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14. ABSTRACT Microglia are the innate immune cells of the brain. They share cell lineage with macrophages, which have been divided into two major subgroups: (i) classical or "M1" macrophages, which promote inflammation and express IL-12, and (ii) alternatively activated or "M2" macrophages, which engulf apoptotic cells, promote wound repair, and (in mice) express arginase-1. We proposed that microglia might also reflect these functional subsets and that activation of microglia by TBI would be detrimental to the extent that it involves M1-like (pro-inflammatory) microglia, but beneficial to extent that it involves the activation of M2-like (reparative) microglia. To test this, we studied TBI in "reporter" mice that allow the identification of M1 or M2 cells by expression of IL12p40 or arginase 1, respectively. Following TBI, neither gene was upregulated in microglia, but 1 day after TBI, 20% of macrophages expressed arginase 1, and these declined thereafter. Examination of gene expression in Arg+ and Arg- cells showed large differences in gene expression but neither represented M1 or M2 cells. Our studies demonstrate subpopulations of brain macrophages following TBI that change over time.					
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INTRODUCTION

Prior studies have provided evidence that traumatic brain injury (TBI) may result in extensive activation of microglia, which phagocytose apoptotic neurons and initiate the process of brain repair (1). Activated microglia, however, can also initiate an inflammatory response, extending brain damage (1,2). Our studies sought to define if TBI induces subsets of microglia that may selectively protect against injury or worsen it. This hypothesis is suggested by studies of peripheral macrophages, which have demonstrated at least two major macrophage subtypes, called M1 and M2 (also called “alternatively activated” macrophages) (3,4). M1 macrophages are pro-inflammatory, while M2 macrophages are generally anti-inflammatory. M1 macrophages also promote insulin resistance and obesity, while M2 cells promote the action of insulin (5).

M1 cells express the p40 component of the IL-12 cytokine (IL12p40), while M2 cells are marked by the expression of arginase-1 (at least in mice). The activation of M2 macrophages is promoted by peroxisome proliferator-activated receptor γ (PPAR γ), a nuclear receptor, and activation of PPAR γ has anti-inflammatory effects both in the periphery and the brain (6-8). The related receptor PPAR δ is also important in the activation of M2 cells (9). *In vitro* studies of microglia suggest that they, like macrophages, may be either proinflammatory or phagocytic, and that expression of the surface receptor TREM-2 promotes phagocytosis while suppressing the production of the inflammatory cytokine TNF- α (10).

Based on these findings, we sought to test the central hypothesis that subtypes of microglia regulate the extent of TBI. To test this hypothesis, we have examined both microglia and macrophages in the mouse brain for the expression of markers that define M1 and M2 cells, and we have then tested the effect of TBI for these markers, using a controlled cortical impact model. We have also examined the effects of activating PPARs on these parameters. As will be discussed, the results of our studies have led us to focus more intensely on the macrophages that infiltrate the brain following TBI, and our most important results regard these cells, but we have continued to examine both microglia and macrophages.

Our original Specific Aims were:

1. Determine the development of microglia into M1 and M2 subtypes in response to TBI at multiple timepoints *in vivo* and establish the function of these microglial subtypes *ex vivo*.
2. Skew the microglial response to TBI towards an M2 phenotype using PPAR γ and TREM-2 agonists as treatments given post-TBI.
3. Define the effects of PPAR γ activation with and without and stimulation of TREM-2 on T cell infiltration and neuronal death in response to TBI.

BODY

Our primary approach to our analysis of microglial subsets is the use of reporter cell mice provided by our collaborator, Richard Locksley (UCSF). There are two strains: (i) yet40 mice, which express the fluor YFP under the control of the IL-12p40 promoter, a marker for M1 macrophages, and (ii) YARG mice, which express YFP under the control of the promoter for arginase-1, a marker for M2 macrophages. Microglia were identified by flow cytometry as expressing CD11b together with intermediate levels of CD45, and macrophages were identified as expressing CD11b together with high levels of CD45.

We did not detect expression of either the IL-12p40 promoter or arginase-1 in microglia either at rest or following TBI (results not shown). We did, however, see early expression of arginase-1 in a subset of brain macrophages, with loss of this subset over the first week following TBI, as discussed below. This is the first demonstration of macrophage subsets in the response to TBI, and it led to a series of discoveries that were recently published (reference 12, attached) and are summarized as follows.

TBI causes an increase in brain macrophages, with a peak 4 days after TBI. Following TBI, we found a rapid increase in macrophages in the injured side of the brain. These peaked 4 days after TBI, when the number of macrophages was increased 77-fold over sham controls (Fig 1). Neutrophils rose even faster with a decline by day 4 (Fig. 1). We have also shown that the rise in macrophages is dependent on CCR2, but these results are not presented as part of this report, as they were done with Christine Hsieh, as part of her VA Career Development Award,

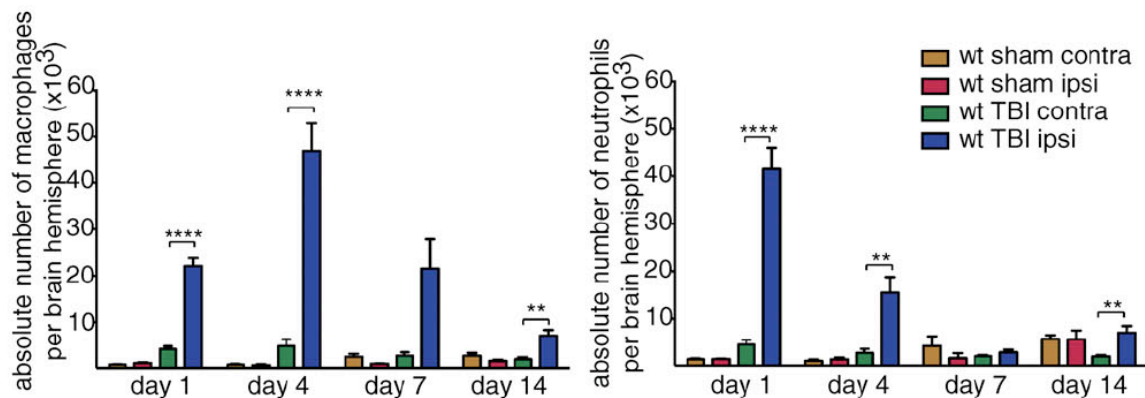


Figure 1. Numbers of macrophages (left panel) and of neutrophils (right panel) in the ipsilateral (injured) or contralateral brain hemisphere following TBI.

Following TBI, a subset of activated macrophages expresses arginase-1. As assessed by the reporter cell mice no macrophages or microglia expressed detectable levels of IL12p40 either in the resting state or following TBI (Fig. 2A, next page, right panels). Microglia also demonstrated little or no expression of arginase-1, either in the resting state or following TBI (Fig. 2A, central panel). Within 1 day following TBI, however, 20-25% of macrophages in the ipsilateral brain expressed arginase-1, as detected by expression of the fluor YFP in YARG mice (Fig. 2A, top row, middle). In contrast, neither microglia nor peripheral blood monocytes expressed detectable levels of arginase-1 (Fig. 2A, middle row and bottom row)

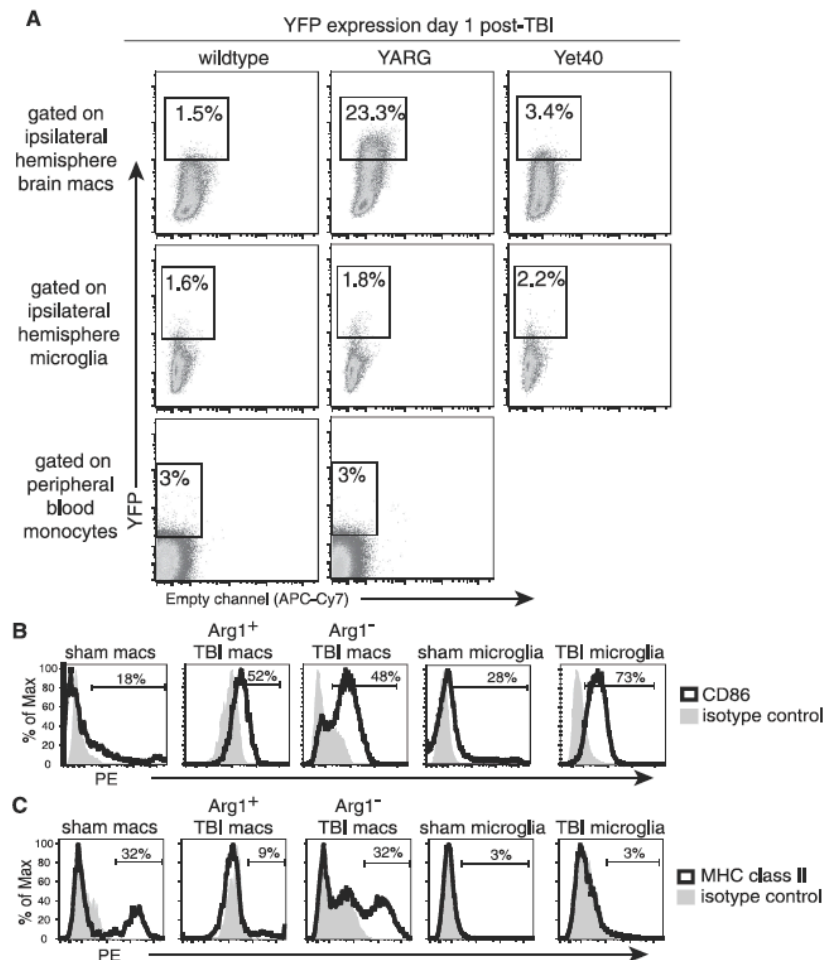


Figure 2. YFP is upregulated in a subset of brain macrophages from YARG mice 1 day post-TBI. (A) Leukocytes isolated from the ipsilateral hemisphere of WT, YARG, and Yet40 mice 1 day after TBI were analyzed for YFP expression in live cells gated on brain macrophages (top), microglia (middle), or peripheral blood monocytes (bottom). YFP expression is set by box gates based on WT controls (left). Data shown are from one experiment that is representative of three experiments with Yet40 mice with controls, and six experiments with YARG mice with controls. (B) Assessment of CD86 expression in Arg1⁺ and Arg1⁻ ipsilateral hemisphere macrophage subsets and in microglia from YARG mice by flow cytometry. Data shown are representative of expression detected at four days post-surgery and are from one experiment representative of three performed. (C) Assessment of MHC class II expression in Arg1⁺ and Arg1⁻ ipsilateral hemisphere macrophage subsets and in microglia from YARG mice. Data shown are representative of expression detected at 4 days-post-surgery and are from one experiment representative of three performed. In these experiments, the background fluorescence for macrophages increases substantially following TBI, because these cells are large and complex.

TBI activates both macrophages and microglia. Following TBI, macrophages were activated, as assessed by expression of CD86 (Fig. 2B). Levels of CD86 were highest on Arg1⁻ macrophages, and these cells also showed an increased level of cells expressing MHC class II antigens, another marker of cell activation (Fig. 2C). Following TBI, both Arg⁺ and Arg⁻

macrophages were large and complex, another measure of cell activation. This is reflected in the increased levels of background fluorescence for these cells (Figs. 2B and 2C). Notably, all microglia also expressed increased levels of CD86, consistent with prior evidence that TBI induces widespread activation of microglia. However, we failed to detect other changes in the expression of surface markers on microglia following TBI, including no change in the expression of type II major histocompatibility antigens (Fig. 2C).

Arg⁺ and Arg¹- cells are similarly distributed in the brain following TBI. To examine the spatial localization of YFP⁺ cells in YARG mice post-TBI, we performed immunofluorescent colabeling for YFP and F4/80 in brain sections days post-TBI, when macrophage infiltration of the brain peaks. (F4/80 detects both microglia and macrophages, and in their activated state these cells cannot readily be distinguished by histology, though from our flow studies, all YFP⁺ cells are macrophages, not microglia). F4/80⁺ macrophages/microglia localized in and around the area of injury (Fig. 3, second row). F4/80 expression was below level of detection by immunofluorescence in sham-injured tissues (data not shown). The Arg¹⁺ cells were scattered among the F4/80⁺ cells in TBI mice (Fig. 3, third row) and were not detectable in the contralateral hemisphere or in sham-treated mice. The majority of the Arg¹⁺ cells costained with F4/80. As suggested from our flow cytometry data in which only a subset of macrophages expresses YFP, the majority of F4/80⁺ cells were Arg¹- (Fig. 3).

