Microglial Physiology: Unique Stimuli, Specialized Responses

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Abstract

Microglia, the macrophages of the central nervous system parenchyma, have in the normal healthy brain a distinct phenotype induced by molecules expressed on or secreted by adjacent neurons and astrocytes, and this phenotype is maintained in part by virtue of the blood-brain barrier's exclusion of serum components. Microglia are continually active, their processes palpating and surveying their local microenvironment. The microglia rapidly change their phenotype in response to any disturbance of nervous system homeostasis and are commonly referred to as activated on the basis of the changes in their morphology or expression of cell surface antigens. A wealth of data now demonstrate that the microglia have very diverse effector functions, in line with macrophage populations in other organs. The term activated microglia needs to be qualified to reflect the distinct and very different states of activation-associated effector functions in different disease states. Manipulating the effector functions of microglia has the potential to modify the outcome of diverse neurological diseases.

INTRODUCTION TO MICROGLIA

Resident tissue macrophages are present in all tissues of the body, and the central nervous system (CNS) is no exception. An extensive literature had debated the origin of the most numerous of the brain macrophages, the microglia, specifically whether they are of myeloid lineage or of neuroectodermal origin. There is little need to rehearse these different points of view; the fact that it took so long to establish the myeloid origin of microglia tells us that these cells have a phenotype that is distinct from other tissue macrophages. Many lines of evidence have converged to establish that the microglia are of myeloid lineage, which was conclusively confirmed by their absence from the CNS of PU.1-null mice (1), although the brains of PU.1-null mice are readily repopulated by microglia following bone marrow transplantation (2). Because microglia and the other populations of macrophages associated with the different structural compartments of the CNS are part of the mononuclear phagocyte system (MPS), we can identify more specific questions: When do macrophages first invade the CNS? Are microglia derived from a specific pool of progenitors or monocytes? How are their numbers maintained in the steady state? What are the signals that lead to microglial activation? What are the phenotypes and functions of these cells in health and disease?

This review relies on studies in humans, mice, and rats. It is worth mention that cells that are functionally equivalent to microglia are observed in the invertebrate CNS. Studies carried out in the leech (*Hirudo medicinalis*) reveal a population of neuroectodermal cells termed small glia or microglia. Leech microglial cells move rapidly to nerve crush lesions, at least partly via purinergic signaling, as described also for mammalian microglia (see below), and they phagocytose debris. Leeches show axonal sprouting and accurate reestablishment of synaptic contacts after nerve crush. Intriguingly, blockade of microglial accumulation at the lesion site impairs axon sprouting (3, 4).

Macrophage Populations of the CNS

Microglia in the adult mammalian CNS have a small cell soma, little perinuclear cytoplasm, and a number of fine, branched processes that are covered in fine protrusions. The cells are readily revealed in rodent and human CNS by lectin cytochemistry or immunocytochemistry for selected antigens, and they occupy a territory that does not overlap with adjacent microglia. The absence or low levels of expression of many antigens typically found on other macrophage populations indicate that the CNS microenvironment plays a critical role in defining the phenotype (see the discussion below in Regulation of the Microglia Phenotype in the Normal Healthy CNS). Despite the downregulated phenotype of microglia in the normal healthy brain, in vivo imaging studies demonstrate that the fine processes of microglia continually palpate and monitor their local microenvironment (5, 6). Microglia are distributed throughout the CNS and vary in density in both rodents and humans, with subtle variations in morphology in different cytoarchitectural regions (7). It is unclear what local factors determine their numerical and morphological variations or whether these variations reflect functional differences, although there are subtle regional diversities in expression of important immune receptors (8).

In addition to the microglia, there are macrophage populations associated with the perivascular space [the perivascular macrophages (PVMs)], the circumventricular organs, the choroid plexus, and the meninges. These macrophages have different phenotypes when compared with the microglia and appear differentially constrained by their local microenvironment. PVMs express antigens not expressed on the microglia. For example, the mannose receptor is detected on PVMs of mouse and human (9) and CD163 in rat and human (10). Similarly, a significant proportion of meningeal macrophages express these receptors. The PVMs and meningeal populations are more overtly phagocytic than are the microglia, and the expression of major

histocompatibility complex (MHC) antigens is more widespread in these populations of cells relative to the microglia. Macrophages are also present in regions of the brain where they are exposed to the blood, including the circumventricular organs and the choroid plexus, and the phenotype of these macrophages is distinct from the microglia and PVMs, perhaps reflecting the role of serum proteins in the regulation of microglia phenotype (11).

An important question regarding the immune privilege of the CNS is whether there exists a population of dendritic cells (DCs), the professional antigen-presenting cells (APCs) of the immune system (12). Since the first reports demonstrating a lack of MHC class II expression in the CNS, there has been no convincing demonstration of DCs, either by expression of relevant DC surface markers or by DC function in the healthy brain parenchyma (13), although DCs are present in the meninges and the choroid plexus. This situation is radically changed in the diseased brain, however (see the section below on Multiple Sclerosis).

Origin of Microglia

In postnatal rodents, immunocytochemical studies using antibodies to F4/80 (ERM1), CR3, and FcγRII/III suggested that the microglia entered the brain from the circulation and presumably were derived from circulating monocytes (14). At earlier stages of development (prior to the development of the vasculature), cells of myeloid origin can be found within the embryonic mouse CNS (embryonic day 8), where they proliferate (15). In the zebra fish, yolk sac–derived macrophages enter the developing brain and retina, where they develop into immature microglia (16). The lineage origin of these cells from the progenitor cells in the yolk sac is unclear, but evidence suggests that these primitive macrophages develop along a PU.1-independent pathway (17). Whether any of these primitive macrophages contribute to the microglial populations that appear later in development or persist into adulthood is not known. In one study (18), more than 20% of microglia isolated from immature brain expressed the hematopoietic stem cell marker CD34, and 90% expressed the B220 antigen, an isoform of CD45 typically associated with B cells and a subset of DCs. These markers are subsequently downregulated during maturation.

Maintaining the Microglial Population

Investigators widely believe that the microglia are a long-lived population of tissue macrophages, but how the resident populations of brain macrophages are maintained in homeostasis and during disease is not yet resolved. Recent studies show that resident tissue macrophage populations may arise from specific subsets of monocytes rather than simply from stochastic recruitment or entry of monocytes into tissues. At least two distinct subsets of monocytes are found in mouse, Gr-1^{hi}/CCR2^{+}/Ly6C^{hi}/CX3CR1^{+} and Gr-1^{lo}/CCR2^{-}/Ly6C^{lo}/CX3CR1^{++} (19). Geissmann et al. (20) reported that Gr-1^{lo}/CCR2^{-} cells may give rise to tissue macrophages, including the microglia, in the steady state. Those investigations did not clarify how many cells enter the brain and into which compartment; furthermore, the cells that apparently entered the parenchyma did not persist for more than a few days. It has been suggested that either CX3CR1 or homeostatic tissue macrophage turnover mediates recruitment, but Cardona et al. (21) demonstrated that CX3CR1 is not required for entry into the brain. Thymidine labeling studies in adult mice demonstrate that microglia undergo DNA synthesis, divide, and contribute to the maintenance of this population (22), akin to the situation found for other long-lived resident myeloid cell populations.

To address the question of the relative contribution of circulating monocytes or intrinsic division to the steady-state population, investigators have typically used radiation chimeras or parabionts. However, the complications introduced by whole body irradiation, including irradiation of the brain, have led to some

confusion. The use of radiation chimeras and parabiosis to study microglia turnover is discussed below in the section on Irradiation Bone Marrow Chimerism and Parabiosis.

The extent to which other populations of brain macrophages, PVMs, or meningeal cells turn over is also poorly understood. Several bone marrow chimera studies have suggested that these cells turn over more rapidly than microglia, but again there are technical issues to consider (discussed below). By injecting India ink into the brain parenchyma, PVMs were labeled that could be identified even two years later (23), suggesting that this population may also be long-lived.

