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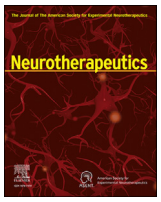

Review

Hematopoietic stem cell gene therapy to halt neurodegeneration

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ABSTRACT

Microglia play fundamental roles in multiple pathological primary and secondary processes affecting the central nervous system that ultimately result in neurodegeneration and for this reason they are considered as a key therapeutic target in several neurodegenerative diseases. Microglia-targeted therapies are directed at either restoring or modulating microglia function, to redirect their functional features toward neuroprotection. Among these strategies, hematopoietic stem cell gene therapy have proven to be endowed with a unique potential for replacing diseased microglia with engineered, transplant progeny cells that can integrate and exert relevant beneficial effects in the central nervous system of patients affected by inherited and acquired neurodegenerative conditions.

Microglia in Neuropathology

Microglia are resident immune cells of the central nervous system (CNS) and represent the most abundant population among the mononuclear phagocytes present in the CNS. Besides exerting key physiological roles during neurodevelopment, microglia act as the first line immune defense in the CNS by actively monitoring and patrolling the microenvironment [1]. Microglia cells exert multiple neuroprotective functions and interact with neurons, astrocytes, oligodendrocytes and infiltrating immune cells to maintain CNS homeostasis [2]. Moreover, microglia respond to CNS diseases or damage with complex and dynamic reactions. Originally, two categories were described to conceptualize microglia state in response to stimuli, with M1 indicating a proinflammatory and neurotoxic state, and M2 being associated to anti-inflammatory and healing activities. However, thanks to recent single cell transcriptomic studies conducted in mice and humans [3–7], the M1/M2 paradigm is now considered inadequate to describe microglia activation that is rather varied and highly dependent from the context [8]. Indeed, multiple microglial states have been recognized related to homeostasis (M0), aging as well as specific disease processes and stages [3] and scRNA-seq studies have identified many microglial transcriptional signatures that are highly dependent on the CNS context. Moreover, remarkable spatiotemporal diversity of microglia has been described in both physiological and pathological conditions.

Microglia are nowadays considered as a key and fundamental player in multiple pathological primary and secondary processes affecting the CNS and ultimately resulting in neurodegeneration. Neurodevelopmental and neurodegenerative conditions arising from abnormal or inadequate microglia function have been described [8]. These conditions include the so-called primary microgliopathies that are associated to the inherited dysfunction of microglia-signature genes resulting in aberrant microglia function, dysmyelination, neurodegeneration and early onset dementia. Examples are Hereditary Diffuse Leukoencephalopathy with Spheroids (HDLS), an autosomal dominant disease causally associated to defects in the colony-stimulating factor-1 receptor (CSF1R) gene [9], or Polycystic membranous Lipomembranous Osteodysplasia with Sclerosing Leukoencephalopathy (PLOSL), also known as Nasu Hakola Disease (NHD), due to the aberrant expression of the genes encoding for the Triggering Receptor Expressed on Myeloid cells 2 (TREM2) or DNAX-activating protein 12 (DAP12) in microglial cells [10].

Primarily impaired microglia function is also reported in other neuropathological conditions, i.e. impaired microglia phagocytosis due to alteration or loss of methyl CpG binding protein 2 (MECP2) expression and consequent accumulation of neuronal debris contribute to the development of Rett syndrome [10]. Inherited lysosomal enzyme deficiencies, such as sphingolipidoses, are frequently characterized by a pronounced dysfunction of microglia cells that are engulfed with storage material, which they are unable to metabolize due to the enzymatic defect, as well as cell debris and residues, and are actively involved in perpetuating the neuroinflammatory and neurodegenerative process ultimately resulting in severe deterioration of neurocognitive and motor functions in the affected patients. Similar mechanisms account for a microglia dysfunction in peroxisomal disorders, such as X-linked adrenoleukodystrophy (X-ALD). Furthermore, recent genetic and functional

