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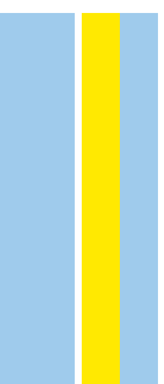

João Pedro da Silva Moreira

Dysregulated inflammation in the pathogenesis of Familial Amyloid Polyneuropathy

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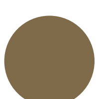

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DOUTORAMENTO

BIOLOGIA MOLECULAR E CELULAR

Dysregulated inflammation in the pathogenesis of Familial Amyloid Polyneuropathy

João Pedro da Silva Moreira

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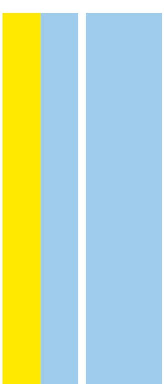
2022



João Pedro da Silva Moreira

Dysregulated inflammation in the pathogenesis of Familial Amyloid Polyneuropathy

Tese de Candidatura ao grau de Doutor em Biologia Molecular e Celular;
Programa Doutoral da Universidade do Porto (Instituto de Ciências Biomédicas de Abel Salazar e Faculdade de Ciências)

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De acordo com o disposto no nº 2, alínea a, do artigo 31º do Decreto-Lei nº 230/2009, utilizaram-se neste trabalho resultados já publicados ou em vias de publicação que a seguir se discriminam:

Moreira, J., Costelha, S., Saraiva, M., & Saraiva, M. J. (2021). The Expression of Chemokines Is Downregulated in a Pre-Clinical Model of TTR V30M Amyloidosis. *Front Immunol*, *12*, 650269. https://doi.org/10.3389/fimmu.2021.650269

Moreira J., Martins, S., Saraiva, M., & Saraiva, M. J. (2022). TLR2/4 signaling pathways are altered in macrophages from V30M TTR mice with downregulated expression of chemokines. (submitted in Frontiers in Cellular Neuroscience)

No cumprimento do Decreto-Lei supra mencionado, o autor desta dissertação declara que interveio na conceção e execução do trabalho experimental, na interpretação e discussão dos resultados e na sua redação. Todo o trabalho experimental foi realizado pelo autor desta tese de doutoramento, João Pedro da Silva Moreira.

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Abbreviation List

AD	Alzheimer's Disease
ANS	Autonomic Nervous System
ApoA-I	Apolipoprotein A1
ASOs	Antisense Oligonucleotides
ATF6	Activating Transcription Factor 6
ATTRv	Hereditary Transthyretin Amyloidosis
ATTR WT	Wild-type Transthyretin Amyloidosis
BDNF	Brain-derived Neurotrophic Factor
BMDM	Bone Marrow Derived Macrophages
cDNA	Complementary DNA
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DAMPs	Danger-associated Molecular Patterns
DRG	Dorsal Root Ganglia
ECM	Extracellular matrix
EGCG	Epigallocatechin-3-gallate
ELISA	Enzyme-linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum-associated Protein Degradation
ERK	Extracellular Signal-regulated Kinase
FAP	Familial Amyloid Polyneuropathy
FAPWTR	Familial Amyloidotic Polyneuropathy World Transplant Registry
FBS	Fetal Bovine Serum
FDA	US Food and Drug Administration

GAGs	Glycosaminoglycans
GalNAc-siRNA	N-acetylgalactosamine Small Interfering Ribonucleic Acid
GAP-43	Growth Associated Protein 43
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial Cell-derived Neurotrophic Factor
HDL	High-density Lipoproteins
HNE	Hydroxynonenal
HSF-1	Heat Shock Factor 1
IDOX	4'-iodo-4'-deoxydoxorubicin
IGF-I	Insulin-like Growth Factor 1
IHC	Immunohistochemistry
IL-10	Interleukin 10
IL-6	Interleukin 6
IL-1β	Interleukin 1 beta
IL-1Ra	Interleukin 1 Receptor Antagonist
iNOS	Inducible Nitric Oxide Synthase
IRE1	Inositol Requiring Enzyme 1
IRF3	Interferon Regulatory Factor 3
ISA	International Society of Amyloidosis
KO	Knockout
LCCM	L929 Cell-conditioned Medium
LDL	Low-density Lipoprotein
LIF	Leukemia Inhibitory Factor
LPS	Lipopolysaccharide
LRP1	Low-density Lipoprotein Receptor-related Protein 1
LT	Liver Transplantation

MCP-1	Monocyte Chemoattractant Protein 1
MMP-14	Matrix Metalloproteinase 14
MMP-9	Matrix Metalloproteinase 9
MRP	Myeloid-related Protein
NF- κB	Nuclear Factor κB
NGAL	Neutrophil Gelatinase-associated Lipocalin
NGF	Nerve Growth Factor
NPY	Neuropeptide Y
NSAIDs	Non-steroidal Anti-inflammatory Drugs
NYHA	New York Heart Association
PAM	Peptidyglycine α-amidating Monooxygenase
PAMPs	Pathogen-associated Molecular Patterns
PBS	Phosphate Buffer Saline
PERK	Protein Kinase R-like ER Kinase
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl Fluoride
PNS	Peripheral Nervous System
RAGE	Receptor for Advanced Glycation end Products
RAP	Receptor-associated protein
RBP	Retinol Binding Protein
RT-PCR	Real-time Polymerase Chain Reaction
SAP	Serum Amyloid P component
SCs	Schwann Cells
SEM	Standard Error of the Mean
siRNA	Small Interfering Ribonucleic Acid
SSA	Senile Systemic Amyloidosis

T3	Tri-iodothyronine
T4	Thyroid hormone thyroxin
TBARS	Thiobarbituric Acid Reactive Substances
TBG	Thyroid Binding Globulin
TBS	Tris Buffer Saline
TLRs	Toll-like Receptors
TNF-α	Tumor Necrosis Factor alpha
TTR	Transthyretin
TUDCA	Tauroursodeoxycholic Acid
UPR	Unfolded Protein Response
WT	Wild-type

Abstract

Amyloid disorders refer to a heterogeneous group of diseases (systemic or localized), characterized by the extracellular deposition of insoluble fibrils in different tissues and organs leading to cellular damage and organ dysfunction. In humans, 24 different proteins are known to self-assemble and form fibrillar amyloid structures. Among them, transthyretin (TTR) amyloidosis is the most common form of hereditary autosomic systemic amyloidosis (ATTRv), caused by the misfolding of protein monomers derived from the tetrameric protein TTR. TTR is a homotetrameric protein synthesized mainly by the liver, choroid plexuses of the brain, pancreas and retinal epithelium, acting as a physiological carrier of thyroxine and retinol. More than 150 reported TTR single point mutations have been associated with ATTRv, being the most common the substitution of a methionine for a valine at position 30 of the polypeptide chain (V30M). ATTRV30M, formerly known as Familial Amyloid Polyneuropathy, is a fatal neurodegenerative disorder, characterized by the extracellular deposition of mutant forms of TTR, particularly in the nerves and ganglia of the peripheral nervous system (PNS). Until now, liver transplantation is the only effective treatment for ATTRV30M amyloidosis but due to its associated limitations other therapeutic strategies that aim to either stabilize the TTR tetramer or to reduce mutant TTR production are currently being explored with special highlight to gene-silencing therapies and immunotherapy technology.

Paradoxically, despite increased expression of the pro-inflammatory cytokines interleukin (IL)-1β and tumour necrosis factor (TNF)-α, especially in endoneurial axons, a downregulated immune response is observed around TTR aggregates in nerves from V30M patients and in V30M transgenic mice comparatively to wild-type (WT) mice, suggesting that mechanisms must operate to prevent or inhibit the correct innate immune response. Therefore, we propose to unravel the molecular mechanisms underlying the lack of inflammatory response observed in the peripheral nervous system of ATTRV30M patients, focusing on the connection between V30M TTR variant and different immune cell populations. Additionally, we will also investigate the impact of V30M variant in the recognition and activation of pattern recognition receptors and their respective signalling pathways.

In the present work, we decided to study the expression of S100A8/A9 proteins, which are referred to as potent initiators of the immune response in injured peripheral nerves; we investigated transversal nerve biopsies from ATTRV30M patients and the sciatic nerve from mouse model of the disease. In both cases, a significant downregulation in the expression

of S100A8/A9 proteins was observed comparatively to the control groups. We also evaluated the impact of the V30M variant in the expression of these proteins in immune cells such as Schwann cells (SCs), which have the ability to regulate the PNS immune response and in bone marrow derived macrophages (BMDM). Supporting our initial findings, we observed a downregulated expression of these molecules in SCs incubated with mutant V30M TTR comparatively with SCs incubated with the WT TTR, as well as, in BMDM derived from the animal model of the disease.