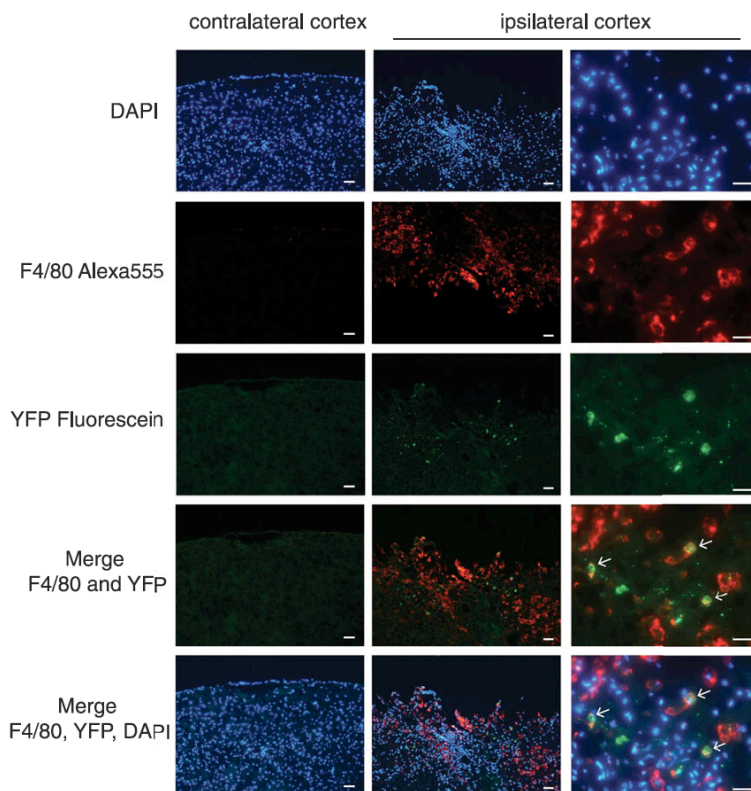


Figure 3. Immunofluorescent microscopy of YFP (green) F4/80 (red) and DAPI (blue) in the cortex of YARG mice 4 days post-TBI. In the first and second columns, the scale bar represents 50 μ m. The top areas of columns 1 and 2, devoid of DAPI staining, are outside the boundary of brain tissue. The third column shows higher magnification of macrophages in the ipsilateral cortex (scale bar 25 μ m). Arrows indicate cells colabeled with anti-YFP and anti-F4/80 antibodies. Data shown are from one experiment and are representative of four experiments performed analyzing three animals, ten sections per animal.

Gene expression by arginase-1-positive and arginase-1-negative macrophages confirmed that these subpopulations differ in the expression of many genes. The expression of YFP by a substantial number of ipsilateral macrophages at day 1 following TBI made it possible for us to

isolate these cells for further study. One day following TBI, microarrays from YFP⁺ and YFP⁻ brain macrophages in YARG mice, were compared to each other and to blood monocytes. In pairwise analyses of differentially expressed genes, both tissue macrophages subsets showed numerous differences from normal blood monocytes, whereas monocytes from injured animals displayed few differences compared to normal monocytes (Fig. 4A, next page). Principal components analysis (PCA) confirmed that distinctions separating macrophages from monocytes was the largest source of variance in the dataset, and that monocytes from injured or normal animals had fewer differences that were not represented in either of the top two principal components (Fig 4B). Scatter plot analysis of gene expression also revealed that a large number of genes (1,360 genes) differed significantly between Arg1⁺ and Arg1⁻ brain macrophages (Fig 4A). PCA demonstrated that Arg1⁺ and Arg1⁻ brain macrophages represented two distinct populations represented in the second most significant principal component (PC2) (Fig 4B).

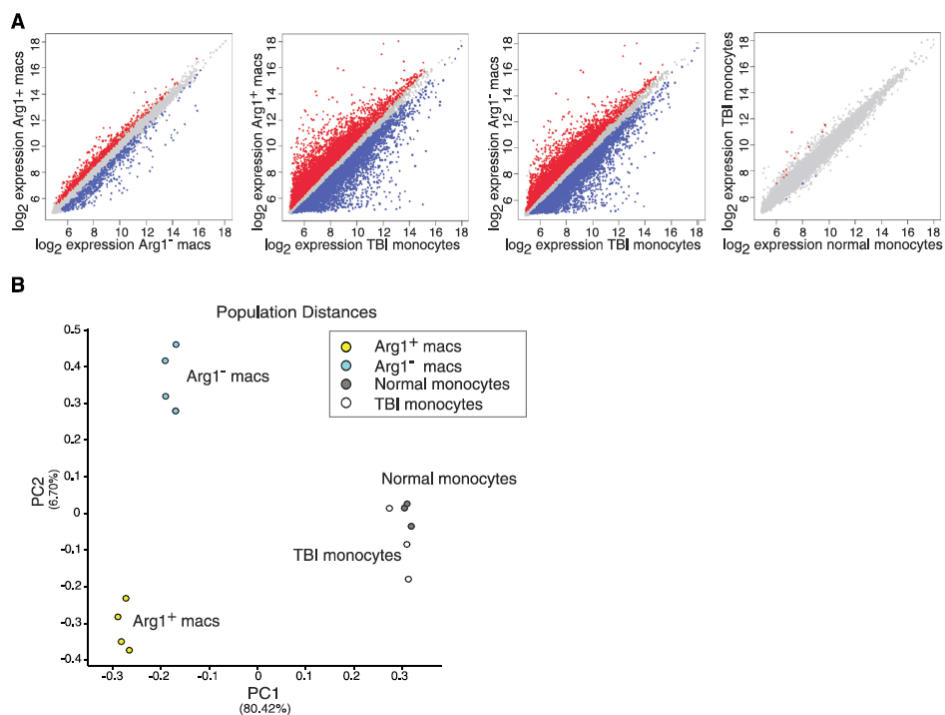


Figure 4. Differences between Arg1⁺ and Arg1⁻ macrophage subsets and peripheral blood monocytes revealed by gene expression profiling. (A) Microarray data comparing gene expression by Arg1⁺ TBI macrophages, Arg1⁻ TBI macrophages, TBI monocytes, and normal monocytes (n = 4 per population) were examined in a pairwise analysis. Red and blue color dots represent genes with significant differences. (B) Principal component analysis using the top 15% most variable genes between all populations analyzed by microarray. The distance between the indicated cell populations is proportionate to their differences in gene expression.

The two subsets of macrophages seen following TBI do not represent M1 or M2 macrophages, but rather are unique cell populations. The gene expression profiles of the YFP⁺ macrophages and the YFP⁻ macrophages indicated that neither cell population represents M1 or M2 cells. The arrays confirmed that the YFP⁺ macrophages expressed high levels of arginase-1, and they also preferentially expressed another marker for M2 cells, *Mrc1*, encoding the mannose receptor/CD206 (Fig. 5, next page). The Arg⁺ macrophages, however, also

preferentially expressed *Nos2*, a gene associated with the M1 phenotype, not the M2 phenotype (Fig. 5). Similarly, although Arg1^- macrophages had increased expression of *Il1b* (interleukin- 1β), an inflammatory cytokine associated with M1 cells, they also preferentially expressed the signature M2 markers, *Retnla* (resistin-like α), and *Clec10a* (C-type lectin domain family 10) (Fig. 5).

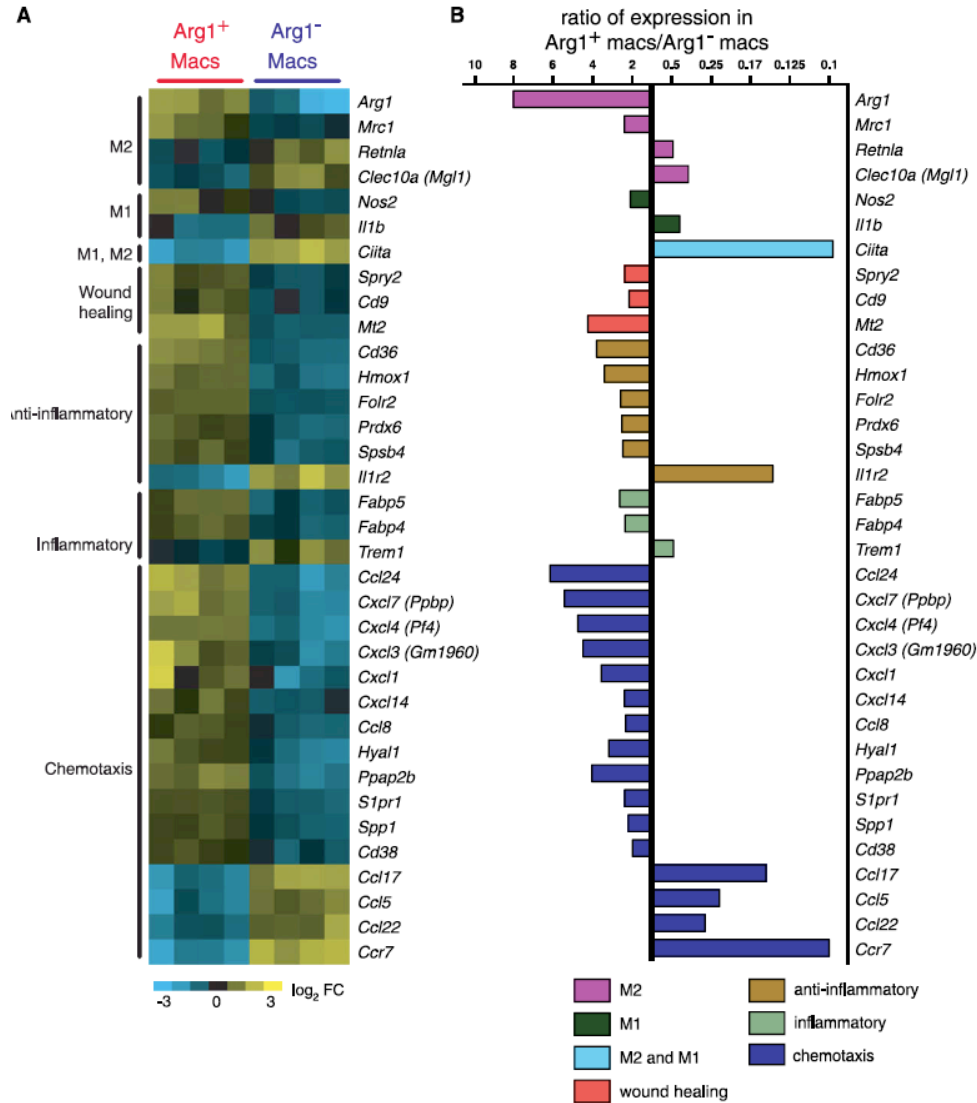


Figure 5. Differences in transcriptional responses between Arg1^+ and Arg1^- brain wound macrophages selecting for genes associated with M1 and M2 polarization and/or with other selected genes involved in macrophage functions. (A) Gene expression analysis comparing Arg1^+ TBI macrophages with Arg1^- TBI macrophages 1 day after injury. Thirty-five selected genes of interest with significant relative differences \geq two-fold are shown. Each column represents a separate experiment. Gene expression was \log_2 transformed and median-centered across genes. Yellow represents a relative increase in expression and blue signifies a relative decrease. Expressed genes are grouped by function. (B) Average gene expression differences from the data in Figure 4A were quantified and ratios of gene expression levels comparing Arg1^+

brain wound macrophages to Arg1⁻ brain wound macrophages are shown, with genes grouped by cell type (M1 or M2) and by function.

