

Developmental plasticity of CNS microglia

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Microglia arise from CD45⁺ bone marrow precursors that colonize the fetal brain and play a key role in central nervous system inflammatory conditions. We report that parenchymal microglia are uncommitted myeloid progenitors of immature dendritic cells and macrophages by several criteria, including surface expression of "empty" class II MHC protein and their cysteine protease (cathepsin) profile. Microglia express receptors for stem cell factor and can be skewed toward more dendritic cell or macrophage-like profiles in response to the lineage growth factors granulocyte/macrophage colony-stimulating factor or macrophage colony-stimulating factor. Thus, in contrast to other organs, where terminally differentiated populations of resident dendritic cells and/or macrophages outnumber colonizing precursors, the majority of microglia within the brain remain in an undifferentiated state.

Parenchymal microglia are ubiquitously distributed in the central nervous system (CNS) where they comprise up to 20% of the total non-neuronal cell population (1). These cells are thought to play a prominent role in infectious, traumatic, inflammatory, ischemic, and degenerative CNS disease processes. The role of microglia as mediators of CNS inflammation is, in part, promulgated through their ability to process and present class II-restricted antigens to CD4⁺ T cells (2, 3).

Microglial cells are derived from CD45⁺ bone marrow precursors of the myeloid lineage, which also can give rise to macrophages, dendritic cells (DC), and granulocytes. Studies by using irradiation chimeras show that the adult brain has two subsets of microglia (3): the resting microglia, which are ramified throughout the brain parenchyma and are mostly a permanent population, and the perivascular microglia, which are periodically replaced by bone marrow-derived elements and are strategically located in the basal lamina of brain capillaries and the choroid plexus. The only known difference between these microglial populations is CD45 expression, which is high on perivascular and low on parenchymal microglia (4). In general, CD45^{high} cells also express more class II MHC and costimulatory molecules. However, it is still unclear whether the parenchymal and perivascular microglia represent the same cellular population at a different activation state, owing to microenvironmental influences, or whether these populations are more distinct.

Traditionally, parenchymal microglia have been considered to be resident macrophages of the brain. However, several pieces of evidence suggest a more complex picture. For example, op/op mice, which are deficient in macrophage colony-stimulating factor (M-CSF) and consequently are depleted of tissue-resident macrophages in several areas, do not exhibit a large reduction in the overall brain microglial population (5–7). Also, previous studies show generation of "DC-like" antigen-presenting cells (APC) from mixed glial cultures (8). Cells clearly displaying macrophage- and DC-like characteristics have been identified from the CNS of mice with experimental allergic encephalitis and toxoplasmic encephalitis (9). Finally, parenchymal microglia are a relatively stable CNS population, exhibiting little or no turnover with other cell compartments (1, 3). This self-renewing capacity is not characteristic of a terminally differentiated

population. Thus, how far along the myeloid lineage pathway microglia have differentiated, and how committed they are toward a DC or macrophage phenotype, is still an open question. The answer to this is essential for evaluating the role of microglia in autoimmune and inflammatory responses within the brain.

Methods

Microglial Cell Preparations. Neonatal microglia were derived from newborn (less than 24 h after birth) SJL/J mice. After removal of the meninges under a dissecting microscope, brains were mechanically disrupted and filtered through 100- μ m cell strainers. Cells were seeded in MEM (GIBCO) supplemented with 10% FCS, 5 μ g/ml insulin (GIBCO), and 2.0 mg/ml L-glucose (Sigma) for 12–14 days. Confluent mixed glial cultures were shaken overnight on an orbital shaker (first shake). Adherent glial cells were trypsinized, split, and reseeded for an additional 10–12 days of culture. The full procedure was repeated twice (second and third shakes). All experiments were performed with cells from the second and third shakes.

Immunohistochemistry. Twenty-micrometer sections from spinal cords of perfused SJL/J mice were fixed in ethyl-3 (3-dimethylaminopropyl) carbodiimide-HCl (EDC) (Pierce), blocked for 1 h with 10% goat serum, and then incubated overnight with 0.3 μ g/ml anti I-A (Y3P, KL-304) or isotype control (mouse IgG2b) in 5% goat serum. Sections then were incubated with 2 μ g/ml secondary mAb [goat anti-mouse F(ab')₂ conjugated with Texas red] (Molecular Probes) in 5% goat serum for 1 h. Sections were stained with KL-304 (0.3 μ g/ml) followed by goat anti-mouse F(ab')₂ conjugated with Alexa 488 and the endothelial cell marker CD31 (PECAM-1), followed by goat anti-rat F(ab')₂ conjugated with Texas red.

