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Review article

Thymosins in multiple sclerosis and its experimental models: moving from basic to clinical application

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Abs
antibodies
Breg
regulatory B cells
CNS
central nervous system
EAE
experimental autoimmune encephalomyelitis
HIV
human immunodeficiency virus
IL
interleukin
MBP
myelin basic protein
miRNA
microRNA
MS
Multiple Sclerosis
OLs
oligodendrocytes
OPCs
oligodendrocyte progenitor cells
PLP
proteolipid protein peptide
RA
rheumatoid arthritis
RRMS
relapsing-remitting Multiple Sclerosis
SLE

ABSTRACT

Background: Multiple sclerosis (MS) afflicts more than 2.5 million individuals worldwide and this number is increasing over time. Within the past years, a great number of disease-modifying treatments have emerged; however, efficacious treatments and a cure for MS await discovery.

Thymosins, soluble hormone-like peptides produced by the thymus gland, can mediate immune and non-immune physiological processes and have gained interest in recent years as therapeutics in inflammatory and autoimmune diseases.

Methods: Pubmed was searched with no time constraints for articles using a combination of the keywords "thymosin/s" or "thymus factor/s" AND "multiple sclerosis", mesh terms with no language restriction.

Results: Here, we review the state-of-the-art on the effects of thymosins on MS and its experimental models. In particular, we describe what is known in this field on the roles of thymosin-α1 (Tα1) and -β4 (Tβ4) as potential anti-inflammatory as well as neuroprotective and remyelinating molecules and their mechanisms of action.

Conclusion: Based on the data that Tα1 and Tβ4 act as anti-inflammatory molecules and as inducers of myelin repair and neuronal protection, respectively, a possible therapeutic application in MS for Tα1 and Tβ4 alone or combined with other approved drugs may be envisaged. This approach is reasonable in light of the current clinical usage of Tα1 and data demonstrating the safety, tolerability and efficacy of Tβ4 in clinical practice.

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systemic lupus erythematosus
Tα1
thymosin-α1
Tβ4
thymosin-β4
TLR
toll-like receptor
Treg
regulatory T cells

1. Introduction

1.1. Multiple sclerosis and treatment strategies

A complex genetic component with multiple susceptibility genes as well as several environmental factors, including UV light exposure or viral infections, have been implicated in Multiple Sclerosis (MS) risk and development (Ascherio and Munger, 2007a, b).

This enigmatic immune-mediated disease of the central nervous system (CNS) displays a progressive neurodegeneration due to immune attacks directly against myelin constituents that cause myelin destruction (Lassmann et al., 2007) in genetically susceptible individuals.

Treatment development in the past years has been extremely active and a great number of potential new therapies have emerged for patients with MS.

MS therapeutic approaches focused for the past three decades on strategies to reduce inflammation and immune activation, acting on immunomodulation, on migration of specific inflammatory cell subsets or on agents directly mediating neuroprotection and neurorestoration.

Disease-modifying therapies have shown beneficial effects in patients with relapsing-remitting MS (RRMS). The majority of these drugs are, however, of little benefit during progressive MS where axonal degeneration following demyelination outweighs inflammation. For obvious reasons, this discrepancy in therapeutic efficacy of approved drugs has sparked great interest in the development of new remyelination therapies aimed at providing neuroprotection and functional recovery.

The monotherapeutic strategy to suppress immune cells from attacking the CNS is insufficient in preventing or ameliorating permanent and accumulating MS deficits (Kremer et al., 2015). Additional therapies for MS are urgently required to enhance remyelination and to reduce axonal damage to improve functional recovery.

Demyelination is a loss of myelin sheaths from axons, which results from damage and death of myelin producing oligodendrocytes (OLs). Remyelination requires oligodendrocyte progenitor cells (OPCs) to differentiate into mature myelinating OLs, because mature OLs in the adult mammalian CNS are post mitotic and, thus, unable to proliferate in response to injury and to form new myelin sheaths (McTigue and Tripathi, 2008; Keirstead and Blakemore, 1997). The failure of newly generated OLs to remyelinate axons and to preserve axonal integrity impedes functional recovery after MS (Kremer et al., 2015). Cell therapies, including stem cell transplantation, have a potential for CNS repair and may be able to provide protection from inflammatory damage caused after injury (Rice et al., 2013).

The glial cell communication network, including astrocytes and microglia, also plays important roles in de/remyelination (Domingues et al., 2016). Thus, it is important to investigate a treatment strategy for MS that would include immunomodulation paired to neuroprotection and neurorestoration. Many new molecules or previously approved drugs developed by high-throughput screens appear to have a positive impact on remyelination and neuroprotection and are in the therapeutic pipeline envisaging their use alone or as part of a combination therapy, including immunomodulatory drugs for the treatment of MS (Harlow et al., 2015). Although the beneficial effects of these drugs on CNS cells are encouraging, careful study of off-target effects will need to be undertaken, given that many of these drugs were originally utilized for non-CNS targets, such as the thymic hormone-like molecules thymosins.

1.2. The use of thymosins in MS

Evidence is emerging that the regulation of thymic peptide hormones, such as thymosins, have anti-inflammatory potential in inflammatory as well as autoimmune diseases (Lunin and Novoselova, 2010).

