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REVIEW

Microglial M1/M2 polarization and metabolic states

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Microglia are critical nervous system-specific immune cells serving as tissue-resident macrophages influencing brain development, maintenance of the neural environment, response to injury and repair. As influenced by their environment, microglia assume a diversity of phenotypes and retain the capability to shift functions to maintain tissue homeostasis. In comparison with peripheral macrophages, microglia demonstrate similar and unique features with regards to phenotype polarization, allowing for innate immunological functions. Microglia can be stimulated by LPS or IFN-γ to an M1 phenotype for expression of pro-inflammatory cytokines or by IL-4/IL-13 to an M2 phenotype for resolution of inflammation and tissue repair. Increasing evidence suggests a role of metabolic reprogramming in the regulation of the innate inflammatory response. Studies using peripheral immune cells demonstrate that polarization to an M1 phenotype is often accompanied by a shift in cells from oxidative phosphorylation to aerobic glycolysis for energy production. More recently, the link between polarization and mitochondrial energy metabolism has been considered in microglia. Under these conditions, energy demands would be associated with functional activities and cell survival and thus, may serve to influence the contribution of microglia activation to various neurodegenerative conditions. This review examines the polarization states of microglia and their relationship to mitochondrial metabolism. Additional supporting experimental data are provided to demonstrate mitochondrial metabolic shifts in primary microglia and the BV-2 microglia cell line induced under LPS (M1) and IL-4/IL-13 (M2) polarization.

LINKED ARTICLES

This article is part of a themed section on Inflammation: maladies, models, mechanisms and molecules. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2016.173.issue-4

Abbreviations

2-DG, 2-deoxy-glucose; AMPK, AMP-activated PK; BBB, blood–brain barrier; CD172 (SIRP1A), signal-regulatory protein; CD206, mannose receptor; EAE, experimental autoimmune encephalomyelitis; FA, fatty acid; Fizz1, found in inflammatory zone 1; HK, hexokinase; iNOS, inducible NOS; MHC-II, major histocompatibility complex-II; NLRP, nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing; NODs, nucleotide-binding oligomerization domains; PPP, pentose phosphate pathway; RNS, reactive nitrogen species; ROS, reactive oxygen species; SR, scavenger receptor; TCA, tricarboxylic acid cycle; TLR, Toll-like receptor.

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TARGETS
Enzymes[a]
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AMPK
Arg-1, arginase 1
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LIGANDS	
CCL2	IL-6
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CCL20	IL-13
CCL22	IL-18
CCL24	Il-21
CX_3CL1	IL-23
CXCL13	IL-33
IFN-γ	LPS
IL-1β	NO
IL-3	TGF-β
IL-4	TNF-α

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

The innate immune response of the body recruits a number of different cells to initiate a response to a novel stimulus such as a pathogen. These various cells of the immune system communicate and cooperate in a complex fashion to successfully complete their assigned tasks to clear the invading factor and return the system back to homeostasis. Such cells include circulating lymphocytes (T-cells, B-cells, NK cells) and monocytes that can develop into either dendritic cells or macrophages. They also include tissue associated bone marrow-derived mast cells (effector cells of allergic reactions) and tissue-specific macrophages. Within this arsenal, cells that phagocytose the initiating factor serve in a workhorse-type capacity. In the periphery, these actions are primarily the function of bone marrow-derived polymorphonuclear leucocytes that constantly circulate in the blood and in tissue-specific macrophages that originate as monocytes from stem cells in the bone marrow. Once the macrophage has phagocytosed the material, it initiates intracellular processes that ensure the destruction of such engulfed material. As this function relates to the clearance of invading microbes, the cell can accomplish this task in two main ways, aerobically or anaerobically. The phagocyte can produce oxygen-based chemicals that, by being reactive, can disrupt the microbe. This is often considered as an oxidative burst or respiratory burst. Alternatively, the cell can kill the microbe without oxygen by either increasing the acidity of the internal environment or by depriving the microbe of iron to inhibit metabolism.

Tissue-specific macrophages can be found in virtually all tissues of the body and are representative of distinct classes (Gautier *et al.*, 2012). In the CNS, microglia function as resident mononuclear phagocytes. In comparison with peripheral tissue macrophages and antigen-presenting dendritic cells that originate from bone marrow-derived monocytes (Parwaresch and Wacker, 1984; Fogg *et al.*, 2006), microglia are derived from primitive yolk sac myeloid progenitors that actively seed the brain parenchyma during mid-embryonic development (Alliot *et al.*, 1999; Ginhoux *et al.*, 2010). Similar to other tissue-specific resident macrophages, microglia represent 10–15% of the total cell population within the brain parenchyma (Carson *et al.*, 2006) and display a level of morphological heterogeneity across regions (Lawson *et al.*, 1990; Mittelbronn *et al.*, 2001; Harry and Kraft, 2012). As resident cells of the brain, microglia are involved in regulatory processes critical for tissue development, architectural refinement, maintenance of the neural environment, response to injury and subsequent remodelling/repair. Similar to macrophages, microglia mount an immune response to pathogens, monitor for tissue changes and maintain tissue homeostasis by clearing pathogens, dying cells, debris or aberrant proteins (Gehrmann *et al.*, 1995; Bruce-Keller, 1999; Stevens *et al.*, 2007; Wake *et al.*, 2009; Nagamoto-Combs *et al.*, 2010; Sierra *et al.*, 2010; Tremblay *et al.*, 2010; Olah *et al.*, 2011; Paolicelli *et al.*, 2011). It is thought that through such capacities, microglia play a role in brain development and in various neurological and neurodegenerative disorders (Kettenmann *et al.*, 2011).

Under normal conditions, microglia assume a neural-specific phenotype (Schmid *et al.*, 2009) and retain a relative quiescent surveillance phenotype for constant monitoring of the brain parenchyma (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). Maintaining microglia in a relatively quiescent

state is, in part, due to signals derived from neuronal- and astrocyte-derived factors (Cardona *et al.*, 2006; Neumann *et al.*, 2009; Ransohoff and Cardona, 2010). This role is attributed to expression of several receptors on microglia including triggering receptor expressed on myeloid cells-2, signal-regulatory protein CD172 (SIRP1A) the chemokine CX_3CL1, colony-stimulating factor 1 receptor and CD200R (Wright *et al.*, 2003; Kierdorf and Prinz, 2013). Healthy neurons accomplish regulatory tasks via secreted and membrane bound signals including CX_3CL1 (Hoek *et al.*, 2000; Barclay *et al.*, 2002; Sunnemark *et al.*, 2005; Lyons *et al.*, 2007), CD200 (Hoek *et al.*, 2000; Frank *et al.*, 2006), neurotransmitters (Pocock and Kettenmann, 2007), neurotrophins and CD22 (Mott *et al.*, 2004), which acts on the CD45R for negative regulation of microglia via inhibition of p44/45 MAPK (Tan *et al.*, 2000). In addition, within the normal CNS environment, microglia express high levels of microRNA-124, reducing expression of CD46, major histocompatibility complex-II (MHC-II) and CD11b serving to maintain the cells in a quiescent yet, surveillance state (Conrad and Dittel, 2011).