To further investigate the lack of inflammatory response in ATTRV30M disease, we performed an RNA sequence analysis, to discriminate differentially expressed genes between the sciatic nerve from V30M mice and the sciatic nerve from WT mice. Interestingly, the pathways that were downregulated in the mouse model of the disease were associated with the immune system process and antigen processing and presentation. In fact, according to the RNA sequence, the expression of several chemokines was significantly downregulated in the sciatic nerve of V30M mice, a finding that we further validated by RT-PCR. Because the expression of chemokines is very limited in V30M mice nerve, we next studied the inflammatory profile of SCs incubated with WT or V30M TTR. Whereas WT TTR activated SCs leading to the secretion of all tested chemokines, V30M TTR failed to do so. This lack of response due to the presence of V30M variant may be the reason underlying the lack of inflammatory infiltrates in the nerves of the animal models and ATTRV30M patients. Moreover, we also demonstrated that the TLR4 signaling pathway is responsible for the expression of chemokines induced by WT TTR stimulation of SCs. Finally, preliminary data regarding to human plasma demonstrated a downregulated tendency in the expression of some chemokines, particularly CCL20 and CXCL2 in a small group of ATTRV30M patients, when compared to healthy donors.

Previous studies using macrophages derived from ATTRV30M patients showed quantitative or qualitative anomalies when compared to healthy donors, which may accelerate TTR amyloid deposition in some organs. Accordingly, we also found a downregulated expression of several chemokines in BMDM generated from a transgenic mouse model for human V30M TTR upon stimulation with agonists for TLR4 and TLR2 receptors. Furthermore, our data also demonstrate an impairment in the p38 signaling transduction pathway in these V30M macrophages. The lower phosphorylation of p38 in mutated macrophages may be associated to the lack of inflammatory cells and chemokine expression observed in V30M patients.

Altogether, our data revealed a severe downregulated expression of several chemokines produced by different cell populations in V30M mice and ATTRV30M patients, that likely

compromises the development of a chemotactic gradient important for the recruitment of immune cells necessary for the clearance of amyloid deposits and fibrils. It will be of importance and interest to investigate other possible reprogramming alterations in other cells populations and how mutated TTR promotes them. Overall, our studies are an important contribute for a better understanding of the molecular mechanisms underlying the lack of inflammatory cellular infiltrates in the nerves of ATTRV30M patients, contributing this way for the development of more effective and targeted therapies for the treatment of ATTR amyloidosis.

Sumário

Amiloidoses são um conjunto heterogéneo de doenças (sistémicas ou localizadas), que se caracterizam pela deposição extracelular de fibras ou agregados insolúveis em diversos tecidos e órgãos, culminado em lesões celulares e disfunção de órgãos. Nos humanos, são conhecidas cerca de 24 diferentes proteínas capazes de formarem estruturas fibrilares de amiloide. Entre elas, a amiloidose provocada pela proteína transtirretina (TTR) é a forma mais comum de amiloidose sistémica autonómica hereditária (ATTRv), que é causada pelo incorreto dobramento dos monómeros da proteína que são derivados da sua estrutura tetramérica. A TTR é uma proteína homotetramérica sintetizada maioritariamente no fígado, no plexo coroide localizado no cérebro, pâncreas e no epitélio da retina, cuja principal função consiste no transporte de tiroxina e retinol. Ao dia de hoje, mais de 150 mutações no gene da proteína TTR foram identificadas, consistindo a mais comum na substituição de uma metionina por uma valina na posição 30 da cadeia polipeptídica (V30M). A amiloidose ATTRV30M, anteriormente conhecida por Polineuropatia Amiloidótica Familiar, é uma doença neurodegenerativa fatal, caracterizada pela deposição extracelular de agregados e fibras de amiloide da proteína, particularmente nos nervos e gânglios do sistema nervoso periférico. Até agora, o único tratamento eficaz para a amiloidose ATTRV30M é o transplante de fígado, mas devido às limitações associadas a este tratamento, estão a ser neste momento investigadas outras terapias alternativas com destaque para terapias de silenciamento de genes e imunoterapia, cujo objetivo principal consiste em estabilizar o tetrâmero proteico ou em reduzir a produção de proteína mutada.

Paradoxalmente, apesar de haver um aumento de expressão quer de interleucina - 1β (IL-1β), quer do fator de necrose tumoral α (TNF-α), especialmente em axónios dos nervos periféricos, é observado em biópsias de nervos de pacientes ATTRV30M e no modelo animal transgénico para esta doença uma clara diminuição da resposta imune em redor dos agregados de TTR, sugerindo que determinados mecanismos devem estar a acontecer para impedir ou prevenir a correta ativação da resposta imune inata.

Deste modo, com este trabalho, propomo-nos a investigar que mecanismos moleculares estão associados à falta de resposta inflamatória observada no sistema nervoso periférico dos doentes ATTRV30M, focando-nos nas interações entre a mutação V30M da proteína com as diferentes populações celulares com funções relacionadas com imunidade no organismo. Além disso, vamos também investigar o impacto da variante V30M da proteína no processo de reconhecimento e ativação de recetores de reconhecimento de padrões (RRP) e as suas respetivas cascatas de sinalização.

Assim sendo, decidimos estudar a expressão das proteínas S100A8/A9, que são referidas na literatura como potentes iniciadores da resposta imune num modelo animal de lesão do nervo periférico, em biopsias transversais de nervo de pacientes ATTRV30M e no nervo ciático do modelo animal para esta doença. Em ambos os casos foram observados um decréscimo significativo da expressão das proteínas S100A8/A9, em comparação com os grupos de controlo. Em seguida, também decidimos avaliar o impacto da variante V30M na expressão das proteínas S100A8/A9 em células do sistema imune, como é o caso das células de Schwann, uma vez que estas células possuem a capacidade de regular a resposta imune do sistema nervoso periférico, e em macrófagos derivados da medula óssea. Em conformidade com os resultados obtidos previamente, foi novamente observado um decréscimo na expressão destas moléculas nas células de Schwann incubadas com a variante V30M, comparativamente com a expressão destas moléculas em células de Schwann incubadas com a proteína TTR sem a mutação, bem como também se obteve uma diminuição da expressão das proteínas S100A8/A9 em macrófagos derivados da medula óssea de animais V30M após incubação com agonistas para diferentes RRP's.

De modo a aprofundar a investigação sobre a ausência da resposta inflamatória característica da doença ATTRV30M, decidimos fazer uma sequenciação completa do transcriptoma de forma a ser possível discriminar quais os genes que estão a ser diferencialmente expressos no nervo ciático de animais V30M e no nervo ciático de animais do tipo selvagem. Interessantemente, as vias de sinalização que se apresentaram particularmente afetadas no modelo animal da doença estavam relacionadas com o sistema imune e com a apresentação e processamento de antigénios. Deste modo, de acordo com a sequenciação, a expressão de diversas quimiocinas estava significativamente diminuída no nervo ciático dos animais V30M, observações que foram posteriormente validadas por RT-PCR. Devido ao facto da expressão das quimiocinas estar bastante diminuída no nervo ciático dos animais V30M, decidimos em seguida estudar o perfil inflamatório das células de Schwann incubadas com a proteína TTR sem mutação ou com a proteína coma mutação V30M. Enquanto que a proteína TTR sem mutação leva a uma ativação das células de Schwann com a posterior produção de quimiocinas, as células de Schwann não são ativadas pela proteína mutada. A falta de resposta das células de Schwann devido à presença da variante V30M pode estar na origem da ausência de infiltrados inflamatórios que são observados nos nervos dos animais V30M e nos nervos dos pacientes ATTRV30M. Para além disto, também demonstramos que a via de sinalização associada ao recetor TLR4 é a via responsável pela expressão de quimiocinas nas células de Schwann após incubação com a proteína sem mutação. Além disto, estudos preliminares com amostras de plasma provenientes de doentes ATTRV30M demonstraram

uma ligeira diminuição na expressão das quimiocinas CCL20 e CXCL2 quando comparados com a expressão destas quimiocinas em indivíduos saudáveis.

Estudos prévios envolvendo macrófagos derivados de pacientes ATTRV30M indicaram que estes apresentavam anomalias qualitativas e quantitativas quando comparados com macrófagos provenientes de dadores saudáveis, sendo este um dos motivos que levava a um aumento da deposição de agregados e fibras de TTR em diversos órgãos. Em concordância com os estudos referidos, também observamos um decréscimo na expressão de diversas quimiocinas em macrófagos derivados da medula óssea de animais V30M após estimulação destes com agonistas para os recetores TLR4 e TLR2. Para além disso, os nossos resultados também indicam uma diminuição na via de sinalização intracelular p38 nestes macrófagos. A menor fosforilação da proteína p38 nos macrófagos provenientes de animais V30M pode estar associado à ausência de infiltrados celulares e expressão de quimiocinas que é observado nos pacientes ATTRV30M.

