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PERSPECTIVE

Magnetofection as a new tool to study microglia biology

Microglia are the resident macrophages of the brain, originally described by Pío del Río-Hortega (a student of Santiago Ramon y Cajal) in a series of studies in 1919. Since those pioneering studies, many others have followed to describe microglia as complex and multitasking cells with many diverse roles under physiological (*e.g.* their key role in synapse pruning during development) or diseased conditions. Microglia exist as sentinels or surveyors of the environment that surround neurons, becoming reactive upon a wide array of stimuli and consequently developing an appropriate inflammatory response. In fact, a neuroinflammatory response driven by microglia is found in virtually every disease process that occurs within the central nervous system. Therefore, a better understanding of the mechanisms governing the microglia response is the key to improving the outcome of neurodegenerative conditions.

Research studies into microglia function have been performed both *in vivo* and *in vitro* using numerous different neurogenerative disease models depending on the disease studied (for example the use of 5xFAD transgenic mice in Alzheimer's disease (AD) research (Keren-Shaul et al., 2017)) . In particular, in vitro approaches, using cell lines or primary cultures, have proven useful to study mechanisms governing microglia activation upon different stimuli (Wang et al., 2015). Many laboratories have been working with murine primary microglia, which have been demonstrated to be a valuable tool with which to perform mechanistic studies and whose generation only requires an easy, short and cost-effective protocol to be followed. Noteworthy is the recent development of protocols to produce human primary microglia generated from induced pluripotent stem cells. Research results obtained using human primary microglia are potentially more translatable to the clinic, although they present the caveat that the procedure required to generate them is still relatively expensive and time consuming.

Despite the intensive use of primary rodent microglia cultures, their transfection with small interfering RNA (siRNA) or plasmids has been considered a challenging process, especially since traditional methods of transfection exhibit low transfection efficiency, affect cell survival and promote an inflammatory response (Smolders et al., 2018). Some alternative methods have been employed to date to try to circumvent these technical difficulties of gene targeting in primary rodent microglia. For instance, the generation of specific transgenic mouse lines from which primary cultures can then be generated is currently used in many studies. The disadvantage of this approach is that the process to generate a specific transgenic mouse line is normally lengthy, arduous and expensive. Another method currently employed is transduction of primary rodent microglia using lentiviral vectors. However, the generation of specific lentiviral vectors is complex and requires several steps including the design of virus, the selection of right bacterial strain to amplify viral vector, and an investment in specific biosafety level equipment and facilities to work with viruses.

Recently, we have described an easy and cost-effective method that does not require specific training and allows the effective transfection of siRNA in primary microglia. In our paper entitled: "Effective knockdown of gene expression in primary microglia with siRNA and magnetic nanoparticles without cell death or inflammation" (Carrillo-Jimenez et al., 2018), we used a method based on the Magnetofection™ principle patented by OZ Biosciences. This method uses specific magnetic nanoparticles that are fully degradable and that have the ability to form complexes with our siRNAs of interest. These nanoparticles, combined with the use of a magnetic field that quickly concentrates those nanoparticles over the microglia, promote the nanoparticle uptake by endocytosis and pinocytosis, two natural biological processes. This approach to assimilate the nanoparticles is less disruptive to the cell than traditional transfection methods which normally form transient pores in the plasma membrane that affects cellular homeostasis (Smolders et al., 2018). Importantly, our study demonstrates that this method allows a high delivery of nanoparticles without causing cell death or activation/priming of the inflammatory response or phagocytic response per se in primary rodent microglia. Although not tested in this study, it is probable that this method could be used successfully to knockdown the expression of target genes in primary human microglia that are now commercially available.

Furthermore, in this paper we studied the specific uptake of such nanoparticles by microglia using fluorescently-labelled RNA oligonucleotide nanoparticles in a mixed primary cerebellar granule cell culture that also included astrocytes and neurons. Though we could observe that the fluorescently-labelled RNA oligonucleotide nanoparticles were taken up by the three different cell types, the majority of microglia (~75%) contained nanoparticles, with lower levels of transfection observed in both astrocytes (40–50%) and neurons (~20%). Therefore, this transfection method could be effective in mixed cultures if a microglia-specific gene is being targeted.