M1 polarization state of macrophages and microglia

Macrophages respond to endogenous stimuli generated following infection or injury and demonstrate both pathogenic and protective roles (Mills, 2012; Boche *et al.*, 2013; Wynn *et al.*, 2013). Upon appropriate stimulation, classically activated, pro-inflammatory (M1) macrophages serve in the first line of defence of the innate immune system occurring often within the first few hours or days. Microglia share phenotypic characteristics with peripheral monocytes. This allows for innate immunological functions. They recognize harmful stimuli using a full array of immune receptors, such as toll-like receptors (TLRs), nucleotide-binding oligomerization domains (NODs), NOD-like receptors and many scavenger receptors (SRs; Ransohoff and Perry, 2009; Ransohoff and Brown, 2012). Within injured tissue, microglia exist in various states of activation and retain the capability to shift their functional phenotype during the inflammatory response (Stout *et al.*, 2005; Graeber, 2010). With injury, resident microglia or macrophages infiltrating from the circulation become polarized towards a pro-inflammatory (M1) phenotype upon exposure to pro-inflammatory cytokines IFN-γ, TNF-α and cellular or bacterial debris. These cells then produce pro-inflammatory cytokines (TNF-α, interleukin (IL)-1β, IL-12), present antigen, and express high levels of inducible NO (iNOS) for NO production (Gordon and Taylor, 2005; Villalta *et al.*, 2009). This action is geared to kill the offending foreign pathogen and polarize T-cells to mount an adaptive immune response. In many experimental models, the M1 response is characterized following exposure to bacterial-derived products such as, LPS or signals associated with infection such as IFN-γ (Martinez and Gordon, 2014). In the absence of microorganisms, a similar, but sterile inflammatory response occurs often as a result of trauma, ischaemia-reperfusion injury or chemical exposure (Chen and Nunez, 2010; Shechter and Schwartz, 2013; McPherson *et al.*, 2014). Like peripheral macrophages, microglia respond by producing M1 associated factors such as, pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-12, IL-23, TNF-α), chemokines, redox molecules (NADPH oxidase, phagocytic oxidase, iNOS), SRs (macrophage receptor with collagenous structure), co-stimulatory proteins (CD40) and MHC-II (Hanisch and Kettenmann, 2007; Henkel *et al.*, 2009; Ransohoff and Perry, 2009; Colton and Wilcock, 2010; Varnum and Ikezu, 2012; Boche *et al.*, 2013). Early work on the MMGT12 murine microglia cell line (Briers *et al.*, 1994) and primary microglia provided a wide-ranging transcription and functional profile using the M1/M2 differentiation spectrum (Michelucci *et al.*, 2009). An extensive transcription profile was examined including LPS or IFN-γ-induced M1 markers, such as *IL-1β*, *IL-6*, *TNF-α*, *NOS2*, *COX-2*, C-C chemokines *CCL2* and *CCL20*, and the receptor *CCR2*. Functional aspects of the polarization showed that phagocytosis was inhibited with M1 polarization. While the majority of work is in rodent microglia, primary microglia obtained from adult human brain can be induced to an M1 phenotype with LPS + IFN-γ (Durafourt *et al.*, 2012).

The outcome of a M1 polarizing event is dependent upon a number of features, not the least of which is whether the response includes a production of iNOS, reactive oxygen species (ROS) or activation of NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome complex (Bordt and Polster, 2014; de Rivero Vaccari *et al.*, 2014). The NLRP3 inflammasome protein complex facilitates production of active caspase-1 for generation of IL-1β and IL-18 from precursor proteins (Netea *et al.*, 2014). NLRP1, NLRP3 and NLRC4 are primary complexes (Martinon *et al.*, 2009) governing caspase-1 activation for proteolytic processing and secretion of pro-inflammatory cytokines (Labbe and Saleh, 2008; Salminen *et al.*, 2008) to activate the full cytokine cascade. Limited data are starting to become available regarding the role of inflammasome activation in CNS injury (de Rivero Vaccari *et al.*, 2014). A role for NLRP3 inflammasomes in IL-1β release has been reported from LPS-primed prion-infected microglia (Shi *et al.*, 2012). In lentivirus-infected microglia, NLRP3 inflammasome activation is an early aspect of infection (Walsh *et al.*, 2014). Chronic exposure to exogenous glucocorticoids primes microglia towards an exacerbated pro-inflammatory response and induces NLRP3 within the hippocampus (Frank *et al.*, 2013).

M2 polarization of macrophages and microglia

While the initial response of macrophages to injury has been known for some time, positive influences on tissue remodelling have been recognized more recently (Longbrake *et al.*, 2007; Ruffell *et al.*, 2009; Deng *et al.*, 2012; Novak and Koh, 2013; Shechter and Schwartz, 2013; Shechter *et al.*, 2013). In the early 1990s, the concept of macrophage alternative activation was developed largely based on work showing a role for IL-4 in the induction of an alternative (M2) activation state (Stein *et al.*, 1992) inducing expression of the anti-inflammatory cytokines (*Il4, Il10, Il13* and *TGF-β*) as well as, arginase-1 (*Arg1*), *CD206* and Chitinase-3-like-3 (*Ym1* in rodents) (Colton, 2009; Henkel *et al.*, 2009). M2 macrophages play a role in allergy response, parasite clearance, inflammatory dampening, tissue remodelling, angiogenesis, immunoregulation and tumour promotion (Sica and Mantovani, 2012). Upon further study, subclasses of M2 activation have been identified. The M2a activation state is induced by para-

sitic products or associated signals (IL-4 and IL-13) with a longer-term function for resolution and repair (Rutschman *et al*., 2001; Gordon, 2003; Lawrence and Natoli, 2011; Mills, 2012; Wynn *et al*., 2013). In this case, signalling occurs through IL-4 receptor α leading to inhibition of NF-κB signalling induced by M1 activation. M2b polarization is observed with triggering of Fcγ receptors, TLRs and immune complexes (Martinez and Gordon, 2014). M2c polarization occurs in response to specific anti-inflammatory factors such as, IL-10, TGF-β and glucocorticoids (Vodovoz *et al*., 1993; Gordon, 2003; Martinez *et al*., 2008; Morris, 2009; Mills, 2012). Other cytokines induce M2 polarization such as IL-3 (Kuroda *et al*., 2009), IL-21 (Pesce *et al*., 2006) and IL-33, (Kurowska-Stolarska *et al*., 2009) as well as the chemokines, CCL2 and CXCL4 (Roca *et al*., 2009; Gleissner *et al*., 2010). In addition, cells can shift from an M2b phenotype to a mixture of M1 and M2a/b (Lisi *et al*., 2014). M2 polarization of microglia is similar to peripheral macrophages (Fenn *et al*., 2012; Liu *et al*., 2012; Chhor *et al*., 2013; Freilich *et al*., 2013), generating different mRNA profiles for IL-4 and IL-10 stimulation including *Arg1*, *Mmr*, *Ym1*, found in inflammatory zone 1 (*Fizz1*) and *Ppar* (Michelucci *et al*., 2009). While these associations have been demonstrated *in vitro*, M2 is induced *in vivo* in sterile wounds in the absence IL-4 or IL-13 (Crane *et al*., 2014) suggesting an alternative stimulus. In this model, M2 macrophages were derived from M1 macrophages that matured into repair macrophages within the tissue after recruitment from the circulation (Italiani *et al*., 2014). Thus, the inherent phenotype of the cells may differ as a function of source and environment.

M2 macrophages facilitate resolution of inflammation through anti-inflammatory factors (e.g. IL-10, IL-13, TGF-β), VEGF, EGF, Arg1) to deactivate pro-inflammatory cell phenotypes and re-establish homeostasis (Gordon, 2003; Gordon and Martinez, 2010; Ortega-Gómez *et al*., 2013). This includes production of IL-10 to down-regulate inflammatory cells, extracellular matrix protecting proteins like Ym1/2, ornithine, polyamines for wound repair and higher levels of receptors associated with phagocytosis (Martinez *et al*., 2009). IL-10 induces STAT3 and downstream genes including *Il10*, *Tgfb1*, macrophage mannose receptor *Mrc1* (Lang *et al*., 2002; Gordon, 2003). Upon STAT6 activation, induction occurred in *Ym1*, *Mrc1* and *Fizz1*. Functional changes associated with M2 activation include increased engulfment of apoptotic cells by cells stimulated with IL-10 (Ghigo *et al*., 2001; 2004; Benoit *et al*., 2008b; Michelucci *et al*., 2009). In contrast, macrophages from IL-10 over expressing mice do not show enhanced phagocytosis, but are unable to clear *Coxiella burnetii* infection. They exhibit an M2-type transcriptional programme with increased mRNA levels for *Arg1*, mannose receptor (*Mr*) and *Ym1/2* and down-modulated inflammatory markers (Meghari *et al*., 2008). Activation of the PPAR-γ characterizes M2 polarization (Benoit *et al*., 2008a; Rajaram *et al*., 2010). M2 cells express chemokines (CCL17, CCL22 and CCL24) (Mantovani *et al*., 2004) and co-express macrophage activation factor with CD68 or CD163 (Barros *et al*., 2013). The specificity of these markers remains in question. For example, CD163 is considered a M2-specific marker (Buechler *et al*., 2000); yet, differential expression of CD163 has not been observed in human disease (Barros *et al*., 2013) or polarized macrophages (Kittan *et al*., 2013). In addition, the primary 'marker' for M2, Arg1, is also induced in M1 macrophages and expressed in some resident and mycobacteria-infected macrophages (El Kasmi *et al*., 2008).