De um modo geral, os dados apresentados nesta tese sugerem uma severa diminuição na expressão de diversas quimiocinas em diferentes populações celulares no modelo animal da doença e em pacientes ATTRV30M, o que, deste modo, compromete o desenvolvimento do gradiente quimiotático necessário para o recrutamento das células imunes que farão a fagocitose dos depósitos e fibras de amiloide. No futuro, será interessante investigar se a mutação V30M promove outras alterações na reprogramação das células e quais as consequências dessas mesmas alterações. Concluindo, este trabalho contribui para uma melhor compreensão dos mecanismos moleculares que estão associados à ausência de infiltrados inflamatórios celulares nos nervos dos pacientes ATTRV30M, fornecendo assim novos dados para o desenvolvimento de terapias mais efetivas para o tratamento da amiloidose ATTRV30M.

General Introduction

General Introduction

Transthyretin

Historically, transthyretin (TTR) was identified in 1942 by two different laboratories and was initially denominated “prealbumin”, as it migrates just in front of the albumin band in electrophoresis gels both in cerebrospinal fluid (CSF) (Kabat et al., 1942) and in plasma (Seibert & Nelson, 1942). The term “prealbumin” persisted until further studies verified the ability of this protein to bind thyroid hormones and retinol (Raz & Goodman, 1969), leading to a change of its name to transthyretin in 1981 by the International Union of Biochemists ("Nomenclature Committee of IUB (NC-IUB) IUB-IUPAC Joint Commission on Biochemical Nomenclature (JCBN). Newsletter 1981," 1981).

In a phylogenetic analysis, TTR is a highly conserved protein across evolutionarily-divergent organisms including mammals, birds, reptiles, amphibians and fish (Power et al., 2000). In addition, TTR-like proteins with functional roles similar to TTR were reported in prokaryotes and lower eukaryote organisms (Eneqvist et al., 2003; Hennebry et al., 2006).

Mutations in the human *TTR* gene are implicated in several fatal forms of amyloidosis, a group of disorders characterized by the extracellular accumulation of amyloid deposits (Westermark, 1998).

Transthyretin structure

TTR molecule is a stable tetramer composed of four identical subunits of 127 amino acids residues each, and a molecular weight of 55kDa (Blake et al., 1974). Each monomer is constituted of 8 β-sheet strands (denoted A-H), organized into 4-stranded β-sheets (DAGH and CBEF), and a short α-helix. The eight β-sheets are designated from A to H according to their sequential order from N- to C-terminal. Strands F and H of each monomer can join edge-to-edge to form a dimer through hydrogen bonding (Figure 1A) (Blake et al., 1978; Blake et al., 1974). The association of two dimers, through hydrophobic contacts, forms the native TTR tetrameric structure (Figure 1B). Non-covalent assembly of the tetramer creates a 50 Å long central hydrophobic channel comprising two symmetrical binding sites for thyroxin with distinct binding constants. As these binding sites exhibit negative cooperativity, due to an allosteric communication between them, only one molecule of thyroxin binds to

TTR tetramer (Irace & Edelhoch, 1978). Another TTR ligand – retinol binding protein (RBP) has four binding sites located at the surface of the TTR molecule (Monaco et al., 1995).

Structurally, TTR is resistant to subunit dissociation and to denaturation with detergents. However, the existence of point mutations may alter the structural stability of the molecule, leading to dissociation of tetramers and partial unfolding of resultant monomers, which may influence the pathogenicity of different TTR variants (Damas & Saraiva, 2000; Kanda et al., 1974). For instance, the substitution of a methionine for a valine at position 30 (V30M variant) forces the β-sheets of the monomer as much as 1Å apart, resulting in a distortion of the T4 binding cavity (Hamilton et al., 1993).

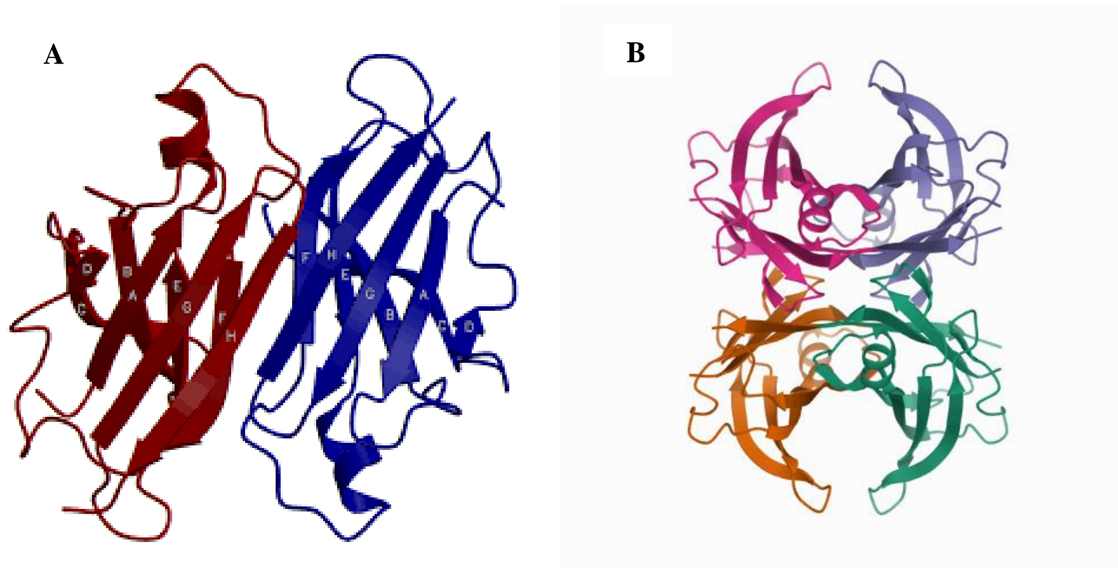


Figure 1. Transthyretin structure. (A) A TTR dimer is formed by the interaction of two monomers. Each monomer contains one small α-helix and eight β-strands (CBEF and DAGH). The eight β-sheets are designated from A to H according to their sequential order from N- to C-terminal. (B) Transthyretin tetramer resulting from the interaction of both dimers, forming a central hydrophobic channel that is 50 Å long. The four identical monomers are represented in different colours. Adapted from Protein Data Bank 1F41.

Transthyretin metabolism

TTR is mainly produced by the liver hepatocytes and the epithelial cells of choroid plexus in the brain, being then secreted into blood and CSF, respectively (Dickson et al., 1986; Soprano et al., 1985). More than 90% of plasma circulating TTR arises from liver production.

The plasma levels are influenced by age, growing after birth until adulthood and starting to decay after the age of 50 (Stabilini et al., 1968). Additionally, TTR levels decrease in situations of malnutrition or chronic inflammation (Dickson et al., 1982), and are reported to be lower in patients with ATTR amyloidosis (Skinner et al., 1985). The choroid plexus is the tissue with the highest concentration of TTR mRNA per gram tissue weight. The adult rat choroid plexus has an 11 times higher concentration of TTR mRNA compared to the liver (Schreiber et al., 1990). TTR represents around 20% of the total CSF proteins and, importantly, more than 90% of the TTR in the CSF is synthetized in the choroid plexus of the brain (Weisner & Roethig, 1983).

The TTR half-life is approximately 2-3 days in humans (Socolow et al., 1965) and is mainly degraded in liver, followed by other tissues and organs such as muscle, skin, fat and kidney (Makover et al., 1988). Additionally, some studies revealed that TTR internalization by the liver and kidney is receptor-mediated. The hepatic uptake of TTR is mediated by a receptor member of a low-density lipoprotein (LDL) family, which is sensitive to receptor-associated protein (RAP). However, this receptor has not yet been identified (Sousa & Saraiva, 2001). On the other hand, based on cell culture studies, TTR internalization by the kidney is mediated through the endocytic receptor megalin (Sousa, Norden, et al., 2000).

Low amounts of the *TTR* gene have been also detected in stomach, namely in gastric ghrelin cells (Walker et al., 2013), heart, skeletal muscle, spleen (Soprano et al., 1985), human placenta (McKinnon et al., 2005), pancreas, retinal pigment epithelium in the eye (Martone et al., 1988) and Schwann cells of the peripheral nerve (Murakami et al., 2010).

Transthyretin physiological functions

TTR has been mainly recognized by its role as a carrier protein of thyroid hormones (Hollander et al., 1962) and retinol (Alvsaker et al., 1967). However, several other functions are associated with this molecule. It has been shown that other molecules can interact with TTR such as noradrenaline, retinoic acid, Lamp-1 and metallothionein 2. However, little is known about the functions assigned by these interactions (Liz et al., 2010). Additionally, more recent studies have implicated TTR as an important molecule in the peripheral and central nervous systems (Vieira & Saraiva, 2014).