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evidence strongly indicate that microglia could be primarily implicated in the pathogenesis of multifactorial, adult-onset NeuroDegenerative Diseases (NDDs) such as Alzheimers' disease (AD), Amyotrophic Lateral Sclerosis (ALS), Parkinsons' disease (PD) and other similar conditions [8,11,12]. These conditions are characterized by age-related deposition of debris and aggregated and misfolded proteins that are recognized by microglia, which in turns reacts in a context and time dependent manner exerting either protective or detrimental effects. Of note, individual carrying hypomorphic and hypofunctional variants in microglia-signature genes, such as TREM2, are known to be at increased risk of developing NDDs, further highlighting the role of microglia in the pathogenesis of these conditions [13].

Based on these and further evidence, microglia is nowadays considered as a key therapeutic target in several neurodegenerative diseases and numerous microglia-targeted interventions and treatments are in development. These microglia-targeted therapies are directed at either restoring or modulating microglia function, to redirect their functional features toward neuroprotection rather than neural damage and inflammation.

Microglia and HSCs Have a Different Ontogeny

Despite the fact that microglia are referred to and classified as brain macrophages, they are distinct from other tissue macrophages because of their unique phenotype, function and origin. Indeed, microglia are now recognized as a unique lineage of tissue macrophages derived from yolk sac (YS) primary erythromyeloid precursors (EMPs), which ultimately give rise to precursors recruited to the brain during embryonic life via blood circulation [14–16]. These cells then turn into CNS microglia without a monocyte intermediate. This origin was recently confirmed also for human microglia employing a single-cell transcriptional profiling approach [17]. Notably, also Hematopoietic Stem Cell (HSC) originate from the hemogenic endothelium, but fetal HSCs arise and migrate from this region to the liver to establish fetal hematopoiesis at a later stage as compared to the wave contributing to microglia [18]. After birth, microglia are mostly maintained through the contribution of CNS-endogenous cells, as they are presumed to be exclusively derived from prenatal sources and to have the ability of self-renewing [19]. Microglia are long-lived cells with an average lifespan of ~4.2 years in humans [20] and maintain their density as a result of a balance between apoptosis and proliferation, which is stochastic in physiological conditions and clonal during pathology [21]. Maintenance of the microglia pool throughout life is also dependent on a constant spatial translocation process mediated by P2Y12 [22]. Besides microglia, CNS-associated myeloid populations also include perivascular, meningeal and choroid plexus macrophages that are present at the interface between the parenchyma and the circulation. Similarly to microglia, perivascular and meningeal macrophages derive from YS precursors, while choroid plexus macrophages have both embryonic and adult hematopoietic origins [23,24].

The contribution of adult hematopoiesis to microgliosis has been extensively investigated, based on the historical view that microglia could be derived from blood monocytes because of similarities in their morphology and phagocytic activity and on the clinical benefit exerted by allogeneic and autologous genetically modified HSC transplantation to patients affected by neurodevelopmental or neurodegenerative diseases [25]. Different approaches have been utilized to this scope, including cell tracking studies in transplantation and parabiosis models in mice, as well as evaluation of post-mortem samples from transplanted patients. Overall, recent research has confirmed that self-renewal of endogenous, CNS resident cells is key for maintenance and reconstitution after depletion of CNS microglia, including following transplantation of HSCs in conventional settings, i.e. by applying irradiation to favor the engraftment of the transplanted cells and their progeny in the recipient (30, 31 32). Importantly, however, preclinical data and clinical practice have also confirmed the appearance of transplant progeny cells in the CNS of recipients [26–30], particularly when favoring transplant conditions have been applied, i.e. when a conditioning regime favoring CNS engraftment of HSCs and their progeny is employed [31,32] or alternative routes for HSC delivery are used [33]. The actual phenotype of these CNS-engrafted, transplant progeny cells and their ability to reproduce at different length the transcriptional and functional complexity of microglia are highly dependent on a definite set of variables that can be controlled and manipulated to favor the integration of highly competent and functional myeloid cells in the CNS of the transplant recipient. Although in most of the cases these cells retain transcriptional features suggestive of tissue macrophages, examples of acquisition of features that are highly consistent with those of *bona fide* microglia are available [32], suggesting that the transplantation of HSCs may hold the possibility to generate a population of cells residing in the CNS that could acquire a microglia-like phenotype and exert relevant functions consistent with this identity to the benefit neurodegenerative or neurodevelopmental disorders.