In a few studies, the effects , particularly on neuroprotection and repair, of M2 microglia have been demonstrated (see Cherry *et al*., 2014a). Butovsky *et al*. (2006) suggested that glatiramer acetate could induce microglia to express insulin-like growth factor 1 for neuroprotective action. In a murine experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis, Ponomarev *et al*. (2007) reported a regulatory role for CNS-derived IL-4 in the induction of Ym1 protein and mRNA. This elevation occurred in the absence of NO production. Clinical signs of EAE were exacerbated in chimera mice deficient in IL-4 suggesting an association with a diminished M2 microglia phenotype (Ponomarev *et al*., 2007). Based upon the temporal expression of M1- and M2-related factors in a mouse spinal cord injury model, Kigerl *et al*. (2009) examined the effect of conditioned media from bone marrow-derived macrophages on dorsal root ganglion cell survival and neurite outgrowth. Conditioned media from LPS + IFN-γ induced macrophages damaged neurons while media from IL-4-induced macrophages-stimulated neurite outgrowth. Additional studies are now being reported linking the M2 microglia phenotype to diminished injury and potential repair; however, the strongest data still remain from spinal cord injury models.

Polarization transitions

Injury-induced inflammatory processes are dynamic, demonstrating spatial and temporal heterogeneity, with the possibility that individual cells express transitional phenotypes. It has been suggested that macrophages transition from a M1 phenotype to a more regulatory or anti-inflammatory M2 phenotypes to promote positive functional outcomes and minimize scar tissue formation. Alternatively, subpopulations of macrophages within an injury environment may express specific phenotypes resulting in concurrent expression of M1- and M2-related factors or mixed M1/M2 phenotypes (Ziegler-Heitbrock *et al*., 2010; Pettersen *et al*., 2011; Wong *et al*., 2011; Vogel *et al*., 2013). *In vitro* studies indicate that human monocytes can polarize to a M1 phenotype then mature into a M2 phenotype as a function of sequential changes in culture conditions (Italiani *et al*., 2014). Following exposure to classic M1 signals, TLR ligands or IFN-γ, M2 macrophages can be reprogrammed to express M1 genes (Stout *et al*., 2005; Mylonas *et al*., 2009). Recent work suggests that histone H3K27me3 demethylase Jumonji domain containing three was essential for microglial M2 polarization and M1 down-regulation (Tang *et al*., 2014). While a shift to an M1 phenotype would be a relatively standard transition, it is considered rare that once activated, peripheral M1 cells would switch to an M2 phenotype. Rather, for peripheral immune cells, it is thought that M1 stage cells become terminal and die during the inflammatory response (Albina *et al*., 1989). However, it has also been shown that inflammatory monocyte-derived M1 macrophages can undergo phenotype conversion and become tissue-resident macrophages (Hashimoto *et al*., 2013; Yona *et al*., 2013). Alternatively, any apparent increase in M2 phenotype cells may be associated with the loss of NO-producing cells and an increase in TGF-β for amplification of M2 polarization. Thus, the question

remains as to whether M1 and M2 macrophages are phenotypically distinct subpopulations that, within different stages of an inflammatory response, would perform different functions (Auffray *et al.*, 2007; Nahrendorf *et al.*, 2007) or rather, shift between functional phenotypes depending upon environmental signals (Arnold *et al.*, 2007; Crane *et al.*, 2014).

Resident microglia versus peripheral macrophages

Evaluation of the M1/M2 paradigm in the CNS becomes complex as compared with other tissues due to the presence of the blood–brain barrier (BBB) that prevents the infiltration of blood-borne monocytes/macrophages. Physical injury or late-stage disease states can lead to a disruption of the BBB allowing monocyte-derived macrophages to infiltrate and influence the injury and repair process. However, a directed response of resident microglia alone can occur in the absence of infiltrating monocytes (Peng *et al.*, 2008; Funk *et al.*, 2011), which may be more reflective of early stages of neurodegenerative disorders. Distinguishing between *in vivo* signals of resident microglia and infiltrating cells that assume a brain macrophage phenotype remains a confounding factor in identifying signals unique to the CNS. It has been suggested that infiltrating cells would be more involved in severe inflammatory injuries, while resident microglia would focus on maintaining tissue homeostasis (Ginhoux and Jung, 2014). Thus, characterization of the stage of an inflammatory response would depend upon the contributing cell type for example microglia versus infiltrating blood-borne monocytes.

While microglia and peripheral macrophages maintain many similar features, they remain uniquely different. Comparison of non-activated microglia to peritoneal macrophages identified a significant number of similarities in gene transcript expression yet, also distinct differences. Of the genes highly enriched in microglia, several were classified as 'sensome' genes allowing cells to sense and interact with the local environment (Hickman *et al.*, 2013). These included putative *P2ry12*, *P2ry13*, *Tmem119*, GPCR 34 (*Gpr34*), the 1-type lectin receptor *Siglec-h*, *Trem2* and *Cx3cr1*. Additional unique transcripts included the enzyme hexosaminidase B (*Hexb*) and the antimicrobial peptides cathelicidin antimicrobial peptide (*Camp*) and neutrophilic granule proteins (*Ngp*). While macrophages expressed a number of 'sensome' genes, the enriched genes included those encoding for fibronectin, CXCL13 and the endothelin B receptor in contrast to microglia. In an elegant study set out to directly compare M1 and M2 polarization capabilities of human microglia and blood-derived macrophages gene expression (PCR array; 26 M1 and 11 M2 genes), microglia were observed to be more restricted in their capacity to adopt an M2 phenotype and cytokine profile, compared with macrophages (Durafourt *et al.*, 2012). Both macrophages and microglia showed a greater induction of gene expression in response to M1, compared with M2 polarization. The majority of genes differentially regulated in M1-polarized macrophages were also observed in M1 microglia compared with their M2 counterparts. Some differences were observed when comparing the M1- and M2-polarized cell populations. Comparison of M1 macrophages with M1 microglia demonstrated that macrophages over-expressed antigen presentation markers, *CD1A*, *1B* and *1C*. *HLA-DM* expression was also increased in M2 macrophages compared with M2 microglia. Upon M2 polarization, microglia and macrophages were found to express similar genes with the exception of *CD64* that was observed in both M1 and M2 microglia.

Metabolism under polarization states

Increasing evidence suggests a role of metabolic reprogramming in the regulation of the innate inflammatory response. Modification of metabolic functions from a growth-promoting capacity (M2) to a killing/inhibitory capacity (M1) allows macrophages to respond with appropriate functions in distinct contexts (Mills *et al.*, 2000; Rodríguez-Prados *et al.*, 2010; Odegaard and Chawla, 2011; Biswas and Mantovani, 2012; Mills, 2012). Under normal oxygen conditions, cells obtain energy via two different mechanisms. In the first, glucose is converted to pyruvate via glycolysis, entering the mitochondrial tricarboxylic acid cycle (TCA) to produce ATP through oxidative phosphorylation (Dashty, 2013). Under hypoxic conditions, anaerobic glycolysis converts pyruvate into lactate. This metabolic switch is promoted by PI3K/Akt signalling and inhibited by AMP-activated PK (AMPK) (Hardie, 2007) and IL-10 (Murray, 2006). Recent evidence suggests that immune cells have the ability to switch from oxidative phosphorylation to aerobic glycolysis; not dissimilar to the Warburg effect seen in tumour cells (Warburg, 1956; Vander Heiden *et al.*, 2009). In this shift, cells preferentially use glycolysis rather than catabolic mitochondrial pathways to conserve and generate metabolic resources that are necessary to meet demands of cellular proliferation and activation while, still producing a sufficient supply of ATP.