TTR as a transport protein

Thyroid hormones are essential for normal mammalian brain physiology and are particularly critical during development (Porterfield & Hendrich, 1993). Thyroid hormone thyroxin (T4) is the principal product synthesized and secreted by the thyroid follicles, while tri-iodothyronine (T3), the biologically active hormone, derives mainly from local deiodination of T4 in tissues (Palha, 2002). In human serum, about 75% of the total circulating T4 is carried by thyroid binding globulin (TBG), as a consequence of its greater affinity for the hormone, with 10–15% bound to both TTR and albumin, (Bartalena & Robbins, 1993). As for CSF, in humans, TTR is the main carrier of T4, transporting up to 80% of T4 in the CSF (Palha et al., 2002). In rodents, TTR is the main carrier of T4 in the plasma as well as in the CSF (Chanoine & Braverman, 1992; Hagen & Solberg, 1974). The native TTR structure and conformation forms a central hydrophobic channel that is 50 Å long and approximately 8 Å in diameter with two binding sites for T4, but as they display negative cooperativity, only one T4 molecule is allowed to bind to the tetramer (Andrea et al., 1980) (Figure 2A).

Studies using TTR knockout mice showed decreased levels of T4 and T3 in plasma and CSF of these knockout mice when compared to the control group. Nevertheless, a normal T4 distribution was observed in brain and other tissues and the animal development was not affected (Episkopou et al., 1993; Palha et al., 1997). In addition, an important role for TTR on T4 transport across the placenta was also described (Landers et al., 2009).

TTR is also involved in the transport of retinol, an essential molecule for several functions such as vision, reproduction, growth and embryonic development (Gudas, 2012). The transport of retinol in circulation occurs through RBP. This molecule is mainly synthetized in the liver and secreted to plasma after retinol binding. TTR associates to the RBP-retinol complex before secretion into the plasma (Kanai et al., 1968). The TTR-RBP complex is a very stable form of retinol transport, allowing its delivery to cells and is important to prevent RBP from being filtered and degraded in the kidney (Noy et al., 1992). TTR tetramer has four RBP-binding sites, two in each dimer at the protein's surface, however steric hindrance prevents the binding of more than two RBP molecules (Figure 2B). Nonetheless, due to the limiting RBP concentration in plasma, only one RBP molecule is transported by TTR under physiological conditions (Monaco et al., 1995). T_4 binding to TTR is not influenced by RBP binding (Raz & Goodman, 1969). Studies using TTR KO mice do not show signs of retinol deficiency in tissues but present lower circulation levels of RBP possibly due to increased renal filtration of the RBP-retinol complex (van Bennekum et al., 2001), suggesting that TTR probably acts as a retinol reservoir, preventing its loss via kidneys.

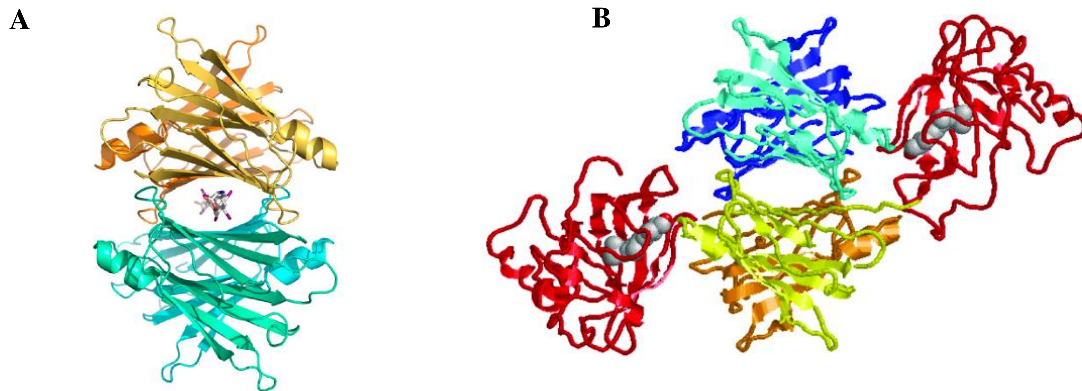


Figure 2. Model of TTR tetramer. Shown with ligand in T4 binding pocket (A). Shown with two molecules of RBP charged with retinol (B). In humans, generally only one of the RBP binding sites is occupied. Adapted from Protein Data Bank 1ICT and 1QAB respectively.

Transthyretin as a novel protease

A small fraction of plasma TTR (1–2%) was found associated with high-density lipoproteins (HDL) through binding to apolipoprotein (apo) A-I (Sousa, Berglund, et al., 2000). Studies have shown that TTR was able to cleave apoA-I in its carboxyl terminal domain, leading to decreased capacity of HDL to promote cholesterol efflux, which can increase the development of atherosclerosis (Liz et al., 2007).

It was reported that neuropeptide Y (NPY), acting as a neurotransmitter in the brain and in the autonomic nervous system (ANS), can be also cleaved by TTR and consequently promote neurite outgrowth (Liz et al., 2009). Only a minor fraction of TTR is active as a metallopeptidase (Liz et al., 2012). Evidence also show that TTR has also the ability to cleave Aβ peptide, suggesting a protective role against Alzheimer's disease. Cleavage of Aβ can occur at several different sites, and the resulting peptides were shown to have decreased amyloidogenic potential when compared with the complete peptide (Costa, Ferreira-da-Silva, et al., 2008).

Transthyretin in the nervous system

So far, several studies have implicated TTR as an important molecule in both peripheral and central nervous system (CNS). Regarding the central nervous system, it was shown

that TTR KO animals presents reduced signs of depressive-like behaviour and increased exploratory activity, suggesting a connection between TTR and behaviour (Sousa et al., 2004). Moreover, mice lacking the *TTR* gene have increased levels of NPY (which among others possesses anti-depressant properties) in the cortex, CSF, the dorsal root ganglia and sciatic nerve, due to upregulation of peptidyglycine α-amidating monooxygenase (PAM), an important enzyme for the maturation of NPY (Nunes et al., 2006). Additionally, TTR KO mice showed memory impairment when compared with WT control group (Buxbaum et al., 2008). Other studies described that TTR KO mice have spatial learning deficits, in Morris water maze testing. So, TTR has also been associated with the maintenance of memory capacities during aging (Sousa et al., 2007).

Similarly, TTR is neuroprotective both for neuronal survival and neurite preservation under excitotoxic conditions, through the activation of neuroprotective megalin-dependent signaling pathways and transcription factors (Gomes et al., 2016). Additionally, TTR also regulates megalin expression, affecting both its mRNA and protein levels in hippocampal neuronal cultures. The interaction between TTR and megalin increases megalin levels, important for the regulation of synaptic plasticity and density, regulation of neurite outgrowth and dendritic spine maturation. Decreased megalin levels results in neurons with impaired neurite length and, consequently, more susceptible to insults (Gomes et al., 2020).

More recently, a nanobody that recognizes both human and mouse TTR was efficiently delivered to the CNS, using a non-invasive route. This anti-TTR nanobody was used as a specific functional tracer that identified motor neurons in the spinal cord as a new cell type that synthesizes and secretes TTR (Gomes et al., 2018).

In the peripheral nervous system, studies in TTR KO and WT mice following sciatic nerve crush, showed a role for TTR in nerve regeneration, as TTR KO mice exhibited diminished capacity of sciatic nerve regeneration, as well as slower recovery of locomotor activity, and reduced numbers of myelinated axons when compared with the WT mice. TTR properties as a nerve regeneration enhancer were further demonstrated when TTR delivery to crushed sciatic nerves rescued the regeneration phenotype of TTR null animals (Fleming et al., 2009; Fleming et al., 2007).

TTR has been suggested to play a key role in the modulation of Aβ aggregation, a major feature of Alzheimer's disease, through interaction between TTR and soluble Aβ, preventing amyloid fibril formation and cellular toxicity (Schwarzman et al., 1994). Several studies reported decreased levels of TTR in CSF and plasma in patients with Alzheimer's disease, suggesting a potential important role for TTR in this disease (Ribeiro et al., 2012; Serot et al., 1997). Additionally, TTR was also able to inhibit and disrupt Aβ fibril formation,

diminishing its neurotoxicity (Costa, Goncalves, et al., 2008). In a mouse model of Alzheymer's disease in a null TTR background, Aβ levels were increased and deposition in the brain was accelerated (Choi et al., 2007).

More recently, a role for TTR in 14-3-3ζ metabolism has been proposed, as hippocampus of young TTR KO mice presented lower levels of 14-3-3ζ protein, but no changes in gene expression. The mechanism proposed for the reduced 14-3-3ζ levels in the absence of TTR was lysosomal degradation (Vieira & Saraiva, 2013). Furthermore, TTR also contributes for gene regulation of insulin-like growth factor I (IGF-I) at the transcriptional and translational levels (Vieira et al., 2015) and to the modulation of low-density lipoprotein receptor-related protein 1 (LRP1) (Alemi et al., 2016).

Presently, TTR is no longer considered to be only a transport molecule, as numerous studies have uncovered several important roles for TTR in nervous system physiology, such as peripheral nerve regeneration, development of Alzheimer's disease (AD) pathology, and prevention of neuronal cell death.