In this context, in early studies conducted using thymosin fraction V to treat old mice (Endoh and Tabira, 1990) or guinea pigs (Woyciechowska et al., 1985) subjected to the model of MS experimental autoimmune encephalomyelitis (EAE), the treatment showed no suppressive effect on incidence and severity of disease. In the first study (Endoh and Tabira, 1990), however, EAE was induced in aged mice, in which susceptibility to disease may be significantly reduced. Indeed, in the same setting, also treatment with another thymic protein, the serum thymic factor, showed no effect, differently from that observed by other groups describing an intensive suppression of EAE symptoms (Nagai et al., 1982). Moreover, thymosin fraction V is a partially purified mixture of 10 major and at least other 30 polypeptides from the thymus gland with extremely varied and important biological properties that may act individually, sequentially, or in concert to influence the development of T cell subsets and that could also mediate inhibitory effects not observed in experiments conducted with single or a mixture of purified thymosins (Hoch and Volk, 2016; Goldstein and Badamchian, 2004).

Indeed, therapeutic benefits were observed in the damaged CNS of neurological disorders, including MS, when the synthetic form of thymosin β4 (Tβ4), a single peptide purified from thymosin fraction V able to pass the blood brain barrier (Mora et al., 1997), was exogenously administered. Using animal models of neurological injury, studies have demonstrated that Tβ4 can target multiple neural cells (including neurons, oligodendrocytes and microglia) and can also provide neuroprotection, immunosuppression, and neurorestoration, including remyelination, synaptogenesis, and axon growth (Zhang et al., 2016a,b; Chopp and Zhang, 2015; Santra et al., 2012; Santra et al., 2016; Wang et al., 2015; Wang et al., 2012; Cheng et al., 2014).

Furthermore, studies showed that purified prothymosin α (ProTα), the precursor of thymosin α1 (Tα1), is able to regulate the defective phenotype of monocytes in MS impacting T cell activation (Reclos et al., 1987; Baxevanis et al., 1990). More recently, the anti-inflammatory potential of Tα1 on the differentiation of regulatory subsets of lymphocytes was also studied in MS (Giacomini et al., 2017).

In this review we will describe in detail what is known on the role of Tβ4 and Tα1 in promotion of remyelination and anti-inflammatory responses in MS and the potential mechanisms of action.

2. Methods

2.1. Search strategy and selection criteria

A literature search was carried out on PubMed/Medline database. The authors deemed important not to miss any potentially relevant

study, therefore a comprehensive search strategy was set and no limits were fixed as to language or date of publication. Based on the review topic, the search strategy included controlled vocabulary and free-text words, synonyms and MeSH terms relating to thymosin "(thymosin/s", "thymus factor/s", "thymus peptide/s", "thymic peptide/s", "thymosin fraction 5", "thymosin beta 4", "thymosin alpha 1", "prothymosin"), combined with the main search filter (multiple sclerosis).

The final reference list was generated considering titles relevant to the review topic based on the online searches updated to June 2018 as well as citations from other bibliographies or authors' suggestions.

3. Thymosins: the old and the new

The thymus gland produces soluble hormone-like peptides that can mediate immune and non-immune physiological processes. Thymosin was originally prepared as a crude extract of mouse or rat thymus gland in 1966. In the next decades, the first biologically active thymic extract was purified and called thymosin fraction V, the fractionation of which led to the isolation of a series of immunoactive polypeptides, thus so named thymosins (Goldstein, 2007). However, these molecules are genetically unrelated while being distributed widely throughout most tissues and play important, yet very different, roles in cells. The active peptides are typically short, highly charged with no or few aromatic amino acids and, therefore, are intrinsically unstructured proteins under natural conditions (Hoch and Volk, 2016).

Starting with fraction V, several main peptides (ProTα, Tα1, polypeptide β1 and different Tβ) were isolated and tested for biological activity (Table 1).

Thymosins are divided into 3 main groups based on the isoelectric focusing pattern of thymosin fraction V: α-thymosins below pH 5.0, β-thymosins between pH 5.0 and 7.0 and γ-thymosins above pH 7. The numerical subscript simply denotes the chronological order of isolation. The first two peptides isolated from fraction V were Tα1 and polypeptide β.

In general, the peptides isolated from the β region of thymosin fraction V do not appear to be thymus-specific products. The most predominant band on the β region is polypeptide β1 that did not show any biological activity, though it was the most prominent component of thymosin fraction V. Later, it was identified as a 74-amino-acid residue fragment of ubiquitin, lacking two glycine residues at the C-terminus (Hoch and Volk, 2016). The next thymosin, which was isolated and sequenced, was termed Tβ4. Successively, β8, 9, 10 and 15 were isolated (Hoch and Volk, 2016).

Many orthologs of human thymosin genes sharing chromosome location and/or sequence similarities have been characterized in different species. For a summary of the main thymosin genes' characteristics and tissue distribution between human and mouse see Table 2.

Several attempts were made in trying to characterize the cellular receptors involved in thymosins' recognition; however, so far, none have been identified (Rinaldi Garaci et al., 1985; Brelinska and Warchol, 1982).

To-date, main members of the thymosin family are considered Tα1 and its precursor ProTα, as well as β-thymosins (Mosoian, 2011).

ProTα is a 12.5-kDa, highly acidic protein, widely distributed in different cell types and expressed at both intracellular and extracellular levels. In humans, a family of 7 genes, 6 of which are considered pseudogenes, encode ProTα (Mosoian, 2011). The major intracellular functions of ProTα are linked to chromatin remodeling, cell proliferation, differentiation and apoptosis, and ProTα was also found overexpressed in different cancer types (Mosoian, 2011). The extracellular ProTα shares many features with interleukin (IL)-1α, thus representing an important endogenous stimulator of the innate immune system related to anti-viral, anti-cancer, anti-fungal and anti-ischemic activities, as well as an adjuvant for vaccines (Mosoian, 2011). Of note, ProTα signaling via toll-like receptor (TLR) 4 is required for its potent anti-human immunodeficiency virus (HIV) activity in macrophages via type I IFN induction (Mosoian, 2011).