M1 polarization

In classically activated M1 macrophages and dendritic cells, metabolism is shifted towards glycolysis and NO and citrulline production. This switch increases glucose uptake and lactate production (Krawczyk *et al.*, 2010; Rodríguez-Prados *et al.*, 2010) with activation of the pentose phosphate pathway (PPP) and decreased mitochondrial oxygen consumption (Haschemi *et al.*, 2012). In M1 macrophages, the Krebs cycle intermediate, succinate, regulates hypoxia-inducible factor 1α to drive a sustained production of IL-1β (Galvan-Peña and O'Neill, 2014). Increased glycolysis is permissive to quickly trigger microbiocidal activity and allows cells to survive in a hypoxic environment.

A key feature of M1 macrophages is associated with their production of ROS to facilitate killing of phagocytosed bacteria (West *et al.*, 2011). Intracellular damage from ROS is limited due to the increased generation of NADPH required for maintenance of reduced glutathione (Kletzien *et al.*, 1994; Salvemini *et al.*, 1999) and also NO production (Bredt and Snyder, 1990; Knowles and Moncada, 1994). NO is synthesized by oxidation of L-arginine by inducible NOS (iNOS) using the electrons supplied by NADPH. At high concentrations NO reversibly inhibits mitochondrial respiration by competing with O_2 in cytochrome c oxidase. With reduced mitochondrial respiration ROS production, in the form of superoxide anion (O_2^-), is increased and converted into H_2O_2 by superoxide dismutase (SOD)3. This then diffuses into the cytoplasm (Fukai *et al.*, 2002). With prolonged production, NO can react with O_2^- to produce peroxynitrite ($ONOO^-$),

irreversibly inhibiting the electron transport chain (Bolaños *et al.*, 2004). NO inhibits the enzyme pyruvate dehydrogenase that converts pyruvate into acetyl CoA before entering the Krebs cycle (Klimaszewska-Łata *et al.*, 2014). Functionally, M1-produced NO serves as an effector molecule with microbiocidal activity and the capacity to inhibit cell proliferation (MacMicking *et al.*, 1997). Using a screening strategy in macrophages, it was shown that the specific modulation of glycolytic energy flux is critical to macrophage activation and is likely to define cell polarization (Haschemi *et al.*, 2012). In this study, a number of non-protein nutrient kinases were reported to have a contributory role including CARKL, a sedoheptulose kinase of the PPP, for repression of LPS-induced macrophage activation. In primary murine macrophages, a decrease in CARKL expression accompanied M1 polarization with only a minimal increase in expression observed following IL-4 or IL-13 (Haschemi *et al.*, 2012).

The majority of the published data on the bioenergetics of polarization states has been generated in peripheral immune cells (Biswas and Mantovani, 2012; O'Neill and Hardie, 2013; Pearce and Pearce, 2013) with only a few studies examining microglia (Moss and Bates, 2001; Chenais *et al.*, 2002; Bernhart *et al.*, 2010; Sohn *et al.*, 2012; Voloboueva *et al.*, 2013; Gimeno-Bayon *et al.*, 2014). The majority of microglia studies were related to the generation of ROS (Innamorato *et al.*, 2009; Ferger *et al.*, 2010; Bordt and Polster, 2014; Chen *et al.*, 2014). As in macrophages, microglia in the surveillance state are likely to rely on oxidative phosphorylation metabolism (Cherry *et al.*, 2014b). Similar to other immune cells, when stimulated with TLR agonists (e.g. LPS), microglia switch from oxidative metabolism towards glycolytic metabolism (Voloboueva *et al.*, 2013; Gimeno-Bayon *et al.*, 2014). Voloboueva *et al.* (2013) showed that upon stimulation by LPS (1 $\mu g \cdot mL^{-1}$, 3 h), BV-2 cells increased lactate production concurrent with a decrease in mitochondrial oxygen consumption and ATP production as measured using an extracellular flux analyzer (Seahorse Bioscience, Billerica, MA, USA). This shift was modulated by the mitochondrial glucose-regulated protein 75/mortalin. Over expression of 75/mortalin attenuated LPS-induced oxidative and metabolic responses, as well as, suppressed pro-inflammatory activation. From these findings the authors proposed that LPS-induced elevations in glycolytic activity and lactate levels contributed to the associated pro-inflammatory response. When the LPS (100 $\mu g \cdot mL^{-1}$) stimulus was augmented by IFN-γ (0.5 $\mu g \cdot mL^{-1}$) a 24-h exposure resulted in an increase in NO formation and a metabolic reprogramming of BV-2 cells based on increased glucose consumption, hexokinase (HK) activity, glucose-6-phosphate dehydrogenase activity, phosphofructokinase-1 activity, lactate dehydrogenase activity and lactate release, suggesting a potentiated glycolysis (Gimeno-Bayon *et al.*, 2014). Time-lapsed confocal imaging of the inner membrane potential by the mitochondrial membrane potential-sensitive dye TMRE showed that the cells