We conclude that Arg1⁺ macrophages and Arg1⁻ macrophages have diverse expression of M2 and M1 properties. To further compare these cell populations to classic M1 and M2 cell populations, we performed a meta-analysis of significant genes differentially expressed between Arg1⁺ and Arg1⁻ TBI brain macrophages with significant genes differentially expressed between IFN γ -stimulated or IL4-stimulated bone marrow-derived macrophages (BMDM), using previously published tables (13). Arg1⁺ and Arg1⁻ macrophages each upregulated genes that were induced in BMDM by either IFN γ or IL-4, supporting the notion that Arg1⁺ and Arg1⁻ TBI brain macrophage subsets have complex expression profiles with a mixed M1 and M2 phenotype.

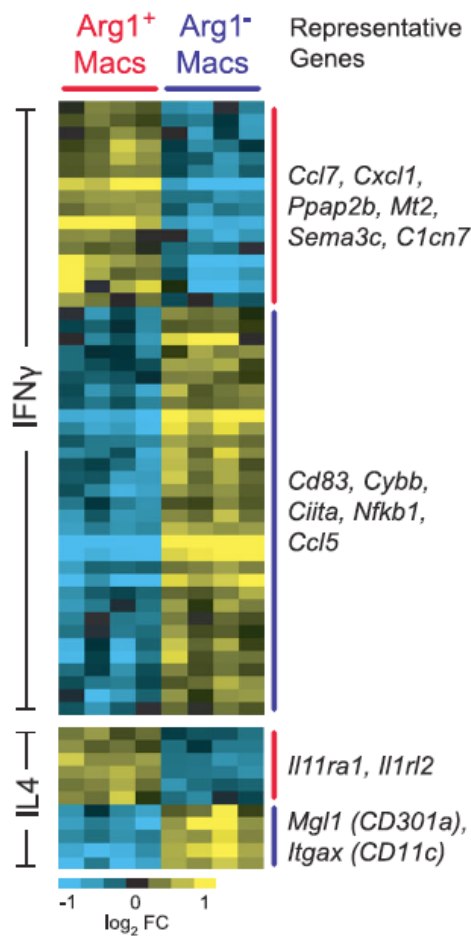


Figure 6. TBI macrophage subsets express genes induced in vitro by IFN- γ or by IL-4 stimulation. Genes that were differentially expressed between IFN- γ - and IL-4-stimulated BM-derived macrophages were compared in a meta-analysis for their expression between Arg1⁺ macrophages and Arg1⁻ macrophages. Each column represents a separate experiment. The data were median-centered across genes. To the right, genes representing each cluster are shown.

Following TBI, Arg1⁺ macrophages and Arg1⁻ macrophages in the brain differ notably in their expression of chemokines. Although the Arg1⁺ macrophages and Arg1⁻ macrophages are neither M1 nor M2 cells, they are clearly distinct from each other. The most striking and perhaps most novel differences between Arg1⁺ and Arg1⁻ macrophages are in their unique chemokine profiles. Arg1⁺ macrophages preferentially express a chemokine repertoire that includes *Ccl24* (eotaxin), *Cxcl7* (pro-platelet basic protein), *Cxcl4* (platelet factor 4), *Cxcl3* (MIP2 β , GRO3), *Cxcl1* (GRO α), *Cxcl14*, and *Ccl8* (MCP-2; 2.3) (Figs. 5A and 5B). Arg1⁻ macrophages have

their own chemokine expression pattern. They preferentially upregulate *Ccl17*, *Ccl5*, *Ccl22*, and *Ccr7* (Figs. 5A and 5B).

In all, the gene expression signatures of Arg1⁺ and Arg1⁻ macrophages suggest the presence of functionally distinct TBI-responsive macrophage subsets with complex roles in promoting and suppressing inflammation as well as possible pleiotropic effects on CNS cells.

Neither PPAR γ nor PPAR δ , either alone or in combination, activates expression of arginase-1 or Il12p40 in the brain. In parallel with the studies above, we pursued Specific Aim 2, to test the role of a PPAR γ agonist in driving microglia to an M2 phenotype. At the suggestion of our collaborator, Ajay Chawla, we used rosiglitazone instead of 15-deoxy $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂), and we used two different protocols. We also added studies using an agonist of PPAR δ , GW0742. This was based on studies in other laboratories, including that of our collaborator Dr. Chawla, demonstrating that the induction of the M2 phenotype in macrophages is dependent not only on PPAR γ but also on PPAR δ (8). Using YARG mice to assess activation of arginase-1 as marker for M2 cells, we did not find that rosiglitazone, either alone or in combination with GW0742, activated cells expressing arginase-1 above control levels in either microglia or macrophages at day 4 following TBI (results not shown). Initially, these studies were confounded by the finding that DMSO alone, which is the vehicle for solubilizing rosiglitazone and GW0742, itself activates arginase-1 expression in macrophages and to a lesser extent in microglia (as assessed by YFP fluorescence). We subsequently overcame this problem by administering the drug by gastric gavage, with the same negative result. For these studies, the mice were given 250 μ g of rosiglitazone and 250 μ g of GW0742 (~10 mg/kg) in 100 μ l of 0.5% methylcellulose by gavage daily beginning 4 days before TBI (including the day of TBI), and again 2 and 3 days after TBI. The mice were then euthanized after 4 days. Despite this improved approach, we saw no increase in arginase-1+ cells compared to controls in 5 out of 6 mice, although in one of the 6 the percentage of arginase-1+ cells was notably increased.

TREM-2 is not expressed on the surface of brain cells with or without TBI. We also examined the expression of TREM-2 on macrophages and microglia. In our proposed work, one approach to activating microglia toward an M2 phenotype was to have been the administration of monoclonal antibodies (mAb) to TREM-2. Despite studies from several labs, including our own, showing that TREM-2 is expressed in microglia within histologic sections from adult brain as well as on the surface of cultured neonatal microglia (11), we found little or no TREM-2 expressed on the surface of freshly prepared microglia from adult brains, with or without TBI, and with or without DMSO or rosiglitazone (results not shown). Therefore the use of anti-TREM-2 to activate M2 cells was not feasible. This led us to substitute studies with GW0742, as discussed above.

Studies in the current (no-cost extension) year. With limited funds (~\$13K) carried over into the current year, we were able first to meet the several additional studies required as controls by the reviewers, including additional immunohistochemical microscopy of macrophages following TBI (stained with anti-F4/80), and additional use of RT-PCR to confirm results from the microarrays (12).

We also completed microarrays of microglia from ipsilateral brains one day following TBI, compared to sham controls. Our initial analysis of these results reveals that 390 genes are upregulated by more than two-fold following TBI. (They also confirmed our findings from YARG and from yet40 mice, showing that microglia do not increase expression of either arginase-1 or IL12p40 following TBI.) The top functional groups of these genes are related to cell division, cell cycling, and DNA repair, indicating that the primary response by microglia to TBI is cell division. One cluster, however, was immune response genes, especially a variety of chemokines, as well as the genes for TNF α , macrophage scavenger receptor 1, IL-1-receptor antagonist, vimentin, and leukocytes Ig-like receptor 4 (Table I). (We had previously detected the rise in TNF α by RT-PCR. Dr. Hsieh will be building on these results as she continues her studies of microglia in TBI.

Table I. Immune response genes increased in microglia by >2-fold one day following TBI.

Functional cluster	Gene names
Secreted cytokines	Spp1, Il1rn, Cxcl2, Ccl5, Ccl7, Cxcl4, Ccl12, Ccl2, Tnf
Transmembrane receptors	Msr1, CD44, CD36, Mmp12, Pilrb1, Pilrb2, Liltrb4, Marco, C5ar1, Siglec1
IFN induced genes	Ifitm2, Ifitm7, Ifi204, Ifi205, Ifitm3, Ifrd2

Work Accomplished:

Year 1

1. **Quantification of microglial subsets in normal mice by use of reporter cell mouse lines yet40 and YARG. Completed.** *Resting microglia do not express detectable transcripts for YFP in either reporter strain.*
2. **Quantification of the expression of TREM2 on microglial subsets in normal mice as identified by use of reporter cell mouse lines yet40 and YARG. Completed.** *Resting microglia from either strain or from wild type mice express little if any TREM-2 on the cell surface. By immunofluorescence microscopy, however, we and others have detected TREM2 within most microglia (by histology), leading to the conclusion that most TREM2 is inside the microglia rather than on the surface.*
3. **Quantification of the production of the cytokine TNF α , as determined by ELISA, by microglial subset from normal mice, as identified by use of reporter cell mouse lines yet40 and YARG. Largely completed, with some changes in approach.** *ELISA proved insufficiently sensitive to detect amounts of TNF α that might be produced by the numbers of microglia obtained. We therefore turned to more sensitive approaches. In brain macrophages following TBI, both Arg⁺ and Arg⁻ cells expressed elevated levels of TNF α , compared to monocytes as assessed both by microarrays at RT-PCR. This was subsequently also shown in microglia.*

Year 2

4. **Quantification of microglial subsets following TBI by use of reporter cell mouse strains yet40 and YARG. Completed.** *TBI alone does not induce detectable activation of YFP in the microglia from either yet40 or YARG mice. By analysis of YARG mice, however, a subset of macrophages expresses arginase-1 at high levels.*

5. **Quantification of TREM2 expression on microglial subsets following TBI, as identified by use of reporter cell mouse lines yet40 and YARG. Completed.** *As with resting microglia (discussed above), freshly prepared microglia from mice subjected to TBI do not express detectable levels of TREM-2 on the cell surface. Because of these findings, we dropped the goal of using anti-TREM-2 to alter the function of microglia (and macrophages) but we added studies to assess the effect of the PPAR δ agonist GW0742.*
6. **Quantification of the expression of the cytokine TNF α as determined by ELISA, by microglial subset following TBI, as identified by use of reporter cell mouse lines yet40 and YARG. Largely completed with necessary modifications.** *See #3 above.*
7. **Quantification of phagocytosis by microglial subsets following TBI, as identified by use of reporter cell mouse lines yet40 and YARG. Change in approach.** *These studies were not possible because TBI did not induce detectable activation of microglia subsets. In subsequent years, however, we used microarrays of microglia to show that we could not detect evidence for increased phagocytic capacity in microglia at day 1 after TBI.*

Year 3

8. **In YARG mice, define the effects of the PPAR γ agonist 15-deoxy $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂) on microglial activation and neuronal death *in vivo*, as assessed by flow cytometry and immunohistochemistry. Studies of microglia completed.** *As advised by our collaborator, Ajay Chawla, we used rosiglitazone as a PPAR γ agonist instead of 15d-PGJ₂, and we added studies testing of the PPAR δ agonist GW0742, both alone and in combination with rosiglitazone. Even the combination of these agents, however, did not alter the profile of YFP expression in either microglia or macrophages from YARG mice, either before or after TBI.*

Year 4

9. **In YARG mice, define the effects of 15d-PGJ₂ with or without antibody to TREM2 (delivered intracranially) on the response to TBI, as assessed by T cell infiltration of the brain and neuronal death. Altered and completed with regard to T cells.** *As discussed above, TREM-2 has not yet been detected on the surface of freshly prepared adult microglia with or without TBI, and this was not altered by the addition of PPAR γ and PPAR δ agonists. We therefore abandoned the use of anti-TREM-2, and substituted studies of the PPAR δ agonist, GW0472. However, even the combination of the PPAR γ agonist rosiglitazone and GW0472 failed to alter the expression of YFP in YARG mice. Further, we could not detect changes in the T cell population. We therefore have not pursued studies of neuronal death following these agents.*

Year 5 (no-cost extension)

Completed controls for publication of studies

Completed microarray studies of microglia one day after TBI.

KEY RESEARCH ACCOMPLISHMENTS

- As detected by the use of the reporter mice, yet40 and YARG, TBI alone in our model does not cause widespread activation of microglia nor expression markers for the M1 or M2 phenotype (IL12p40 or arginase-1, respectively).
- TBI alone however, induces a large influx of macrophages.

- The influx of macrophages is largely dependent on CCR2, evidence that they are recruited from the periphery and providing a mean of studying the importance of these cells in TBI.
- As detected by expression of YFP in the YARG reporter mice, about 20% of the infiltrating macrophages express arginase-1 within a day following TBI. Although the number of macrophages is higher at days 4 and 7, the percent with detectable expression of YFP is highest at day 1, declines at day 4 and is not detected at day 7.
- Gene expression arrays confirm that the Arg⁺ and the Arg⁻ macrophages identified by YARG reporter cell mice differ in the expression of many genes, notably cytokines. Thus they represent distinct cell populations.
- Neither the Arg⁺ nor the Arg⁻ macrophages in are M1 or M2 macrophages, as assessed by the gene expression profiles.
- As a direct result of this DoD work, Christine Hsieh, the postdoctoral fellow who has pursued these studies (her first of TBI) was awarded a VA Career Development Award to pursue the importance of CCR2 in the functional consequences of TBI. This began in January 2011. Further, she been appointed as an Assistant Professor at UCSF and has PI status at the VA.