Brain Capillaries. Brain capillaries were obtained from perfused brains of SJL/J mice as described (10). Capillaries were seeded on poly-L-lysine-coated coverslips (Biocoat) and fixed with 20 mg/ml EDC. Staining was performed with biotin-conjugated CD45 followed by Texas red-conjugated streptavidin and KL-304 (0.3 μ g/ml) followed by goat anti-mouse F(ab')₂ conjugated with Alexa 488.

Surface Biotinylation and Immunoprecipitation. N9 cells were washed in Hanks' balanced salt solution and surface biotinylated with 1 mg/ml Sulfo-NHS-LC Biotin (Pierce) Hanks' balanced salt solution for 30 min on ice. After blocking excess biotin with 50 mM glycine, cells were lysed (50 \times 10⁶/ml) in 1% Nonidet

Abbreviations: CNS, central nervous system; DC, dendritic cells; M-CSF, macrophage colony-stimulating factor; GM-CSF, granulocyte/M-CSF; APC, antigen-presenting cells; EDC, ethyl-3 (3-dimethylaminopropyl) carbodiimide-HCl; RT, reverse transcription; TNF α , tumor necrosis factor α ; LPS, lipopolysaccharide.

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P-40/200 mM NaCl/50 mM Tris/HCl protease inhibitor mixture (Roche Molecular Biochemicals) for 60 min on ice. Postnuclear supernatants were precleared for 2 h with normal mouse serum/protein A agarose and for 2 h with protein A agarose alone (Amersham Pharmacia). Immunoprecipitation was performed for 2 h at 4°C by using a mixture of mAbs to class II MHC protein (clone Y3P, N22, and KL-304) crosslinked to protein A/G followed by elution in gentle elution buffer (Pierce). The eluate was reimmunoprecipitated with the same mixture of mAb overnight at 4°C. Immunoprecipitates were washed in 10 mM Tris/150 mM NaCl and eluted in SDS/PAGE sample buffer containing 2% SDS at room temperature. Proteins were transferred to nitrocellulose membranes, probed with 1:5,000 dilution of streptavidin-horseradish peroxidase, and visualized by chemiluminescence (Pierce).

Confocal Microscopy. For confocal analysis, cells were fixed with EDC1 (20 mg/ml) and permeabilized with 0.1% saponin at room temperature for 30 min before staining. Cells were stained with 10 µg/ml KL-304 followed by goat anti-mouse F(ab')₂ Alexa 488 (Molecular Probes) or biotin-labeled Y3P followed by streptavidin-Texas red 1:1,000 (Molecular Probes).

Western Blot Analysis. Cells were pelleted and lysed in 1% Triton X-100/50 mM NaPO₄, pH 4.2/1 mM EDTA/3 mM DTT for 1 h at 4°C. Cells were incubated with and without IFN-γ (100 units/ml) for 72 h before analysis. Microglia, bone marrow DCs, and splenocytes were isolated as per established protocols. Peritoneal macrophages were harvested by peritoneal lavage 72 h after i.p. injection of 1 ml of thioglycollate. Supernatants were collected by centrifugation, and protein content was normalized for all samples in each experiment. Cell lysates prepared as above were incubated with ¹²⁵I-JPM for 1 h at 37°C. Labeled cysteine proteases were then analyzed by 13% SDS/PAGE followed by autoradiography to visualize the active proteases. For cathepsin F and L immunoblotting, blots were first probed for cathepsin F (11), stripped, and reprobed with an antibody directed against cathepsin L (gift of A. Erickson, University of North Carolina, Chapel Hill).

Reverse Transcription (RT)-PCR. mRNA from mouse lung (Lu), spleen, thymus (Thy), BM DC, N9 cells, and microglia was prepared, measured, and normalized to a concentration of 0.5 µg/µl. Two micrograms (4 µl) of each mRNA was used in an RT reaction (total reaction volume, 25 µl). One microliter of each RT reaction was used for PCR (forward primer, GCTGAGC-CGGAATGAAAATA; reverse primer, CTTGATGGCCCAG-TAAGGAA; T melting 96°C for 30 s, T annealing 55°C for 30 s, T elongation 72°C for 1 min, 30 cycles).