HSC Transplantation and Gene Therapy in Neurodegeneration: Mechanisms of Benefit

Based on the proven contribution of HSCs to CNS-associated myeloid populations, cell-based therapies by means of allogeneic HSC transplantation (HCT) from healthy compatible donors has been applied over the past 30 or more years to halt or curb the progression of inherited neurometabolic disorders such as peroxisomal diseases and Lysosomal Storage Disorders (LSDs) [34,35]. Indeed, upon engraftment in the patient's CNS (and perypheral tissue), the myeloid cells derived from the transplant restore the defective metabolic activity as well as normal scavenging functions thus contributing to the rescue of storage (Fig. 1). In the case of LSDs caused by enzymatic deficiencies, the allogeneic myeloid cells also release functional lysosomal enzymes in the CNS and in other tissues, which are then taken up by the recipient's enzyme-deficient cells via mannose 6-phosphate and other mannose receptors present on the plasma membrane in a mechanism known as cross-correction. Notably, the molecular defects at the basis of these conditions because of the intracellular accumulation of undegraded metabolites lead to severe alterations of cell-homeostasis and cell-signaling, with consequent oxidative stress and neuroinflammation, which represent the principal mechanisms of neurodegeneration and/or demyelination [36]. Neuroinflammation typically occurs in response to a significant activation of resident microglia and astrocytes, and can be characterized by the recruitment of peripheral macrophages infiltrating the brain in the late stages of the neurodegenerative pathology [37]. Based on this pathogenic mechanism, CNS engraftment of transplant-derived, donor myeloid cells could also contribute to a reduction and/or mitigation of neuroinflammation in favor of the establishment of a neuro-protective environment (Fig. 1) [37] and, therefore, result in neuroprotection, prevention of tissue damage and clinical benefit. Despite this sound rationale, clinical efficacy of HCT has been documented only a limited number of conditions. Among these, the childhood cerebral form of the peroxisomal disease X-linked adrenoleukodystrophy (ccALD) and Mucopolysaccharidosis type I (MPS I) are the neurometabolic diseases for which therapeutic efficacy of HCT is more robust (35, 36]. At present, allogeneic HCT is the standard of care for boys with ccALD and children affected by the most severe MPS I variant in early-stage of their cerebral disease. Moreover, HST is considered for Alpha Mannosidosis (AM), based on limited published evidence [38] and for early, pre-symptomatic cases of infantile Globoid Cell Leukodystrophy (GLD) [39]. Indeed, only if performed when the neurologic and neuropsychological deficits are minimal, HCT can arrests the disease progression providing a stable clinical efficacy. Indeed, in a typical scenario, neuropathology will continue to progress clinically for ~12–18 months after the transplant before slowing down and possibly arresting.

These same favorable effects described for allogeneic HCT have been also hypothesized and proven in the case of the transplantation of autologous and gene corrected HSCs. Importantly, HSC gene therapy (overcomes the most severe immunological limitation of allogeneic HCT