REPORTABLE OUTCOMES

- The studies of macrophage subsets following TBI have been published (12, attached). Dr. Hsieh presented her DoD-sponsored studies regarding the effects of TBI on macrophages as a poster at the 10th Annual Meeting of the International Society of Neuroimmunology, Barcelona, October 26-30, 2010, and as a poster at the Annual Meeting of the National Neurotrauma Society, Nashville, TN Aug 4-8, 2013.

CONCLUSIONS

Our studies have shown that resting microglia do not express markers for an M1 or an M2 phenotype, as detected by reporter mice, nor are these markers induced by TBI. TBI, however, induces a marked influx of macrophages, which is dependent on CCR2, and as detected by YARG reporter mice, about 20% of these cells express arginase-1 at high levels one day after TBI, suggesting that they may be of M2 phenotype. Gene expression analysis of these cells, however, indicates that they are neither M2 nor M1 cells, but instead have a unique phenotype. They differ from the Arg⁻ in mice in the expression of many genes, especially the expression of chemokines.

Initially, we did not detect activation of microglia following TBI as assessed by the expression of surface markers. Subsequently, with a larger impact and with improved FACS methodologies, we could detect CD86 on most microglia following TBI, consistent with widespread activation of microglia. Our recent microarray studies of microglia following TBI indicate as well the upregulation of other immune-related genes, although we could not detect clear evidence for the upregulation of genes involved in phagocytosis.

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APPENDICES

List of Personnel. This was unchanged during the past year. Personnel include:
William E. Seaman, M.D. Principal Investigator
Mary C. Nakamura, M.D. Investigator
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SUPPORTING DATA

A reprint of our recent publication (ref 12) is attached.

Traumatic brain injury induces macrophage subsets in the brain

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Traumatic brain injury (TBI) elicits innate inflammatory responses that can lead to secondary brain injury. To better understand the mechanisms involved in TBI-induced inflammation, we examined the nature of macrophages responding to TBI in mice. In this model, brain macrophages were increased >20-fold the day after injury and >77-fold 4 days after injury in the ipsilateral hemisphere compared with sham controls. TBI macrophage subsets were identified by using a reporter mouse strain (YARG) that expresses eYFP from an internal ribosome entry site (IRES) inserted at the 3' end of the gene for arginase-1 (Arg1), a hallmark of alternatively activated (M2) macrophages. One day after TBI, 21 ± 1.5% of ipsilateral brain macrophages expressed relatively high levels of Arg1 as detected by yellow fluorescent protein, and this subpopulation declined thereafter. Arg1⁺ cells localized with macrophages near the TBI lesion. Gene expression analysis of sorted Arg1⁺ and Arg1⁻ brain macrophages revealed that both populations had profiles that included features of conventional M2 macrophages and classically activated (M1) macrophages. The Arg1⁺ cells differed from Arg1⁻ cells in multiple aspects, most notably in their chemokine repertoires. Thus, the macrophage response to TBI initially involves heterogeneous polarization toward at least two major subsets.

Keywords: Alternative activation · Inflammation · Macrophage · Traumatic brain injury



Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

Traumatic brain injury (TBI) is the leading cause of morbidity and mortality from childhood to age 44 [1]. Following the initial trauma, inflammatory responses can expand brain damage [1]. TBI rapidly leads to activation of microglia, macrophages, and neutrophils, and to local release of inflammatory cytokines [1–5]. Understanding the inflammatory events that occur during this crit-

ical window is an important step toward developing interventions targeting the immune response [6].

Following brain injury, the host response has the potential for both benefit and harm. While inflammatory mechanisms may be required for wound sterilization, the response can extend neuronal cell death and impair recovery. Macrophages have previously been studied in models of CNS injury including experimental autoimmune encephalitis, ischemic stroke, and spinal cord injury as well as TBI, and there is conflicting evidence as to whether macrophages are overall harmful or beneficial to the brain. A detrimental role for macrophages has been found in most

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neuroimmunologic studies [7–13]. However, the inflammatory response is also important for clearing necrotic debris and for wound repair [14]. In support of this, macrophages have also been shown to suppress inflammation and were critical for recovery in one model of spinal cord injury [15]. Moreover, in EAE, macrophages that suppress inflammation through the production of IL-10 and TGF- β are beneficial [16]. These differing roles for macrophages may reflect different functional states of macrophage activation.

In vitro and in vivo studies have demonstrated that macrophages can be activated into two major subsets: classically activated (M1) and alternatively activated (M2) macrophages [17–19]. M1 macrophages directly incite inflammation by releasing IL-12, TNF- α , IL-6, IL-1 β , and nitric oxide (NO) in response to microbial pathogens or LPS. In contrast, M2 cells are activated in response to helminths, to allergens, by adipose tissue, and in vitro by IL-4 [20, 21]. M2 macrophages suppress inflammation and promote wound healing [14]. They express increased levels of arginase-1 (*Arg1*), CD206 (mannose receptor), Clec7a (dectin-1), CD301, resistin-like alpha (REL μ), and PDL2. Additional macrophage subsets have been identified [17, 18]. In vivo studies demonstrate that macrophages may differentiate along a spectrum of phenotypes that do not adhere to well-defined in vitro phenotypes [14, 17, 22, 23]. Furthermore, macrophages may shift from one phenotype to another [17].

In considering the role of macrophages in brain injury, it may be important to distinguish between macrophage subsets. Thus, in vitro studies have demonstrated that M1 macrophages are neurotoxic, while M2 macrophages promote regenerative neuronal growth [24]. CCL2, which is expressed post-TBI in the brain and cerebrospinal fluid, has been thought to elicit primarily M1 macrophages, and the presence of macrophages/microglia early after TBI by histology is often associated with the expression of TNF, IL-6, and IL-1 [1, 13, 25–27]. These findings previously suggested that there is a prominent M1 phenotype in early macrophage recruitment following TBI. Characterization of macrophages in TBI by histology has been complicated by difficulty in distinguishing them from microglia; there is no known marker that is expressed by macrophages but not microglia or vice versa. By flow cytometry, however, the two cell populations can be distinguished by the level of CD45 expression. Using this approach, we have examined the nature of macrophages responding to TBI in mice. To facilitate macrophage subset identification, we examined TBI in YARG mice, in which yellow fluorescent protein (YFP) is expressed under the promoter for the M2 marker, *Arg1* [28, 29], and Yet40 mice, in which YFP is expressed under the promoter for the M1 marker, IL-12p40. We here demonstrate that a subset of brain wound macrophages upregulate *Arg1* and home to the site of injury. At day 1 after injury, 21 \pm 1.5% of the ipsilateral hemisphere macrophages express high levels of *Arg1*, but the number of *Arg1*⁺ cells falls thereafter and cannot be detected after 1 week. Whole genome expression analysis of *Arg1*⁺ and *Arg1*⁻ macrophages following TBI revealed that these macrophage subsets differ in their expression of over 1300 genes, with notable differences in genes encoding chemokines. The pattern of gene

expression in neither population is characteristic of in vitro derived M2 or M1 cells. Our results indicate that the macrophage response to TBI is heterogeneous, and the early response includes at least two distinct subsets. As assessed by expression of *Arg1*, the ratio of these subsets changes with time.

Results

Macrophages are recruited to the lesion site in large numbers early post-TBI

To assess the immune response following TBI, we used an adult murine controlled cortical impact model. Histological analysis of brain sections following TBI confirmed cortical injury, which extended into the hippocampus (Fig. 1A). Hematoxylin and eosin (H&E) staining revealed increased cellular recruitment to cortical tissues adjacent to the lesion (Fig. 1A). Immunohistochemical staining for F4/80 showed that macrophages/microglia are widely present at the pericontusional site (e.g. in areas of the cortex adjacent to the lesion) (Fig. 1B).

To assess leukocyte subset frequencies present in the brain following TBI, single-cell suspensions from TBI and sham-injured brain contralateral and ipsilateral hemisphere tissues were analyzed by flow cytometry. Flow cytometry permitted discrimination of macrophages from microglia based on levels of CD45 expression; both microglia and macrophages express CD11b, but macrophages express a higher level of CD45 [30, 31]. In our analyses of macrophages and microglia, neutrophils (which also express CD45 and CD11b) were consistently excluded by using an antibody against Ly6G (Clone 1A8). Blood leukocytes were excluded by perfusing the brain prior to cell recovery.

Flow cytometry plots of cell preparations from brain tissues 4 days following TBI of WT mice showed that macrophages are a major part of the inflammatory response to TBI primarily on the side of injury (Fig. 1C); macrophages comprised 40 \pm 2% of all CD45⁺ leukocytes in the ipsilateral TBI hemisphere compared with 5.7 \pm 1.5% of CD45⁺ cells in sham control tissues ($p < 0.001$).

Quantification of the kinetics of macrophage numbers that accumulate in brain hemispheres after TBI revealed that macrophage infiltration in ipsilateral hemispheres of TBI mice increased by 21-fold on day 1 (mean \pm SEM, 22 115 \pm 1732), and by 77-fold on day 4 (46 968 \pm 5918) compared with sham controls (1081 \pm 151 and 613 \pm 205, respectively) (Fig. 1D). On day 7, WT ipsilateral TBI macrophage numbers declined but were still 25-fold higher than levels in sham controls, and on day 14 macrophage numbers were fourfold higher (Fig. 1D).

On the first day following TBI, there was also a substantial increase in neutrophils (CD45^{hi}CD11b⁺Ly6G⁺) in the brain (41 520 \pm 4533 compared with 1419 \pm 94 in sham controls), with a decline thereafter (Fig. 1D). These findings are similar to the recent findings of Jin et al. [32], although our results add quantification of absolute cell numbers as well as proportions, and we find that macrophage levels are higher on day 4 than on day 1.