Results and Discussion

To investigate the differentiation of microglia along the myeloid lineage the expression of receptors for stem cell factors, c-kit-ligand (c-kit-L) and flt-3-ligand (flt-3) on primary cultures of microglia isolated from neonatal mouse brain was examined. These receptors are expressed on myeloid progenitor cells but not on fully mature myeloid DC or resident macrophages (12). Committed DC and macrophage precursors are c-kit⁺ flt3⁺ or c-kit⁺ flt3⁻, the latter being more differentiated as flt3 is down-regulated at an earlier stage than c-kit (12). As shown in Fig. 1A, primary cultures of microglial cells expressed c-kit, whereas flt3 was almost undetectable. Thus, microglial cells appear to be partially, but not completely, differentiated along the myeloid lineage (13, 14).

Uncommitted myeloid precursors, but not fully differentiated cells, respond to lineage growth factors, such as c-kit-ligand (c-kit-L), flt3-ligand (flt3-L), granulocyte/M-CSF (GM-MCSF), and M-CSF (12, 15). To further investigate the lineage

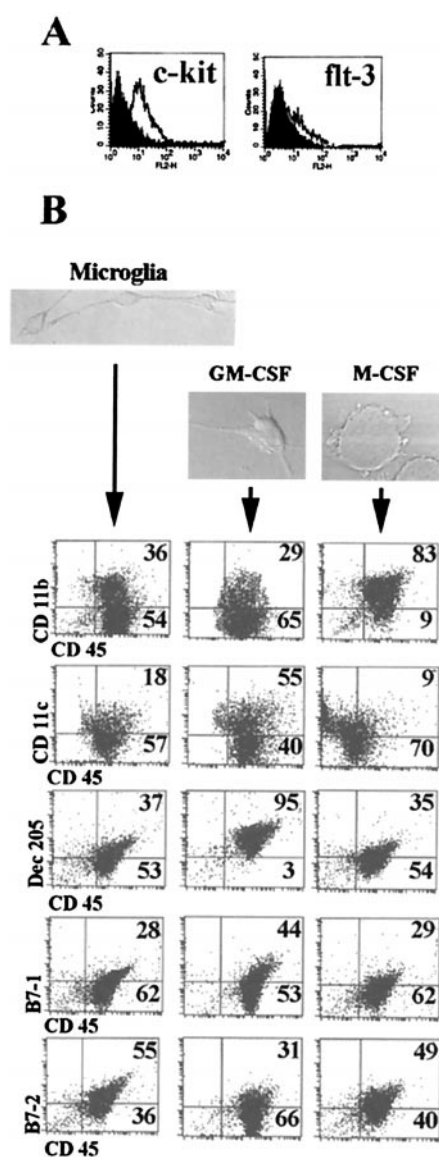


Fig. 1. Neonatal microglia can be skewed toward a DC-like or macrophage-like phenotype by GM-CSF or M-CSF. (A) Neonatal microglia express c-kit, but not flt3, as analyzed by flow cytometry (c-kit, clone 2B8; flt3, A2F10.1). (B) Phenotypic markers are differentially expressed on neonatal microglia after administration of myeloid growth factors. Neonatal microglia were cultured with or without 5 ng/ml GM-CSF or 1 ng/ml M-CSF from the beginning of the mixed glial culture. (Upper) Phase-contrast confocal microscopy of microglia skewed as described above (magnification: microglia, ×69; GMCSF and MSCF, ×100). (Lower) Cell-surface expression of CD11b (clone M1/70), CD11c (clone HL3), mannose receptor (clone Dec205), B7-1 (clone 16-10A1), and B7-2 (clone GL1) was analyzed by flow cytometry.

commitment of CNS microglia, cultures were grown in the presence of these growth factors, and microglial cell-surface expression of CD11b, CD11c, Dec 205, B7-1, and B7-2 was analyzed by flow cytometry. Microglial cells cultured on an astrocyte monolayer (mixed glial culture) in the presence of c-kit-L and/or flt3-L did not show any morphologic or phenotypic alterations as compared with untreated cells (data not shown). However, GM-CSF-treated microglial cells dramatically expanded in number (10-fold as compared with untreated cells) and up-regulated the DC cell-surface markers Dec-205 and CD11c (Fig. 1B Center). GM-CSF also increased surface expression of B7-1, a marker reported to be highly expressed on

immature DC. In contrast, M-CSF skewed the neonatal microglial population toward a more defined macrophage phenotype (CD11b^{high}, CD11c^{low}) (Fig. 1B Right) and did not induce significant microglial cell expansion. Thus, neonatal microglial cells are immature myeloid precursors at a stage subsequent to the branching out of granulocytes (GR1^{neg}, data not shown) but not yet committed to a defined macrophage or DC phenotype. In this respect, neonatal microglia resemble circulating monocytes that colonize the brain a few days after birth and can be differentially biased by GM-CSF or M-CSF (15–17).