Transthyretin amyloidosis

Amyloidosis are a large group of diseases that can either be focal or systemic, characterized by tissue deposition of abnormally folded proteins, leading to organ dysfunction (Westermark, 1998). Many mechanisms may trigger the predisposition of a protein to misfold, for example its intrinsic biochemical properties, increased protein synthesis, and pathogenic gene mutations (Merlini & Bellotti, 2003). Amyloid can accumulate in liver, spleen, kidney, heart, nerves, and blood vessels causing different clinical syndromes including cardiomyopathy, autonomic dysfunction, neuropathy, renal failure and corneal and vitreous abnormalities. TTR is one of the human proteins associated with systemic amyloidosis. Besides TTR, twenty different proteins have been identified as amyloidogenic agents including amyloid-beta (in Alzheimer´s disease), α-synuclein (in Parkinson´s disease), among others (Cheng et al., 2018).

The nomenclature committee of the International Society of Amyloidosis categorized ATTR in different sub-types: wild-type ATTR amyloidosis (ATTR WT) and hereditary ATTR amyloidosis (ATTRv) associated with TTR variants (Benson et al., 2020).

Wild-type ATTR amyloidosis

TTR WT protein has a tendency to aggregate with ageing, depositing primarily in the heart, but also in the gut and carpal tunnel, a condition denominated as ATTR WT, previously known as senile systemic amyloidosis (SSA) (Merlini & Westermark, 2004; Rapezzi et al., 2009; Westermark et al., 1990). The prevalence of this type of amyloidosis was estimated to be approximately 10% in individuals older than 80 years old, and 50% at ages of 90 years and over (Pomerance, 1965; Westermark et al., 2003).

Symptoms usually start after the age of 60 and are mostly associated with cardiomyopathy. Amyloid deposits in the heart make the heart wall stiffen and work inefficiently. Eventually, this leads to congestive heart failure with symptoms such as shortness of breath, fatigue, nausea and an irregular heartbeat. Carpal tunnel syndrome is another common manifestation that often occurs at an initial state of the disease (Sekijima, 2011).

Hereditary ATTR amyloidosis

ATTRv, occurs through single-residue substitutions in the coding region of the *TTR* gene, mostly producing less stable TTR variants (Saraiva, 1995). Nowadays, over 140 mutations in the primary sequence of TTR have been identified, most of them being associated with amyloidosis (Connors et al., 2003). TTR-related amyloidosis are phenotypic heterogeneous and although the main clinical feature is sensorimotor polyneuropathy, cardiomyopathy, carpal tunnel syndrome, vitreopathy and leptomeningeal involvement can occur as well. Focusing in the severe cases, the mutation appears in a heterozygous context with individuals expressing both normal and variant TTR (Benson & Kincaid, 2007). Till now, 15 TTR variants without clinical manifestations have also been described (Connors et al., 2003). One of the most common TTR variants is V30M, that results from the substitution of a valine for a methionine in the position 30 of the amino acid sequence (Saraiva et al., 1984).

The most amyloidogenic variant results from the replacement of a leucine by a proline at position 55 (L55P), with both cardiac and neurological phenotype (Jacobson et al., 1992). The most predominant TTR variant targeting the heart is the valine to isoleucine substitution at position 122 (V122I), which is also the main mutation in the United States affecting 3-4% of African Americans and leading to a late-onset cardiomyopathy (Jacobson et al., 2015). Furthermore, 9% of TTR mutations give rise to leptomeningeal amyloidosis, including those

derived from the TTR mutation L12P, considered as one of the most aggressive variants (Brett et al., 1999).

The study of pathogenic and non-pathogenic TTR variants is important for the understanding of TTR-related amyloidosis, and to provide novel insights regarding therapeutic strategies.

Familial Amyloidotic Polyneuropathy

The first form of hereditary amyloidosis was described in 1952 by Corino de Andrade, in the north of Portugal (Andrade, 1952). The patients developed the initial clinical symptoms in the third or fourth decade of life and ultimately leading to death within 10-20 years (Andrade, 1952). First clinical manifestations included impairment of temperature and pain sensations in the lower limbs. With the disease progress, patients progressively lose motor control, suffer from general weakness, aggravated gastrointestinal dysfunction and cardiac insufficiency. Subsequently, this disease was named familial amyloidotic polyneuropathy (FAP). The cause of this disease was unknow until a study reported that the amyloid fibril protein accumulated in tissues of Portuguese FAP patients consisted of an abnormal form of TTR (Costa et al., 1978). Later, Saraiva and colleagues identified the molecular abnormality in TTR as a substitution of methionine for valine at position 30 of the polypeptide chain (Saraiva et al., 1984). Recently, the Nomenclature Committee of the International Society of Amyloidosis (ISA) made specific recommendations regarding the nomenclature used in this context. Amyloid transthyretin is named ATTR and the name "hereditary amyloidosis" is recommended rather than "familial amyloidosis". Therefore, the disease was renamed as ATTRV30M (Benson et al., 2018).
When first discovered, the disease was believed to be restricted to endemic areas. Nowadays, it is known that this disorder is distributed worldwide with cases diagnosed in Spain, Italy, Sweden, United States of America, Japan and Portugal (Ando, 2005b). The estimated global prevalence of ATTRV30M is around 10,000 people worldwide (Schmidt et al., 2018). Patients with ATTRV30M can be classified as early-onset and late-onset. The penetrance rate of V30M mutation varies with the age of onset and coexists in the same family. Indeed, younger generations are usually associated with more severe phenotypes and disease anticipation (Lemos et al., 2014). The onset of clinical symptoms generally occurs between the third and fourth decade of life but with great variations across different populations (Ando et al., 2013). Patients with an early onset of symptoms are far more susceptible to the effects of the disease while in carriers with the late onset, the

polyneuropathy developments slowly with different pathological presentations (Koike et al., 2004). Among first symptoms of the disease is sensory loss, with pain and temperature sensations being the most severely affected, and eventually all sensory modalities are impaired. There is also a progressive loss of reflexes with upper limb involvement occurring months to years after lower limb manifestations. The majority of ATTRV30M patients have early and severe autonomic nervous system dysfunction, involving gastrointestinal, urogenital and cardiovascular system (Sousa & Saraiva, 2003). Cardiac involvement occurs in about 50-80% of patients leading to amyloid restrictive cardiomyopathy with episodes of arrhythmias. Additional symptoms often found include vitreous opacity, dry eye, glaucoma, nephritic syndrome, renal failure and carpal tunnel syndrome (Ando et al., 2013; Ando et al., 2005; Sousa & Saraiva, 2003). In patients with the late-onset, the polyneuropathy progresses slowly with different pathological presentations (Figure 3) (Koike et al., 2004).
The standard protocols for the diagnosis of ATTRv amyloidosis include tissue biopsy for the detection of amyloid deposits and genetic studies for the identification of the TTR mutation (Koike et al., 2012).

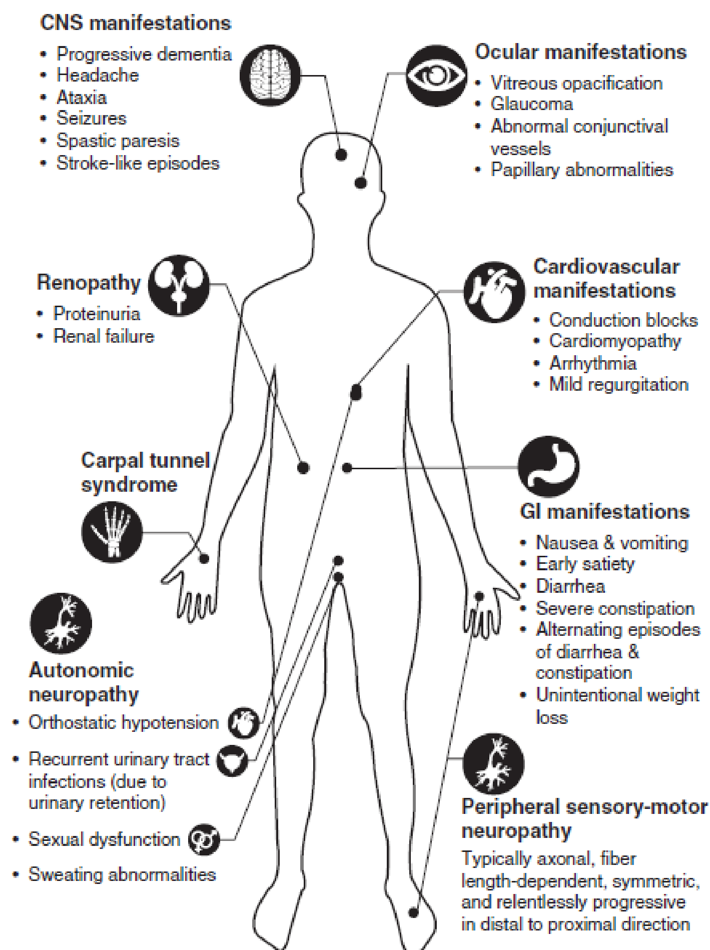


Figure 3. Clinical features associated with ATTRV30M. From (Conceicao et al., 2016).