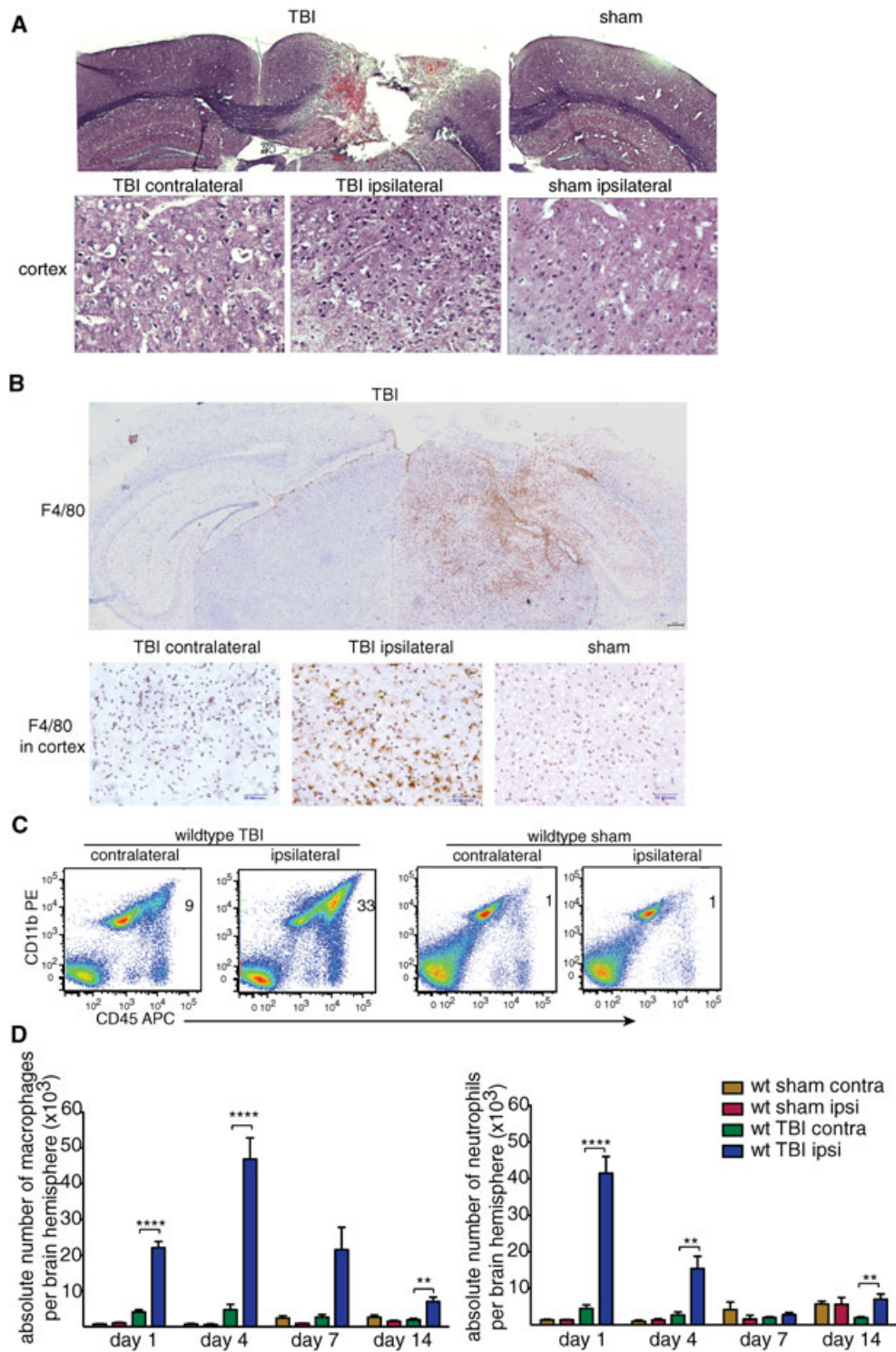


Figure 1. TBI induces a significant macrophage response near the lesion site. (A) Controlled cortical impact (CCI) model of murine TBI 4 days postsurgery. Coronal brain sections were stained with H&E (top). Scale bars represent 500 μm . Images of H&E staining of cortical brain tissues 4 days after surgery (bottom). Scale bars represent 50 μm . (B) Representative immunohistochemical staining of coronal brain sections for F4/80⁺ macrophages/microglia (brown) and counterstained with hematoxylin (light purple) 4 days post-TBI. Images of staining in cortex are also shown (bottom). Scale bars represent 200 μm (top) and 50 μm (bottom). (A, B) Data shown are representative of three experiments performed. (C) Representative flow cytometry data of brain leukocytes isolated 4 days post-TBI ($n = 8$) or sham surgery ($n = 4$) were stained for CD45-allophycocyanin and CD11b-PE. Density plots shown are of live cells that are negative for the Ly6G granulocyte marker. The polygram gate highlights the proportion of macrophages defined as CD45^{hi} CD11b⁺Ly6G⁻Sytox blue⁻ in the total cell preparation. (D) The absolute numbers of macrophages from the contralateral and ipsilateral brain hemispheres quantified by flow cytometry following TBI on days 1, 4, 7, or 14 or sham surgery are shown as mean \pm SEM of 16 (day 1), 8 (day 4), 4 (day 7), 8 (day 14), or 2–4 (sham) mice. Data shown are pooled from 14 experiments performed. **** $p < 0.0001$, ** $p < 0.005$, unpaired t-tests with Welch's corrections.

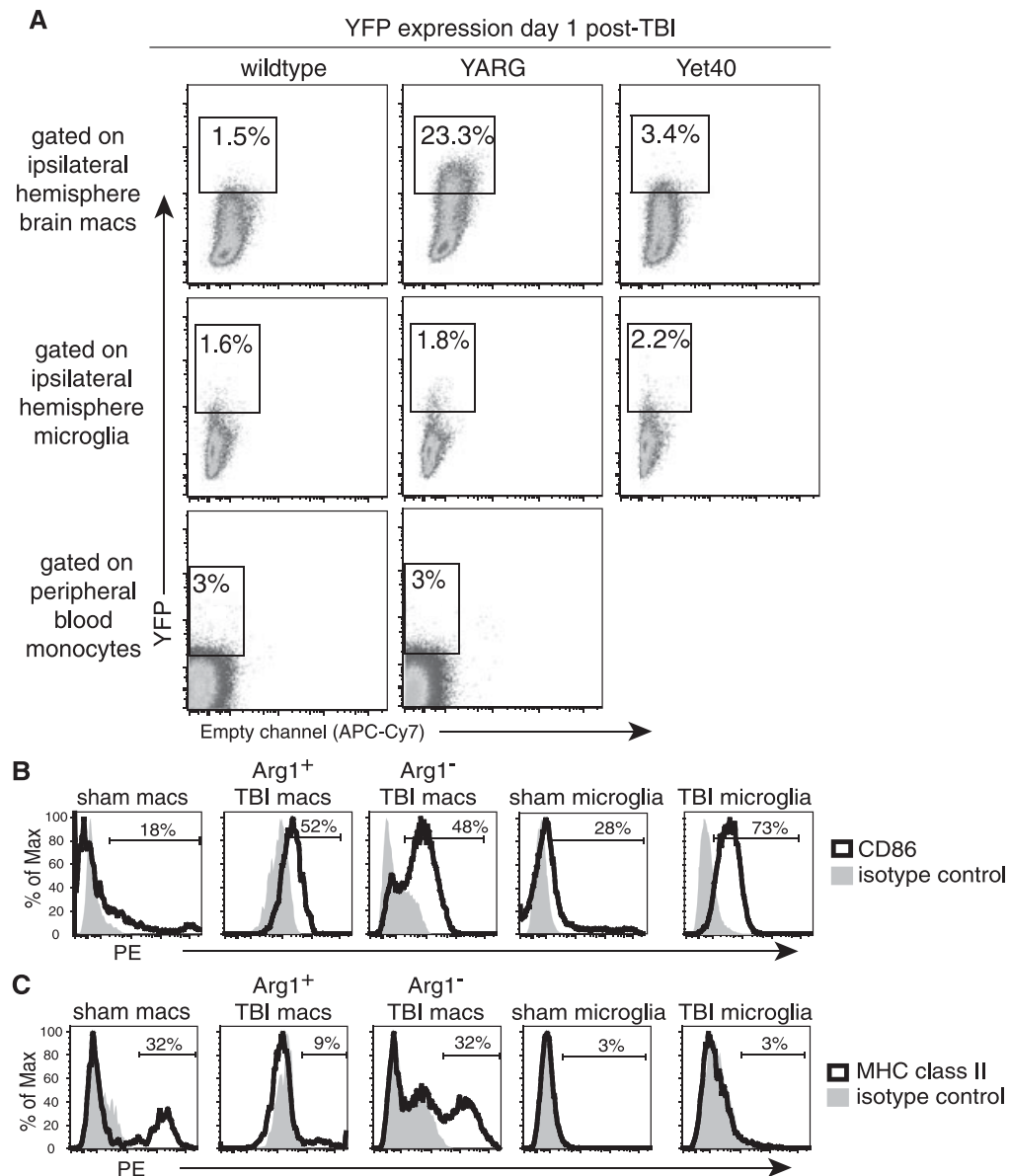


Figure 2. YFP is upregulated in a subset of brain macrophages from YARG mice 1 day post-TBI. (A) Leukocytes isolated from the ipsilateral hemisphere of WT, YARG, and Yet40 mice 1 day after TBI were analyzed for YFP expression in live cells gated on brain macrophages (CD45^{high} CD11b⁺ Ly6G⁻, top), microglia (CD45^{lo} CD11b⁺ Ly6G⁻, middle), or peripheral blood monocytes (CD11b⁺F4/80⁺Ly6G⁻, bottom). YFP expression is set by box gates based on WT controls (left). Data shown are from one experiment that is representative of three experiments with Yet40 mice with controls, and six experiments with YARG mice with controls. (B) Assessment of CD86 expression in Arg1⁺ and Arg1⁻ ipsilateral hemisphere macrophage subsets and in microglia from YARG mice by flow cytometry. Data shown are representative of expression detected at four days postsurgery and are from one experiment representative of three performed. (C) Assessment of MHC class II expression in Arg1⁺ and Arg1⁻ ipsilateral hemisphere macrophage subsets and in microglia from YARG mice. Data shown are representative of expression detected at 4 days postsurgery and are from one experiment representative of three performed.

Early macrophage response to TBI includes Arg1⁺ and Arg1⁻ subsets

To examine macrophage polarization post-TBI, we first sought to trace the genetic expression of *Arg1*, which is highly expressed during M2 polarization, or of *Il12b*, the gene for IL-12p40, a signature of M1 polarization. To do this, we took advantage of two reporter mouse strains, YARG (YFP-Arginase-1) and Yet40 (YFP-enhanced transcript for IL-12p40) [28, 33]. TBI was performed in

YARG and Yet40 mice, and YFP expression in brain and peripheral blood leukocytes was compared by flow cytometry to WT animals, which lack YFP expression.

One day after TBI, $21 \pm 1.5\%$ (mean \pm SEM, $n = 6$) of ipsilateral hemisphere brain macrophages in YARG mice expressed YFP (Fig. 2A), but brain macrophages in the contralateral hemisphere and from either hemisphere of sham animals uniformly lacked YFP (data not shown). YFP expression in YARG brain macrophages peaked on day 1 after TBI, fell to 4–7% of the macrophage

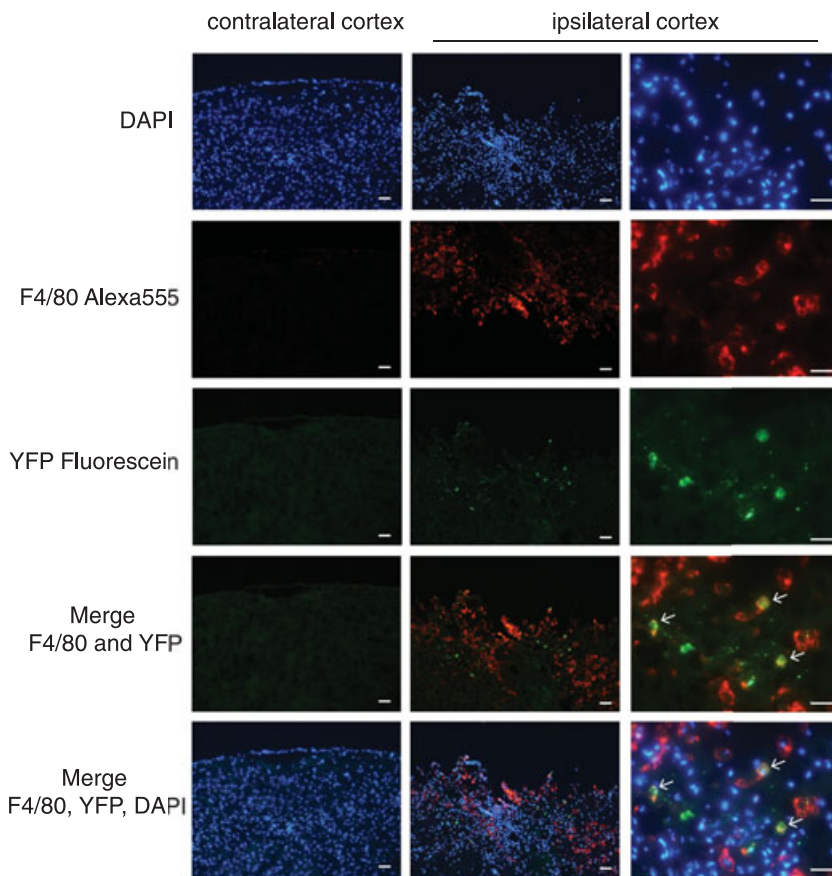


Figure 3. Immunofluorescent microscopy of YFP and F4/80 costaining in the cortex of YARG mice post-TBI. Representative images from colabeling experiments of YFP, F4/80, and DAPI on the contralateral (first column) and ipsilateral cortex (second column) of brain tissue 4 days post-TBI. Scale bar represents 50 μm . The top areas of columns 1 and 2, devoid of DAPI staining, are outside the boundary of brain tissue. The third column shows higher magnification of macrophages in the ipsilateral cortex; scale bar 25 μm . Arrows indicate cells colabeled with anti-YFP and anti-F4/80 antibodies. Data shown are from one experiment performed and are representative of four experiments performed analyzing three animals, ten sections per animal.

population by day 4, and was undetectable on days 7 and 14 (data not shown). YFP expression could not be detected in microglia following TBI at any time point. F4/80⁺ blood monocytes isolated from the same injured YARG animals also lacked expression of YFP (Fig. 2A), suggesting that TBI induces macrophage differentiation after localization in the tissue. Brain macrophages and blood monocytes from TBI animals differed markedly not only in YFP expression but also in their gene expression profiles as assessed by microarray (Fig. 4 and Supporting Information Fig. 1), confirming that macrophages isolated from brains were not significantly contaminated by blood monocytes. Yet40 mice subjected to TBI had little or no upregulation of YFP in macrophages or microglia on days 1, 4, 7, and 14 (day 1 is shown), and this was subsequently confirmed for macrophages by microarray analysis for IL-12p40 on day 1 where all comparison ratios were close to 1, indicating no change in expression in comparison to blood monocytes or between brain macrophage subsets. Thus, TBI rapidly induces a macrophage response that is characterized at early time points by at least two major subsets of cells that differ in Arg1 expression, and these are hereafter called Arg1⁺ and Arg1⁻ cells.