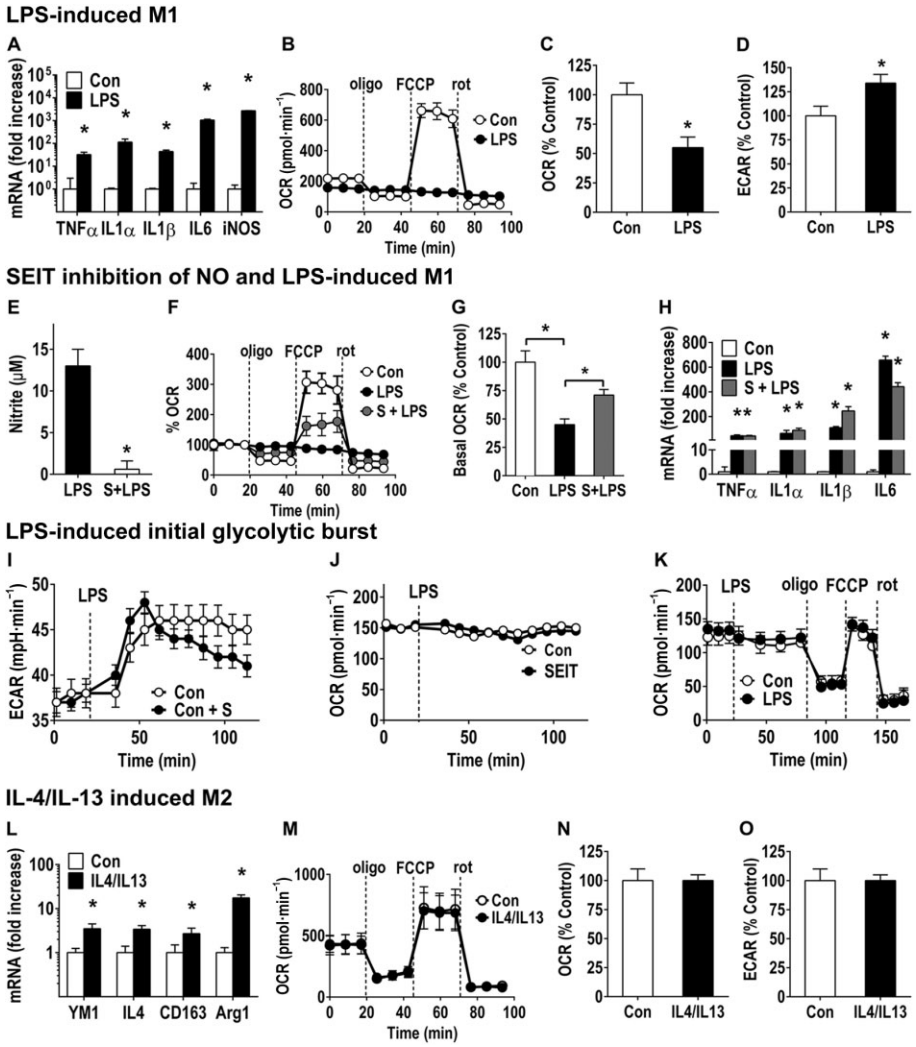
Figure 1

LPS and IL-4/IL-13 stimulation of BV-2 cells. (A–D) LPS-induced M1 phenotype of BV-2 cells. BV-2 cells were plated in six-well tissue culture plates for mRNA (2×10^5 cells per well) or in 24-well Seahorse plates (2.5×10^4 per well). 24 h post-plating, cells were exposed to LPS (100 $ng \cdot mL^{-1}$ final concentration; 24 h) or media (Con). (A) Total RNA was isolated by Trizol and mRNA levels for M1-related genes determined by qRT-PCR (Supporting Information). Threshold cycle values were determined, GAPDH was used for normalization, and the mean fold changes over saline controls were calculated according to the $2^{-\Delta\Delta C_T}$ method. Data represent mean ± SEM ($n = 6$). (B) Representative example of a bioenergetics profile (Seahorse Bioscience; Supporting Information) shows a normal response pattern for control BV-2 cells (2.5×10^4 cells per well) for basal respiration (first three readings), and following addition of the mitochondrial stressors oligomycin (oligo; 0.75 μM), FCCP (0.75 μM) and rotenone (rot; 1 nM). LPS-exposed cells showed a decrease in basal respiration and were unresponsive to the different mitochondrial stressors, suggesting an impairment of mitochondrial function. Calculation of (C) OCR and (D) ECAR as a percentage of control demonstrated a significant difference between controls and LPS-exposed cells suggesting an increased extracellular acidification of the media. Data represent mean ± SEM calculated as a percentage of control (six to seven individual wells across three independent experiments) ($n = 3$). *$P < 0.05$, significantly different from control; Student's *t*-test. (E–H) Response of BV-2 cells to LPS following inhibition of iNOS with SEIT. BV-2 cells (nitrite release; mRNA: 2×10^5 cells per well per six-well; 24-well Seahorse plate (2.5×10^4 per well) were pre-exposed to the iNOS-inhibitor, SEIT (S, 200 μM) for 1 h followed by LPS (100 $ng \cdot mL^{-1}$, 24 h) exposure. (E) LPS-induced nitrite release into the media, as determined by Griess reaction, was significantly inhibited by SEIT (S). *$P < 0.05$, significantly different from LPS alone; Student's *t*-test. (F) Seahorse bioenergetics profile showed that inhibition of iNOS partially blunted the mitochondrial impairment induced by LPS as demonstrated by the cellular response following FCCP. (G) Calculation of OCR indicated a significant decrease with LPS exposure and a blunting of this effect with SEIT. (H) mRNA levels for M1-related genes as determined by qRT-PCR showed no significant difference between cells exposed to LPS and those exposed to SEIT + LPS. Data represent mean ± SEM ($n = 6$). *$P < 0.05$, significantly different from control; ANOVA with Bonferroni's test. (I–K) LPS-induced polarization provokes a glycolytic burst in BV-2 cells. (I–J) BV-2 cells (2.5×10^4 per well Seahorse plate) were exposed to the iNOS-inhibitor, SEIT (S; 1 h, 200 μM) or media (Con) followed by LPS (100 $ng \cdot mL^{-1}$ final concentration; 24 h). (I) Cells showed an increase in ECAR following LPS. SEIT dosed cells showed a decrease in ECAR over time as compared with controls suggesting a role for iNOS in maintaining elevated ECAR. (J) SEIT alone showed no effect on basal respiration (OCR). (K) To examine mitochondrial function during the initial LPS-induced glycolytic burst, BV-2 cells were administered LPS (100 $\mu g \cdot mL^{-1}$ final concentration) or media (Con) at the 20 min time point after recording basal respiration (line indicating dosing). No significant differences were observed in response to oligomycin, FCCP or rotenone, indicating that cells maintained normal mitochondrial function during the initial glycolytic burst. Data represent mean ± SEM calculated as a percentage of control (six to seven individual wells across three independent experiments) ($n = 3$). (L–N) IL4/IL13 induction of M2 phenotype. 24 h post-plating, BV-2 cells (mRNA: 2×10^5 cells per well per well plate; 2.5×10^4 per well per 24-well Seahorse plate) were exposed to IL4/IL13 (10 $ng \cdot mL^{-1}$ final concentration of each) or media (Con) for 24 h. (L) Total RNA was isolated by Trizol and mRNA levels for M2-related genes determined by qRT-PCR (Supporting Information). Threshold cycle values were determined, GAPDH was used for normalization, and the mean fold changes over saline controls were calculated according to the $2^{-\Delta\Delta C_T}$ method. Data represent mean ± SEM ($n = 6$). *$P < 0.05$, significantly different from control; Student's *t*-test. (M) Seahorse bioenergetics profile shows no significant effect of M2 polarization by IL4/IL13 as compared with media controls and (N) OCR and (O) ECAR were not altered with the addition of IL4/IL13. Data represent mean ± SEM calculated as a percentage of control from seven independent wells from duplicate experiments.

maintained their electron transport chain usage. In comparison, stimulation with IL-4 (0.5 μg·mL^{-1}) for 24 h resulted in a reduction of glucose consumption and lactate production. The authors suggested that this shift is association with phagocytic actions of the cells and the reduction in the need for anabolic reactions. These data suggest that different metabolic programming is associated with the different phenotype states of microglia.

To provide specific experimental data on the bioenergetics profile of microglia following polarization, we examined specific features of the profile under different polarization states using a XF24 extracellular flux analyzer (Seahorse Bioscience; see Supporting Information for detailed methods). In murine BV-2 microglia cells, LPS (100 μg·mL^{-1}; 24 h) exposure resulted in an elevation in M1-related pro-inflammatory genes (Figure 1A). Under this M1 stimulatory condition, the cells shifted from a primary oxidative metabolic state towards glycolytic metabolism (Figure 1B–D) and showed no evidence of cell death. LPS-polarized BV-2 cells were unresponsive to the mitochondrial stressors oligomycin, FCCP and rotenone, highlighting the loss of mitochondrial function (Figure 1B). Similar to findings in the work of Voloboueva *et al*. (2013) shifting of BV-2 cells to a glycolytic metabolism decreased mitochondrial oxygen consumption (OCR) (Figure 1C) and, as a consequence of lactate release, the cells increased their extracellular acidification rate (ECAR) (Figure 1D). Under the stimulatory conditions induced by LPS, the data suggest that in BV-2 cells, a switch to glycolysis appears to serve as a survival response to maintain ATP levels, after inhibition of oxidative phosphorylation by NO. This finding is similar to that obtained by Everts *et al*., (2012) with dendritic cells (DCs).

The response of dendritic cells to LPS is characterized by a rapid increase in glycolytic flux that occurs within minutes of TLR activation, independent of NO (Everts *et al*., 2014). This serves to stimulate *de novo* synthesis of fatty acids (FA) and secretion of proteins critical for cell activation (Everts *et al*., 2014). In comparison, a similar phenomenon was observed in BV-2 cells. To examine the role of NO in the metabolic shift of microglia, NO production in LPS-stimulated BV-2 cells was blocked by pre-treatment with the iNOS-inhibitor S-ethyl-isothiourea (SEIT). Pretreatment of cells with SEIT (S, 200 μM) for 1 h prior to LPS (100 μg·mL^{-1}, 24 h) exposure was effective in inhibiting LPS-induced nitrite production (Figure 1E), partially blunting mitochondrial impairment (Figure 1F and G). The inhibition of iNOS, however, did not alter the LPS-induced elevation in mRNA levels for M1-related genes