Can microglia isolated from brains of adult mice expand after GM-CSF stimulation, or is this solely a property of neonatal cells? Microglia isolated from adult mouse brain by density-gradient centrifugation, as expected, exhibited both CD45^{low} and CD45^{high} populations, expressed CD11b, CD11c, low levels of B7-1, and undetectable B7-2 (data not shown). When cultured in the presence of M-CSF or GM-CSF, no significant expansion was observed, even though these cells were viable in culture for over a month. However, when adult microglia were cultured over an astrocyte monolayer, GM-CSF (but not M-CSF)-treated cells rapidly expanded as compared with untreated controls (data not shown). Thus, adult microglial cells retained the capacity to proliferate in the presence of GM-CSF, albeit only when cocultured with astrocytes. These data further support the hypothesis that adult, as well as neonatal, microglia are immature myeloid precursors. Also, the requirement of astrocytes for microglial cell proliferation indicates that other factor(s), in addition to GM-CSF, are necessary to elicit this response (18).

Immature DC are unique among “professional” APC in expressing “empty” or peptide-receptive class II MHC molecules (19). Macrophages and mature DC, on the other hand, primarily express peptide-loaded class II. To further characterize the maturational stage of CNS microglia, surface expression and conformation of class II MHC molecules were analyzed, using both microglial cells derived from a primary mixed glial culture and two stable transformed cell lines: N9, derived from fetal

microglia (20), and N1P, derived from neonatal microglia (21). Cell-surface class II MHC molecules expressed on neonatal microglia, N9, and N1P were recognized by mAb KL-304 (Fig. 2A), an antibody that recognizes the empty or peptide-receptive class II conformation (19). These same cells stained weakly, or not at all, with Y3P, an mAb that is selective for peptide complexes of I-A (Fig. 2A). Expression of empty class II MHC also was investigated in the adult CNS. Immunostaining of adult mouse brain demonstrated that KL-304 reactivity occurs throughout the parenchyma and is particularly prominent in the submeningeal and perivascular spaces (Fig. 2B and C). Near the brain capillaries, KL-304 immunostaining did not colocalize with the endothelial cell marker PECAM, as assessed by confocal microscopy. However, in capillaries dissociated from perfused adult brain, KL-304 staining colocalized with CD45, indicating expression of empty class II MHC molecules on perivascular microglial cells (Fig. 2D). These data suggest that resting microglia most closely resemble an immature myeloid precursor and may have important implications as to potential pathways for antigen presentation. Notably, antigenic determinants derived from myelin basic protein have been reported to be presented in the CNS through a pathway that does not involve internalization and endosomal processing (22). Presentation of exogenous proteins has previously been shown to occur with bone marrow-derived immature DC (23). The presence of empty class II MHC molecules on the cell surface of microglial cells implies that these molecules may play an important role in recognition and antigen presentation of these proteins within the CNS.

Stimulation of both “nonprofessional” and “professional” APC with IFN- γ markedly increases expression of class II and class II-related molecules. To characterize the cell-surface class II MHC conformation expressed by microglial cells both before and after stimulation with IFN- γ , class II MHC molecules were immunoprecipitated from surface-biotinylated N9 cells using a mixture of mAbs (Fig. 3A). Empty class II $\alpha\beta$ dimers and class II-Ii complexes dissociate into their respective polypeptide