RESEARCH METHODS—THEIR STRENGTHS AND LIMITATIONS

In Vitro

In vitro microglial culture. In vitro systems provide a critical tool for exploring many aspects of microglia biology. Removal of these cells from their microenvironment will release them from the normal constraints that play such a critical role in their phenotype. Thus, using in vitro systems assumes we recognize either that the cells must be studied rapidly after isolation or that the investigator must attempt to recapitulate features of the CNS microenvironment that will in turn give clues about the factors regulating their phenotype. A significant problem is the identification of the microglia phenotype in vitro, as there is no single microglia marker and the microglial phenotype is defined in vivo by a combination of morphology and often lack of or low expression of multiple macrophage antigens. This circumstance has led to a relatively loose use of the term "process bearing" or other terms for the characterization of microglia in vitro, and all too often no attempt is made to address whether they differ from macrophages. Despite these caveats, many studies rely on the isolation and expansion of microglia from the neonatal brain as a model for the study of resident microglia function. Another limitation of studying neonatal microglia is that they have not experienced the CNS milieu in vivo in the context of an intact, mature blood-brain barrier (BBB). Ponomarev et al. (24) recently described a protocol for isolating adult rodent microglia.

The importance of astrocyte-microglia interactions as part of the environmental regulation is well illustrated in experiments by Rosenstiel and colleagues (25), who have shown that microglia and other macrophage populations grown on an astrocyte monolayer develop a highly ramified morphology that is associated with a downregulation of nuclear factor-κB (NF-κB). Whether expression of a spectrum of cell surface antigens is also downregulated has not been systematically studied. Isolation of adult microglia and neonatal microglia shows these cells to be poor or immature APCs, although treatment with granulocyte macrophage colony-stimulating factor (GM-CSF) will lead to differentiation to a more DC-like phenotype (26). Isolation of adult microglia and subsequent culture in low levels of macrophage colony-stimulating factor-1 (M-CSF) lead to proliferation of the surviving cells that then develop a modestly ramified morphology that could be maintained for many weeks (24), consistent with the notion that M-CSF is a key component of tissue macrophage survival and phenotype. Mice lacking M-CSF do exhibit normal morphology and apparent function of microglia, however (27).

Elucidation of many aspects of macrophages and microglia clearly can be readily accomplished in vitro, but the direct relevance of these observations needs to be established through in vivo studies before accepting them at face value. In most in vitro studies, there has been no attempt to replicate the CNS microenvironment, and the state of the cells grown on glass or tissue culture plastic is likely more relevant to inflammatory cells rather than to steady-state microglia.

Brain slices. An intermediate approach to in vivo characterization or the isolation of microglia from their natural environment is to investigate their properties in accessible acute

brain slices or organotypic cultures. Acute brain slices suffer from the obvious consequences of the slicing process, and one might imagine that the microglia would be rapidly activated, but in contrast to isolated in vitro microglia, the ion channel expression in the slice cultures is distinct from that found in cells in vitro and consistent with a downregulated or resident phenotype (28). Organotypic cultures permit the study of microglia with a resident phenotype after several days in vitro, and subsequent injury to these slices leads to somewhat predictable responses given what we know of the in vivo condition and the responses of macrophages (29). Few reported studies have used slice culture, and wider exploitation of this approach might be productive, given the evident limitations of in vitro culture of isolated microglia.

In Vivo

Immunohistochemistry and in situ hybridization. Given the marked limitations imposed by studying microglia in vitro (see above), much effort has been devoted to evaluating microglial biology in the intact CNS. Immunohistochemistry (IHC) has been widely used to characterize microglia, both in human tissues and in those from experimental animals. The strengths of this approach are manifold, and no other technique can provide a comparable wealth of information. Different from RNA analysis [such as in situ hybridization (ISH), Northern blotting, or reverse transcriptase-coupled polymerase chain reaction (RT-PCR)], IHC detects protein, which is more likely to be functionally relevant than mRNA. Furthermore, unlike Western blotting or enzyme-linked immunosorbent assay (ELISA), IHC enables protein both to be detected and to be localized to specific cell types with regional differentiation. With proper controls, IHC supports quantitative morphometry. In addition, using high-resolution techniques such as confocal microscopy with immunofluorescence, subcellular localization can be achieved, occasionally with functional implications. For example, stimulus-dependent transcription factors, such as the signal transducers and activators of transcription (STATs) and the NF-κB components, translocate to the nucleus upon ligand engagement of upstream receptors. Therefore, the detection of STATs in microglial nuclei implies ligation of relevant cytokine receptors (30). IHC can accordingly yield an incomparably vivid depiction, albeit static, of physiological and pathological processes.

Against these strengths lies the cardinal weakness that IHC is fraught with artifact, much of which is deceptively subtle and the causes of which are legion. Artifactual immunostaining can appear crisp and specific with low or absent background. Because of IHC's importance in clarifying neurobiological processes, guidelines for validating antibody staining in neuroscience have been proposed (31, 32). These guidelines are useful both for those performing experiments and for those evaluating experimental results. At the outset, it is evident that simple technical controls such as omission of primary antibody represent only a first step in this crucial process. Pertinent suggestions include use of Western immunoblotting to verify presence of the antigenic target in tissue lysates and application of multiple independent antibodies directed against a single target to verify cellular localization. Where possible, antibodies can be used in negative control experiments to stain tissues of animals [such as gene-targeted mice or patients with gene mutations (33)] that definitively lack the target antigen. These guidelines also recommend that subcellular localization of target antigen should correspond to the known function of the protein; immunoreactivity of receptor antibodies should typically demonstrate plasma membrane distribution, for example.

Parallel performance of ISH with IHC is extremely useful for confirming that target-encoding mRNA resides in the same cell, region, and physiological context as the immunoreactivity. This analysis can be assisted in silico using the Allen Brain Atlas (**http://www.brain-map.org/welcome.do**), a searchable online compendium of ISH experimental results, that can be used to localize mRNAs in the

mouse brain with impressive anatomical detail. Definitive experiments combine IHC and ISH.

In summary, tissue IHC is a centrally important technique for microglial research because the most valid observations of these enigmatic cells need to be conducted in the intact CNS. At the same time, it has been famously difficult to apply IHC to characterize microglia owing to their relatively low expression of MPS markers. Adherence to guidelines for validating IHC results is therefore critical for research into microglia.

Two-photon imaging. Excitation fluorescence imaging allows visualization of neural structures to a depth of 100–200 μm, through a thinned-skull preparation. This approach has been successfully applied to neurobiological questions related to dendritic physiology for more than a decade (34). A major advantage is that this technique affords prolonged imaging sessions, without photobleaching or photodamage. As noted above, two groups used two-photon fluorescence imaging to study mice in which CX3CR1, the receptor for chemokine CX3CL1, was replaced with enhanced green fluorescent protein (EGFP) (*Cx3cr1*$^{+/GFP}$ mice). Both groups made the same surprising observation: that microglia in the intact, healthy CNS continually remodel their processes, in apparent surveillance of the extracellular milieu (5, 6). This landmark finding provided an entirely new view of parenchymal cells previously stigmatized as resting microglia (35). Their dynamic surveillance of the CNS involved processes at all levels of branching, without movement of the soma, and was calculated to monitor the entire tissue every several hours. Contacts with astrocytes, vascular elements, and neurons were visualized. Blocking voltage-gated sodium channels (and thus neuronal action potentials) with tetrodotoxin did not affect process motility. Further research motivated by these findings showed that microglia walled off laser lesions extremely rapidly through process extension, again with minimal initial displacement of the soma (5). Extracellular nucleotides/nucleosides such as ATP or ADP were required for microglial process movement in response to injury (but not in surveillance of the healthy CNS) and signaled through the metabotropic purine receptor P2Y12 (5, 36). The signals that underlie physiological process movement remain to be established (35). *Cx3cr1*$^{+/GFP}$ mice were ideally suited for these studies, as the EGFP fluorescence was expressed in all cortical microglia, as judged by colocalization with microglial markers such as ionized calcium-binding adapter molecule 1 (iba-1), and was restricted to microglial cells, as determined by lack of colocalization with lineage markers for neurons and glial subpopulations (21). Interpretation of data gained by the application of two-photon imaging to microglial biology will depend on which promoter-reporter is used and on detailed knowledge of promoter activity under physiological and pathological conditions. In this context, it bears repeating that no microglial-specific promoter has yet been identified.

