and comparison of the signaling mechanisms of the Toll/interleukin-1 receptor family. *Biochim Biophys Acta*. 2002; 1592:265–280. [PubMed: 12421671]
- Mauney J, Olsen BR, Volloch V. Matrix remodeling stem cell recruitment: A novel in vitro model for homing of human bone marrow stromal cells to the site of injury shows crucial role of extracellular collagen matrix. *Matrix Biol*.
- Miller JT, Bartley JH, Wimborne HJ, Walker AL, Hess DC, Hill WD, Carroll JE. The neuroblast and angioblast chemotactic factor SDF-1 (CXCL12) expression is briefly up regulated by reactive astrocytes in brain following neonatal hypoxic-ischemic injury. *BMC Neurosci*. 2005; 6:63. [PubMed: 16259636]
- Monje ML, Toda H, Palmer TD. Inflammatory blockade restores adult hippocampal neurogenesis. *Science* (New York, N.Y.). 2003; 302:1760–1765.
- Nagao M, Sugimori M, Nakafuku M. Cross talk between notch and growth factor/cytokine signaling pathways in neural stem cells. *Mol Cell Biol*. 2007; 27:3982–3994. [PubMed: 17371842]
- Ohab JJ, Fleming S, Blesch A, Carmichael ST. A neurovascular niche for neurogenesis after stroke. *J Neurosci*. 2006; 26:13007–13016. [PubMed: 17167090]
- Pardridge WM. Blood-brain barrier genomics. *Stroke*. 2007; 38:686–690. [PubMed: 17261716]
- Pluchino S, Muzio L, Imitola J, Deleidi M, Alfaro-Cervello C, Salani G, Porcheri C, Brambilla E, Cavasinni F, Bergamaschi A, Garcia-Verdugo JM, Comi G, Khoury SJ, Martino G. Persistent inflammation alters the function of the endogenous brain stem cell compartment. *Brain*. 2008a; 2564–2578.
- Pluchino S, Muzio L, Imitola J, Deleidi M, Alfaro-Cervello C, Salani G, Porcheri C, Brambilla E, Cavasinni F, Bergamaschi A, Garcia-Verdugo JM, Comi G, Khoury SJ, Martino G. Persistent inflammation alters the function of the endogenous brain stem cell compartment. *Brain*. 2008b; 131:2564–2578. [PubMed: 18757884]
- Purow BW, Haque RM, Noel MW, Su Q, Burdick MJ, Lee J, Sundaresan T, Pastorino S, Park JK, Mikolaenko I, Maric D, Eberhart CG, Fine HA. Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation. *Cancer Res*. 2005; 65:2353–2363. [PubMed: 15781650]
- Rahpeymai Y, Hietala MA, Wilhelmsson U, Fotheringham A, Davies I, Nilsson A-K, Zwirner J, Wetsel RA, Gerard C, Pekny M, Pekna M. Complement: a novel factor in basal and ischemia-induced neurogenesis. *EMBO J*. 2006a; 1364–1374.
- Rahpeymai Y, Hietala MA, Wilhelmsson U, Fotheringham A, Davies I, Nilsson AK, Zwirner J, Wetsel RA, Gerard C, Pekny M, Pekna M. Complement: a novel factor in basal and ischemia-induced neurogenesis. *EMBO J*. 2006b; 25:1364–1374. [PubMed: 16498410]
- Rasmussen S, Imitola J, Ayuso-Sacido A, Wang Y, Starossom SC, Kivisakk P, Zhu B, Meyer M, M. G-VJ, Khoury SJ. *Anna Neurol*. submitted.