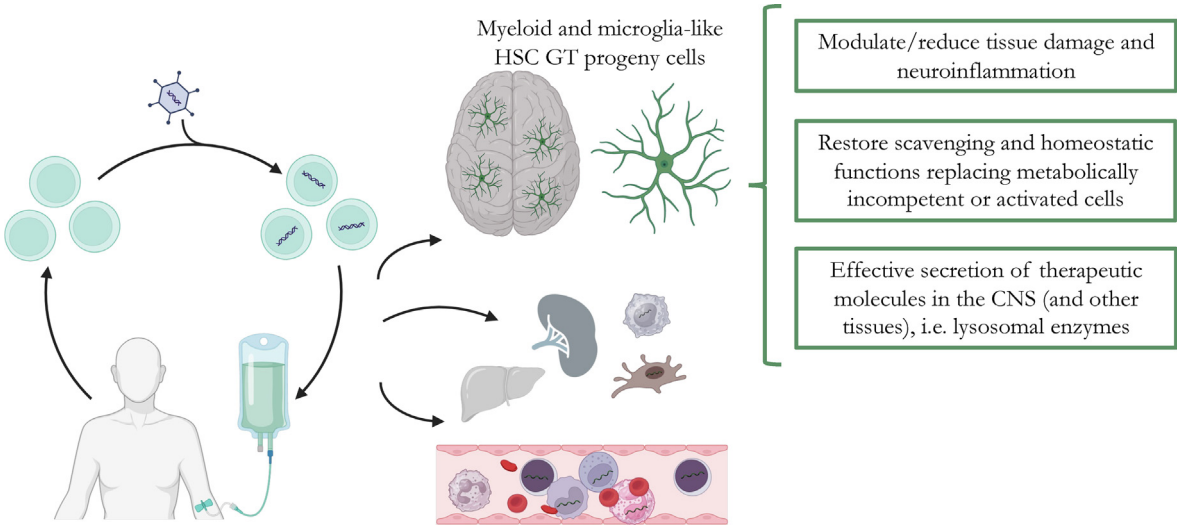

Fig. 1. Mechanisms of benefit associated to the use of HSC GT in neurodegenerative diseases.

represented by Graft *versus* Host Disease (GvHD) thus eliminating the need for immunosuppression in the recipient and offers to every candidate patient a suitable donor. Moreover, genetic engineering of autologous HSCs may allow these HSCs to correct the genetic defect in the patients' cells, but also to achieve over-expression of a functional protein or lysosomal enzyme in the transplant-derived cells, potentially resulting in a robust cross-correction of tissue resident cells and in anticipated metabolic correction and benefit, as observed in preclinical studies [40–44].

Importantly, many of the (dys)functional roles exerted by microglia and of the pathogenic cascades activated in the CNS of patients affected by inherited neurometabolic diseases are also recognized in the brain of patients affected by multifactorial, adult-onset neurodegenerative disorders where activated and neural damaging microglia cells are typically identified. Therefore, therapeutic approaches based on microglia replacement via HSC transplantation are being explored with the rationale of achieving microglia (and CNS tissue) targeting of therapeutics, restoration of physiological microglia functions and modulation of neuroinflammation.

Examples of HSC GT Efficacy in Inherited Neurodegenerative Disorders of Childhood

Two HSC-based gene therapy drug products for neurodegenerative diseases have been approved for commercialization: elivaldogene autotemcel in USA by the FDA since 2022 and atidarsagene autotemcel by both the FDA in 2024 and EMA in 2020. These drug products are intended for the treatment of two inherited neurometabolic disorders of childhood, ccALD and Metachromatic Leukodystrophy (MLD), respectively.