In our study, we demonstrated the effectiveness of this transfection method by targeting the expression of triggering receptor expressed on myeloid cells 2 (TREM2) and CD33, two AD risk genes identified by massive genome analysis (Bettens et al., 2013). The critical roles of these genes in microglia function, especially TREM2, are supported by recent massive transcriptomic analysis of microglia at the single cell level under different disease and physiological conditions, including AD and amyotrophic lateral sclerosis (Keren-Shaul et al., 2017; Krasemann et al., 2017). A common TREM2-dependent microglia molecular signature has been identified and named as disease-associated microglia (DAM) (Keren-Shaul et al., 2017) or microglia neurodegenerative phenotype (Krasemann et al., 2017). Importantly, this common disease-associated reactive phenotype highly differs from the classical M1/M2 polarization states and highlights the critical importance of TREM2 in governing microglia reactivity under neurodegenerative conditions. In the study of Keren-Shaul and colleagues, the microglia molecular signature of brains obtained from WT and transgenic mouse models of AD and ALS was analyzed. They identified three main microglial clusters; a large group (group I), associated with homeostatic microglia, and two small groups (groups II and III) showing major transcriptional changes in lipid metabolism and phagocytic genes. They found that the cellular transcriptomes of groups II and III were uniquely found under disease conditions and were hence termed DAM. The use of 5xFAD/TREM2 KO mice led the authors to suggest that the transition from group II to III is TREM2-dependent and therefore implicated TREM2 as a critical receptor governing the microglia immune response

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under neurodegenerative conditions. A subsequent study by Krasemann et al. (2017) identified a TREM2/ApoE-dependent switch from homeostatic to disease-associated microglia, which was associated with neuritic dystrophy in AD mouse models. Very recently, Lee et al. (2018) developed bacterial artificial chromosome-mediated transgenesis to overexpress TREM2 in 5xFAD mice (5xFAD/TREM2). In this study, the authors investigated how TREM2 overexpression affected AD pathology in 5xFAD mice. They observed that overexpression of TREM2 in fact confers neuroprotection. The authors found that TREM2 overexpression reduced amyloid burden and neuritic dystrophy. At present, the exact roles of reactive microglia under disease conditions (beneficial vs. deleterious or both, probably depending on the environment) is a challenging issue (Wang et al., 2015; Keren-Shaul et al., 2017) and TREM2 is suggested to play a critical role. Using selective and efficient knock-down methodologies, such as the one we have developed, may help to decipher the wide array of microglial-associated functions ascribed to TREM2. We should consider for instance that TREM2 R47H heterozygous variants are associated with reduced total TREM2 expression around amyloid plaques (Cheng-Hathaway et al., 2018), hence the need for easily feasible gene knock-down in primary cells. Interestingly, our results using magnetofection in primary murine microglia showed that a 50% decrease in TREM2 expression induces a different inflammatory response (induced by LPS) and phagocytic response (induced by neuronal debris) compared with studies where TREM2 was completely abrogated (using TREM2 KO mice). Our results mimic those effects obtained using mice lacking one of the two TREM2 alleles (Ulrich et al., 2014). This demonstrates that changes in TREM2 levels in microglia may induce different phenotypes.

So what is next for this technology. The fact that we can manipulate primary microglia cultures without causing death or priming opens an array of possibilities for targeted genome editing using CRISPR Cas9 technology. In particular, we could use primary microglia cultures generated from the Cre-dependent Cas9 knockin mouse (Platt et al., 2014) that is commercially available. Using our method, we could transfect a single guide RNA (sgRNA), consisting of both the crRNA and tracrRNA as a single construct in primary microglia that already possess Cas9, to edit the DNA sequence of our targeted gene. Using this approach would allow us to perform CRISPR Cas9 editing of single or multiple gene targets in primary microglia, rendering results in a short time. The foreseeable disadvantage of this method compared to directly isolating primary gene-edited microglia from CRISPR Cas9 generated mice is that not 100 % of the primary transfected microglia would have acquired the sgRNA. Even so, it could be enough for preliminary studies to decide whether or not to invest time and money to generate a CRISPR Cas9 mouse with the desired gene-edited target.

Overall, this transfection method adds an important research tool to the scientific community studying microglia biology. The possibility of effectively silencing genes in primary microglia will allow us to unravel many diverse roles of the multi-tasking microglia in different neurodegenerative conditions.

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