Tα1 peptide, which is only 28 amino acids-long, is contained at the N-terminus of its precursor ProTα, nearly 100 bases-long. It was shown that a lysosomal asparaginyl endopeptidase (the so-called legumain) is able to proteolytically process the asparagynil-glycine residues Asn^{28}-Gly^{29} of ProTα to generate Tα1 (Sarandeses et al., 2003).

Since its discovery, investigations on Tα1 were mainly performed in the area of infectious diseases. Tα1 administration was studied in a wide variety of animal and human settings and its pharmacologic effects were shown to enhance cellular immunity inhibiting viral replication of different viruses. Based on these data, Tα1 was then used for the treatment of chronic hepatitis B and C (Iino et al., 2005; You et al., 2006), cytomegalovirus infection (Bozza et al., 2007) and invasive aspergillosis (Romani et al., 2004). In addition, the post-marketing data on Zadaxin® (SciClone) clearly confirmed Tα1 immunomodulatory activities and related therapeutic potential also in cancer (such as Hepatocellular carcinoma, lung cancer, and melanoma), infectious diseases (sepsis, infections after bone marrow transplant, lung infections including chronic obstructive pulmonary disorder, Severe acute respiratory syndrome, and HIV) (King and Tuthill, 2016; Liu et al., 2016a; Matteucci et al., 2017; Jia et al., 2015) and improvement in immune responses of elderly immunocompromised patients (for example for enhancement of response to vaccines) (Tuthill et al., 2012).

The Tβ family is composed of 20 short (40–44 amino acids) peptides; among these only three of them have been characterized in detail, Tβ4, Tβ10 and Tβ15. However, while Tβ15 was found only up-regulated in different malignancies, such as the human prostate cancer, representing a potential biomarker (Bao et al., 1996), in a healthy human body only Tβ4 and Tβ10 are expressed (Huff et al., 2001). These peptides play numerous different functions. Among others, they affect the processes of carcinogenesis, differentiation and angiogenesis, influence metalloproteinase activity and accelerate wound healing.

Tβ4 is a highly conserved, 43-amino acidic peptide, which is widely distributed in mammalian tissues, including the CNS. Tβ4 is the major G-actin-sequestering molecule, and its primary physiological function is to regulate cell motility (Bock-Marquette et al., 2004). During

Table 1
Thymosin proteins and their functions.

Name	Species	Role	References
PROTHYMOSIN α (ProTα)	mice, rat, human	chromatin remodeling, transcriptionally regulation, cell proliferation and survival	(Ueda et al., 2017; Samara et al., 2017; Samara et al., 2016; Ueda et al., 2012)
THYMOSIN-α1 (Tα − 1)	rat, mice, humans	immunoregulation	(You et al., 2006; Romani et al., 2004; King and Tuthill, 2016; Liu et al., 2016a; Romani et al., 2017; Garaci et al., 2007)
THYMOSIN-β4 (Tβ − 4)	mice, rats, humans, cattle, chimpazees	actin polymerization, angiogenesis, cell migration, collagen deposition, wound healing, fibrosis, neovasculogenesis, tissue repair and regeneration	(Chopp and Zhang, 2015; Kuzan, 2016; Goldstein and Kleinman, 2015)
THYMOSIN-β10 (Tβ − 10)	rat, mice, humans, cattle,	cytoskeleton organization and morphology, proliferation, motility, anti-inflammatory effects, insulin secretion	(Sribenja et al., 2009; Zhang et al., 2017b)
THYMOSIN-β15 (Tβ − 15)	rat, mice, human	motility, progression and metastatis of non-small cell lung cancer	(Banyard et al., 2007)

Table 2
Thymosin genes: chromosome location and tissue distribution in human and mouse
Source: https://www.ncbi.nlm.nih.gov/gene/

Name (*gene*)	Species	Chromosome	Location	Tissues
Prothymosin-α (*PTMA*)	*Homo sapiens*	2	NC_000002.12	Bone marrow, lymph node, appendix, endometrium, thyroid and urinary bladder
	Mus musculus	1	NC_000067.6	CNS, limb, liver, thymus, adult ovary and placenta
Thymosin beta 4 X-linked (*TMSB4X*)	*Homo sapiens*	X	NC_000023.11	spleen, lymph node, lung, appendix, bone marrow
	Mus musculus	X	NC_000086.7	bladder adult, placenta adult and CNS
Thymosin beta 4 Y-linked (*TMSB4Y*)	*Homo sapiens (homolog of TMSB4X)*	Y	NC_000024.10	testis, prostate and colon
	No ortholog in Mus musculus			
Thymosin beta 10 (*TMSB10*)	*Homo sapiens*	2	NC_000002.12	appendix , colon, spleen, lymph node, lung, ovary
	Mus musculus	6	NC_000072.6	CNS and placenta adult
Thymosin beta 15a (*TMSB15A*)	*Homo sapiens*	X	NC_000023.11	prostate, endometrium, testis, thyroid
	Mus musculus	X	NC_000086.7	Restricted expression toward testis adult
Thymosin beta 15b (*TMSB15B*)	*Homo sapiens*	X	NC_000023.11	ovary, endometrium and prostate
	No ortholog in Mus musculus			