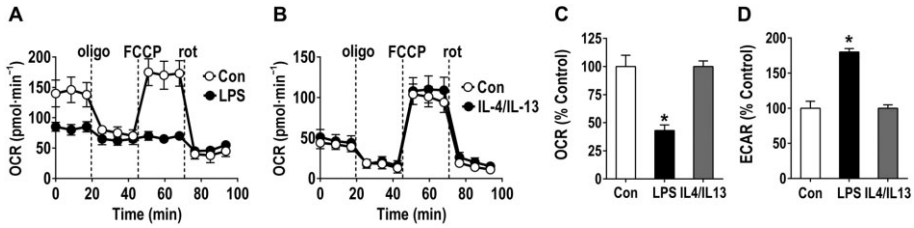


Figure 2

Representative mitochondrial function analysis of polarized primary microglia cells. Primary microglia cells were seeded in a Seahorse XF plate (Seahorse Bioscience, Supporting Information) at a density of 1.25×10^5 cells per well and polarized *in situ* for 24 h with LPS (100 ng·mL^{-1}) or a combination of IL-4 and IL-13 (10 ng·mL^{-1} each). After signal stabilization (three measures) the cells were sequentially exposed to the mitochondrial stressors oligomycin (0.75 μM), FCCP (0.75 μM) and rotenone (rot, 1 μM). (A) Representative bioenergetics profile demonstrates a normal response pattern for control primary microglia. LPS-exposed cells showed a decrease in basal respiration and were unresponsive to the different mitochondrial stressors, suggesting an impairment of mitochondrial function. (B) Following exposure to IL-4/IL-13, cells maintained a bioenergetics profile similar to controls. Calculation of (C) OCR and (D) ECAR as a percentage of control showed that LPS exposure decreased basal respiration, and increased extracellular acidification of the media, respectively, with no changes observed with IL-4/IL-13 exposure. Data represent mean ± SEM calculated as a percentage of control (seven individual wells each condition). *$P < 0.05$, significantly different from control; Student's *t*-test. All studies were conducted under an animal protocol approved by National Institute of Environmental Health Sciences Animal Care and Use Committee.

(Figure 1H) and was ineffective in preventing the initial glycolytic burst observed within the first hour following LPS (Figure 1I). Under these conditions, OCR was not altered with the inhibition of NO (Figure 1J) suggesting an increase in glycolytic metabolism while maintaining mitochondrial function. Everts *et al*. (2014) reported that in DCs, the increase in glycolysis and the stimulation of pyruvate flux into the TCA contributes to an increased spare respiratory capacity. In contrast, while BV-2 cells demonstrated an initial glycolytic burst, there was no indication of an increase in spare respiratory capacity (Figure 1K). However, similar to peripheral immune cells such as, dendritic cells, the transition from oxidative phosphorylation to glycolysis in microglia appears dependent upon NO.

BV-2 cells offer significant advantages over primary microglia with regards to their ability to generate sufficient cells to conduct biochemical studies. However, they are limited and do have some significant differences as compared with primary microglia. To confirm if the findings on mitochondrial bioenergetics observed in BV-2 cells would also be observed in primary microglia, we conducted a focused investigation in primary murine microglia following exposure to LPS (100 μg·mL^{-1}, for 24 h). We demonstrated that under LPS stimulatory conditions, the primary microglia replicated the effects observed for BV-2 cells (Figure 2) including a loss of mitochondrial function (Figure 2A), decreased basal respiration (Figure 2C) and increased ECAR (Figure 2D).

Integration of the data on peripheral immune cells with the limited data on microglia lead to the proposal of a two-stage process during activation following TLR signalling (Figure 3). It is proposed that, while in the initial stage, cells are capable of utilizing both oxidative and glycolytic metabolism yet, at the same time, activating the PPP. In the second stage, microglia shift to rely on glycolytic metabolism for survival and activation of the PPP.

M2 polarization

M2 macrophages use oxidative metabolism for the more long-term functions involved in tissue repair and wound healing (Biswas and Mantovani, 2012). The M2 phenotype has also been considered as the 'default' polarization of resident macrophages (Murray and Wynn, 2011) with the production of ornithine to promote cell proliferation and repair through polyamine and collagen synthesis, fibrosis and tissue remodelling functions (Pesce *et al*., 2009). In M2 macrophages, glucose consumption is significantly lower as compared with M1 (Rodríguez-Prados *et al*., 2010) and the sedoheptulose kinase CARKL is critical for regulating the PPP (Galvan-Peña and O'Neill, 2014). IL-4 induced M2 macrophages utilize FA oxidation and oxidative respiration for energy production (Odegaard and Chawla, 2011). Under these conditions, arginine metabolism is shifted to ornithine and polyamines (Mills *et al*., 2000). This contributes to phagocytosis by regulating energy demands and membrane fluidity.

Currently, there are limited data on the role of mitochondria as associated with M2 activation of microglia. Gimeno-Bayon *et al*. (2014) reported that IL-4-stimulated (0.5 μg·mL^{-1}; 24 h) BV-2 cells decreased glucose consumption and lactate production. The authors suggested that this shift was association with phagocytic actions of the cells and the reduced need for anabolic reactions. When we examined the response of BV-2 cells following stimulation with IL-4/IL-13 (10 μg·mL^{-1}, 24 h), mRNA levels for a number of M2-related genes were found to be elevated (Figure 1L). When the bioenergetic state was examined upon such stimulation, the cells were found to remain within an oxidative metabolic state (Figure 1M) maintaining OCR and ECAR at levels similar to non-stimulated cells (Figure 1N,O). These observations are in contrast to peripheral macrophages where IL-4 stimulates glucose uptake in addition to FA metabolism and mitochondrial biogenesis (Vats *et al*., 2006). When similar dynamics were examined in primary murine microglia, it was found that upon stimulation with IL-4/IL-13, cells remained within an oxidative metabolic state (Figure 2B). They showed no alteration in basal respiration and OCR (Figure 2C) and ECAR (Figure 2D) were similar to non-stimulated cells. In peripheral macrophages, inhibition of mitochondrial respiration inhib-