Irradiation bone marrow chimerism and parabiosis. Hematopoiesis research has long relied on introducing labeled donor cells, with or without functional alterations, into recipient animals. One widely used technique is to subject a recipient host to lethal irradiation and provide rescue by transferring bone marrow containing hematopoietic stem cells. An alternative is parabiosis, involving anastamosis of the circulations of host and recipient, allowing for mixing of the circulating elements. Irradiation bone marrow chimerism to study the immunology of the CNS was first reported in the early 1980s and addressed questions such as the identification of CNS cells that expressed the MHC class II antigens (37). Irradiation chimerism was suitable for studies of this type because the polymorphic MHC antigens enabled differentiation between the host and donor cells, and the experiments gave unambiguous answers. It was noted early on that parenchymal microglia did not display the markers of transferred cells, although perivascular MPS cells did (38), yielding a preliminary suggestion that perivascular

and parenchymal MPS cells turned over with differing kinetics.

The technique of irradiation chimerism was soon adapted to address key questions in CNS immunity and inflammation. A wide spectrum of research employed the model disease experimental autoimmune encephalomyelitis (EAE), involving sensitization of rodents with myelin protein fragments or adoptive transfer of myelin-specific T cells. In 1988, a seminal study used irradiation chimerism and adoptive-transfer EAE to demonstrate that PVMs of the CNS could stimulate myelin-reactive T lymphocytes adequately to bring about demyelination (39). These results were updated recently, using additional controls to show that PVMs of the CNS were necessary and sufficient for adoptive-transfer EAE (40).

Irradiation chimerism was used to address central questions regarding the turnover and proliferative capacity of microglia and perivascular cells (41–43). The response of engrafted (infiltrating) and resident cells to varied disease or injury models was extensively compared. Irradiation chimerism seemed well adapted for examining the comparative roles of the resident microglial cells and the infiltrating blood-derived elements.

It now appears that data from these earlier irradiation chimerism experiments need reevaluation in light of studies that directly address potential confounds arising from the preparation (44–46). Three sources of confound are recognized: (*a*) Use of bone marrow cells to reconstitute the hematopoietic system might lead to nonphysiological numbers of hematopoietic stem cells or progenitors in the circulation. (*b*) Lethal irradiation-induced cell death in the hematopoietic system is associated with enormous fluxes of cytokines through the circulation and in tissues. (*c*) Irradiation of the CNS causes vascular changes, permanently affecting competence of the BBB. Together, these constituents of the irradiation chimerism protocol appear to lead to nonphysiological transmigration of cells into the CNS parenchyma.

Informative studies have used the facial-axotomy model, which results in brisk microglial reaction in the ipsilateral facial nerve nucleus, after facial nerve crush in the periphery (47, 48). Previous studies showed that facial axotomy of irradiation chimeric rodents leads to incorporation of donor-derived cells in the microglial reactive population (43), and parabiotic animals showed few if any donor-derived microglia under physiological conditions (49). To address mechanisms for recruiting donor microglia into the axotomized facial nerve nucleus, parabiotic animals were generated, and the CNS vasculature of the recipient was conditioned by lethal irradiation, yielding recipients in which actin-GFP-labeled donor cells predominated in the circulation of the recipient but did not contain bone marrow elements beyond those found circulating physiologically (44). After facial axotomy, no GFP^+ microglia were found in the reactive microglial population of the facial nerve nucleus. These results indicated that irradiation alone was not sufficient to allow circulating cells to become parenchymal microglia after this type of injury (44). In complementary experiments, irradiation chimerae were generated, using a protocol that spared the cranial vasculature, and then subjected to facial axotomy (45). Again, no donor-derived microglia were found in the axotomized facial nerve nucleus. The interpretation was that irradiation was required (although not sufficient) (44) to enable circulating cells into the parenchymal microglial pool. Therefore, based on direct comparisons between parabiotic and irradiation-chimeric preparations, investigators now recognize that nonphysiological processes seed the CNS with donor microglia following irradiation chimerism (46). These two complementary observations pointed to the conclusion that both cranial irradiation and transfer of bone marrow cells into the circulation were required for the nonphysiological entry of donor cells into the parenchymal microglial population (46). Corollary conclusions are that parenchymal microglia are replaced by proliferation of resident cells and that microgliosis can be exceedingly impressive in the absence of a contribution from circulating cells.

PET scan. Microglia are embedded in a dense network of neural interactions and are intimately associated with neurons. Owing to its networked nature, the CNS demonstrates unique, distributed pathological patterns, by contrast to other tissues. Damage to an axon excites a brisk (retrograde) response by its remote cell body; damage to an axon also causes an (anterograde) reaction by local microglia that invests the terminus of the injured cell process; most remarkably, when a nerve cell loses input owing to elimination of a distant projecting neuron, there is a trans-synaptic response, including microglial reaction in the vicinity of the deprived neuron.

Neuropathological description of these phenomena captures neither their extent nor their kinetics. Positron emission tomography (PET) scanning has been a useful adjunct in neurological practice, showing, for example, hypometabolism in affected regions of brain in neurodegenerative disease through the application of [^{18}F] 2-fluoro 2-deoxy-D-glucose (FDG) as a PET tracer. Labeled R-enantiomer of an isoquinolone [^{11}C] (R)-PK11195 binds selectively to a mitochondrial membrane translocator protein (TP)-18, part of a protein complex previously termed the peripheral benzodiazepine-binding site (PBBS). This binding activity is expressed selectively by myeloid cells in the CNS, as shown by correlation of IHC and autoradiography. PET studies using PK11195 take advantage of the partition of this moiety into brain across the competent BBB, as well as its selectivity for activated, in contrast to surveillant, microglia. The binding activity remains relatively constant across the spectrum of morphological variations of microglial activation (activated, rod-like, amoeboid) and also binds to infiltrated macrophages. The technique requires significant postprocessing and is challenged by confounds in circumstances of BBB disruption. Having accepted these limitations that, to date, preclude routine clinical application, PET with PK11195 has provided proof-of-principle for microglial activation in neurodegenerative disorders, where trans-synaptic processes must be operative, as well as in the lateral geniculate projection fields of optic nerves of multiple sclerosis (MS) patients suffering from severe optic neuritis (50–52). Furthermore, this technique demonstrates early microglial activation as well as remarkably prolonged activation in neurodegeneration. Documenting microglial activation in these clinical settings has been a prominent catalyst for ongoing research, but the phenotype associated with PK11195 upregulation has not been defined.

Genomics, Proteomics, and Other "Omics"

MPS cells originate from a clonogenic bone marrow progenitor that gives rise to macrophages and DCs (53). Despite their common origin, components of the MPS are markedly heterogeneous, beginning with the circulating elements: As noted above, inflammatory and resident populations of blood monocytes have been tentatively identified in both humans and mice using surface markers (20). Although the two monocyte populations are believed to originate from a bone marrow progenitor by cell-autonomous mechanisms, differential properties of tissue macrophages probably derive mainly from environmental cues (54). The properties of tissue macrophages reflect the demands placed on them by host tissues. Cutaneous Langerhans cells express functions associated with pathogen recognition and entrainment of host defenses. In bone, osteoclasts reflect the requirement for continual tissue remodeling. Pulmonary alveolar macrophages express host-defense functions associated with airborne pathogens and particulate matter, whereas thymic macrophages need high-capacity ability to engulf apoptotic cells. Growth factors related to the generation and maintenance of some of these populations have been described: Mice lacking M-CSF exhibit grossly defective osteoclast development but only subtle alterations in microglia, whereas those deficient for GM-CSF show faulty alveolar macrophage function resulting in alveolar proteinosis. Deficiency for the ets family

transcription factor PU.1 results in failed development of the myeloid lineage and, as noted above, provided proof of the myeloid origin of microglia (1).

It is tempting to consider how concepts related to macrophage heterogeneity could be informative for microglial physiology. Macrophage functions are evidently adapted to host tissues. Even within a single organ such as spleen, regional macrophage heterogeneity has been described. The CNS MPS populations exhibit phenotypic heterogeneity, plausibly related to function. For example, parenchymal microglia may infrequently be exposed to viral pathogens but rarely to bacteria, fungi, parasites, or particulate matter. Their Toll-like receptor (TLR) complement comprises at minimum TLR1 through TLR9, but relative expression levels and signaling efficiency have not been defined (55). Adult parenchymal microglia would, however, be confronted with lipid-rich tissue debris and, to a lesser extent, with apoptotic cells. Appropriate receptors, including scavenger receptors (SRs) and apoptosis-recognition components, appear to be expressed by parenchymal microglia. Surveillant microglia also express P2Y12, a receptor for ATP and ADP, which mediates process extension in reaction to tissue damage (36).