Mechanisms for transthyretin amyloid formation

Although significant improvement has been made regarding TTR amyloid formation, the exact molecular mechanisms underlying amyloid fibril formation are not completely understood. It is now accepted that several factors might be involved in the pathogenesis of TTR amyloidosis. Several studies revealed that under high temperatures or acid conditions, WT and mutant TTR tetramers can dissociate into non-native monomers with low stability originating soluble aggregates (Colon & Kelly, 1992; Quintas et al., 2001). Structural X-ray studies were also fundamental to elucidate the influence of point mutations in protein stability, as amino acid substitutions usually destabilize the primary protein (Benson & Kincaid, 2007). This work showed the importance of protein stability as part of the fibril formation process, as TTR variants are more prone to aggregation than the wild-type form. Another hypothesis for amyloid fibrils formation is related to proteolytic cleavage. This hypothesis is supported by findings of the presence of TTR peptide fragments as well as the full-length protein in amyloid fibrils extracted from patients and composed by TTR V30M. However, not all studies detected the presence of the referred proteolytic fragments (Saraiva et al., 1984; Tawara et al., 1983). Currently, the stability of the TTR tetramer has been pointed out as the most important factor for amyloid fibril initiation. Truthfully, there is a correlation between the thermodynamic stability of TTR variants and their potential to form modified monomers and soluble aggregates. In fact, X-ray diffraction studies identified a molecule structurally resembling the TTR monomer as the basic unit in the structure of fibrils found in vitreous humour of ATTRV30M patients (Inouye et al., 1998). Pathogenic mutations in the *TTR* gene were shown to favour both cleavage and dissociation, for example the L55P variant is associated with a 10 times faster disintegration of tetrameric TTR in vitro (Hammarstrom et al., 2002). Additionally, mutation V30M results in a reduced affinity to thyroid hormone thyroxine and lower protein stability due to the distortion of thyroxine binding pocket and consequently dissociation of the tetramers into monomers (Hamilton et al., 1993). Contrarily, the benign variant T119M creates additional hydrogen bonds between serine residues of adjacent monomers and therefore increases the tetramer stability (Almeida et al., 2000). Because monomer denaturation is the critical step preceding fibril formation and amyloid deposition, the disease severity and penetrance might not only depend on the tetramer, but also on the monomer stability influenced by the respectively underlying mutation. Therefore, the currently accepted theory for fibril formation specifies that mutations cause protein destabilisation and less stable TTR tetramers dissociate into unfolded monomers which undergo subsequent partial refolding, forming insoluble amyloidogenic intermediates (Figure 4) (Quintas et al., 1999; Quintas et al., 2001; Si et al., 2021).

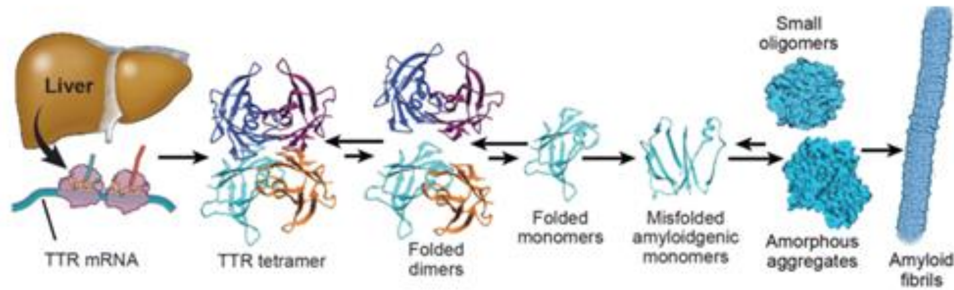


Figure 4. Model for TTR amyloid formation. Tetramer dissociation into non-native monomers is the first and rate-limiting step for amyloid fibril formation. This unfolded monomer is prone to aggregation, leading to the formation of intermediate species such as oligomers/aggregates finally ending in insoluble amyloid fibrils. Adapted from (Hendren et al., 2020).

Transthyretin amyloid deposits

In ATTRV30M patients, TTR amyloid deposits are not composed entirely of the amyloidogenic protein. Amyloid fibrils also contain other components, such as glycosaminoglycans (GAGs), proteoglycans, serum amyloid P component (SAP) and several membrane constituents, like laminin and fibronectin (Kisilevsky, 2000). The role of these components in amyloidogenesis is not entirely clear, however it is possible that they may affect *in vivo* fibril dynamics. Indeed, affinity between TTR and GAGs was previously demonstrated, with evidence that GAGs may be involved in TTR deposition (Bourgault et al., 2011; Inoue et al., 1998). GAGs are a family of complex carbohydrates that display a variety of important biological roles, such as cell adhesion, growth factor signalling and collagen structure (Mikami & Kitagawa, 2017). GAGs are expressed ubiquitously and abundantly on the extracellular matrix (ECM), attached to a core protein, as proteoglycans (Hacker et al., 2005). Heparan sulphate proteoglycans might be present extracellularly or at the cell surface and have been demonstrated to be a significant component of amyloid formation by increasing fibril stability and protecting fibrils from proteolysis (Dember, 2005; Noborn et al., 2011). Actually, Noborn and colleagues showed that heparan sulfate or heparin are able to prompt TTR aggregation and fibrillogenesis both *in vitro* and in a transgenic *Drosophila melanogaster* model of amyloidosis. Additional *in vitro* studies demonstrate that larger GAGs are able to interact with multiple TTR aggregates, enhancing its self-assembly (Bourgault et al., 2011).

In its turn, SAP is a plasma glycoprotein always present in amyloid deposits, contributing to amyloidogenesis, most likely due to stabilization of amyloid fibrils and retardation of their

clearance (Pepys, 2001). Both SAP and proteoglycans have been found as components of amyloid deposits extracted from nerves of ATTRV30M patients (Inoue et al., 1998).

Animal models of human amyloidosis

Generation of animal models that mimic the pathology of protein conformation diseases is tremendously important, since most protein misfolding diseases are experimentally difficult to study in detail except from autopsy and biopsy samples. Consequently, to study *in vivo* the molecular mechanisms related to ATTRV30M amyloidogenesis and to search for pharmacological therapies, some ATTRV30M animal models have been generated.
The first mouse model generated for the disease used cDNA with the coding sequence for TTR V30M driven by the mouse metallothionein promoter. The expression of TTR was induced by zinc but circulating levels were too small and no amyloid deposition was detected (Sasaki et al., 1989; Sasaki et al., 1986). Subsequently, transgenic mice with the intact human *TTR* V30M gene, with a 6.0 kb of sequence upstream to the initiation site, were generated. These mice presented TTR protein levels in plasma similar to what is found in humans and the protein was expressed in liver, yolk sac and the choroid plexus. Additionally, showed deposition in the gastrointestinal tract, heart, skin and kidney at 9 months of age. However, no TTR deposition was observed in both peripheral and autonomic nervous system (Nagata et al., 1995; Takaoka et al., 1997).
Gene depletion (KO) technology allows for gene silencing, generating animals with a different phenotype. In 1993, Episkopou and colleagues developed a TTR KO mouse to investigate the TTR physiological roles (Episkopou et al., 1993). Since then, this strain has been widely used to address several aspects of TTR biology. In a later collaboration, TTR KO mice were crossed with the 6.0-TTR V30M transgenic mice, generating animals with the human TTR V30M variant but lacking the endogenous mouse TTR gene. These mice contained 30-60 copies of the human gene and at 24 months of age, TTR deposition resembled that of human FAP patients, but still without deposition in both peripheral and autonomic nervous system (Kohno et al., 1997). Even though amyloid deposition was still not detected in the peripheral nerve, this model proved useful for a better understanding of amyloidogenesis and for the research of molecules able to modulate this process.
Transgenic animals carrying TTR variants other than V30M have also been generated, such as L55P (Teng et al., 1996). Nevertheless, this strain displayed no deposition phenotype even at 2 years of age. A TTR mice model for this mutation in a null endogenous TTR background was later on was developed. Non-fibrillar TTR deposition was found earlier in

both gastrointestinal tract and skin, while amyloid deposits started to accumulate in these organs approximately at 8 months of age. Still, no TTR deposition was detected in the peripheral nervous system (Sousa et al., 2002). These findings suggested that endogenous TTR inhibited aggregation and deposition of the highly unstable human TTR L55P variant, which was later corroborated by Reixach who reported that human-murine TTR heterotetramers are kinetically stable and non-amyloidogenic (Reixach et al., 2008). Then, in 2010 a new mouse model emerged by crossing animals with the V30M mutation, in a *TTR* null background, with mice KO or heterozygous for the heat shock factor 1 (*HSF-1*). The impaired chaperone response led to relatively early TTR gastrointestinal deposits and, for the first time, protein deposition in the PNS (Santos et al., 2010). TTR/HSF-1 mice show an increased and more extensive TTR deposition in the gastrointestinal tract, particularly in the stomach, related to HSF-1 disruption. This higher penetrance was also evident in TTR/HSF-1 transgenic mice heterozygous for HSF-1, indicating that HSF-1 modulates and increases TTR tissue deposition (Santos et al., 2010). Moreover, this novel model displayed signs of inflammatory and oxidative stress replicating nerve pathology of asymptomatic carriers of the disease (Santos et al., 2008).

