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PERSPECTIVE

Ghosts in the shell: identification of microglia in the human central nervous system by P2Y12 receptor

Microglia are the tissue resident macrophages of the brain and represent the sole immune population located in the parenchyma of the central nervous system (CNS). These cells are hidden between neurons, astrocytes as well as oligodendrocytes and account for only 5–10% of CNS cells. Even though microglia were already identified in 1913 by the Spanish neuroanatomist Ramon y Cajal and further seminally investigated by his student Pio del Rio Hortega, the specific identification of these 'ghostly' cells was, and still is, problematic. The problem arises due to other CNS-related, but non-parenchymal macrophage populations like perivascular and meningeal macrophages that are present in the physiological CNS and which are possibly complemented by bone marrow-derived cells in humans. Recent advantages in microglia biology through animal research gave us a first impression about murine microglia origin, function, turnover as well proliferation rates and microglial lifespan. However, our understanding of human microglia remains limited, partly due to the lack of specific research tools to distinguish microglia from other myeloid populations in humans. Here I would like to give a short summary about microglia development, function and recent advantages in their identification in humans, especially by P2Y12 receptor expression.

Compared to other tissue macrophage populations, microglia – at least in mice – originate entirely from primitive yolk sac-derived erythro-myeloid progenitors, which seed the brain rudiment early during embryogenesis (Ginhoux et al., 2010). In the mouse embryonic CNS, microglia undergo high rates of proliferation in order to colonize the whole CNS parenchyma. At this stage, microglia are phagocytic and show phenotypic signs of activation ("activation" is used throughout this perspective as the definition for morphological and transcriptional transformation of microglial cells during development, injury or disease irrespective of the M1 or M2 paradigm). During adulthood, microglia have self-renewal capacity (Ajami et al., 2007), which is independent of bone marrow-derived cell replenishment (Mildner et al., 2007).

To date, the origin and turnover rate of microglia in humans remain unknown, even though a tight balance between proliferation and cell death was recently predicted for human microglia (Askew et al., 2017) and is supported by the fact that human microglia numbers remain stable throughout lifetime (Mildner et al., 2017). Based on the assumption that a small percentage (around 2%) of human microglia show active proliferation (based on Ki67 and IBA1 (allograft inflammatory factor-1 (AIF1, also known as IBA1, an EF hand motif-bearing phosphoprotein involved in inflammation) staining), the calculated value for a whole replenishment of the CNS microglial compartment exceeds hundreds of renewal cycles during a lifetime of around 80 years (Askew et al., 2017). However, further modelling experiments are required to accurately discern the turnover of microglia, for example retrospective birth dating with ^{14}C isotopes (Spalding et al., 2005). A similar strategy with a reliable microglia-specific nucleus marker might bring to light the lifespan and self-renewal capacity of human microglia and their turnover in different CNS areas.

Microglia are rather commonly classified as cells with restricted immune functions during infectious and/or inflammatory conditions. This classification, however, seems too naïve. Emerging evidence indicates that these innate immune cells (and tissue resident macrophages in general) play a vital role in the development and homeostasis of the host tissue under physiological conditions. Beside classical functions as phagocytes – like removal of cellular debris – microglia were shown to control neuronal differentiation and are involved in the formation of synaptic connections through synaptic pruning, which is dependent at least in part on microglial *cx3cr1* expression (Paolicelli et al., 2012). However, whether this function of microglia in synapse formation is important only during development or is also performed during adulthood has not been resolved. Although efforts have been made to shed light on the homeostatic role of microglia through microglia depletion experiments, these experiments have severe side effects like massive microglial cell death and are, due to the high replenishment rate of microglia, difficult to control (see for review: Waisman et al., 2015). More subtle genetic approaches would be necessary to investigate this in more detail. Nevertheless, the important role of microglia during development and adulthood makes them an integrative ingredient of the homeostatic and healthy brain.

As immune sentinels of the CNS, microglia activation is a hallmark of almost every pathogenic CNS condition and is commonly accompanied by morphologic changes. These changes were already observed in 1919 by Rio del-Hortega in patients with meningitis and other CNS disorders. However, under specific host conditions, like inflammatory processes within the CNS that lead to the disruption of the blood-brain barrier integrity, peripheral monocytes are able to migrate into the CNS parenchyma (Mildner et al., 2009). In these circumstances, infiltrated peripheral monocytic cells and activated microglia acquire a similar morphology and share common myeloid marker expression (*e.g.*, macrosialin (CD68; a transmembrane glycoprotein), IBA1, major histocompatibility complexes class II (MHCII) and integrin alpha M (CD11b; part of alpha-M beta-2 integrin complex)). However, the two myeloid populations may have completely different functions during CNS diseases and therefore a reliable identification system for either cell type is critical to investigate in detail their respective contribution to human CNS diseases.