development of CNS, Tβ4 regulates neurogenesis, tangential expansion, tissue growth and hemisphere folding (Lever et al., 2017; Wirsching et al., 2012, 2014). Tβ4 was initially employed as an anti-inflammatory agent (Badamchian et al., 2003; Girardi et al., 2003) and, subsequently, to inhibit proliferation and induce differentiation and apoptosis of leukemic cells (Huang et al., 2006). Recently, Tβ4 healing properties were described in skin, cornea and cardiac repair (Zhang et al., 2016a; Chopp and Zhang, 2015; Bock-Marquette et al., 2004; Badamchian et al., 2007; Li et al., 2017; Kim et al., 2017; Goldstein et al., 2012).

4. The established role of Tβ4 as a restorative therapy for MS

4.1. The effect of Tβ4 on neuroprotection in the MS animal model

Neuroprotection is a well-investigated effect of Tβ4 (Santra et al., 2016; Xiong et al., 2012). In spinal cord injury and traumatic brain injury models, Tβ4 treatment significantly improved locomotor and sensorimotor functional recovery and spatial learning, as well as increased survival of neurons and OLs, and reduced cortical lesion volumes after neurological injuries (Cheng et al., 2014; Xiong et al., 2012). In vitro, exogenous Tβ4 treatment significantly reduced apoptosis of the neural progenitor cells subjected to oxygen glucose deprivation (Santra et al., 2016). Moreover, Tβ4 may also drive neuroprotection in EAE. The EAE model is widely employed in investigation studies of MS since it exhibits significant neurological functional deficits as well as obvious pathological changes, including demyelination and inflammatory infiltration (Procaccini et al., 2015; Eng et al., 1996). When Tβ4 was administered as a prophylactic treatment on the day of proteolipid protein peptide ($PLP_{139-151}$) immunization, Tβ4 treatment significantly delayed the EAE onset, and evoked a significantly improved neurological functional recovery. Since mature OLs and myelin support axonal integrity and function (Nave, 2010), further investigation found that robust functional improvement accompanied increased numbers of mature OLs in the CNS of the EAE mice (Zhang et al., 2009). Since the EAE model is induced by an autoimmune response, the anti-inflammatory and immunomodulatory properties of Tβ4 (Badamchian et al., 2003; Girardi et al., 2003; Sosne et al., 2007) via the suppression of nuclear factor-kappa B (NF-kB) activation (Sosne et al., 2007) may protect OLs from damage and death. In addition to inflammatory cells (which infiltrate from peripheral circulation), microglia, the resident innate immune cells of the CNS, are activated by neuroinflammation and play a pivotal role in onset and pathological changes of disease. Thus, the effect of exogenous Tβ4 treatment on inhibition of microglial activation after damage may contribute to reduce the secretion of inflammatory mediators (Zhang et al., 2016b; Zhou et al., 2015), and thereby prevent and/or reduce damage of OLs after EAE by attenuating immune onslaught (neuroprotection).

The most important aspect of our study is in the novel evidence showing that prophylactic Tβ4 treatment may contribute to remyelination, since this treatment was able to promote an increase of new OLs generated from OPC proliferation and differentiation (Zhang et al., 2009).

4.2. The effects of Tβ4 on OPC differentiation and remyelination in the demyelination models

To further investigate remyelination effects of Tβ4, the treatment window was delayed to permit the full development of demyelination damage in the CNS of EAE animals. When the therapeutic treatment of Tβ4 was administered after EAE symptoms' onset instead of on the day of PLP immunization, functional outcomes revealed that this Tβ4 treatment approach evoked significant functional benefit (Zhang et al., 2016a), and concurrently increased OLs in the demyelinating CNS. At the early stage of EAE (day 7 after onset), the protein level of myelin basic protein (MBP) and the numbers of OLs were strongly decreased. Conversely, OLs were significantly increased after Tβ4 treatment, suggesting that the newly generated OLs formed new myelin and contributed to improved functional recovery. This hypothesis was supported by results that also OPC differentiation was significantly induced, axons were re-wrapped, and axonal damage was reduced after Tβ4 treatment at the late stage of EAE (day 30 after onset), and that OPC differentiation significantly correlates with the neurological functional score (Zhang et al., 2016a).

This Tβ4 treatment approach increased myelin areas and improved functional outcome in the late disease stage, demonstrating the presence of a remyelination effect of Tβ4, in addition to its neuroprotective and anti-inflammatory effects. Complementing the EAE model, an additional demyelination model -induced by cuprizone diet- was employed to further confirm that the remyelination effect of Tβ4 derives from its direct action on OPCs. The cuprizone model is extensively used to study in vivo toxicity-induced demyelination (Zhang et al., 2017a) with less infiltrated immune cells as compared to what found in EAE (Procaccini et al., 2015). Thus, this experimental model has utility for directly investigating the effects of a therapeutic agent on OPC differentiation and remyelination. Cuprizone-fed mice with demyelination treated by Tβ4 exhibited a significant increase of remyelination, accompanied with a robust increase of newly generated OLs, and elevation of MBP density in the demyelinating corpus callosum (Zhang et al., 2016b). In concert, these results obtained from both the EAE and cuprizone models indicate that the in vivo effects of Tβ4 on OPC differentiation and remyelination are independent of its systemic anti-inflammatory effect.