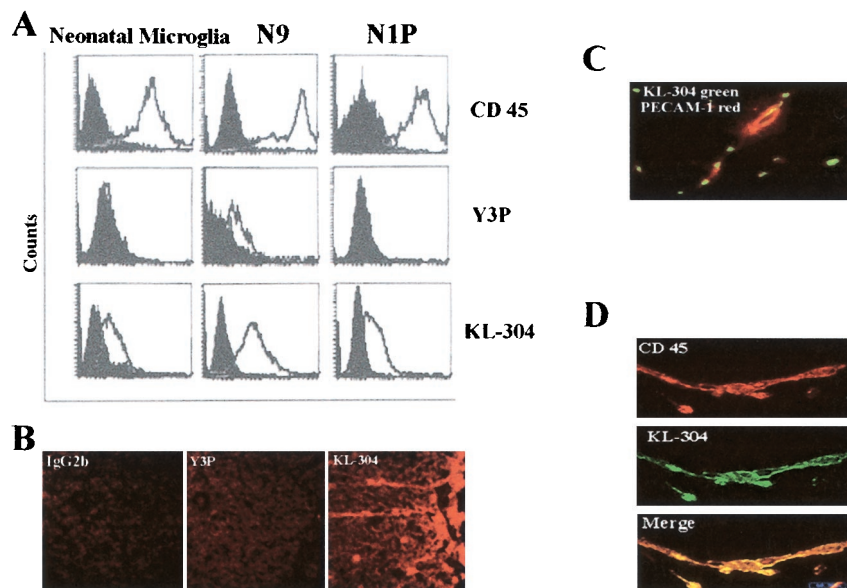


Fig. 2. Microglial cells express surface-empty class II MHC molecules. (A) Resting neonatal microglia express empty, but not peptide-loaded class II MHC molecules, as detected by flow cytometry by using mAb KL304 (empty) and Y3P (peptide-loaded). Primary cultures of neonatal microglial cells and two microglial cell lines (N9, N1P)²¹ were stained with CD45, a marker for bone marrow-derived cells, to distinguish microglia from other CNS components before class II analysis. (B) Empty/peptide-receptive class II MHC molecules are expressed throughout the brain parenchyma, but are particularly evident in the submeningeal spaces. Brain sections were immunostained with anti I-A (Y3P, KL-304) or isotype control (mouse IgG2b) to detect MHC class II conformation. (C) Empty class II molecules do not colocalize with PECAM-1, a marker for endothelial cells. Brain sections were double-stained with CD31 (PECAM-1) and KL-304. (D) Empty MHC class II do colocalize with CD45 in isolated brain capillaries, implying expression of empty/peptide-receptive class II MHC on perivascular microglia.