Parenchymal microglia encounter T lymphocytes only under pathological conditions (56). Other CNS MPS cells, including PVMs, choroid plexus, epiplexus, and meningeal macrophages, may, however, be called upon to present antigen to T lymphocytes within the subarachnoid space (57). Accordingly, parenchymal microglia express MHC class II determinants at a low level compared with other CNS MPS cells; furthermore, MHC class II on parenchymal microglia appears cytoplasmic rather than associated with the plasma membrane when localized by confocal microscopy (58). Therefore, one can envision diverse MPS cells in the CNS, analogous to those found throughout the organism.

Defining how the varied but closely related cell populations of the MPS differ at the levels of gene expression, protein production, and signaling has proven a tantalizing and productive field of investigation (59). As one example, the Alliance for Cell Signaling (AfCS, **http://www.signaling-gateway.org**) conducted an extensive analysis of the responses of RAW 264.7 cells (a murine macrophage-like, Abelson leukemia virus–transformed cell line on the BALB/c background). For this study, RAW cells were exposed individually to each of 22 separate ligands and, subsequently, to 231 pairwise combinations of ligands (60). Evaluation of response included cytokine secretion, second messenger generation, and signaling protein phosphorylation. Applying matrix analysis of these outputs to address how cells respond to multiple simultaneous inputs was revealing: There were remarkably selective cytokine outputs in response to combinations such as TLR- plus G protein–coupled receptor (GPCR)-mediated signaling. Evaluation following varied ligand combinations including purinergic- and prostaglandin-responsive GPCRs provided sufficient information to derive a dynamic scheme for cross-regulation of cAMP and Ca^{2+} mobilization. Initial application of the brute-force approach was required to obtain this type of information, as existing data sets would not have allowed generation of a broad concept relating cAMP levels and Ca^{2+} in a dynamic interaction.

Use of these multifold approaches to analyze CNS MPS cells is constrained by considerations described above for in vitro approaches to microglial biology. The most robust physiological data can likely be generated only from cells analyzed immediately ex vivo, but such cell preparations are both labor intensive and resource consumptive (61). Keeping these limitations in mind, data sets combining high-density oligonucleotide microarrays and proteomics platforms have been described for cultured microglia exposed to disease-relevant stimuli such as nitrated alpha-synuclein aggregates, a moiety present in the CNS of individuals with Parkinson's disease (62). Comparing results from such studies with those obtained through examination of other MPS populations subjected to other stimuli will be informative (63).

Systems biological studies, in which computational bioinformatics tools are applied to exploit fully the information in unbiased gene expression data sets, have provided novel insights into the MPS and will also be essential for research into microglia. One instructive report began by compiling a kinetic description of LPS-induced genes in macrophages. Cluster analysis yielded the unexpected prediction that there was an identifiable group of transcripts predicted to be regulated by the cAMP response element binding-protein (CREB) family member activating transcription factor (ATF)-3 (64). Network analysis suggested that ATF-3, not previously ascribed an immune or inflammatory function, modulated a subset of NF-κB-induced transcripts that were induced by TLR4 ligation in macrophages. Specifically, ATF-3 seemed to be a negative regulator of the late wave of NF-κB-induced genes such as IL-6. Biochemical studies validated this hypothesis both by showing closely approximated binding sites for ATF-3 and NF-κB transcription factor complexes in the promoters of the putative regulated genes and by demonstrating physical interactions between ATF-3 and NF-κB components. This type of research, beginning with unbiased descriptive compilation of regulated genes and culminating in delineation of regulatory pathways, should be applicable to dissecting how microglia respond to the distinctive stimuli with which they are confronted within their unique environment. Microarray experiments using cultured microglia have been reported but have not taken systematic advantage of the strengths of kinetic description of gene-expression changes after stimulation and have not been comprehensively exploited with bioinformatics tools (65, 66).

Integrated molecular, bioinformatics, and biochemical approaches will be essential for characterizing microglial responses to stimuli and will benefit from existing rich data sets. As one example, the Innate Immune Database (**http://www.innateimmunity-systemsbiology.org**) contains a searchable database of more than 150 microarray experiments in which TLR ligands were used to stimulate murine macrophages from a single inbred strain (67). The pathway to use these tools to explore microglial biology has been ably blazed by the MPS research community and now awaits utilization for CNS MPS studies.

AFFERENT SIGNALS FOR MICROGLIAL ACTIVATION—NEURODEGENERATION AND INFLAMMATION

Regulation of the Microglia Phenotype in the Normal Healthy CNS

One of the striking features of microglia beyond their morphology is their distinct downregulated phenotype when compared with other tissue macrophage populations. Surprisingly, only recently have factors emerged that might be responsible for this phenotype.

Soluble Factors

A first place to seek factors that regulate microglia phenotype is in the interstitial fluid, the solution that bathes them. In contrast to most other macrophages, these cells are shielded from serum proteins that might lead to their activation, and there is evidence that serum constituents can selectively and potently activate macrophages (68). Furthermore, the cytokine mediator profile in the healthy adult CNS is consistent with a downregulated phenotype with relatively readily detectable levels of cytokines such as transforming growth factor-β (TGF-β) and prostaglandin E_2 (PGE_2). A cytokine both soluble and bound that plays a key role in tissue macrophage survival, proliferation, and differentiation is M-CSF. Mice deficient in M-CSF (op/op) show reduced numbers of microglia with minor alterations in morphology (27), but no difference in the PVM population.

Cellular Interactions

The microglia are in intimate contact with cells in their immediate environment, and the

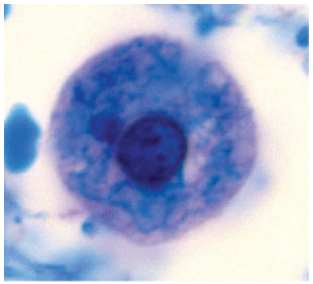
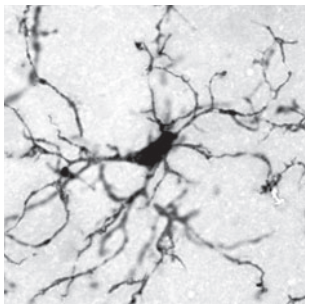
expression of receptor-ligand pairs on the microglia and their neighbors will have a potent effect on their phenotype. The state of activation of myeloid cells is determined in part by the relative levels of expression of receptors expressing immunoreceptor tyrosine-based activation motifs (ITAMs) and immunoreceptor tyrosine-based inhibition motifs (ITIMs) (69). Following receptor ligation, receptors bearing an intracellular ITAM consensus sequence are phosphorylated by Src family protein kinases, which leads to docking of SH2 domains of Syk protein kinases and an intracellular activation cascade. In contrast, ITIM-bearing receptors are phosphorylated by Src with recruitment of SHP-1 and SHP-2 phosphatases, with consequent decreased intracellular activation. The receptor CD200R is expressed on microglia and has an ITIM motif. In mice lacking CD200, normally expressed on neurons, the microglia show constitutive upregulation with changes in morphology and expression of MHC class II, and these changes also lead to more severe disease in EAE (70). There are a number of other receptor-ligand pairs that might be expected to play a similar role. CD172a/SIRPa expressed on macrophages binds the relatively ubiquitous ligand CD47, leading to downregulation of phagocytosis via an ITIM motif (71). Neumann and colleagues (72) have shown that microglia express TREM2 (triggering receptor expressed on myeloid cells-2) and that this receptor is involved in the phagocytosis of debris and downregulation of proinflammatory cytokine expression. Loss or ablation of this receptor leads to deficient removal of cellular debris from apoptotic cells but enhanced expression of inflammatory mediators. Precisely how signaling via TREM2 leads to inactivation of the macrophage is as yet unclear because this receptor signals via the adaptor molecule DAP12 normally associated with cell activation. Recent studies show that deletion of TREM2 or of DAP12 from macrophages leads to identical phenotypes (namely, enhanced inflammatory cytokine production) when challenged with TLR agonists (73). Importantly, homozygous deficiency of either TREM2 or DAP12 in patients leads to adult-onset dementing leukoencephalopathy, providing proof-of-principle that microglial functions are required for CNS homeostasis. The expression of these receptors and others such as the Siglecs (74) will likely contribute to maintaining the state of inactivation of the microglia. Accordingly, disturbances or loss of their ligands during pathological processes will contribute to microglial activation.