- Rasmussen S, Imitola J, Ayuso-Sacido A, Wang Y, Starossom SC, Kivisakk P, Zhu B, Meyer M, M. G-VJ, Khoury SJ. Reversible Neural Stem Cell Niche Dysfunction In A Model Of Multiple Sclerosis. *Anna Neurol*. 2010;1–42. in press.
- Rasmussen S, Wang Y, Kivisakk P, Bronson RT, Meyer M, Imitola J, Khoury SJ. Persistent activation of microglia is associated with neuronal dysfunction of callosal projecting pathways and multiple sclerosis-like lesions in relapsing remitting experimental autoimmune encephalomyelitis. *Brain*. 2007;2816–2829. [PubMed: 17890734]
- Robin AM, Zhang ZG, Wang L, Zhang RL, Katakowski M, Zhang L, Wang Y, Zhang C, Chopp M. Stromal cell-derived factor 1alpha mediates neural progenitor cell motility after focal cerebral ischemia. *J Cereb Blood Flow Metab*. 2006; 26:125–134. [PubMed: 15959456]
- Serini G, Bussolino F. Common cues in vascular and axon guidance. *Physiology (Bethesda)*. 2004; 19:348–354. [PubMed: 15546852]
- Shao M, Bi ZG, Sun G. The in vitro differentiation and the variant expression of protein of bone marrow stromal stem cells when treating the spinal cord injury. *Zhonghua Wai Ke Za Zhi*. 2008; 46:1823–1826. [PubMed: 19094798]
- Shen Q, Goderie SK, Jin L, Karanth N, Sun Y, Abramova N, Vincent P, Pumiglia K, Temple S. Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. *Science*. 2004;1338–1340. [PubMed: 15060285]
- Shinjo N, Stahlberg A, Dragunow M, Pekny M, Pekna M. Complement-derived anaphylatoxin C3a regulates in vitro differentiation and migration of neural progenitor cells. *Stem Cells*. 2009; 27:2824–2832. [PubMed: 19785034]
- Stark A, Lin MF, Kheradpour P, Pedersen JS, Parts L, Carlson JW, Crosby MA, Rasmussen MD, Roy S, Deoras AN, Ruby JG, Brennecke J, Curators HF, Project BDG, Hodges E, Hinrichs AS, Caspi A, Paten B, Park S-W, Han MV, Maeder ML, Polansky BJ, Robson BE, Aerts S, Van Helden J, Hassan B, Gilbert DG, Eastman DA, Rice M, Weir M, Hahn MW, Park Y, Dewey CN, Pachter L, Kent WJ, Haussler D, Lai EC, Bartel DP, Hannon GJ, Kaufman TC, Eisen MB, Clark AG, Smith D, Celniker SE, Gelbart WM, Kellis M, Crosby MA, Matthews BB, Schroeder AJ, Sian Gramates L, St Pierre SE, Roark M, Wiley KL Jr, Kulathinal RJ, Zhang P, Myrick KV, Antone JV, Gelbart WM, Carlson JW, Yu C, Park S, Wan KH, Celniker SE. Discovery of functional elements in 12 *Drosophila* genomes using evolutionary signatures. *Nature*. 2007;219–232. [PubMed: 17994088]
- Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol*. 2001; 17:463–516. [PubMed: 11687497]
- Thored P, Heldmann U, Gomes-Leal W, Gisler R, Darsalia V, Taneera J, Nygren JM, Jacobsen SE, Ekdahl CT, Kokaia Z, Lindvall O. Long-term accumulation of microglia with proneurogenic phenotype concomitant with persistent neurogenesis in adult subventricular zone after stroke. *Glia*. 2009; 57:835–849. [PubMed: 19053043]
- Trapp BD, Nave K-A. Multiple sclerosis: an immune or neurodegenerative disorder? *Annu Rev Neurosci*. 2008;247–269. [PubMed: 18558855]
- Trapp BD, Ransohoff R, Rudick R. Axonal pathology in multiple sclerosis: relationship to neurologic disability. *Curr Opin Neurol*. 1999; 12:295–302. [PubMed: 10499174]

Figure 1. Systematic Strategy of SVZ Microglia isolation and Microarray analysis

Schematic representation microglia isolation from the SVZ and microarray strategy. Microglia were isolated from RR EAE during the acute and chronic disease phase (red arrows) and from CFA injected healthy control mice, followed by dissection of the SVZ with flow cytometry sorting of CD11b/CD45 dim microglia. Subsequent microarray and data analysis was performed after 2 rounds of RNA amplification.

Figure 2. Venn Diagramm

Comparison of the significant genes identified as differentially expressed in acute EAE, chronic EAE and acute and chronic EAE. (a) Identification of 1024, 28 and 111 upregulated genes for acute, chronic and common EAE respectively. (b) Identification of 147, 14 and 90 downregulated genes for acute, chronic and common EAE respectively.

Figure 3. Ontology analysis of differential gene expression in EAE signature

IPA analysis of Gene Ontology. (a) Pie-diagram of the cellular location of genes from each up- and downregulated signature set for acute, chronic and common EAE, with each number representing the number of genes in a given cellular location. (b) Gene Ontology functions of up-regulated genes with location in extracellular space and plasma membrane for acute, chronic and common EAE. Top 5 significant GO functions categorized into “Molecular and Cellular Functions”, Physiological System Development and Functions” and “Diseases and Disorders” are displayed as pie-diagrams with each number representing the number of molecules associated with the given function. (c) Top 3 canonical pathways associated with upregulated genes of secreted and plasma membrane expressed molecules for the signature sets in common EAE, acute EAE, and chronic EAE and their dynamics in each disease phase. Displayed are the significance ($-\log(p\text{-value})$; blue bars; left y-axis) and the ratio (number of signature genes present in the canonical pathway / total number of genes associated with the canonical pathway; orange line; right y-axis). The red lines show the threshold for significance ($-\log(p=0.05)$)

Figure 4. Functional network analysis

(a) Top scoring network for signature genes of secreted and plasma membrane expressed molecules in acute EAE, many of which are involved in neural- or oligodendroglial precursor function, neuroprotection, neurite outgrowth or angiogenesis. (b) High scoring network with an enrichment of complement factors. Nodes represent genes, with their shape representing the functional class of the gene product and edges indicate the biological relationship between the nodes. Genes that were selected as target genes for microarray validation are highlighted with red borders.

Figure 5. Correlation of microarray with RT-PCR

The microarray data was validated by RT-PCR, correlating the Log2 ratio of the microarray genes with the Log2 ratio of given genes as measured by quantitative RT-PCR. The data show a good linear fit with a correlation coefficient of 0.78 and a slope of 1.18.