Data obtained from other models of neurological injury, which induce demyelination and white matter damage and result in

neurological deficits, including stroke, peripheral neuropathy and traumatic brain injury, likewise show the benefits of Tβ4 on OPCs and myelin after delayed Tβ4 treatments. Rats with middle cerebral artery occlusion treated with Tβ4 demonstrated a significant overall improvement in functional outcome (Morris et al., 2010). Although lesion volumes were not reduced, Tβ4 treatment increased myelinated axons in the ischemic boundary, and augmented remyelination, which was associated with an increase of OPCs and myelinating OLs (Morris et al., 2010). In a model of diabetes-induced peripheral neuropathy, extended Tβ4 treatment of diabetic mice significantly improved neurological function, and was closely associated with increased axonal regeneration and remyelination in peripheral nerves (Wang et al., 2015). Traumatic brain injury remains a leading cause of mortality and morbidity worldwide with no effective pharmacological treatments. Tβ4 treatment of traumatic brain injury in rats amplified endogenous remyelinating processes including oligodendrogenesis, neurogenesis and axonal remodeling, which appeared to drive functional recovery (Xiong et al., 2012). In vitro experiments provide evidence of the direct effects of Tβ4 on OPCs and neurons. After Tβ4 treatment of primary cultured OPCs, the differentiation of OPCs into mature OLs identified by the protein level of MBP significantly increased (Zhang et al., 2016a; Santra et al., 2014). Thus, data generated from these in vivo and in vitro studies in concert, confirm the remyelination effect of Tβ4. Due to the combined benefits in neuroprotection and remyelination (summarized in **Fig. 1**), Tβ4 is an excellent candidate for the treatment of demyelinating diseases also based on data demonstrating safety, tolerability and efficacy of Tβ4 in clinical practice (Crockford, 2007; Vasilopoulou et al., 2015; Marks and Kumar, 2016).

In vivo and in vitro studies have also given mechanistic insights into the therapeutic effects of Tβ4. The epidermal growth factor receptor and the TLR signal transduction pathways may contribute to CNS remyelination and recovery of function induced by Tβ4 (Zhang et al., 2016a; Santra et al., 2014). Hedgehog signaling pathway was shown to be involved in activation of stem cells after Tβ4 treatment, which may contribute to therapeutic benefit (Kim et al., 2017).

Furthermore, having in mind that microRNA (miRNA)−146a may directly induce OPC differentiation to OLs (Zhang et al., 2017a; Liu et al., 2016b), Tβ4 treatment may prominently stimulate the expression of this mRNA in primary cultured OPCs, thus promoting the proliferation and differentiation of OPCs and OLs (Santra et al., 2014), which may serve as a common remyelination therapeutic mechanism. In addition to increasing miR-146a expression in OPCs, Tβ4 remarkably also increased miR-146a expression in microglia and significantly inhibited secretion of pro-inflammatory mediators (Zhou et al., 2015), all of which may promote remyelination (Crockford, 2007).

5. Tα1: a newcomer in MS field

5.1. Tα1 as a pleiotropic immunoregulator

Tα1 has been shown to have beneficial effects on numerous immune system parameters, related to both innate and adaptive immune cells, including macrophages, neutrophils, natural killer cells and DC in addition to the well-characterized effects on the differentiation and maturation of T cells (Serafino et al., 2012). For these features, Tα1 was used as an adjuvant or immunotherapeutic agent to treat disparate human diseases, including viral infections, immunodeficiencies and malignancies (Romani et al., 2012). Tα1, however, demonstrated to be a powerful pleiotropic molecule able not only to induce anti-viral and pro-inflammatory responses, but also to promote a regulatory milieu depending on the context, as others' and our data showed (Romani et al., 2006; Giacomini et al., 2015). These observations are consistent with the fact that Tα1 can negatively control inflammation during immunopathological pro-inflammatory infections and diseases, exerting an interesting, and until now not-completely understood, role as a homeostasis regulator (Romani et al., 2007).

In this context it is important to underline another interesting property of Tα1 that relies on the modulation of regulatory T cell (Treg) function by acting on signals delivered through TLR in response to pathogen associated molecular patterns (Romani et al., 2007; Montagnoli et al., 2006). This characteristic was also recently tested in cystic fibrosis where Tα1 increases the altered maturation of the cystic fibrosis transmembrane conductance regulator and reduces the chronic inflammation caused by the excessive activation of the innate immune response (Romani et al., 2017). Indeed, owing to its ability to activate the tolerogenic pathway of tryptophan catabolism via the immunoregulatory enzyme indoleamine 2,3-dioxygenase 1 (Puccetti and Grohmann, 2007), Tα1 specifically potentiates immune tolerance in the lung, breaking the vicious circle that perpetuates chronic lung inflammation in response to a variety of infectious noxae (Romani et al., 2006).

Collectively, these data suggest that Tα1 represents a promising molecule to control inflammation, immunity and tolerance in a variety of clinical settings, including organ transplantation, tumors as well as autoimmune diseases, such as MS (Serafino et al., 2012).