Defining Microglial Activation

2008 marked the 150th anniversary of a seminal description of microglia by Virchow, and morphological accounts of resting and activated microglia were embedded in that first report. Subsequent studies of microglial activation began with a premise that activated microglia emerged from a resting state and underwent morphological transformation from ramified to varied activated forms, including amoeboid, rodlike, phagocytic, and so on. The notion of alternative forms of microglial activation seemed implicit in these studies but remain incompletely defined. At present, one can begin to integrate contemporary macrophage biology with further understanding of microglia to attempt to reframe concepts of microglial activation (**Table 1**).

Studies of the MPS have led to varied formulations of macrophage activation. Early during a pathological process, tissue macrophages may be stimulated either by nonself pathogens (stranger signals) or by injured-self components (danger signals). Both stimuli can activate pattern-recognition receptors such as the TLRs, SRs, and the NOD system. Effector outputs of these stimuli focus on clearance of tissue debris, generation of cues for tissue restoration, and resistance to pathogens. Together, these reactions comprise innate immune responses. Subsequent events may require establishment of responses, including lymphocyte effector functions, antibodies, and immunological memory, collectively termed adaptive immunity. MPS cells contribute to this process by antigen presentation, including the instruction

Table 1 Alternate activation of macrophages and microglia[a]

Macrophage	Stimulus	Receptor	Signaling	Activation pattern	Output
	LPS, peptidoglycan, dsRNA	TLR	NF-κB/IRFs	Innate stranger	Inflammatory cytokines, ROS, RNS
	Endogenous TLR ligands (dsDNA, HSPs)	TLR	NF-κB/IRFs	Innate danger	Inflammatory cytokines, ROS, iNOS/RNS
	IFN-γ + LPS	IFNGR plus TLR4	Jak/STAT + NF-κB/IRFs	Classical (M1)	Inflammatory cytokines, ROS, iNOS/RNS, increased antigen presentation via upregulated MHC II and costimulatory molecules, bacteriocidal activity, iNOS
	IL-4 and IL-13	IL-4/IL-13R	Jak/STAT	Alternative (M2a)	Endocytic activity, mannose receptor, β-glucan receptor, fibrogenic cytokines, parasitocidal activity, arginase
	TGF-β, IL-10, glucocorticoids, CD200	TGF-βR, IL-10R, GC-R, CD200L	Smads, Jak/STAT, nuclear hormone receptor, unknown	Alternative-deactivating (M2c)	Reduced MHC class II, reduced inflammatory cytokines, anti-inflammatory prostaglandins
Microglia	**Stimulus**	**Receptor**	**Signaling**	**Activation pattern**	**Output**
	Serum proteins such as fibrinogen	CD11b/CD18 (Mac-1)	Rho-family GTPases	Danger	Cytoskeletal rearrangement, enhanced phagocytosis
	Glutamate	mGluR2	GPCR/NF-κB	Neurotoxic	TNF-α
	ATP, ADP (brief exposure)	P2Y12	GPCR	Danger	Process extension
	UDP (prolonged exposure)	P2Y6	GPCR	Danger	Phagocytosis
	Peripheral nerve injury	Unknown	Src-family kinases	Axotomy and loss of input	Via P2X4, mediate neuropathic pain
	CX3CL1	CX3CR1	GPCR	Inhibition	Reduced IL-1, ROS, iNOS/RNS
	CD200	CD200L	ITIM	Contact-dependent inhibition	Reduced inflammatory cytokines, less MHC class II

[a]Abbreviations: GPCR, G protein–coupled receptors; iNOS, inducible nitric oxide synthase; IRF, interferon regulatory factor; ITIM, immunoreceptor tyrosine-based inhibition motif; LPS, lipopolysaccharide; RNS, reactive nitrogen species; ROS, reactive oxygen species; TLR, Toll-like receptor.

of T cells to adopt varied effector programs (Th1, Th2, Th17) and in some cases directing them to the tissue from which pathogenic material originated. As part of the immunological effector program, MPS cells respond to the products of activated T cells, including IFN-γ, TNF, and IL-1.

One useful exercise has been to organize MPS effector responses along varied pathways by which macrophages can be polarized by exposure to pathogen or to T cell–derived products (**Table 1**). Classically (or M1) activated macrophages respond to LPS plus IFN-γ, whereas alternatively activated macrophages "see" either IL-4/IL-13 (M2a); immune complexes along with IL-1 (M2b); or a variety of modulatory stimuli, such as glucocorticoids, TGF-β, or IL-10 (M2c) (75). Microglia differ decisively from peripheral macrophages, and their activation likely does not follow these precise pathways; nevertheless, it is instructive to consider how the underlying concept of macrophage heterogeneity might apply to microglial responses.

Given the results discussed above from two-photon imaging (35), microglia may now be regarded as surveying the healthy CNS and engaging varied modes of progression from the surveillant state to effector microglia. This concept should replace the notion that microglia proceed from resting to activated (76). Next, stimuli for microglia are analogous to, but clearly distinct from, those confronted by peripheral macrophages. Three (at least) clear distinctions between microglia and macrophages apply here:

1. Microglia reside behind the BBB so that serum products represent danger signals indicating BBB breach.
2. Microglia are MPS family members but need also to be equally regarded as brain glial cells, so that altered synaptic activity with perturbation of neurotransmitter availability will affect microglial activation states and effector properties. Regulation by neurotransmitter signaling may intermittently affect peripheral macrophages, as described for the cholinergic anti-inflammatory response (77), but neurotransmitter effects are more diverse and pervasive for microglia.
3. Microglial cells, perhaps as a consequence of living among fragile, nonrenewing neurons, exhibit an actively repressed phenotype, so that removal of (mainly neuronally derived) inhibitory constraints constitutes a type of danger signal, indicating that neuronal function is impaired.

Here, we cite examples of each of these special considerations for microglial activation.

BBB disruption as a danger signal. Serum constituents activate microglia (which is a key limitation of using in vitro cultures to examine their physiology). One molecular interaction that underlies microglial activation during demyelinating disease may be exposure to fibrinogen, which engages CD11b/CD18 integrin heterodimers (68). Suppressing this stimulatory pathway reduces the severity of EAE.

Microglial responses to neurotransmitter alterations. Glutamate is an excitatory neurotransmitter, and excess glutamate stimulation is neurotoxic. Glutamate excess can result from increased release (by neurons) or decreased clearance (which is mainly the responsibility of astrocytes). Glutamate receptors can be coupled to calcium entry (ionotropic) or to GPCR signaling (metabotropic or mGluRs). Microglia express mGluRs and respond to fluxes in extracellular glutamate. Microglial responses to acute and chronic injury are regulated in part by selective activation of mGluRs, culminating either in neurotoxic or neuroprotective outcomes (78, 79). Adding complexity, extrinsic inflammatory stimuli regulate mGluR expression.

Danger signals from damaged cells and from altered neurotransmitter levels. As a composite example of specialized danger responses by microglia, ATP is a purinergic

neurotransmitter, signaling both to P2X ionotropic and P2Y metabotropic receptors. Microglia are endowed with both P2X and P2Y purinergic receptors. As noted above, extracellular ATP, released in part from damaged neurons, acts through microglial P2Y12 to mediate process extension after a cortical laser lesion (5, 36). Therefore, P2Y12 can be regarded as a specialized PRR for danger signaling within the CNS. Neuronal cell death or injury confronts microglia with a requirement to function as phagocytes, and another purinergic receptor, P2Y6, acting in a longer time frame than P2Y12 (which transduces signals within seconds), appears to play a significant role in microglial phagocytosis (80). Intriguingly, the endogenous danger ligand for P2Y6 is extracellular UDP, again providing an instance of selectivity in the microglial reaction to injury.

Microglial activation by loss of neural input. P2X4 receptors are induced on spinal microglia via Src family kinases after peripheral nerve injury and are critically involved in the CNS mediation of neuropathic pain (tactile allodynia) in pain models, although the specific mechanism by which this effect occurs is uncertain (81–83). This phenomenon, given its specificity, can be viewed as an alternative mode of microglial activation, evidenced by selective upregulation of a purinergic receptor.