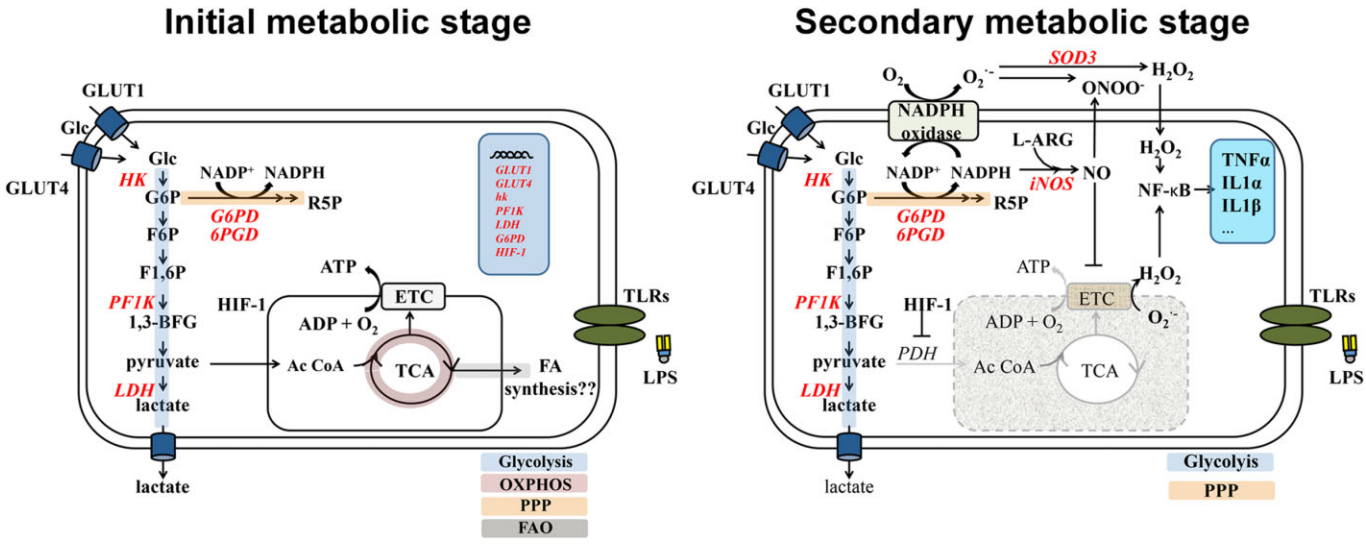


Figure 3

Schematic representation of LPS-induced BV-2 polarization. Activation of TLRs by LPS provokes dramatic changes in the metabolism of microglia, inducing a glycolytic switch that decreases mitochondrial O_2 consumption and increases extracellular acidification via production of lactate. As observed in other immune cells (Everts *et al.*, 2014), the data suggest that activation of microglia goes through two metabolic steps. In the first step, immediately after LPS stimulation, glycolytic metabolism is enhanced independently of NO production, increasing intracellular glucose (Glc) via glucose transporter (GLUT)-1 and GLUT-4 and production of several glycolytic enzymes. The PPP is induced via expression of its rate-limiting enzyme, 6-phosphogluconate dehydrogenase (G6PD). The electron transport chain (ETC) remains functional and the cells rely on oxidative phosphorylation and glycolysis for energy production. While LPS provokes a rapid glycolytic burst in microglia, there is little evidence of increased oxidative phosphorylation for synthesis of new molecules, as observed in DCs (Everts *et al.*, 2014). In the second stage, NADPH, generated through the PPP, is used to produce ROS. H_2O_2 is then used as a bactericidal and also as a second messenger to modulate NF-κB. In the presence of NADPH, iNOS oxidation of L-arginine (L-ARG) produces NO to inhibit Cytochrome *c*. In addition, HIF-1α inhibits pyruvate dehydrogenase (PDH) and thus, the conversion of pyruvate into acetyl CoA, forcing a sole reliance on glycolysis for cell survival. As a consequence, mitochondrial dysfunction provokes the generation of additional mitochondrial ROS that are transported to the cytoplasm activating NF-κB to exacerbate the pro-inflammatory response. Abbreviations: 1,3-BFG, 1,3-biphosphoglycerate; ETC, electronic transport chain; FAO, fatty-acid oxidation; F6P, fructose-6-phosphate; F-1,6P, fructose-1,6-biphosphate; G6P, glucose-6-phosphate; $NADP^+$, oxidized form of NADPH; OXPHOS, oxidative phosphorylation; PF1K, phosphofructose-1-kinase; R5P, ribose-5-phosphate; SOD3, superoxide dismutase 3.

its induction of arginase activity and minimizes the anti-inflammatory effects of IL-4 on LPS-induced secretion of IL-6 and TNF-α (Vats *et al.*, 2006). Ferger *et al.* (2010) found that non-toxic doses of the mitochondrial electron transport chain inhibitors, rotenone or 3-nitropropionic acid, impaired IL-4 stimulation of M2-related genes and inhibited LPS-stimulated IL-6 and TNF-α release. In contrast to peripheral macrophages, primary microglia showed no inhibition of IL-4 down-regulation of LPS-induced secretion of IL-1β protein (Ferger *et al.*, 2010). In mixed glia cultures, IL-4 was found to enhance LPS-induced IL-1β production suggesting that, under these conditions, IL-4 could activate the NLR inflammasome (Cao *et al.*, 2007).

Modifications of the innate immune response

The innate immune system is shaped and conditioned to subsequent responses for days or months following activation or immunological signals (Netea *et al.*, 2011; Kleinnijenhuis *et al.*, 2012). This has been associated with increased non-specific resistance to infectious agents following exposure to microbial agents. An augmented innate immune response occurring upon a secondary infection or challenge has been termed 'trained innate immunity' (Netea *et al.*, 2011) and a lymphocyte-independent shaping of innate immunity has been termed 'memory' (Kurtz and Franz, 2003; Kleinnijenhuis *et al.*, 2012). Such a memory can occur in macrophages previously exposed to IFN-γ with an elevated response to LPS (Nathan *et al.*, 1984; Bosisio *et al.*, 2002), potentially for a protective inflammatory response (Netea, 2013; Quintin *et al.*, 2014). In contrast, preconditioning can occur with the development of endotoxin tolerance or hypo-responsiveness to a subsequent challenge as a defence strategy to limit damage (Medzhitov *et al.*, 2012). One could consider that stimuli encountered by microglia on a regular basis may serve to provide a pool of memory-like cells enhancing performance upon a subsequent challenge. LPS-induced tolerant macrophages express a transcriptional profile similar to M2 polarization with *Il10*, *Arg1*, *Ccl17* and *Ccl22* rather than a diminished M1 response, suggestive of a reorientation of function (Biswas and Lopez-Collazo, 2009; Porta *et al.*, 2009). While inhibition of mitochondrial function can be influenced by LPS stimulation, the reverse also occurs in that mitochondrial inhibition can influence the level of stimulation induced by LPS (Park *et al.*, 2013). Questions remain as to whether microglia resume a normal functional phenotype once activated. If they conserve a memory of past inflammatory activation, do they shift response to a new challenge or react as naïve cells? Earlier work suggested

that oxidative metabolism primed macrophages for a less-inflammatory mode of activation (Vats *et al.*, 2006). Thus, how this relates to mitochondrial functioning and shifts in metabolism with polarization warrants further examination.

Disruption of mitochondrial function is linked to a range of cellular effects in the nuclear genome including loss of heterozygosity, chromosome instability and epigenetic modifications (Veatch *et al.*, 2009; Seoane *et al.*, 2011). Thus, such shifts in the microglial activation state could lead to a broader cascade of effects as they relate to cell function over the lifespan. Warburg (1956) initially suggested that respiratory insufficiency was irreversible. However, the absence of cell death with microglia M1 polarization and down-regulation to a quiescent phenotype with return to homeostasis suggests that microglia survive respiratory insufficiency. As an example, 2-deoxy-glucose (2-DG), a glucose analogue that blocks glycolysis by inhibiting HK activity, is capable of blunting TNF-α and IL-6 production by inhibiting NF-κB signalling in primary microglia (Wang *et al.*, 2014). However, inhibition of the glycolytic metabolism in M1 microglia, during which the cell relies solely on glycolysis for survival, could prove fatal. For example, Lyons and Kettenmann (1998) reported that the substitution of glucose by 2-DG under hypoxic conditions was lethal to 90% of cultured microglia. In a more recent study, Vilalta and Brown (2014) reported that 2-DG killed microglia when co-cultured with neurons. Rather than directly inhibiting the glycolytic metabolism, an alternative approach would involve preventing the metabolic switch or in enhancing oxidative metabolism. Something similar has been observed in DCs with the anti-inflammatory cytokine, IL-10 (Krawczyk *et al.*, 2010) and may be translated to microglia. A better understanding of the inflammatory-associated metabolic state and changes that occur with a polarization shift should help identify appropriate targets for modulating and regulating actions of microglia.