5.2. Tα1 and the anti-inflammatory effect in MS: focus on B cells

Due to the Tα1 ability to establish a regulatory environment for balance of inflammation and tolerance, we recently hypothesized a possible role for this molecule in novel therapeutic applications towards

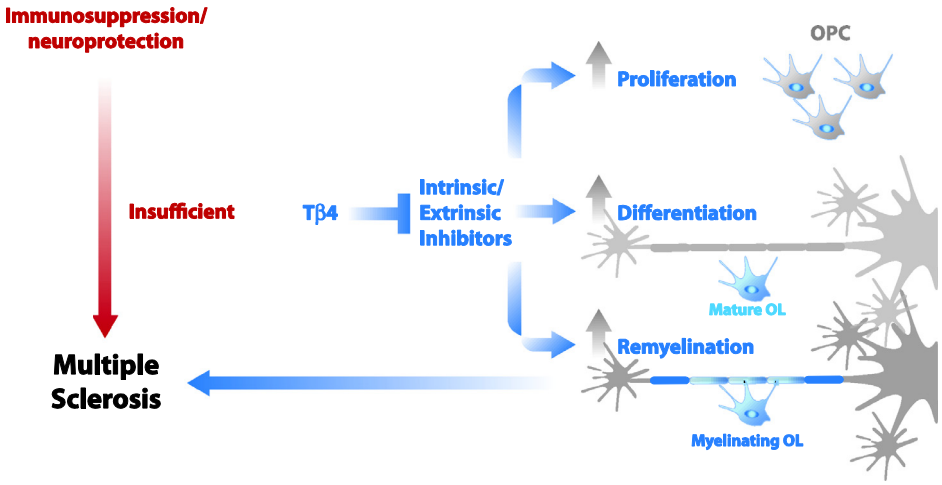

Fig. 1. Scheme of impact of Tβ4 on the promotion of OPC differentiation and remyelination via blocking the intrinsic and extrinsic inhibitors, thereby promoting MS recovery.

autoimmune diseases, and in particular for MS (Giacomini et al., 2017).

Concentration of Tα1 in human serum is very high in fetuses and newborns, when the immune system is first developing, but rapidly drops in early childhood coincident with the maturation of T cells in the body remaining to a steady-state level throughout adulthood. However, deregulation in Tα1 serum concentrations was found in different types of cancers or infectious diseases and, more recently, in patients affected with chronic inflammatory autoimmune diseases such as psoriatic arthritis, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), that have a much lower level of serum Tα1 as compared to healthy controls (Pica et al., 2016). Similarly, we also recently found that serum Tα1 is significantly lower in RRMS patients than in matched controls (Giacomini et al., 2017) and we hypothesized that the deficient endogenous Tα1 level found in patients' sera could be related to MS-associated altered inflammatory status.

As for Tα1 always studied as a modulator of T cell responses, MS was considered by far a T cell-mediated autoimmune disease. Nonetheless, over the past few years B cells have been increasingly recognized as disease-relevant in MS and recent evidence on their immunopathogenic support on MS development has been collected, highlighting their central role (Franciotta et al., 2008), historically overshadowed by the emphasis on T cell research.

Traditionally, B cells have been implicated in MS for their ability to produce pathogenic antibodies (Abs) or auto-Abs, found to be present in CSF and brain tissue of MS patients. Accordingly, characteristic oligoclonal IgG bands in their CSF are thought to be a quite specific marker for MS (Walsh et al., 1985). Numerous B lymphocytes were also described together with T lymphocytes, DC and plasma cells in white matter lesions, with a very high frequency in acute lesions (Nyland et al., 1982). However, new impetus on the central role of B cells in MS pathogenesis was received from the identification in the meninges of SPMS patients of ectopic lymphoid follicles enriched with B and plasma cells, whose establishment in the brain of these individuals could provide a microenvironment in which B cell expansion and maturation, and hence local Ig production, may occur (Serafini et al., 2004). Nowadays, however, important Ab-independent pathogenic roles for B cells are emerging, also in light of successful results of B cell-depleting therapies in MS (Barun and Bar-Or, 2012). In particular, selective depletion of B cells *via* monoclonal Abs against the B cell lineage specific surface marker CD20 (i.e. rituximab, ocrelizumab, and ofatumumab) proved to be remarkably effective in the induction of long-lasting suppression of lesion activity and clinical relapses but showed no effect on plasma cell differentiation and Ab production (Barun and Bar-Or, 2012).

Importantly, B cells can efficiently present antigens to T cells and modulate local immune responses by secreting soluble factors. In humans, different B cell subsets were described producing distinct effector cytokines. In particular, CD19 + CD27- naïve B cells release mainly the anti-inflammatory IL-10; while CD19 + CD27 + memory B cells largely express pro-inflammatory factors, such as lymphotoxin (LT), TNF-α and IL-6. In human B cells the effector cytokine profile is stringently context-dependent with a reciprocal regulation of pro- and anti-inflammatory response. In MS, this cytokine network is dysregulated, with a much lower production of the anti-inflammatory factor IL-10 (Duddy et al., 2007). Furthermore, B cells of MS patients exhibit aberrant pro-inflammatory responses, with increased LT:IL-10 ratio and exaggerated LT and TNF-α secretion, that may mediate 'bystander activation' of disease-relevant pro-inflammatory T cells, resulting in new relapsing MS disease activity (Bar-Or et al., 2010). The term "regulatory B cells" was first introduced by Mizoguchi and Bhan to indicate a subset of B cells with the ability to produce IL-10 and to suppress inflammatory cellular immune responses (Mizoguchi and Bhan, 2006). Since then the family of B reg is expanding with many subsets identified and shown to arise at different stages of B cell differentiation in a context-dependent manner (Mauri and Menon, 2015). In particular, among many others, two main B reg populations have been deeply characterized: CD24 + CD38hi transitional-immature B reg arising from CD27- B cell compartment (Blair et al., 2010) and CD24low/negCD38hi plasmablast-like B reg cells differentiating from CD27 + memory B cells (Matsumoto et al., 2014). While much evidence indicate that B cell-derived IL-10 production is strongly downregulated in MS patients (Duddy et al., 2007; Bar-Or et al., 2010), only few studies have characterized so far B reg populations in MS by using different Ab cocktails leading to contrasting results (Knippenberg et al., 2011; Michel et al., 2014; Li et al., 2015; Habib et al., 2015).