Analysis of markers for cell activation and for antigen presentation on macrophages from YARG mice revealed that both Arg1⁺ and Arg1⁻ populations upregulated the activation marker CD86 compared with sham control macrophages (Fig. 2B). Few

Arg1⁺ macrophages, however, expressed MHC class II antigens (MHCII; Fig. 2C), a marker that has been described on both M1 and M2 cells [17,34]. In contrast, 25–30% of Arg1⁻ macrophages expressed MHCII (Fig. 2C). This is similar to the proportion of macrophages that express MHCII in sham brains (Fig. 2C), and it suggests that the Arg1⁻ cells include at least two subpopulations, one lacking and the other expressing MHCII.

Although microglia from TBI brains did not express detectable MHCII (Fig. 2C), virtually all microglia upregulated CD86 following TBI (Fig. 2B). This finding is consistent with previous observations that TBI induces widespread activation of microglia [35,36].

To examine the spatial localization of YFP⁺ cells in YARG mice post-TBI, we performed immunofluorescent colabeling for YFP and F4/80 in brain sections days post-TBI, when macrophage infiltration of the brain peaks. F4/80⁺ macrophages/microglia localized in and around the area of injury (Fig. 3, second row). F4/80 expression was below level of detection by immunofluorescence in sham-injured tissues (data not shown). The Arg1⁺ cells were scattered among the F4/80⁺ cells in TBI mice (Fig. 3, third row) and were not detectable in the contralateral hemisphere or in sham-treated mice. The majority of the Arg1⁺ cells costained with F4/80. As suggested from our flow cytometry data in which only a subset of macrophages expresses YFP, the majority of F4/80⁺ cells were Arg1⁻ (Fig. 3).

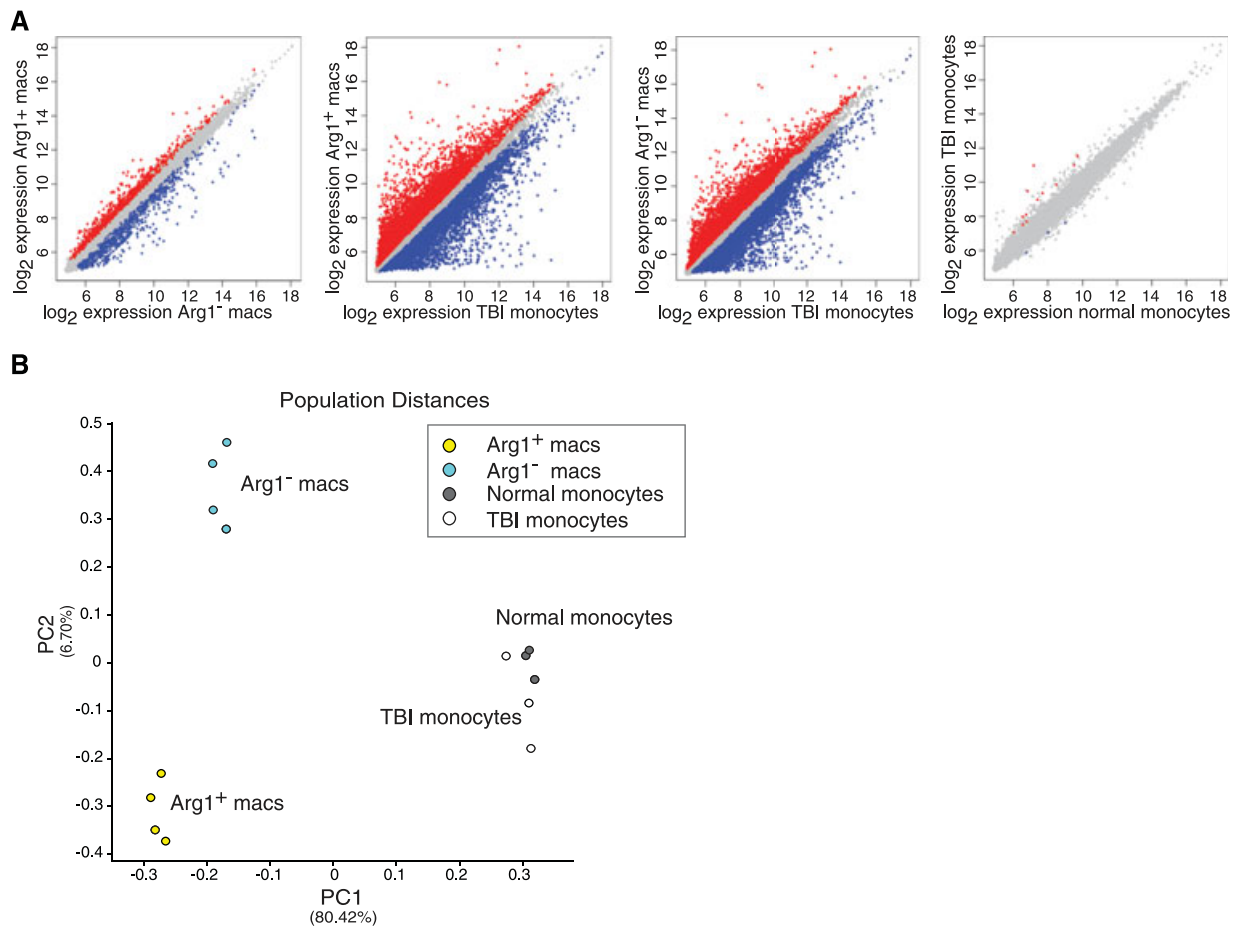


Figure 4. Differences between Arg1⁺ and Arg1⁻ macrophage subsets and peripheral blood monocytes revealed by gene expression profiling. (A) Microarray data comparing gene expression by Arg1⁺ TBI macrophages, Arg1⁻ TBI macrophages, TBI monocytes, and normal monocytes ($n = 4$ per population) were examined in a pairwise analysis. Red and blue color dots represent genes with significant differences. (B) Principal component analysis using the top 15% most variable genes between all populations analyzed by microarray. The distance between the indicated cell populations is proportionate to their differences in gene expression.

Arg1⁺ and Arg1⁻ brain wound macrophages represent at least two distinct subsets

To further characterize the nature of brain macrophages following TBI, we sorted the Arg1⁺ and Arg1⁻ cell populations from the ipsilateral hemisphere of YARG mice 1 day after TBI (when the proportion of Arg1⁺ cells peaked) and performed gene expression analysis of both cell populations. We also examined gene expression by peripheral blood monocytes from injured animals to assess the expression state of monocytes prior to their infiltration into the brain and differentiation into macrophages. As a control, peripheral blood monocytes from uninjured animals were also analyzed. It was not technically feasible to perform arrays on brain macrophages from sham animals, because there were insufficient cells to generate adequate amounts of RNA. Pairwise analyses of differentially expressed genes showed that Arg1⁺ and Arg1⁻ brain macrophages differed in the expression of 1360 genes, and both populations showed even greater differences from TBI monocytes (11 799 genes differed between Arg1⁺ macrophages and TBI monocytes; 9932 genes differed between Arg1⁻ macrophages)

(Fig. 4A). TBI monocytes displayed few differences compared with normal monocytes (15 genes) (Fig. 4A). Principal component analysis (PCA), an analytical technique that uses dimensionality reduction to identify dominant patterns within highly multivariate data, was performed. PCA confirmed that distinctions separating macrophages from monocytes were the largest source of variance in the dataset (principal component (PC) 1), and that the monocyte populations had fewer differences that were not represented in either of the top two PCs (Fig. 4B). PCA also confirmed that Arg1⁺ and Arg1⁻ brain macrophages represented two distinct populations, representing the second most significant PC (PC2) (Fig. 4B).

TBI-induced macrophages exhibit transcriptional responses distinct from known macrophage subsets

Although robust Arg1 expression is often used as a marker for alternative activation of macrophages, we observed that Arg1⁺

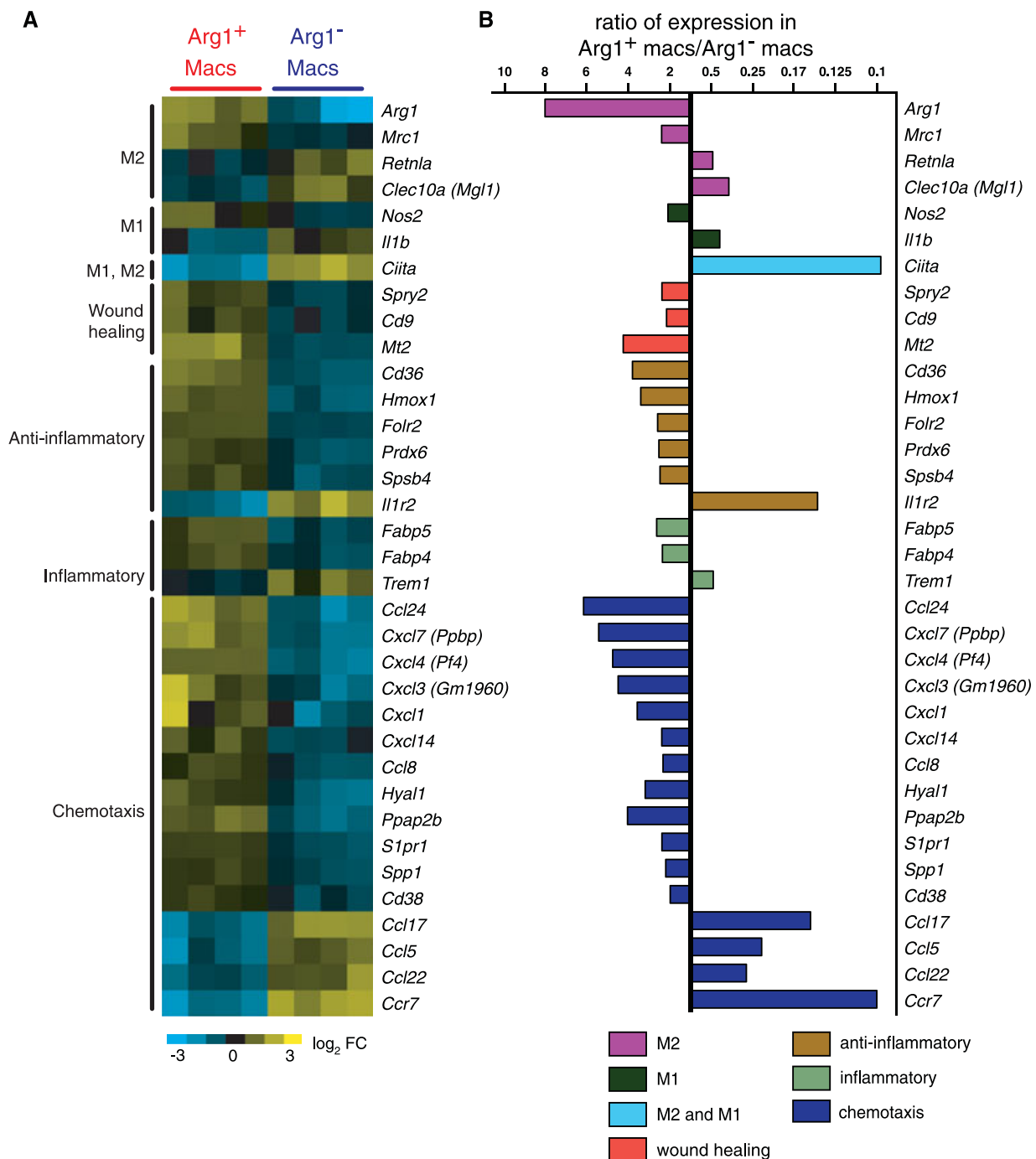


Figure 5. Differences in transcriptional responses between Arg1⁺ and Arg1⁻ brain wound macrophages selecting for genes associated with M1 and M2 polarization and/or with other selected macrophage functions genes. (A) Gene expression analysis comparing Arg1⁺ TBI macrophages and Arg1⁻ TBI macrophages 1 day after injury. Thirty-five selected genes of interest with significant relative differences of close to twofold or higher are shown. Each column represents a separate experiment. Gene expression was \log_2 transformed and median-centered across genes. Yellow represents a relative increase in expression and blue signifies a relative decrease. Expressed genes are grouped by function. (B) Average gene expression differences from the data in Figure 4A were quantified and ratios of gene expression levels comparing Arg1⁺ brain wound macrophages to Arg1⁻ brain wound macrophages are shown.