To study the process of age-related fibrillogenesis and TTR deposition in the heart, a different transgenic mice model was generated by overexpressing human WT TTR. These animals showed non-fibrillar TTR deposits prior to 18 months of age and amyloid deposits are frequently found in heart and kidney, similar to lesions characterizing age-related fibrillogenesis in humans (Teng et al., 2001).

The animal models are very useful to test the efficacy and safety of several TTR stabilizers, like tafamidis or diflunisal. Although several mouse models have been generated, they all express mouse RBP. Thus, human TTR associates with mouse RBP, resulting in different kinetic and thermodynamic stability profiles of TTR tetramers. Therefore, a double-humanized mouse strains, $TTR^{hWT\ TTR/hWT\ TTR}$: $RBP4^{hRBP/hRBP}$ and $TTR^{hWT\ TTR/hV30M\ TTR}$: $RBP^{hRBP/hRBP}$ were generated (Li et al., 2018). Amyloid deposits were first noticed in the gastrointestinal tract in both strains, at 12 and 18 months, increasingly with age. At 24 months, amyloid deposition was observed in the heart of both lines with more deposits in the mutant mice. Notably at 24 months, Congo red positive amyloid deposits were only found in the perineurium of the sciatic nerve of the mutant line (Li et al., 2018). This study further reveal the impact RBP might have on amyloid deposition as it is known to prevent TTR amyloid formation at lower pH (White & Kelly, 2001).

Moreover, to analyze the ATTRv metabolisms including after liver transplantations, a transgenic rat possessing the human ATTR V30M gene was generated using the albumin promoter (Ueda et al., 2007). The human ATTR V30M mRNA was strongly expressed in liver and brain. Additionally, the transgenic rat was the first animal model which shows the

expression of human ATTR V30M in the ocular tissues (Ueda et al., 2007). However, only in the submucosal lesions of the large intestine human ATTR V30M deposits were observed, even in older animals. Therefore, this transgenic rat turns out to be an animal model to analyze ATTR V30M metabolism and amyloid formation mechanisms in the tissue.
In addition to the animal models above described, other models were used to study ATTR amyloidosis, namely *Drosophila melanogaster* and *Caenorhabditis elegans*. Transgenic Drosophila expressing V30M TTR variant showed protein aggregation, reduced lifespan and neurological impairment, while flies with the human WT TTR presented a mild phenotype. V30M TTR flies exhibited a congo red positive staining in brains (Berg et al., 2009). In addition, similar studies were performed using different TTR variants, namely L55P (Pokrzywa et al., 2007). Overall, the *Drosophila* system can be used as an invertebrate model to complement studies on TTR amyloid formation, as well as allowing to screen for pharmacological agents, but the comparison of a highly mitotic model to mammals might be problematic as cellular and molecular phenotypes could have major differences. A model of *Caenorhabditis elegans* was also created expressing the human TTR in the body wall muscle. Transgenic *Caenorhabditis elegans* models of TTR amyloidosis that exhibit aggregation and quantifiable cell nonautonomous neuronal phenotypes, including impaired pain sensation, as seen in humans, and altered neuronal morphology (Madhivanan et al., 2018). The ability to probe for tissue-specific mechanisms of toxicity and do genetic screens in *Caenorhabditis elegans* should help to identify the genetic and molecular pathways by which peripheral tissues, including heart or sensory neurons, are selectively impaired in the TTR amyloidosis.
In summary, it is unquestionable that murine transgenic models for TTR amyloidosis have provided interesting insights into the pathogenesis of these disorders.

Cellular toxicity induced by TTR

It was suggested in the past that only amyloid fibrils induce tissue damage in TTR amyloidosis by direct compression and local blood circulation failure. However, an electron microscope study of the peripheral nerve from patients revealed that fibrillar and non-fibrillar aggregates were present (Coimbra & Andrade, 1971a, 1971b). Using microscopic and cell viability assays, Sousa and colleagues demonstrated the presence of non-fibrillar TTR aggregates in nerve biopsies from ATTRV30M patients at an early stage of disease before detection of amyloid fibrils, while at later stages both fibrillar and non-fibrillar aggregates were observed (Sousa, Cardoso, et al., 2001). The authors also showed that mature

amyloid fibrils were unable to cause cellular toxicity in cell culture while non-fibrillar aggregates did. Later, it was demonstrated that TTR amyloid fibrils with size higher than 100kDa were not toxic, whereas monomeric or very low molecular mass TTR aggregates were cytotoxic (Reixach et al., 2004).

Altogether, these data suggest that cellular toxicity starts at early stages of the disease, predominantly consequent to the presence of non-fibrillar TTR aggregates. Although the precise molecular mechanisms mediating cytotoxicity in FAP are not yet fully understood, several pathways, including ER stress, oxidative stress and inflammation, have been implicated.

Endoplasmic reticulum stress and structural destabilization

The unfolded protein response (UPR) is a stress-responsive signalling pathway that regulates endoplasmic reticulum (ER) quality control in response to developmental signals, environmental or aging related insults that increase accumulation of misfolded proteins in the ER (Ron & Walter, 2007). The UPR is a collective term for three stress signalling pathways activated downstream of the ER stress-sensing proteins Protein Kinase R-like ER kinase (PERK), inositol requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) (Xu et al., 2005). The activation of transcription factors enhances the folding capacity of ER, promote ER-associated degradation (ERAD) and inhibit mRNA translation, with the ultimate goal of restoring the normal ER function (Maattanen et al., 2010). If this response proves insufficient to counteract ER stress, apoptosis occurs. Clinical presentation of TTR amyloid diseases is influenced by the ability for ER quality control pathways to identify destabilized TTR variants and prevent their hepatic secretion.

Different studies have demonstrated that ER stress is activated in ATTRV30M amyloidosis. Indeed, increased levels of the ER-resident chaperone BiP were found both in tissues from ATTRV30M patients and from a mouse model of the disease, in close relation with TTR deposition (Teixeira et al., 2006). The same authors also reported calcium mobilization to the cytosol. Calcium release from ER stores can modulate cell death in several ways, including activation of caspases (*via* activation of Bax and Bid and inhibition of Bcl-2), induction of mitochondrial dysfunction or autophagy (*via* Bcl-2 activation). In fact, many of the Bcl-2 family members are associated with ER stress by regulation calcium homeostasis (Pinton et al., 2008). The intervention of apoptosis in ATTRV30M toxicity has been

demonstrated, as the effector caspase-3 was seen activated in nerve biopsies of ATTRV30M patients (Sousa, Cardoso, et al., 2001).

Patients expressing highly-destabilized, highly-aggregation prone TTR variants such as D18G TTR present with a relatively mild systemic amyloid disease pathology that is inconsistent with the extremely high aggregation propensity of these variants (Hammarstrom, Sekijima, et al., 2003; Sekijima et al., 2005). Interestingly, these highly-destabilized, highly-aggregation-prone TTR variants are recognized by ER quality control pathways in the liver and targeted for degradation (Sato et al., 2007). The recognition of these variants decreases their secretion and subsequently serum concentrations, slowing proteotoxic aggregation of these highly-destabilized TTRs, as opposed to TTR WT or TTR V30M variant (Sato et al., 2012). Further studies showed that V30M TTR variant is present in serum of patients in an *N*-glycosylated form, being post-translationally modified and secreted, escaping the ER quality control (Teixeira & Saraiva, 2013). Because *N*-glycosylation can improve the thermodynamic and kinetic stability of proteins, the role of this post-translational modification in TTR amyloidosis is yet to be clarified (Figure 5).