Microglia are actively repressed. CX3CL1 is a membrane-tethered chemokine that is tonically released from CNS neurons through the action of ADAM proteases. Microglia receive tonic inhibitory inputs through the CX3CL1 receptor, CX3CR1 (21). If this tonic inhibition is removed, microglial neurotoxicity is unleashed in response either to systemic inflammatory stimuli or to damage to resident neurons (21). Release from constitutive inhibition is also believed to underlie the devastating neurological consequences of deficiency either for microglial TREM2 or its intracellular adaptor DAP12 (84, 85). Although TREM2 is a transmembrane receptor–like molecule with clear-cut inhibitory function for varied stimuli, its endogenous ligands remain to be fully characterized.

EFFECTOR FUNCTIONS OF MICROGLIA: DEVELOPMENT AND REPAIR

The early entry of macrophages into the embryonic brain was described above (Origin of Microglia section). It is uncertain whether these cells persist and give rise to adult microglia, but their potential roles in development have attracted interest. Microglial developmental functions may be informative for roles in disease, and conversely.

Synaptic Remodeling: Pruning, Stripping, and Plasticity

During CNS development, axonal connections with synaptic targets exceed those required and are reduced in a process termed pruning. For some years, it has been clear that synaptic pruning is aberrant in mice lacking specific MHC class I determinants, although mechanisms continue to unfold (86). Recently, Stevens et al. (87) found that mice lacking complement components C1q or C3 exhibit defects in visual system synaptic refinement that are similar to those found in MHC class I–deficient animals. They and others (86, 87) proposed assigning microglia an effector role in this aspect of CNS development, as microglial cells are the CNS elements that express the appropriate complement receptors.

Stevens et al. (87) also provided evidence that synaptic loss might be associated with aberrant engagement of complement pathways in a mouse glaucoma model. The opposite possibility has also been proposed: specifically, that microglia assist in synaptic repair after brain lesions. Synaptic repair is incorporated along with learning under the rubric of synaptic plasticity. By extrapolation from developmental synaptic pruning, Cullhein & Thams (88) suggested that microglia contribute to plasticity after lesions, by pathways similar to those used for pruning excess synapses during development and facial axotomy, but direct evidence is lacking.

Inducing Apoptosis in Supernumerary Purkinje Cell Neurons During Development

CNS development is characterized by waste: excess production of synaptic connections and generation of a surplus of cells. Supernumerary cells undergo apoptotic cell death and are engulfed without generating inflammation by microglia (14, 89, 90). It was long speculated that microglia might also provide inputs that promote developmental neuronal apoptosis (89), and data have been forthcoming to support this contention. Cerebellar slice cultures from neonatal mice demonstrate apoptosis of Purkinje cell neurons, which are elaborately invested with microglial processes. Furthermore, elimination of microglia with toxic liposomes reduces the efficiency of Purkinje cell commitment to apoptosis (91).

Do Microglia Produce Neural Growth Factors?

Can microglia also provide growth factors for neurons during development? Here the data are indirect and rely solely on observations in adult mice. PU.1-deficient mice lacking microglia exhibit grossly intact CNS structure and function. However, studies of adult neural stem cell niches provide tantalizing hints of supportive microglial-neural progenitor relationships. In adult mice, neural stem/progenitor cells (NSPCs) are localized in the hippocampal subgranular zone (SGZ) and in the rostral subventricular zone (SVZ). In culture, NSPCs give rise to both neurons and glia, thereby fulfilling criteria of multipotency. Conditioned medium of microglial cultures contained factors capable of supporting prolonged neurogenesis, implying an instructive function for microglia toward NSPCs of the adult SVZ (92). Microglia secrete factors that direct NSPC migration in vitro (93). Microglia also promoted neuronal differentiation in these studies (93). Taken together, these data suggest a role for microglia in promoting CNS lesion repair by NSPCs. This concept was given relevance for neurodegenerative disease, through studies of microglia from transgenic mice expressing mutant forms of human presenilin-1 (PS1) associated with familial Alzheimer's disease (AD). NSPCs in these mice fail to exhibit environmental enrichment-mediated proliferation and neurogenesis. In vitro, NSPCs from transgenic mice expressing either mutant or wild-type PS1 proliferated and differentiated equally. By contrast, co-culture of microglia from mice expressing mutant PS1 with wild-type NSPCs recapitulated the phenotype of impaired proliferation and neuronal differentiation. This effect was mediated by microglial-derived secreted factors. The data supported an important role for microglia in regulating NSPCs during physiology and pathology (94, 95).

Vascular Development

It is not clear whether microglia exert functions that promote CNS vascularization. The notion that they might exert such functions comes by inference from two observations: first, the provocative finding that microglia are present in the CNS before vasculogenesis and that invading vessels are closely invested with microglia; second, that experimental CNS tissue implants are colonized by microglia before they are vascularized (89).

PATHOLOGY

Introduction

Microglia are exquisitely sensitive to disturbances of their microenvironment, and they have been dubbed the sensors of pathology (96). The early and rapid response of microglia is entirely consistent with the role of tissue macrophages as the first line of defense against infection or injury. The response of the microglia is not a linear process varying simply by degree, but rather, as has been documented in other macrophage populations, their response is dictated by the nature of the stimulus, the receptor repertoire that is engaged, and the prior state of the macrophage (97) (see section on Defining Microglial Activation). In the context of neurodegeneration, a number of important variables must also be considered in the

temporal and spatial domains. The two most common neurodegenerative conditions of the CNS are stroke and AD. In the former, the death of neurons is extremely rapid, a matter of minutes; in the latter, the pathology driven by the presence of a misfolded protein may lead to death of a slowly increasing number of neurons over years or even decades. The former stimulus is a step function, the latter a slow ramp function. The slow degeneration of neurons—their synapses, soma, axons, and myelin sheath—may continue for many years, providing a stimulus that must lead to adaptive changes in the surrounding microglia. The adaptive changes may then be influenced by other comorbidities and systemic influences that communicate with the brain (98).

Neurodegeneration versus Neuroprotection

A major theme in studies of the role of microglia in neuropathology is the dichotomy between their contributions to neurodegeneration versus neuroprotection. The role of macrophages in wound repair is well documented and part of the innate immune response (99). The thinking behind this dichotomization is that if we can understand the two components we can minimize the harmful and capitalize on the beneficial (100, 101). This is a hugely ambitious aim when we recognize that this has yet to be achieved for significant clinical advantage in any organ, let alone within the complex context of the CNS. Furthermore, the CNS is an immune-privileged organ in which evolutionary pressures have ensured that the innate and acquired immune responses are tightly controlled. Overcoming the immune privilege per se is unlikely to benefit the host except perhaps in combating infections. Finally, microglia themselves might be targeted by a pathological process, which also affects adjacent tissues. Recent data from studying the genetically determined inflammatory demyelinating metabolic disorder X-linked adrenoleukodystrophy suggest that such a mechanism might play a part in the cerebral form of this disease (102).

SPECIFIC NEUROLOGICAL DISORDERS

Alzheimer's Disease

It is now nearly 20 years ago that an innate inflammatory response in AD, the presence of morphologically activated microglia, was described, rapidly followed by studies indicating that people taking nonsteroidal anti-inflammatory drugs were in some way protected from the onset or progression of AD. The role of inflammation in AD has been extensively researched and exhaustively reviewed (103, 104), but the contributions to the disease of the microglia and the associated innate inflammatory response are by no means clear. A wide variety of inflammatory mediators are expressed by microglia when they are challenged in vitro with the Aβ peptides derived from the amyloid precursor protein (APP). However, there is a lack of consensus on which cytokines or other mediators are indeed present in the postmortem brain tissues of patients that died with AD. In APP transgenic mice (which mimic the deposition of Aβ amyloid in the brain, a hallmark of AD pathology), the microglia do indeed appear morphologically activated, particularly in the immediate vicinity of the plaques. But again, there is little agreement on the cytokine profile associated with these microglia (98), which is consistent with the difficulty in performing assays of low abundance and highly soluble proteins such as the cytokines (see above).