and Arg1⁻ brain macrophages after TBI did not represent clear M2 and M1 macrophages, respectively, but instead each subset expressed markers of both M1 and M2 cells. Comparison of gene expression between Arg1⁺ and Arg1⁻ macrophages confirmed

that the former expressed much higher levels of Arg1 (eightfold) as well as higher levels of *Mrc1* (2.4-fold), which encodes the mannose receptor/CD206 [17] (Fig. 5). Increased expression of these two genes is a feature of M2 cells. The expression of

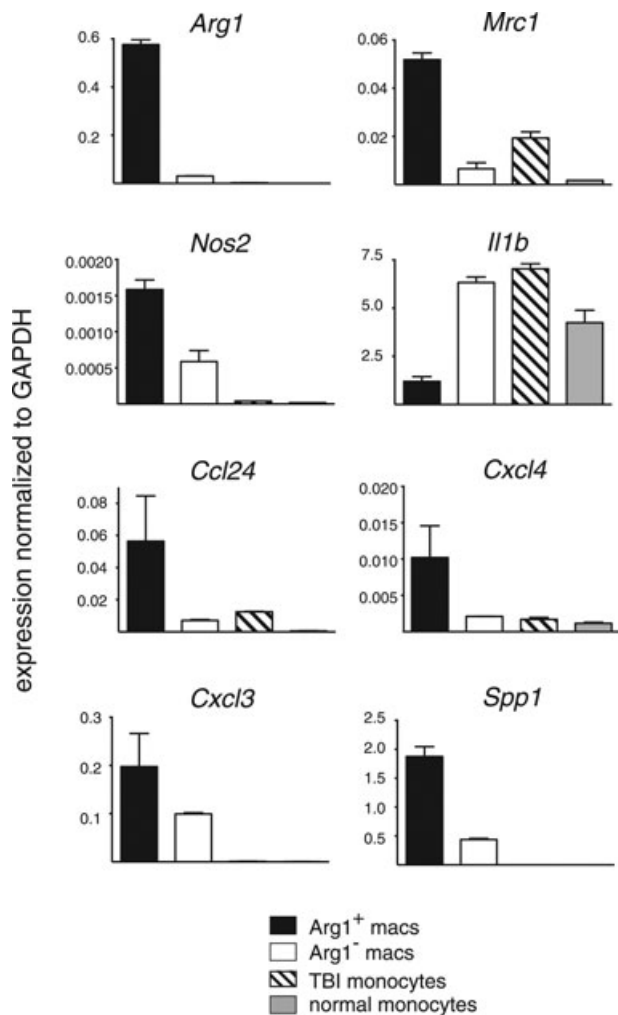


Figure 6. Semiquantitative real-time PCR data of selected M2-, M1-, and chemotaxis-associated genes identified by microarray analysis to have significant differences between the macrophage populations. Data were normalized to GAPDH levels and are shown as mean +SEM of triplicate values. Data shown are from one experiment representative of two experiments performed on separate animals.

other genes, however, indicated that Arg1⁺ macrophages were not identical to M2 cells. For example, Arg1⁺ macrophages preferentially expressed *Nos2* (2.1-fold), an M1-associated gene [17] (Fig. 5). Similarly, although Arg1⁻ macrophages had increased expression of *Il1b* (IL-1 β) (2.4-fold), they also preferentially expressed signature M2 markers, notably *Retnla* (resistin-like α) (2.1-fold) and *Clec10a* (C-type lectin domain family 10, member A)/CD301 (2.9-fold) [17, 37] (Fig. 5). The relative increases in expression levels of *Arg1*, *Mrc1*, *Nos2*, and *Il1b* were confirmed by real-time PCR, demonstrating that relative to GAPDH, these genes were indeed transcriptionally active (Fig. 6). In accordance with flow cytometry data (Fig. 2C), gene expression analysis of MHCII, a molecule thought to be on both M1 and M2 cells, revealed that the Arg1⁻ macrophage population as a whole expressed much higher levels of MHCII transcripts (not shown) and higher levels of *Ciita* (class II, MHC, transactivator) than the

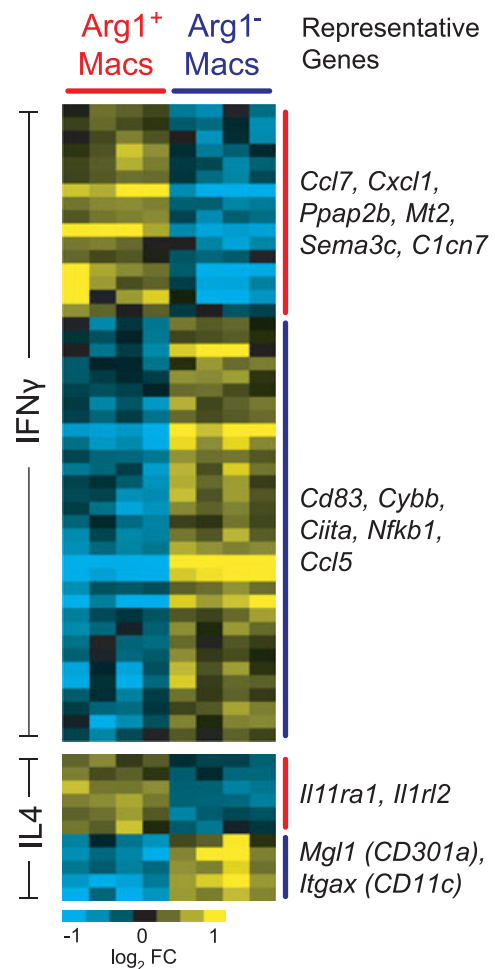


Figure 7. TBI macrophage subsets express genes induced in vitro by IFN- γ or by IL-4 stimulation. Genes that were differentially expressed between IFN- γ - and IL-4-stimulated BM-derived macrophages were compared in a meta-analysis for their expression between Arg1⁺ macrophages and Arg1⁻ macrophages. Each column represents a separate experiment. The data were median-centered across genes. To the right, genes representing each cluster are shown.

Arg1⁺ macrophages (Fig. 5). The MHCII⁺ Arg1⁻ macrophages may thus have increased capacity to present antigen to CD4⁺ T cells.

Taken together, we conclude that Arg1⁺ and Arg1⁻ macrophages each have mixed expression of M2 and M1 properties, and under the conditions of TBI Arg1 cannot be used as a marker for conventional M2 cells. To further compare Arg1⁺ and Arg1⁻ TBI brain macrophages with M1 and M2 macrophages, we performed a meta-analysis of genes differentially expressed between Arg1⁺ and Arg1⁻ TBI brain macrophages compared with genes differentially expressed between IFN- γ - or IL-4-stimulated bone marrow derived macrophages (BMDMs) stimulated in vitro with IFN- γ or with IL-4, representing M1 and M2 cells, respectively [38]. Arg1⁺ and Arg1⁻ macrophages each upregulated a variety of genes that were also expressed by BMDMs in response to either IFN- γ or IL-4 (Fig. 7). Thus, Arg1⁺ and Arg1⁻ TBI brain macrophage subsets have features of both M1 and M2 phenotypes

(Fig. 7). There are at least two explanations for these findings, not mutually exclusive: (i) individual brain macrophages may have features of both M1 and M2 cells (including cells that are incompletely polarized or are in transition from between different states of polarization and (ii) there may be subsets of cells within the Arg1⁺ and Arg1⁻ cells that have different expression of M1 and M2 markers. Regardless, the gene expression profiles demonstrate that Arg1⁺ and Arg1⁻ macrophages differ by many genes other than just *Arg1*.

The most striking and novel differences between Arg1⁺ and Arg1⁻ macrophages were in their unique chemokine profiles. Arg1⁺ macrophages preferentially expressed a chemokine repertoire that included *Ccl24* (which is also secreted by M2 cells; 6.2-fold), *Cxcl7* (5.4-fold), *Cxcl4* (2.4-fold), *Cxcl3* (4.5-fold), *Cxcl1* (3.6-fold), *Cxcl14* (2.4-fold), and *Ccl8* (2.3-fold) (Fig. 5). Arg1⁻ macrophages, in contrast, preferentially upregulated *Ccl17* (6.8-fold), *Ccl5* (4.4-fold), *Ccl22* (3.7-fold), and *Ccr7* (tenfold) (Fig. 5).

Although the gene profile of the Arg1⁺ macrophages suggests that they are not typical or homogeneously polarized M2 cells, they may have a role in promoting wound healing and in suppressing inflammation. Thus, Arg1⁺ macrophages preferentially expressed *Spry2* (sprouty2; 2.4-fold), *Cd9* (2.2-fold), *Cd38*, and *Mt2* (metallothionein-2; 4.2-fold, Fig. 5). Sprouty2 and CD9 have protective roles in wound healing in skin injury models [39, 40]. *Mt2* and *Cd38* have been implicated in neuroprotection during brain injury [41, 42]. Arg1⁺ brain macrophages also preferentially expressed several other genes that are associated with protection against tissue injury, including *Cd36* (3.8-fold), *Hmox1* (heme oxygenase 1; 3.4-fold), *Folr2* (folate receptor-2; 2.6-fold), *Prdx6* (periredoxin-6; 2.5-fold), and *Spsb4* (SPRY domain and SOCS box containing protein 4; 2.5-fold) (Fig. 5) [43–49]. If Arg1⁺ cells do have the potential for neuroprotection following TBI, this may be overwhelmed by Arg1⁻ cells, which are greater in number and are less transient.

Discussion

Our findings demonstrate a heterogeneous macrophage response to TBI that changes over time. Expression profiling of Arg1⁺ and Arg1⁻ macrophage subpopulations demonstrate that they do not exemplify previously described in vitro derived macrophage subsets [17]. They also differ from macrophages that accumulate in skin wound macrophages [50]. Skin wound macrophages, such as TBI-induced Arg1⁺ cells, both express *Arg1* and *Mrc1*. However, skin macrophages additionally upregulated *Clec7a*, and do not express *Nos2*, features that distinguish them from TBI-induced Arg1⁺ cells.

It may not be surprising that the macrophage response to TBI differs from macrophage polarization induced in vitro or in other organs and other in vivo conditions. It is likely that macrophages can assemble their functions and products in a variety of combinations with great diversity. Our findings do demonstrate the heterogeneity of the macrophage response to TBI and they suggest that *Arg1* should not in isolation be used as a marker for M2

cells. In this regard, *Arg1* expression can be induced by pathways independent of IL-4/STAT6 [51].