Based on this background and having in mind the strong immunomodulatory and pleiotropic activity of Tα1 molecule as well as our previous data showing a TLR7-driven dysregulation of B cell response in MS patients (Giacomini et al., 2013; Rizzo et al., 2016), we set up an in vitro PBMC-based experimental procedure to study differentiation of B reg subsets and the impact of Tα1 in this context (Giacomini et al., 2017). In particular, differently from studies on

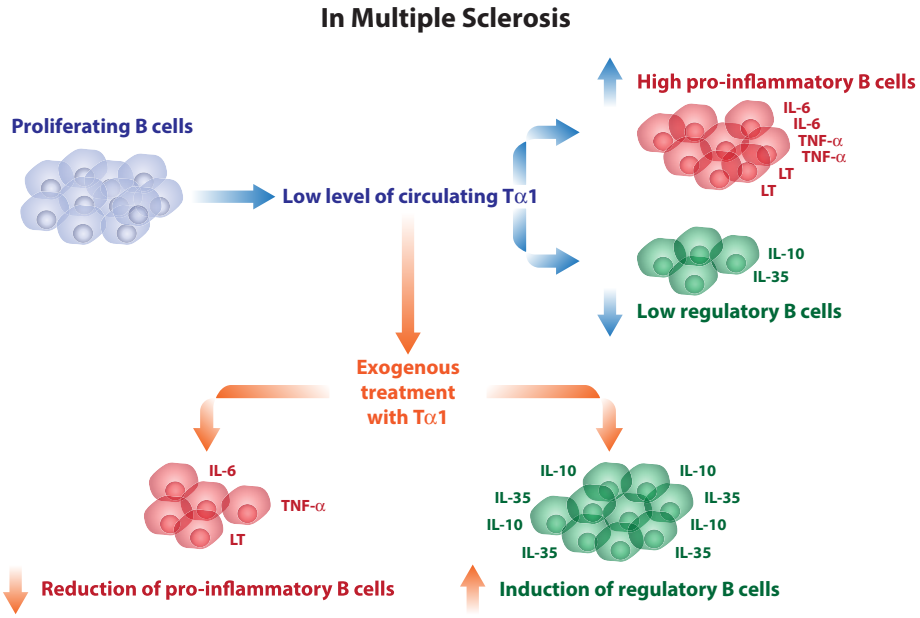

Fig. 2. Scheme of impact of synthetic Tα1 treatment on B cell differentiation in MS.

purified B cells, the mixed cell population of PBMC resemble the in vivo scenario and, upon stimulation with a specific TLR7 agonist (the so-called Imiquimod), could account for cytokine production as well as triggering of CD40 signaling generating an in vitro B cell proliferating milieu unique for either healthy individuals or patients. By using this setting, our study demonstrated a striking difference in the ability of B cells from RRMS patients to differentiate into both CD24 + CD38hi transitional-immature and CD24low/negCD38hi plasmablast-like B reg subsets, as compared to matched controls (Giacomini et al., 2017). Very interestingly, in vitro exposure to Tα1 drastically reduced the TLR7-induced production of the pro-inflammatory cytokines IL-1β, IL-8 and IL-6, while increasing expression of the anti-inflammatory mediators IL-10 and IL-35. Accordingly, Tα1 treatment restored the ability of RRMS B cells to differentiate into IL-10-producing transitional-immature B reg and regulatory plasmablasts to the level found in healthy controls (**Fig. 2**). Furthermore, the B reg subsets expanded by Tα1 treatment display a suppressive activity reducing both IFN-γ and IL-17 production found in TLR7-treated MS patients-derived PBMC cultures (Giacomini et al., 2017). Unfortunately, there is no evidence on the role of Tα1 in EAE. However, Janeway and colleagues first observed that B10.PL mice lacking B cells suffered an unusually severe and chronic form of EAE, suggesting that anti-inflammatory B cells in charge of negatively regulating inflammatory reactions may be depleted (Wolf et al., 1996). Therefore, we envisage that treatment with Tα1, impacting induction of both B and T regulatory cell subsets, may exert protective effects during EAE by reducing disease severity.

In concert, these findings highlight the therapeutic potential of Tα1 in MS as well as in other autoimmune conditions that show reduced differentiation of B reg subsets and chronic immune activation.

6. Conclusive remarks

Ongoing research in MS therapeutics seeks strategies to target or modulate the pro-inflammatory responses into a more anti-inflammatory scenario, in which antigen-specific immune tolerance may be induced and, in this regard, manipulating B reg subsets may be successful. However, since approved treatments for MS work by reducing immune system activity or blocking entry of immune cells into the CNS, thus reducing relapse rate and severity of attacks, but they do not repair immune-mediated damage to the myelin sheaths surrounding axons, drug repurposing or development of new treatments to promote myelin repair and neuronal protection is desirable.

In this scenario, we reviewed the state-of-the-art on thymosins and MS, with particular attention to the neuroprotective and remyelinating action of Tβ4 and to the more recently characterized anti-inflammatory activity of Tα1.