A more direct route to assessing precisely which molecules are synthesized by microglia that might either promote or delay disease is to study their impact on the plaque load. The plaques in various mouse models, and in human AD patients, inexorably increase with age, and the conclusion must be that the microglia are rather inefficient phagocytes, which is perhaps not surprising given their very slow removal of neuronal and myelin debris after a stroke (105). However, indirect activation of the microglia by intracranial challenge with LPS leads to a decrease in plaque load (106). Others suggest that bone marrow–derived macrophages selectively invade the region of the plaques and play a role

in removal of the amyloid material (107). How such findings translate to the clinical situation is unclear because these models are not progressive neurodegenerative disease models. The use of bone marrow chimeras in these studies will also have to be reassessed.

An exciting development in the attempts to manipulate the immune system for benefit in AD comes from studies in APP transgenics, which show that systemic immunization against Aβ leads to removal of the amyloid plaques (108). Several hypotheses have been suggested as to how systemic antibodies might achieve this, including microglia phagocytosis via FcRs. Alternatively, antibody-mediated clearance of systemic/circulating Aβ would promote increased drainage of Aβ from the brain and inhibit Aβ fibril aggregation (109). These mechanisms are not mutually exclusive, and it is by no means clear that the microglia are essential for effective removal of the amyloid. This intriguing experimental approach has been rapidly translated into human: Despite the fact that it is unclear how the amyloid is removed, this approach does permit a direct test of the hypothesis that removal of the amyloid plaques might slow disease progression. A recent neuropathological analysis of the brains from a small number of patients who were vaccinated in the AN1792 Elan Pharmaceuticals trial shows a positive correlation between the degree of plaque removal and the antibody response. However, clinical analysis of the immunized cohort revealed evidence neither of improved survival nor of a delay in the time to severe dementia (109a). It is too early to conclude that a vaccination strategy will not work, and it is possible that earlier or different vaccination strategies will be required to achieve a benefit.

Multiple Sclerosis

MS is an acquired inflammatory demyelinating disorder of the CNS and is the leading cause of nontraumatic disability among young adults in the United States and Europe. The cause of MS is unknown but includes both environmental and genetic factors, some of which have been identified (110). MS research has depended largely on an autoimmune animal model, EAE, that exhibits features of CNS inflammatory demyelination and paralysis that resemble aspects of MS. The model disease begins with peripheral immunization, usually with peptide epitopes of myelin proteins. EAE is a T cell–dependent disease, most commonly mediated by $CD4^+$ T cells, including those that express both Th1 and Th17 cytokine profiles. EAE proceeds either by monitoring animals until disease onset around two weeks postimmunization (active EAE) or by isolation and transfer of primed cells from draining lymph nodes or spleen (adoptive-transfer EAE).

Roles of microglia in EAE illustrate their ability to contribute to adaptive (auto)immune reactions that target CNS antigens. We have long known that, to elicit EAE after adoptive transfer, T cells require restimulation with antigen in the CNS compartment. Classic experiments used radiation bone marrow chimeras to generate animals in which bone marrow–derived cells expressed MHC determinants distinct from those present on parenchymal microglia. Adoptive transfer of myelin-specific T cells that were restricted by the parenchymal cells did not cause disease, whereas those that recognized antigen in the context of PVMs derived from the bone marrow inoculum led to EAE (39). In follow-up studies, eliminating all potential APCs except for $CD11c^+$ PVMs produced mice that remained susceptible to adoptive-transfer EAE (40). A variant form of adoptive transfer, in which EAE was primed by one myelin epitope and mice with ongoing disease received unprimed TCR transgenic T cells specific for a different epitope, was used to show that myelin-specific T cell proliferation occurred mainly in the CNS after disease onset (111). This experiment extended concepts of the capabilities of CNS DCs, which arise in the context of active CNS inflammation (112). Nevertheless, it has yet to be shown that the healthy CNS parenchyma contains DCs competent to initiate immune responses by presenting antigen to naive T cells in vivo (113).

Following priming, do parenchymal microglia contribute to neurobehavioral impairment in EAE? This issue was addressed by generating CD11b-HSV-TK mice that expressed a lineage-restricted Herpes virus thymidine kinase (HSV-TK) suicide gene in myeloid cells, so that administration of gancyclovir would eliminate proliferating $CD11b^+$ cells. Radiation chimerism with wild-type bone marrow produced animals in which only the radiation-resistant parenchymal microglia remained susceptible to gancyclovir-mediated death. Combining EAE immunization with gancyclovir treatment led to mice exhibiting EAE with microglial paralysis. These mice exhibited mild EAE signs, indicating a role for microglia in the severity of EAE (114).

Are these studies informative for MS? The journey from EAE mechanism to MS pathogenesis is notoriously tortuous, the route baffling, and the destination uncertain. Proof-of-principle that MS is autoimmune has not been forthcoming. For that reason, immunopathologic mechanisms of EAE seem more directly pertinent for MS than do applications of the principles of autoimmunity. From EAE, we could predict that MPS cells would exhibit DC-like characteristics in MS tissues, a hypothesis with experimental support (115, 116). In white matter, actively demyelinating MS lesions are identified by the presence of myelin debris in macrophages. Early-active zones in which demyelination began within a few days can be distinguished from late-active zones in which demyelination has been present for 2–4 weeks, by defining whether labile or relatively stable myelin breakdown products are found within phagocytes (117). Early-active MS lesions contain a mixture of infiltrating hematogenous monocytes and microglia, which are differentiated by morphology, localization, and surface markers; late-active MS lesions are typified by a monomorphic population of phagocytic macrophages (117). Numbers of MPS cells in individual early-active and late-active MS lesions are identical, suggesting that phagocytic macrophages in late-active regions arose both from monocytes and microglia. Enumeration of monocytic and microglial cells in early-active zones suggests that between one-half and two-thirds of phagocytic macrophages in late-active MS white matter lesions arise from microglia (117, 118).

Recently, it has become clear that MS is not exclusively a white matter disease: Remarkably, cortical (or gray matter) demyelination is more extensive, as a fraction of total myelin, than is white matter demyelination. The largest and most mysterious demyelinating foci of MS gray matter affect the most superficial three layers of the cortical ribbon and are termed subpial lesions (119). Among inflammatory demyelinating diseases, subpial lesions are relatively specific for MS (120). Although the mechanisms underlying subpial lesions remain unclear, the inflammatory characteristics of such lesions in chronic-progressive disease are noteworthy: There is a paucity of lymphocyte or monocyte infiltration, as evidenced by sparseness of perivascular cuffs, whereas microglial activation is remarkably robust (119). The data suggest the possibility that microglia are key effectors in the process of subpial demyelination, possibly activated by diffusible factors from the cerebrospinal fluid. Subpial demyelination may be a critical determinant of disability in MS patients (121), so its mechanisms are crucially important for therapeutic development. Equally vital, data to date comprise only chronic material; imaging studies indicate that cortical damage occurs very early during the course of MS, and the mechanisms underlying cortical demyelination in the initial phases of disease have yet to be characterized. Roles of microglia, which seem prominent in chronic cortical demyelination, may be different in the earlier stages of MS.

Can microglia promote repair of MS lesions? Remyelination is clearly a prevalent and poorly understood attribute of MS lesions (122). Many factors elaborated by activated microglia might support oligodendroglial survival or function (123). However, present MS therapy aims at complete elimination of MS-associated neuroinflammation. Until this goal is shown by evidence to be impossible, it is most

logical and straightforward to strive to abrogate CNS inflammation, rather than allow damage while trying simultaneously to bias the process toward enhanced repair.

Prion Disease

Prion disease offers a tractable laboratory model to study many aspects of fatal neurodegeneration associated with protein misfolding and has the additional attraction that it is the same disease in both humans and animals. In murine prion disease, the microglia become activated early in the disease process, even in the absence of widespread histologically detectable amyloid protease–resistant PrP^{sc} deposits (124). The cytokine profile associated with this activated phenotype was not dissimilar from that observed in APP transgenics, featuring low levels of inflammatory cytokines but, in addition, readily detectable levels of TGF-β and PGE_2, a phenotype previously associated with macrophages that had phagocytosed apoptotic cells (125). This phenotype appears prior to the onset of neuronal apoptosis but in association with synaptic loss (126), indicating first that prion disease is a member of diseases with an early synaptic degeneration (a synaptopathy), and second that this may lead to an atypical microglial activation that has been referred to as anti-inflammatory or benign. In prion disease induced on a wild-type background, there is no evidence that the enhanced levels of PGE_2 are detrimental, nor that TGF-β is injurious. However, when TGF-β is neutralized by decorin, delivered by an otherwise harmless adenoviral vector, this leads to acute and marked neuronal degeneration, highlighting the importance of anti-inflammatory regulation in chronic neurodegenerative disease (127).