In order to identify possible candidates for the specific staining of human microglia, we investigated the expression of the purinergic P2Y12 receptor in human CNS tissue. It was shown previously in mice that expression of metabotropic, G-protein coupled P2Y receptors in microglia senses extracellular ADP and ATP, which is released during cellular injury or cell death and are important for the site-directed movement of microglial extensions towards cellular injury (Davalos et al., 2005). Two of these receptors, *p2ry12* and *p2ry13*, are highly expressed in murine microglia (Butovsky et al., 2014) and it was shown that *p2ry12*-deficient microglia are characterized by significantly reduced directional branch extension toward sites of cellular CNS injury (Haynes et al., 2006). We therefore hypothesized that human microglia might be characterized by a similar expression pattern. Staining of various brain regions from healthy individuals with the antibody HPA014518 (Sigma) against P2Y12 receptor specifically detected homeostatic human microglia under physiological conditions throughout development (Mildner et al., 2017). Furthermore, all microglial cells in various regions of the brain, including cortex, hippocampus, basal ganglia and cerebellum, expressed P2Y12 receptor. As mentioned before, the numbers of microglial cells detected by P2Y12 receptor remained stable throughout the ageing process in the cerebellum as well as cortex.

Interestingly, following irradiation, bone marrow-derived $IBA1^+$ "microglia" in mice do not express P2Y12 receptor (Butovsky et al., 2014). Therefore, if over a lifetime prenatally-derived microglia are exchanged by bone marrow-derived "microglia" in humans, we would expect during ageing a gradual loss of endogenous $P2Y12^+$ microglia in the brain, while cell numbers of bone marrow-derived P2Y12-negative $IBA1^+$ cells would increase. This was not the case; $P2Y12^+$ cell numbers were stable over lifetime and consistently overlapped with $IBA1^+$ cells. This finding argues for the self-renewal capacity of human microglia as observed in mice (Mildner et al., 2007).

Importantly, compared to other commonly used microglial markers like IBA1, CD68 or MHCII, the P2Y12 receptor was absent on

meningeal macrophages or perivascular myeloid cells (Mildner et al., 2017). Therefore, the P2Y12 receptor, as recognized by the Sigma HPA014518 antibody, provides a novel tool for future immunohistochemical investigations to visualize the microglial network in humans under physiological conditions.

An interesting observation was that certain P2Y12 receptor-expressing cells were evident outside of the CNS parenchyma in the choroid plexus of fetal brain sections (11 gestation weeks). It is possible that these cells reflected migratory microglia, which enter the brain parenchyma through the brain-ventricular choroid plexus. However, further studies are necessary to investigate the migratory path of human microglia during fetal development in more detail.

We next focussed on microglial expression of the P2Y12 receptor in different CNS pathologies. We first analyzed the autoimmune disease multiple sclerosis (MS), in which autoreactive T-cells recognize CNS self-antigens like myelin peptides leading to a strong blood-brain barrier damage with subsequent infiltration of monocytes. P2Y12 receptor-expressing cells were detectable at the lesion borders of infiltration, while P2Y12 was lacking in the inflammatory centre. Since *p2ry12* expression is strongly affected by cellular injury as observed upon trauma-induced activation (Haynes et al., 2006), it is likely that reactive microglia in MS down-regulate *p2ry12* expression in the inflammatory foci.

We next tested patients with Alzheimer's disease (AD) as representative cases of neurodegeneration. Extracellular accumulation of cleaved amyloid-beta (Aβ) peptides and the deposition of neurofibrillary tangles are hallmarks of AD pathogenesis. Experimental evidence in mice indicates that microglia but not bone marrow-derived myeloid cells are recruited to Aβ plaques (Mildner et al., 2011). We therefore investigated the expression of P2Y12 receptor on microglia in cortical tissues of human AD patients. Interestingly, microglia associated with Aβ plaques did not show any detectable P2Y12 expression, while these cells were still positive for IBA1 (Mildner et al., 2017). There are two possibilities that might explain this observation: either peripheral P2Y12-negative cells infiltrate the AD brain and accumulate around Aβ plaques or endogenous microglia down-regulate *p2ry12* expression as in MS. In regard to this, recent genome-wide association studies identified a mutation in the triggering receptor expressed on myeloid cells 2 (Trem2), which substantially increases the risk of developing AD in humans. It was subsequently shown in a seminal study that Trem2-deficiency in AD mice leads to exacerbated symptoms of AD including increased Aβ plaque load, while microgliosis and recruitment of microglia towards Aβ deposits was decreased (Wang et al., 2016). Trem2 was further shown to be selectively expressed by microglia in proximity to Aβ plaques and identified as a receptor for several lipids that are exposed during Aβ deposition as well as during glial and neuronal cell death (Wang et al., 2016). It is therefore likely that P2Y12-negative cells in our human AD cases represent reactive Trem2-expressing microglia. Due to the absence of markers for the identification of human plaque-associated microglia, cell isolation with fluorescent activated cell sorting (FACS) for further analysis becomes challenging. The identification of markers like the P2Y12 receptor in combination with Trem2, however, might overcome this limitation and allow us for the first time to enrich specifically for plaque-associated microglial cells that can be used for instance for RNA-sequencing. Alternatively, single cell RNA sequencing would provide an unbiased approach to study microglia in pathological contexts.

It has become clear over the past years of microglia research that the precise identification and subsequent characterization of these cells and their reactive states are not trivial, albeit necessary for us to decipher their contribution to both homeostasis and disease. Identification of microglia by their expression of the P2Y12 receptor will help us to uncover these 'ghosts in the shell/CNS' in more detail, especially when it comes to studying them in humans.

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