The clinical usage of Tα1 in the past in cancer or chronic infections or as adjuvant in vaccine formulations (Garaci et al., 2007), in which an immune potentiation is needed, may seem difficult to reconcile with its use in autoimmune conditions where an anti-inflammatory action is desirable. However, our results on Tα1 potentiation of B reg differentiation in MS (Giacomini et al., 2017), together with its well-characterized pleiotropic activity able to shift inflammation toward a more anti-inflammatory milieu depending on the stimuli or pathological conditions (Romani et al., 2012; Giacomini et al., 2015) and its capacity to induce Treg generation and IL-10 production (Romani et al., 2006; Romani et al., 2017), indicate beneficial and advisable functions for a drug to be used in autoimmune diseases. These data may pave the way for a repurposing potential of Tα1, alone or in combination with other approved drugs, for treatment of MS or other autoimmune conditions that display reduced differentiation of B reg subsets and chronic immune activation.

In addition, Tβ4 has a broad net of protective and restorative effects on neurological degeneration and injury in the CNS. Recent studies investigated the remarkable capacity of Tβ4 not only on promotion of OPC proliferation, but also on OPC differentiation and remyelination, and demonstrated significant improvement of functional and behavioral outcomes in animal models of MS. The ability of Tβ4 to target many diverse processes via multiple molecular pathways that drive oligodendrogenesis and axonal remodeling may also be mediated by miRNAs, particularly, miR-146a (Santra et al., 2014).

Thus, Tβ4 has substantial potential for clinical translation as a multiple-target therapy for MS/EAE or other neurological demyelinating diseases, with the remyelination effect supplementing its anti-inflammatory and neuroprotective role, as previously found in studies involving Tβ4 prophylactic treatment (Zhang et al., 2009).

Since low level of endogenous Tα1 is present in sera of patients affected with chronic inflammatory autoimmune diseases such as psoriatic arthritis, RA and SLE (Pica et al., 2016) as well as in RRMS patients (Giacomini et al., 2017), an interesting approach might also be to quantify the levels of serum Tβ4 in the same categories of patients to investigate whether a deregulation of both these thymic peptides may be related to MS-associated autoimmune responses. Tβ4 level in the MS population has been reported to be down-regulated in CSF (Liguori et al., 2014) and this down-regulation is a potential biomarker of MS.

Furthermore, one may also envisage that treatment with currently approved disease modifying therapies would affect a dysregulated endogenous thymosin system in MS.

Thus, it may be interesting to investigate whether endogenous Tα1 and/or Tβ4 expression level in cells or sera are modified in patients undergoing treatment with disease-modifying therapies. In particular, immune-modulatory therapies, such as IFN-β, glatiramer acetate or teriflunomide, could impact the low circulating level of Tα1 and/or Tβ4 in MS patients. Or possibly, the combination of synthetic Tα1 with B cell-directed therapeutics, as the specific anti-CD20 mAbs ocrelizumab and ofatunumab or those mAbs, including natalizumab and alemtuzumab, affecting both T and B lymphocytes, may complement their ability to affect the B cell compartment. The profound and rapid depletion of B cells that arises after treatment with these mAbs is then followed by repletion of lymphocytes with a more regulatory phenotype. However, in some cases (as for example following CD52 depletion) there is a hyperpopulation of immature B cells that may be responsible for secondary autoimmunity, a major drawback of alemtuzumab therapy (Baker et al., 2017). As in the combination regimens of chemotherapeutics plus Tα1 adopted for treatment of many cancer types (Garaci et al., 2015), in MS a combination of disease modifying therapies with synthetic Tα1 may help in addressing and correctly modulating the repopulation of B cell compartment towards a more regulatory and anti-inflammatory phenotype.

Furthermore, the induction of miR-146a mediated by Tβ4 can modulate the innate and adaptive immune response (Wu and Chen, 2016). For example, the increased level of miR-146a can suppress IFN-γ-dependent Th1 responses and reduce Th1-mediated lesions by Tregs inhibition of signal transducer and activator of transcription 1 (Lu et al., 2010), and diminish adhesion of T cells to endothelial cells (Wu et al., 2015). Currently, FDA-approved disease modifying therapies for MS mainly focus on immunomodulation, and there is lack of remyelination treatments. In this review, Tβ4 has been demonstrated to promote remyelination, synaptogenesis and axon growth, in addition to its effect on immunosuppression. In future clinical treatment, Tβ4 may be employed as a monotherapy or combined with other disease modifying therapies to treat MS patients. Thus, therapeutic approaches should attempt to decrease severity of disease by immunosupression in order to reduce the damage to myelin and axons as well as to promote repair of damaged myelin and axons and, thereby, enhance functional recovery of MS patients.

We believe that further studies will be needed to address this very interesting aspect of thymosin biology, such as the effects of Tβ4 on MS patients, and that of Tα1 on the EAE model, having in mind that treating MS patients with Tα1 and Tβ4 alone or together with other approved drugs may meet the requirements for a desirable but-still-

unknown therapy for MS acting on both immune dysregulation and CNS damage.

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Conflict of interest

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Enrico Garaci is a patent holder.

Henry Ford Hospital has a Material Transfer Agreement with RegeneRx Biopharmaceuticals, Inc., Rockville, MD. A US Provisional Patent 61/163,556 has been filed for use of Tβ4 in neurological injury.

The other authors have no conflict to declare.

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