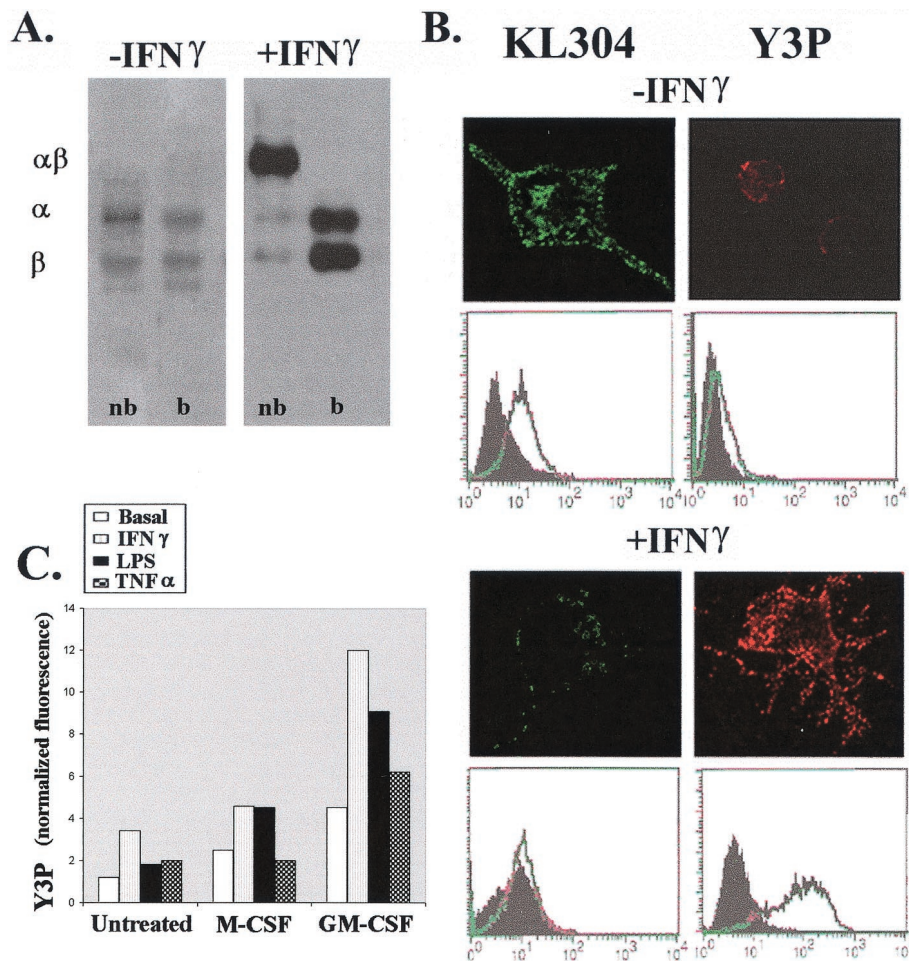


Fig. 3. Expression and conformation of class II MHC molecules are regulated by proinflammatory cytokines. (A) IFN- γ induces expression of SDS-stable MHC class II on N9 cells. Cells were incubated with or without 100 units/ml IFN- γ for 48 h before the MHC class II proteins were immunoprecipitated ($\alpha\beta$ -SDS stable MHC class II complexes; α - α chain of class II, and β - β chain of class II; nb, nonboiled; b, boiled). (B) IFN- γ up-regulates peptide-loaded class II MHC and down-regulates empty class II surface expression on N9 cells. Confocal microscopy (Upper) and flow cytometry (Lower) of similarly treated cells, using anti I-A mAb KL304 (green) and Y3P (red). (C) Microglia cells, differentially skewed by myeloid growth factors, are able to translocate class II MHC protein to their surfaces in response to proinflammatory cytokines. Neonatal microglial cells incubated with M-CSF and GM-CSF as described in Fig. 1 were washed and recultured with 10 ng/ml IFN- γ , 100 ng/ml LPS, or 100 units/ml TNF α for 48 h. Cells were collected and analyzed for surface MHC class II expression by staining with the Y3P mAb. Normalized fluorescence represents the ratio between the geometric mean of the Y3P staining divided for the geometric mean of the isotype control.

chains when incubated in SDS at room temperature, whereas the majority of class II-peptide complexes remain intact and migrate with a higher molecular weight (24). When boiled before SDS/PAGE, class II-peptide complexes also dissociate. Surface-labeled class II MHC $\alpha\beta$ heterodimers expressed on nonstimulated microglial cells were unstable in the presence of SDS (Fig. 3A), consistent with the presence of cell-surface empty class II. After IFN- γ stimulation, surface class II MHC protein levels rose significantly, with the vast majority of the class II MHC molecules now stable to SDS, indicative of class II-peptide complex formation (Fig. 3A Right). A similar set of observations was obtained by using confocal microscopy (Fig. 3B). Unstimulated N9 cells expressed empty class II MHC proteins on their surface as detected by mAb KL-304 but not peptide-loaded class II MHC molecules, detected by mAb Y3P, which were retained intracellularly (Fig. 3B Upper). After stimulation with IFN- γ , cell-surface expression of empty class II MHC molecules markedly decreased, whereas surface expression of peptide-loaded complexes was dramatically enhanced (Fig. 3B Lower). Thus, resting microglial cells expressed predominately empty or peptide-receptive class II MHC molecules at the cell surface,

whereas microglia activated with IFN- γ expressed predominately peptide-loaded cell-surface class II molecules.

A feature that distinguishes professional from nonprofessional APC is the capacity of professional APC to increase class II MHC surface expression in an IFN- γ -independent fashion. In macrophages and DC, the proinflammatory cytokine tumor necrosis factor- α (TNF α), and the bacterial product lipopolysaccharide (LPS) are able to induce translocation of class II MHC proteins from endosomal compartments to the surface (15, 25). Can this same phenomenon be observed in microglia? To investigate this, neonatal microglial cells were first cultured in the presence of GM-CSF and M-CSF to skew these cells toward a DC- or macrophage-like phenotype, extensively washed, and recultured with IFN- γ , LPS, or TNF α (Fig. 3C). In naive microglial cells (not exposed to either M-CSF or GM-CSF), only IFN- γ was capable of inducing surface expression of class II MHC protein, albeit at a low level. This behavior is consistent with the effects of IFN- γ in activating or priming microglia. In cells treated with M-CSF, higher basal levels of class II MHC molecules were observed, which increased even further after IFN- γ and LPS treatment (but not TNF α). In cells treated with GM-CSF, basal MHC class II protein expression