Future directions

Translation to chronic conditions

The current review focuses primarily on cellular responses initiated upon acute or short-interval exposures. It is from such models that experimental data support an association between the activation state and cellular bioenergetics. These events are likely to reflect those that occur in brain or spinal cord injury with traumatic events or stroke. However, gaining a better understanding of the acute response capability of microglia may become critical for understanding associations with neurodegenerative diseases. This will require a better understanding of the complex nature and heterogeneity of cellular responses as they occur within more chronic conditions. The association of neuroinflammation and activation state with various neurodegenerative diseases including Alzheimer's disease (Varnum and Ikezu, 2012; Tang and Le, 2015), Parkinson's disease (Blandini, 2013; Kannarkat *et al.*, 2013; Moehle and West, 2014), Huntington disease (Ellrichmann *et al.*, 2013; Crotti *et al.*, 2014), amyotrophic lateral sclerosis (Evans *et al.*, 2013; Zhao *et al.*, 2013; Hooten *et al.*, 2015), prion disease (Gómez-Nicola *et al.*, 2014) and multiple sclerosis (Goldmann and Prinz, 2013; Strachan-Whaley *et al.*, 2014) suggests a contribution to disease progression (Boche *et al.*, 2013). Whether the contribution of microglia actions in progressive neurodegenerative diseases is associated with an elevation in the M1 pro-inflammatory phenotype or a diminished ability of the cells to differentiate into an M2-type phenotype remains an issue under current study. Given the diverse pathological patterns of each of these diseases, questions remain as to whether the contribution of microglia and the associated inflammatory response follows a general pattern or a level of specificity as may be influenced by the environmental niche. In addition, it is not clear if responses of individual microglia within such disease states may follow an acute response pattern or a prolonged cellular shift. In human patients, data are limited with regards establishing a temporal progression of cellular changes and inflammatory responses. Restrictions also exist in the various animal models of specific aspects of human neurodegenerative diseases with regards to examination of the temporal aspect of associations with classical pro-inflammatory activation and alternative or repair associated phenotypes. In addition, within such disease-oriented conditions there exists the potential confounding factor of a perivascular macrophage or infiltrating blood-borne immune cell contribution occurring with tissue degeneration (Mildner *et al.*, 2011; Funk *et al.*, 2013). This then brings an entirely different dynamics to the environment as compared with a response limited to resident microglia (Jung and Schwartz, 2012; Yamasaki *et al.*, 2014). Hypotheses have been put forward that (i) under chronic degenerative conditions, microglia maintain a high pro-inflammatory state leading to an enhanced and prolonged generation of low MW mediators such as NO, $ONOO^-$, and ROS, and (ii) that pro-inflammatory molecules impair the ability of microglia to clear excess or aberrant proteins, which could then extend the stimulating environment. How either of these two situations would result in an alteration of the bioenergetics of microglia has yet to be examined; however, it is likely that the ability of microglia to shift their bioenergetic profile will significantly influence the final outcome (Urrutia *et al.*, 2014).

Classification for microglia phenotyping

The initial concept of individual macrophages having either a M1 or M2 phenotype has effectively set a framework for experimental examination of inflammation (Figure 4). Currently available data demonstrate a more complex phenotyping, especially *in vivo* with influence of various cell types comprising the inflammatory niche.

A transcriptomic analysis of macrophages following discrete stimulation show activation pathways outside the standard M1/M2 polarization paradigm (Hume and Freeman, 2014) recommending the use of a combination of markers rather than isolated canonical markers of any specific activation state. It is becoming clear that any effort to determine whether or not macrophages exist within distinct activation or polarized states cannot rely on only one or two 'markers', but rather will require the examination of several markers including the mitochondrial bioenergetics of the cell. Further proposals for advancing our understanding of macrophage states and the heterogeneity of cellular responses include

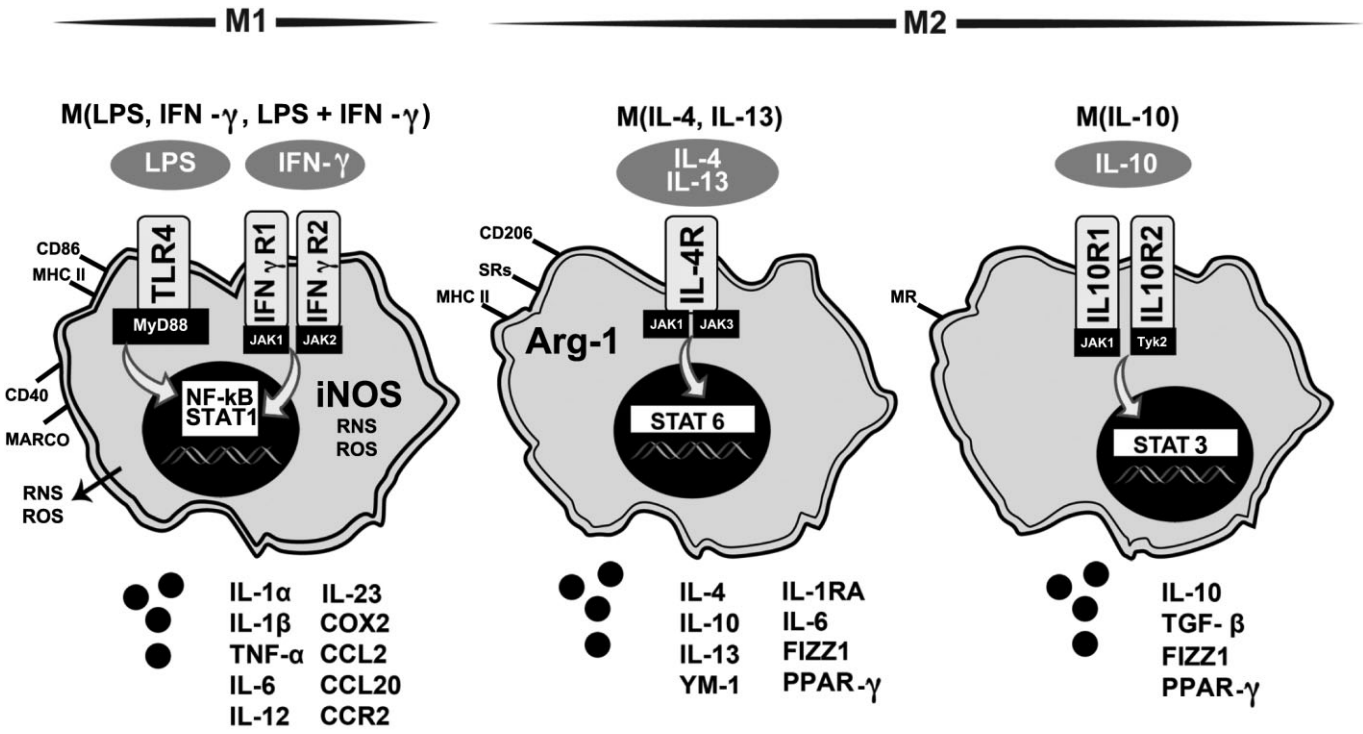


Figure 4

Diagram of activation states of microglia based on inflammatory profile and effector function. Based upon peripheral macrophage nomenclature, M1 and M2 polarization states of microglia have been proposed as a framework to evaluate the heterogeneity of responses (Colton, 2009; Mosser and Edwards, 2008) with recent evaluations suggesting a framework focused on the inducing stimuli (Martinez and Gordon, 2014; Murray *et al.*, 2014). This diagram is based upon these papers and adapted from Martinez and Gordon (2014). Under the classic M1 state, exposure to LPS and/or IFN-γ stimulates TLR4 or IFN-γ receptors 1 and 2, respectively, leading to activation of transcription factors NF-κB and STAT1 and increased expression of CD86 and MHC-II. The increase in iNOS produces a burst of ROS and reactive nitrogen species (RNS) and the release of pro-inflammatory cytokines, such as IL-1α, IL-1β, TNF-α, IL-6, IL-12, IL-23, the chemokines CCL2 and CCL20, and the receptor CCR2, macrophage receptor with collagenous structure (MARCO) and COX2.
M2 states have been proposed following various stimulatory factors. For example, upon stimulation with IL-4/IL-13, binding of the IL-4 receptor (IL-4R) initiates activation of STAT6, shifting the cells towards an anti-inflammatory phenotype with an increase in Arg-1, expression of CD206 and mannose receptor (MR), and release of anti-inflammatory factors (IL-4, IL-6, IL-10, IL-13, IL-1RA, FIZZ1, and PPARγ. Exposure of the cells to IL-10 activates STAT6 via stimulation of IL-10 receptors 1 and 2. This serves to shift the cells to a primary immunosuppressive state with an expression of CD206 and the release of IL-10, TGF-β, FIZZ1 and PPARγ.

recommendations for the use of nomenclature linked to the activation standards rather than an M1/M2 classification (Murray *et al.*, 2014). To translate these recommendation to microglia, efforts towards characterization would include assessments of morphological phenotype, discrimination between resident and infiltrating macrophages, metabolism, and where possible, functional features of the cells. Gaining a better understanding of the link between mitochondrial function and inflammation will support any future efforts to develop therapeutic approaches to support the normal and well-regulated function of these dynamic cells.

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Conflict of interest

None.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Representative schematic of mitochondrial function analysis using the Seahorse Bioscience extracellular flux analyzer (XF24) (Seahorse Bioscience, Billerica, MA, USA).
Table S1 Quantitative real-time PCR primers sequences.