Although we were able to identify macrophage subsets by using *Arg1* as a marker in YARG mice, we could not detect robust expression of IL-12p40 by flow cytometry on days 1, 4, 7, or 14 in any macrophages or microglia by using Yet40 mice or by gene expression profiling comparing Arg1⁺ and Arg1⁻ macrophages, as assessed by gene profiling. This suggests that IL-12p40 may not be a major effector cytokine promoted by brain macrophages or microglia in TBI, and that early in TBI, IL-12p40 is not inversely proportional to *Arg1* expression. Other M1 genes are detected, however, both in Arg1⁺ and Arg1⁻ cells. Thus, the use of a single marker to define M1 and M2 cells in TBI appears not to be sufficient, and the functional consequences of the Arg1⁺ and Arg1⁻ cell populations on the course of TBI remain unknown.

Our findings do not exclude the possibility that there are more than two subsets of responding macrophages, and this is clearly supported by the bimodal expression of MHCII in Arg1⁻ macrophages. Also, despite the extensive differences in gene expression between these cell subsets, particularly, in the expression of chemokines, it is also possible that Arg1⁺ and Arg1⁻ macrophages may have a shared lineage and/or be partially polarized and that one subtype could become or becoming the other.

Before conducting the microarrays, we initially considered that the Arg1⁺ cells might be M2 macrophages, whose formation relies on the transcription factors, PPAR- γ and PPAR- δ (peroxisome proliferator-activated receptor γ and δ) [17]. We therefore treated YARG mice both before and after TBI with PPAR agonists, rosiglitazone, and GW0742, but we observed no increase in generation of YFP⁺ cells. This may reflect our subsequent demonstration that the Arg1⁺ cells are not, in fact, typical homogeneous M2 cells. Other studies of TBI have shown a beneficial effect of rosiglitazone during TBI, which was associated with reduced presence of myeloid cells, although mechanisms directly involving macrophages were not established [52].

Our findings expand our knowledge on chemokines expressed during TBI. Prior gene expression arrays analyzing cortical brain tissue found that IL-8, CCL2, CCL3, CCL4, CCL6, CCL9, CCL12, CXCL10, and CXCL16 were upregulated [5]. Our results identify macrophage subsets as a source of several additional chemokines (Fig. 5) that differ from those that have been previously described, in addition to showing that production of chemokines varies between macrophage subsets.

Macrophages and microglia have distinct roles during homeostasis and pathogenic diseases [11, 53]. Our studies took advantage of flow cytometry to distinguish macrophages from microglia [30]. It is difficult to make this separation by immunohistochemistry, because microglia and macrophages share many markers. Using YARG and Yet40 reporter mice, we did not detect arginase-1, IL-12p40, or MHCII expression in microglia before or after TBI. Thus, microglial activation in TBI was dissimilar from macrophages, despite a broad increase in CD86 expression in both cell types.

In summary, our studies demonstrate that TBI induces a robust infiltration of macrophages that differentiate into at least two

subpopulations in the brain. The two subsets colocalize near the site of injury. They express distinct repertoires of chemotactic molecules, including some that were not previously associated with TBI. In studying the effect of macrophages on the consequences of TBI and in designing strategies to alter these effects, it may be important to consider the role of different macrophage subsets in shaping protective versus pathological responses.

Materials and methods

Animals

C57BL/6 WT males (age 10–16 weeks) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). YARG and Yet40 knockin mice were generated from C57BL/6 mice as previously described [28, 33] and bred in the AALAC-approved transgenic animal facility of the San Francisco VA Medical Center. YARG mice express enhanced YFP from an internal ribosome entry site (IRES) inserted at the 3' end of the *Arg1* gene, leaving the gene and regulatory regions intact, and Yet40 mice express enhanced YFP from an IRES inserted at the 3' end of the *IL-12p40* promoter. Where indicated, mice were administered LPS at 10 mg/kg i.p. and euthanized 4 days later.

Surgery

Controlled cortical impact surgery or sham surgery was performed on anesthetized animals under a protocol approved by the San Francisco VA Medical Center Animal Care Committee. Briefly, bupivacaine was administered subcutaneously above the skull, and an incision was made followed by a 2.5 mm circular craniectomy. TBI was inflicted by a 2 mm circular, flat pneumatic piston traveling at 3 m/s, penetrating 1.5 mm, for 150 ms (Amsci Instruments, Richmond, VA, USA with extensive modifications by H&R Machine, Capay, CA, USA). Target brain coordinates for the center of injury were 1.5 mm lateral, 2.3 mm posterior to the bregma point. After minor bleeding had ceased, the skin was clipped together and animals were monitored for recovery. Sham animals received all surgical procedures without piston impact. As needed, animals were given rehydration therapy for the first 3 days.

Brain and blood leukocyte isolation

Brain leukocytes were harvested according to previously published methods [30]. Briefly, following perfusion brain tissues were obtained and mechanically disassociated through a 100 μ m cell strainer. Washed cells were treated with 400 U/mL DNase I (Sigma-Aldrich) and 0.5 mg/mL collagenase type I (Worthington) at 37°C for 30 min. Leukocytes were isolated by separation on a Percoll gradient (Amersham Biosciences). For PBL isolation,

mononuclear cells were separated from peripheral blood using ficoll-hypaque (GE Healthcare).

Flow cytometry and antibodies

Fc receptors were blocked with 10% rat serum (Sigma) and cells were stained with fluorescent antibodies. Leukocyte analysis used a combination of the following antibodies: anti-CD45 (clone Ly5) allophycocyanin (eBioscience), anti-CD11b (clone M1/70) PE (Invitrogen) or PE-Cy5 (eBioscience), anti-Ly6G (clone 1A8) PE-Cy7 (BD Biosciences), F4/80 (clone BM8) FITC or PE-Cy5 (eBioscience), MHCII (clone M5/114.15.2) PE (eBioscience), CD86 (clone GL1) PE (eBioscience). SYTOX Blue (Invitrogen) was used to gate out dead cells. Cells were sorted on a FACSARIA (BD Biosciences) and data were analyzed using FlowJo Software (Treestar). All data represent mean \pm SEM.

Histology

Brains were perfused with saline followed by 3.7% formaldehyde. After a 2-h fixation, brains were incubated in 30% sucrose overnight and frozen in tissue-freezing medium (Sakura, Inc.). For H&E staining, brains were sectioned 10 μ m thick onto glass slides, heat-dried, and stained (at least three animals per group were analyzed, five sections per animal). For F4/80 staining, 5 μ m sections that were quenched for endogenous peroxidases and blocked with streptavidin and biotin (VectorLabs) were immunostained with an anti-F480 antibody (Clone BM8, eBioscience), followed by goat anti-rabbit biotinylated antibody and visualized using a Vectastain ABC elite kit (VectorLabs) (three animals per group and at least five sections per animal were analyzed).

For immunofluorescent labeling of YFP and F4/80, a biotinylated goat anti-YFP antibody (Abcam) and streptavidin-HRP (Perkin Elmer) were used and amplified by fluoresceinated tyramide (Perkin Elmer). After an additional round of quenching and blocking, mounted sections were further stained with a biotin-conjugated anti-F4/80 (Clone BM8, eBioscience) antibody followed by streptavidin-HRP and Alexa fluor-555 conjugated tyramide (Invitrogen). DAPI (Invitrogen) was used at 300 nM to identify cellular nuclei. Sections were mounted by using Fluorogel (Electron Microscopy Services). All sections were imaged using either a Nikon Eclipse 80i microscope or an Olympus BX-51 microscope. Three TBI animals were analyzed and at least five sections per animal were analyzed.

Microarrays

For gene expression profiling of macrophages from YARG mice, $Arg1^+$ (YFP⁺ CD45^{hi} CD11b⁺ Ly6G⁻ SYTOX Blue⁻) and $Arg1^-$ macrophages (YFP⁻ CD45^{hi} CD11b⁺ Ly6G⁻ SYTOX Blue⁻) were isolated by flow cytometry from ipsilateral brain hemispheres at day 1 following TBI ($n = 4$ for each cell sample). Monocytes

(CD11b⁺ F4/80⁺) from peripheral blood were also collected. Sorted cells were immediately lysed in denaturation buffer and frozen. RNA was isolated by using an RNAqueous Micro kit (Ambion). Further sample preparation, labeling, and array hybridizations were performed according to standard protocols from the UCSF Shared Microarray Core Facilities and Agilent Technologies. RNA quality was assessed using a Pico Chip on an Agilent 2100 Bioanalyzer (Agilent Technologies), and RNA was amplified by use of a whole transcriptome amplification kit (Sigma-Aldrich). Subsequent Cy3-CTP labeling was performed by using a NimbleGen one-color labeling kit (Roche-NimbleGen, Inc.). The quality of the amplified products was assessed by using an Agilent 2100 Bioanalyzer and Nanodrop ND-8000 (Nanodrop Technologies, Inc.). The products were hybridized to Agilent whole mouse genome 4×44K microarrays according to the manufacturer's protocol. Arrays were scanned with an Agilent microarray scanner, and raw signal intensities were extracted with Feature Extraction v10.5 software. Data were normalized by using the quantile normalization method [54]. No background subtraction was performed, and the median feature pixel intensity was used as the raw signal before normalization. A one-way ANOVA linear model was fitted to the comparison to estimate the false discovery rate for each gene for the comparison of interest, and genes with a false discovery rate < 0.05 were considered significant. Scatter plots compared averaged log₂ gene expression from each group. PCA was performed using the top 15% of genes exhibiting the most variance across all samples, using the PopulationDistances module of GenePattern (PMID: 16642009). For heatmaps, data were log₂ transformed and median centered across genes. Replicates were hierarchically clustered (PMID: 16939791). Heatmaps of genes selected from the top 15% most variable genes that exhibited interesting pairwise comparisons were visualized using Java Treeview (<http://sourceforge.net/projects/jtreeview/files/>) (PMID: 15180930).

Meta-analysis of transcriptional responses of brain wound macrophages to BMDMs stimulated by either IFN- γ or IL-4 was performed using previously published tables [38]. Macrophage genes with significant changes in expression upon IFN- γ or IL-4 stimulation were compared with genes with significant expression differences between Arg1⁺ brain macrophages versus Arg1⁻ brain macrophages.

Accession number

Microarray data were deposited in Gene Expression Omnibus (GEO) under accession number GSE39759.

Semiquantitative real-time PCR

Total RNA was isolated from sorted cell populations, including macrophages from injured brain hemispheres and monocytes from peripheral blood, by using an RNAqueous micro kit (Ambion). RT was performed using oligo dT primers and Superscript II reverse

transcriptase (Invitrogen). Amplicons were amplified using SYBR green (New England Biolabs) and the rate of amplification was measured using a 7500 real-time PCR machine (Applied Biosystems). Relative transcript levels for each gene were normalized to GAPDH controls by calculating delta cycle of threshold values. The following primers were used for: Arg1 5'-CTCCAAGCCAAA GTCCTTAGAG-3', 5'-GGAGCTGTCATTAGGGACATCA-3'; Mrc1 5'-CTCTGTTCAGCTATTGGACGC-3', 5'-TGGCACTCCCAAACATA ATTTGA-3'; Nos2 5'-TGTGGCTGTGCTCCATAGTT-3', 5'-CCAGGG CTCGATCTGGTAGT-3'; Il1b 5'-GCAACTGTTCTGAACTCAACT-3', 5'-ATCTTTTGGGGTCCGTCAACT-3'; Ccl24 5'-TCTTGCTGCAC GTCCTTTATT-3', 5'-CTAACCCTCGGTTTTCTGGAAT-3'; Cxcl4 5'-CCTGGGTTTTCCGGACTGGGC-3', 5'-CCGCAGCGACGCTCATGT CA-3'; Cxcl3 5'-CAGAGCTTGACGGTGACGCC-3', 5'-CCAGACA CCGTTGGGATGGA-3'; Spp1 5'-ATCTCACCATTGCGATGAGTCT-3', 5'-CTTGTGTACTAGCAGTGACGG-3'; GAPDH 5'-ATTCAACG GCACAGTCAAGG-3', 5'-TGGTTCACACCCATCACAAA-3'.

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Abbreviations: BMDM: bone marrow-derived macrophage · MHCII: MHC class II · PC: principal component · PCA: principal component analysis · TBI: traumatic brain injury · YFP: yellow fluorescent protein

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