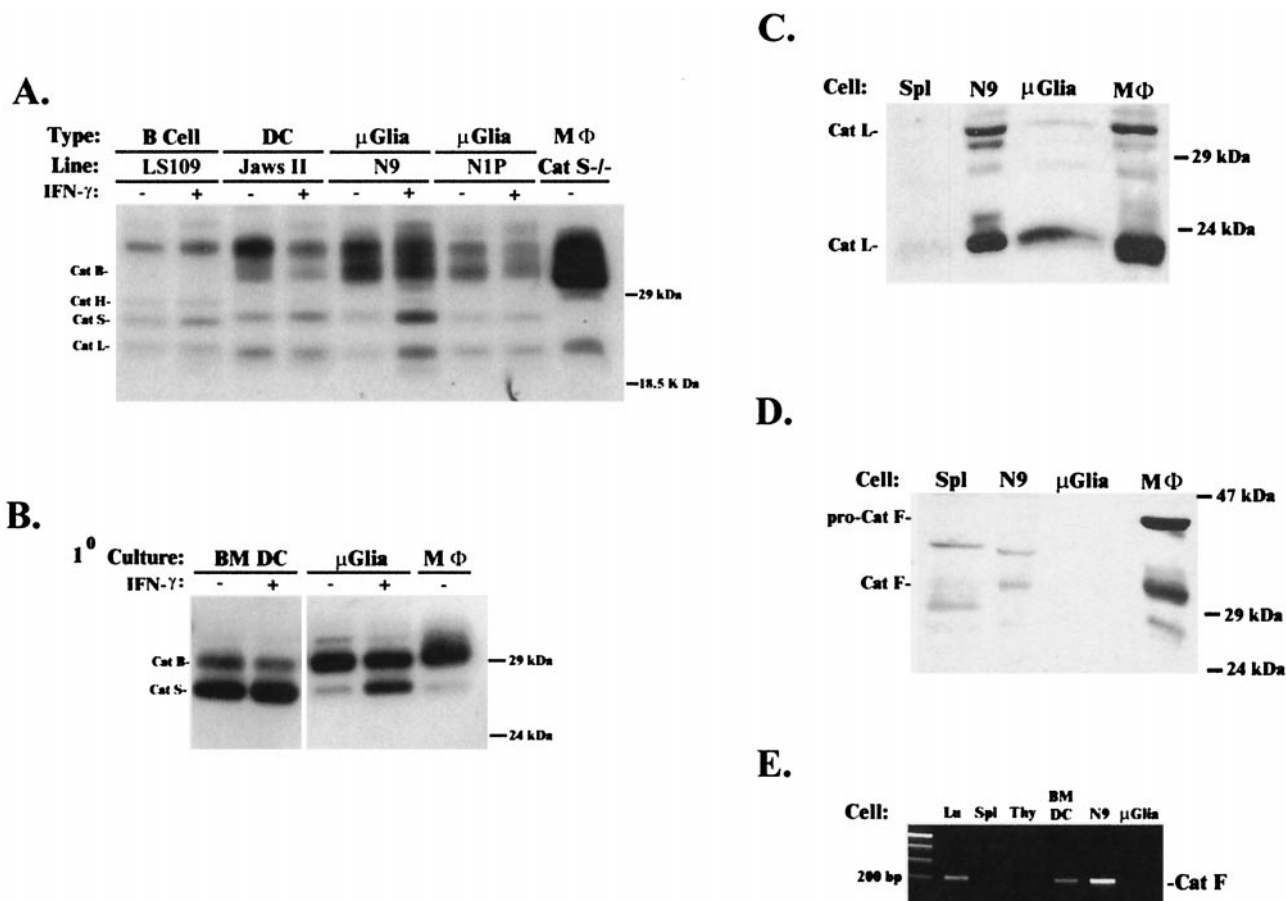


Fig. 4. Microglia express cathepsins S and L but not F. (A and B) Cell lysates from cell lines (LS109, Jaws II, N9, and N1P), primary cultures of neonatal microglia (μ Glia), bone marrow-derived DC (BM DC), splenocytes (Spl), and peritoneal macrophages (M Φ) were labeled with 125 I-JPM and analyzed by 13% SDS/PAGE. All APC express active cathepsins S and B. Up-regulation of cathepsin S with IFN- γ is seen in many, but not all, cell lines. (C) Cathepsin L Western blot of cell lysates showing the active form of cathepsin L in N9 cells, microglia, and macrophages but not splenocytes. (D) Cathepsin F Western analysis of the same blot as in C demonstrating expression of active cathepsin F in macrophages and, to a lesser extent, in N9 cells. Microglia, but not other cells, were stimulated with IFN- γ (100 units/ml) for 72 h before analysis to maximize expression of cathepsin F. (E) RT-PCR of cathepsin F from mouse tissues and cell suspensions. Cathepsin F mRNA is more highly expressed in N9 cells and lung (Lu) compared with neonatal microglia, thymocytes (Thy), and splenocytes.

was again up-regulated, increasing dramatically after stimulation with IFN- γ but less extensively with LPS and TNF α . Thus, both M-CSF and GM-CSF are capable of inducing maturation of microglial cells, which are able to respond to proinflammatory stimuli by increasing surface expression of class II MHC proteins in an IFN- γ -independent manner (18).