X-ALD is caused by loss of function mutations in the ABCD1 gene that encodes for the ALD protein (ALDP), a peroxisomal membrane transporter, that result in impaired transport and metabolism of very long-chain fatty acids (VLCFAs) into peroxisomes and consequent inflammatory demyelination and neurodegeneration. CcALD is the most severe disease variant characterized by onset of symptoms in early childhood and precocious decline of neurologic function and death within few years from symptom onset. As discussed above, when performed at an early stage of brain disease allogeneic HCT from compatible healthy donor can halt disease progression and provide a curative opportunity to the affected boys. However, donor availability and allogeneic transplant side effects and risks, i.e. GvHD, limit the applicability of this treatment. To address this unmet need, HSC gene therapy based on a third generation, self-inactivating Lentiviral Vector (LV) encoding for the functional human ABCD1 cDNA has been developed for patients lacking a fully compatible HSC donor. First in human testing started in 2006 in France with a pioneering Phase I/II clinical trial (NCT01896102) that enrolled four boys in whom functional human ALDP expression and disease stabilization were observed after myeloablative conditioning and transplantation of engineered autologous HSCs [45]. This initial trial was followed by a larger multicenter study (STARBEAM; NCT01896102) in which gene therapy was shown to be a safe and effective alternative to allogeneic HCT in the boys treated in early-stage cerebral disease, with prevention of major functional disability and minimal clinical symptoms in most of the enrolled and treated patients [46]. Interestingly, widespread and sustained normalization of white matter permeability and microvascular flow was also shown in the treated patients, in whom an inverse correlation between gene dosage and lesion growth suggested an active role of the gene corrected cells in contributing to long-term to remodeling of brain microvasculature [47]. Based on these and other results, elivaldogene autotemcel was approved under accelerated path by the FDA for marketing in September 2022 for the treatment of asymptomatic or mildly symptomatic (neurologic function score, NFS $\leq$1) ccALD boys who have gadolinium enhancement on brain magnetic resonance imaging and Loes scores of 0.5–9. Of note, the drug label reports about a risk of hematologic malignancy because of myelodysplastic syndrome occurred in a small subset of the treated boys and suggests monitoring patients closely upon treatment.

In May 2010 another Phase I/II HSC gene therapy clinical trial based on LVs had started for the inherited neurometabolic disease Metachromatic Leukodystrophy (MLD, OMIM #250100), known as a prototypical LSD severely affecting the CNS not amenable to benefit from allogeneic HCT. MLD is an autosomal recessive disease caused by deficient activity of the lysosomal enzyme arylsulfatase A (ARSA), resulting in sulfatides accumulation and subsequent demyelination, neuroinflammation and neurodegeneration affecting the CNS and the peripheral nervous systems (PNS) at different length based on the age at symptom onset. Different clinical forms of MLD are described (late infantile, early and late juvenile, and adult-onset forms) that constitute a continuum with increasing severity of symptoms and rapidity of the progression of neurological deterioration [48]. The first-in-human trial (NCT015601821) was intended for the treatment of late infantile MLD (LI-MLD) and pre or early symptomatic patients with early juvenile MLD (EJMLD) with the expectation of obtaining prevention of disease onset and/or progression based on preclinical data that had shown a superior benefit of HSC gene therapy *versus* allogenic HCT in the preclinical disease model mediated by an ARSA encoding LV [49–51]. This increased benefit, that was likely based on the expression of the ARSA enzyme above normal levels in transplant-derived progeny, was confirmed in the initial patients' cohort. In these subjects, who received the autologous

engineered HSCs after administration of a myeloablative busulfphan dose, enzyme activity recovery in the CNS was shown as early than 6 months post-transplant with normal ARSA levels in CSF measured by 6 months post-treatment. This metabolic correction was paralleled by as well as a significant improvement in survival in the absence of severe motor impairment, particularly in pre-symptomatic patients, and normal cognitive development or mild alterations of the dysexecutive functions (long term analysis are still missing). Effect on the PNS appears only partial. Based on this evidence, this gene therapy product now known as atidarsagene autotemcel was approved for marketing by EMA in 2020 with indication for use in children with the LI or EJ forms of MLD, who are carriers of the defective gene but have not yet developed symptoms, and to early symptomatic EJMLD patients who still can walk independently and have not yet developed a cognitive deficit. FDA has also recently approved atidarsagene autotemcel for marketing with the same indications of EMA. The pre-symptomatic patients who might be eligible to treatment can be identified based on familiar history or by neonatal blood screening (NBS) as soon as it will be available. Notably, pilot studies for MLD newborn screening combining biochemical and genomic analysis are currently on going (USA, Germany, UK and France) [52].

Besides these advanced products, clinical testing of this same approach is ongoing for two other LSDs with neurodegenerative features, namely Mucopolysaccharidosis type I and IIIA (MPS I and MPS IIIA, respectively). While patients affected with MPS I and CNS involvement are considered primary candidates to allogeneic HCT, MPS IIIA patients do not benefit from such procedure independently from the stage of the disease at diagnosis, likely because of disease-specific factors. With the scope of increasing the benefit associated to allogeneic HCT [43] or providing a potential for benefit [53]. HSC gene therapy has been tested in patients (NCT03488394, NCT06149403, NCT04201405) with promising preliminary results [54].