To investigate how the activated microglia phenotype might be affected by systemic inflammation, a common occurrence in clinical neurodegenerative disease, mice were challenged systemically with endotoxin to mimic an intercurrent infection. This maneuver led to a dramatic switch in the microglial phenotype with an aggressive inflammatory cytokine profile, exacerbation of the sickness behavior associated with endotoxin challenge, and increased neuronal apoptosis (128). Investigators have proposed that the microglia in the prion diseased brain are primed by the ongoing pathology and that a secondary stimulus, the signaling of systemic inflammation across the BBB, switches these cells to an aggressive phenotype (98). Whether microglia switched to an inflammatory phenotype phagocytose or degrade the prion protein by secretion of relevant proteases remains unclear.

The concept of the rapid switching of the microglia phenotype is of course entirely in keeping with what we know of the considerable degree of plasticity of the cells of the macrophage lineage. Systemic inflammation has a profound impact on a number of other animal models of neurological disease (98) and accelerates cognitive decline in Alzheimer's patients (129).

Amyotrophic Lateral Sclerosis (ALS)

ALS is a fatal neurodegenerative disease that can involve toxic gain-of-function of a mutant protein. In contrast to prion diseases, in which the protein accumulates predominantly extracellularly, the toxic protein in ALS is an intracellular protein and exerts its action intracellularly. Approximately 5–10% of ALS is an autosomal-dominant familial form of the disease, of which a significant proportion has mutations in the enzyme superoxide dismutase-1 (SOD-1). Microglial activation is present in the vicinity of the degenerating motorneurons (130). Transgenic mice expressing the mutant SOD-1 have been a valuable model for understanding disease pathogenesis, revealing that the demise of the motorneurons is not a cell-autonomous event, but rather depends on the surrounding non-neuronal cells (131). Dissection of the role of microglia has come from several different approaches.

Mice in which the microglia are no longer able to respond to the downregulatory influence of the chemokine CX3CL1 have been

crossed with SOD-1-expressing mice, and these mice show a modest shortening of survival time (21). More dramatic effects have been uncovered in studies in which mice expressing Cre-recombinase driven by the CD11b promoter are crossed with a LoxSOD1^{G37R} mouse, leading to levels of mutant SOD-1 that are significantly reduced in microglia (132). These mice lived longer than LoxSOD1^{G37R} mice, and, in particular, the late stage of the disease was prolonged. The role for microglia expressing SOD-1 in accelerating disease progression was also demonstrated by another group using a different approach in which SOD-1 mice crossed with PU.1 mice were transplanted with either wild-type or SOD-1-expressing bone marrow that would then populate the tissues, including the brain, with either wild-type or SOD-1-expressing macrophages (2). The mice transplanted with wild-type cells lived longer than those transplanted with SOD-1 bone marrow, and again it was the later stage of the disease that appeared to be prolonged. SOD-1-expressing microglia generated more superoxide and nitric oxide when challenged with LPS in vitro than did wild-type microglia. The general principle, namely that action of a mutant gene in microglia can promote selective loss of neuron subpopulations, has since been validated in other diseases (133).

In both these studies, the impact of the Cre-recombinase or bone marrow transplantation will affect not only the microglia, but also the systemic macrophage populations and any monocytes that might be recruited to the vicinity of the degenerating motor neurons. The differential contributions of the local microglia and of the systemic macrophages have yet to be separately assessed and may be consequential for studies on prion disease, as described above.

Tumor

Primary brain tumors are primarily derived from the astroglial lineage. These lesions are infiltrative and typified by vigorous angiogenesis; their metastatic potential is relatively low. Considerations of the roles of microglia in brain tumor follow from a robust literature about tumor-associated macrophages (TAMs) (75). TAMs are considered to provide a variety of proneoplastic functions, while residing in an immunosuppressive environment, which restrains antitumor properties such as capacity to generate reactive oxygen and nitrogen species. Investigators have proposed that TAMs represent variant M2 macrophages that have been exposed to immunosuppressive prostaglandins, TGF-β, and IL-10. Beyond inhibiting their antitumor potential, these environmental cues could stimulate TAMs to produce growth and angiogenic factors for tumor cells. Such principles translate readily to an evaluation of the roles of microglia in primary brain tumors. Microglia were detected in brain tumors more than 80 years ago (134). Subsequent research focused on three issues: (*a*) Why do tumor-associated microglia appear quiescent rather than tumoricidal? (*b*) What microglial factors promote tumor growth or survival? (*c*) How can microglia be stimulated to express tumoricidal functions? As a generalization, immunosuppressive factors present in other tumor beds are also detected in brain tumors, prominently including TGF-β, prostaglandins, and IL-10. The origin of tumor-associated microglia is uncertain. Glioma cells exuberantly express CCL2, so much so that the human protein was first isolated from glioma culture fluid (135). CCL2 acts toward hematogenous monocytes, but CCR2, the relevant receptor, is not expressed on microglia. Furthermore, flow cytometric analysis of human tumor samples revealed infiltrating CD45^{hi} cells, unlike the mixed population of CD45^{hi} and CD45^{dim} cells found in rodent glioma models (134). It is plausible therefore that tumor-infiltrating MPS cells are mainly monocytic in origin, attracted through an impaired BBB by chemoattractants such as CCL2. Tumor-infiltrating microglia, stimulated by local IL-6, produce IL-10, which suppresses macrophage antitumor effector functions and also promotes Type 2–related angiogenic factors. Type 2 macrophage/microglia may also support invasiveness by expression of matrix metalloproteases. The presence of

MPS cells within brain tumors has elicited therapeutic endeavors, mainly aimed at stimulating antitumor immunity through peripheral immunization with DCs. To present tumor antigens, DCs are pulsed with antigenic peptides or programmed to express glioma cell cDNAs (136). One particularly interesting approach involves use of cDNA from glioma stem cells (136). These strategies rely in part on the APC properties of resident MPS cells to restimulate tumor-specific $CD4^+$ T cells within the CNS. Microglia may also be called upon to execute tumoricidal functions following stimulation by activated $CD4^+$ T cells.

Human Immunodeficiency Virus (HIV)

Infection with HIV-1 imposes a vast burden of neurological disease, exclusive of that caused by opportunistic infection and metabolic compromise (137, 138). During early years of the pandemic, HIV-associated dementia (HAD) was the most feared and most prominent of these complications. HAD is a clinical syndrome comprising cognitive, affective, and motor symptoms. With highly active antiretroviral therapy (HAART) and prolonged survival, the incidences of HAD have lessened and those of subtle cognitive impairment along with that of painful sensory neuropathy have increased. Two considerations placed microglia and infiltrating MPS cells squarely in the center of HAD pathogenesis: First, mononuclear phagocytes are the major infected population in the CNS, and second, neurons, whose function is most evidently impaired, are very poorly infectable and are not grossly reduced in number. Given their prevalence and severity, HAD and related conditions represent the most significant disease for which primary pathogenic pathways involve microglia.

HIV-1, like other lentiviruses (139), enters the CNS in Trojan horse mode within trafficking MPS cells. In HAD tissue sections, most cells containing viral antigens are macrophages and microglia. In vitro studies suggest that microglial physiology is disrupted by infection, so that potentially neurotoxic functions are engaged and neuroprotective responses are blunted. Microglia can elevate levels of excitotoxins such as glutamate and quinolinic acid by diverse mechanisms, many of which seem active in HAD; combined with oxidative stress, these components may lead to neuronal structural pathology and functional compromise (62, 140). Microglial activation may also operate through production of inflammatory cytokines to recruit astrocytes and BBB elements into the HAD process. There is evidence for unapparent BBB dysfunction and for the participation of astrocytes in mediating neuronal dysfunction. Understanding mechanisms underlying HAD is regarded as significant for therapeutics, for extension to other dementing illnesses, and for addressing neuron-glial interactions during disease.

DISCLOSURE STATEMENT

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