Cysteine proteases (cathepsins) play a critical role in class II MHC protein trafficking and antigen presentation in all APC thus far investigated. These enzymes are critical for mediating Ii proteolysis during maturation of class II MHC molecules, permitting subsequent class II-peptide association and antigen presentation (26). Although all bone marrow-derived APC constitutively express the potent endoprotease, cathepsin S, the exact repertoire and roles of the cysteine proteases vary among different APC. B cells and DC use cathepsin S exclusively to mediate the terminal step of Ii breakdown (27–29). Inhibition of cathepsin S activity, either via pharmacologic means or genetic deletion, produces a profound defect in Ii degradation resulting in abnormal class II MHC protein trafficking and antigen presentation. Alternatively, thymic cortical epithelial cells use cathepsin L for this purpose (30). Genetic deletion of cathepsin L activity in mice results in abnormal CD4 $^{+}$ T cell selection secondary to defective class II-Ii processing within thymic cortical epithelial cells. Finally, peritoneal and alveolar macrophages do not rely on either cathepsin S or L to mediate Ii

processing. These APC express a third cysteine protease, cathepsin F, which is capable of degrading the Ii alone (11).

What cysteine proteases are expressed in microglial cells, and how does this cysteine protease profile compare with B cells, DC, and macrophages? The cysteine protease profile of cells was visualized by labeling of cell lysates with 125 I-JPM (Fig. 4A and B) (11). This molecule irreversibly binds to the active site of cysteine proteases in proportion to their activity, making visualization on SDS/PAGE possible. All of the bone marrow-derived APC, including neonatal microglia and microglial cell lines, expressed cathepsin S. Most of the cells (B cell line LS109, DC line Jaws II, fetal microglia line N9, fresh DC SJL, and fresh microglia) exhibited up-regulation of cathepsin S activity with IFN- γ . To investigate expression of cathepsins L and F, immunoblot analysis is required as the migration of these proteases on SDS/PAGE overlaps other cysteine proteases, making clear delineation with 125 I-JPM difficult. Both N9 cells and fresh fetal microglia expressed active cathepsin L by immunoblot (Fig. 4C). Interestingly, N9 cells, but not fresh fetal microglia, expressed a small amount of active cathepsin F, a cysteine protease thus far found only in resident tissue macrophages (Fig. 4D). Semiquantitative RT-PCR confirmed these results, showing much less cathepsin F mRNA in fresh microglia as compared with N9 cells or lung mRNA (Fig. 4E), in the latter case presumably present in alveolar macrophages. Bone marrow-derived DC also showed

some cathepsin F message, possibly from contaminating bone marrow macrophages. The finding that neonatal microglia from primary cultures expressed cathepsins S and L, but not F, supports the hypothesis that these cells more closely resemble immature DC, and not resident tissue macrophages found, for example, within the alveolar and peritoneal spaces.

These data suggest that brain-resident microglia, in contrast to resident APC in other tissues, are not terminally differentiated along the myeloid lineage. Instead, they remain in a primitive stage and may be termed prodendritic cells. They can be skewed toward an immature DC-like or macrophage-like phenotype by GM-CSF or M-CSF, respectively (8, 13, 16).

The immature state of parenchymal microglial cells may account for their capacity for self-expansion, which, in contrast to perivascular microglia, is not mediated by infiltrating bone marrow-derived elements (3, 31). Also, the immature state of

microglial cells would explain their “graded” response to activation (2, 21, 32) as well as their enormous plasticity and capacity to assume different morphologies and phenotypes under degenerative, inflammatory, and autoimmune conditions (2, 31). Maintenance of their immature state is likely related to the cytokine milieu of the brain microenvironment, which is generally biased toward an immunologically nonresponsive state (33). In addition, the immature state of microglial cells, with low expression of class II MHC and costimulatory molecules, could contribute to the brain’s “immunoprivileged state” by inducing tolerance in circulating myelin autoreactive T cells (13, 21, 33).

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