New Indications and Future Directions

Research is currently exploring new NDD indications that may benefit from HSC gene therapy approaches. Firstly, new LSDs with CNS involvement are being considered for future clinical testing, which include neuronal ceroid lipofuscinoses [55], other MPSs [56,57] and possibly other forms of leukodystrophy. Importantly, also inherited microgliopathies could represent a relevant future target indication for HSC gene therapy. Information on the use of HCT for the treatment of these conditions may guide future treatment development. Allogeneic HCT has been increasingly offered to patients with CSF1R-HLDS and of the patients who had undergone the procedure, the majority have experienced stabilization of neurologic symptoms beginning at approximately 6 months after transplant, as well as decrease/stabilization of white matter lesions at brain magnetic resonance imaging. Other minor clinical improvements tended to be empirical and some of the patients developed transplant complications, such as GvHD [58–60]. Notably, also in this setting patients with a higher burden of cognitive symptoms were most likely not to benefit from HSCT. Based on this evidence, HSC gene therapy could be developed for CSF1R-HLDS and other similar microgliopathies, such as TREM2-NHD, with the scope of replacing dysfunctional microglia with gene corrected myeloid cells derived from the transplant, without undergoing the risks of an allogeneic procedure. Based on the critical role that microglia exert in the pathogenesis of NDDs and on the potential for therapeutic transcript delivery via cell replacement, HCT and HSC gene therapy are also being preliminarily explored in these indications, with first evidence accumulating in preclinical animal models of Alzheimer's and Parkinson's disease [61–64]. Interestingly, restoration of wild type microglia in relevant Alzheimer's animal models was sufficient to mitigate some of the disease manifestations, including amyloid accumulation. Notably, delivery of GDNF to the Parkinson's disease mouse brain via the transplantation of genetically engineered HSCs could be beneficial and attenuate disease manifestations. Whether the same approach, besides and inspiring future development of therapeutics based on HSCs in NDDs, could be employed to approach also neurodevelopmental conditions characterized by inherited *versus* acquired microglia dysfunctions remains object of interesting future research.

Interestingly, new modalities are also being explored in the preclinical arena intended at optimizing the outcome and potentially anticipating the benefit of HSC transplantation in patients affected by NDDs. Testing alternative and novel drug regimens to enhance the engraftment of the myeloid progeny of the transplanted cells in the CNS. Among these strategies, of interest is the Colony-stimulating factor 1 receptor (CSF1R) targeting being CSF1R crucial for the survival of microglia and macrophages in rodents and humans [65]. At present, regimens that combine CSF1R inhibition with myeloablative total body irradiation or busulfan have been reported to result in ameliorated replacement of microglia turnover with transplant-derived cells in mice [29,66], providing early indications that in the future may guide inclusion of CSF1R inhibition in pre-transplant conditioning protocols for NDDs.

HSC gene therapy nowadays stands as a therapeutic strategy uniquely capable of reaching the brain to prevent or halt a neurodegenerative process *via* a population of cells endowed by multiple relevant functions. These functions could be enhanced and modulated *via* genetic engineering that could be further empowered thanks to the use of novel vectors and/or of gene editing, as well as by innovative protocols for patients' conditioning before transplant. These innovations may ultimately provide now opportunities for extending the use of HSC gene therapy to refractory or complex diseases, as well as anticipating benefit, as at present the best clinical results are obtained upon treatment administration to pre- or very early symptomatic patients. Development of gene therapies targeting pathogenic mechanisms of neurodegeneration and not restricted to the gene defect could also be of great relevance for enabling further applications in NDDs.

Author Contributions

Alessandra Biffi, MD, is the only authors who wrote this manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alessandra Biffi reports a relationship with Orchard Therapeutics that includes: consulting or advisory and funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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