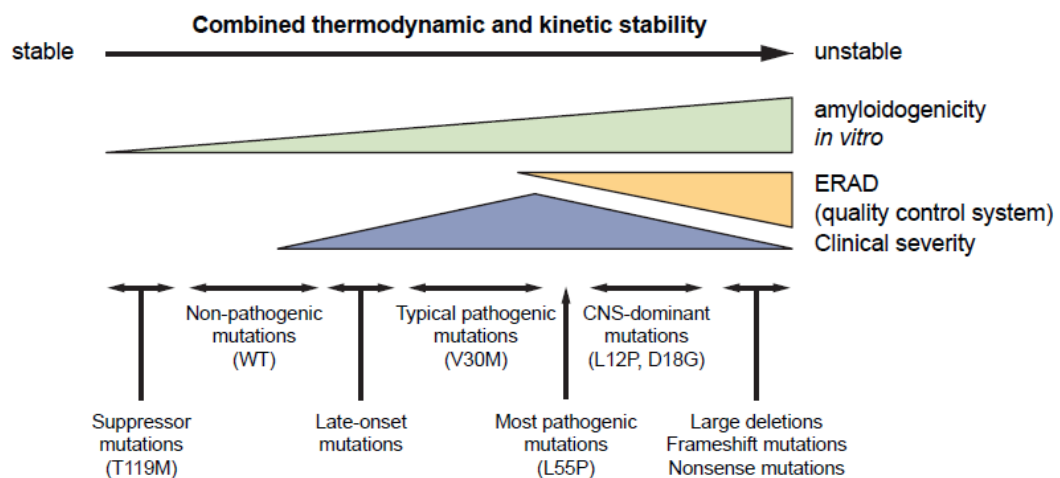


Figure 5. Schematic relationship between disease phenotype and combined thermodynamic and kinetic stability of TTR variants. Highly destabilized mutations like large deletions, frameshift mutations or nonsense mutations, are not usually found in TTR, most likely due to the cellular quality control mechanisms. Adapted from (Sekijima et al., 2008).

Extracellular matrix remodeling

The ECM is a highly dynamic structure that is present in all tissues and continuously undergoes controlled remodeling (Hynes, 2009). This process involves quantitative and qualitative changes in the ECM, mediated by specific enzymes that are responsible for ECM degradation, such as metalloproteinases. The ECM interacts with cells to regulate diverse functions, including proliferation, migration and differentiation (Bateman et al., 2009; Jarvelainen et al., 2009). Dysregulation of ECM composition, structure, stiffness and abundance contributes to several pathological conditions, such as fibrosis (Lu et al., 2011). Excessive ECM dynamics is also a feature of ATTRV30M amyloidosis. Through microarray analysis of salivary glands from ATTRV30M patients, increased expression of ECM related genes was observed, specifically biglycan, neutrophil gelatinase-associated lipocalin (NGAL) and matrix metalloproteinase 9 (MMP-9) (Sousa et al., 2005). Interestingly, although MMP-9 has the ability to degrade TTR aggregates and fibrils *in vitro*, authors observed that in the presence of SAP, TTR fibrils become resistant to MMP-9 proteolysis. More recently, a different study observed that matrix mettaloproteinase 14 (MMP-14) was significantly overexpressed in degenerated ATTRV30M nerves presenting massive amyloid deposits and was also upregulated in sciatic nerves from a preclinical transgenic mouse model (Martins et al., 2017).

Histochemical analyses of cardiac tissue of ATTRV30M amyloidosis patients also revealed that fibronectin, laminin, collagen IV and heparan sulfate in close association with amyloid fibrils deposition in cardiomyocytes (Misumi et al., 2009).

Inflammation and oxidative stress

Neuroinflammation is a protective immune response concerning host cells, proteins and other mediators, aiming elimination of the initial cause of injury, for cell repair. When neuroinflammation is an established response, persisting long after the initial insult, result in a continued release of pro-inflammatory mediators and increased oxidative stress. Neurodegenerative disorders, including Alzheimer's, Parkinson's and ATTRV30M diseases have been associated with chronic neuroinflammation with increased levels of pro-inflammatory cytokines (Frank-Cannon et al., 2009).

Pro-inflammatory mechanisms are upregulated in ATTRV30M, especially in endoneurial axons, with increase expression of TNF-α, macrophage-colony stimulating factor and IL-1β

since earlier stages of disease and increase with the ongoing neurodegenerative process (Sousa, Cardoso, et al., 2001; Sousa, Du Yan, et al., 2001). The proposed mechanism underlying these observations was the binding of TTR species to the receptor for advanced glycation end products (RAGE) with activation of extracellular signal-regulated kinases (ERK) 1/2, nuclear factor κB (NF-κB) translocation and consequent transcription of pro-inflammatory proteins (Monteiro, Sousa, et al., 2006; Sousa, Du Yan, et al., 2001; Sousa, Yan, et al., 2000). NF-κB is a transcription factor essential on several biological processes, including cell proliferation, metastasis, response to DNA damage, apoptosis, and immune response through its vast target genes (Bottex-Gauthier et al., 2002). Therefore, NF-κB is tightly correlated with human diseases such as inflammation, cancer, and autoimmune diseases (Taniguchi & Karin, 2018). In accordance with this, one of the NF-κB subunits was found upregulated, with a predominant nucleus distribution, in tissues from ATTRV30M patients (Sousa, Yan, et al., 2000).

Previous work with nerve biopsies from ATTRV30M patients shows the close association between production of inflammatory mediators and TTR pre-fibrillar aggregates or amyloid fiber deposition suggesting a prominent role for inflammatory pathways in the progress of the disease (Sousa, Du Yan, et al., 2001). In fact, despite local increased cytokine production by axons, such as IL-1β, TNF-α or NF-kB activation, no immune inflammatory cellular infiltrate is observed around TTR aggregates in nerves from V30M patients, which might contribute to disease progression and suggesting that mechanisms must operate to prevent or inhibit the correct innate immune response (Misu et al., 1999; Sousa, Du Yan, et al., 2001). In line with this observation, a non-optimal activation of Schwann cells (SCs) was noted, since upon the injury stimulus provided by TTR aggregates, these cells had no ability to express chemokines and neurotrophic factors, important for tissue regeneration (Sousa, Du Yan, et al., 2001). Thus, this lack of Schwann cells responsiveness may contribute for neuronal dysfunction present in ATTRV30M. To explore the eventual balance between pro and anti-inflammatory events in this disease, histological analysis for the interleukin-10 (IL-10) was performed in human sural nerve biopsies. Surprisingly, expression of IL-10 was noticed only in advanced cases of disease, suggesting that activation of anti-inflammatory mechanisms might occur too late, being therefore insufficient to control inflammation (Sousa et al., 2005).

Oxidative stress has similarly been associated in the pathogenesis of neurodegenerative disorders as the nervous system is particularly vulnerable to this type of stress associated with the generation of reactive oxygen and nitrogen species (Contestabile, 2001). In ATTRV30M, different studies support the notion of oxidative stress involvement. For instance, in human colon biopsy samples of ATTRV30M patients were found increased

levels of HNE (hydroxynonenal) and TBARS (thiobarbituric acid reactive substances), markers of lipid peroxidation, as well as the levels of protein carbonyls, a marker of protein modifications by free radicals (Ando et al., 1997). A subsequent study revealed an increased expression of inducible form of nitric oxide synthase (iNOS) and 3-nitrotyrosine, in nerves from patients and asymptomatic carriers (Sousa, Du Yan, et al., 2001). Other indicators of oxidative stress, like 8-hydroxydeoxyguanosine, marker of nuclear and mitochondrial DNA oxidative damage and glutaredoxin 1, that measures cellular redox homeostasis and acts as regulator of oxidative stress and apoptosis, were detected increasingly expressed in tissues from patients and mouse models of disease (Macedo et al., 2007).

Therapies for ATTRV30M

The treatment of ATTRV30M is complex and requires a neurological and cardiological multidisciplinary coverage. It includes specific treatments to control the progression of the systemic amyloidogenesis, the symptomatic treatment of the peripheral and autonomic neuropathy and the treatment of organs severely involved by amyloidosis. Therefore, several therapies are currently being developed from suppression of TTR synthesis (gene silencers), to prevention of tetramer dissociation (stabilizers) and elimination of deposits (disruptors) as illustrated in Figure 6. Many have already been approved for use in patients with ATTRV30M neuropathy.

Liver transplantation

Liver transplantation (LT) became available for the suppression of mutant TTR synthesis in the early 1990s, through the surgical removal of the source organ and its replacement by a liver secreting wild-type TTR (Holmgren et al., 1993). The liver produces the majority of circulating TTR and therefore LT is an attractive treatment strategy for ATTRV30M as it significantly reduces the production of mutant protein. LT if performed in the early stage of disease, it has been reported to halt the progression of clinical manifestations, since it eliminates more than 95% of the variant amyloidogenic TTR from circulation. Therefore, the primary aim of LT is to stop disease progression, not to reverse established symptoms (Adams et al., 2000). LT has been proven to prolong survival of ATTRV30M patients

(Yamashita et al., 2012). In 1995, the Familial Amyloidotic Polyneuropathy World Transplant Registry (FAPWTR) was formed by a group of investigators aiming to monitor international experience with liver transplantation. FAPWTR reports overall survival rates of 77 % at 5 years and 55.3 % at 20 years after LT (Ericzon et al., 2015; Herlenius et al., 2004).
Despite the relatively high survival numbers, this procedure carries some limitations the most important being the limited organ availability. Additionally, many patients are not candidates for this invasive procedure mainly because of age or advanced disease. Moreover, this is an invasive technique that forces patients to be on a lifetime of immunosuppressants, still with an associated risk of organ rejection. The continued secretion of mutated TTR in the eye and choroid plexus accounts for the ineffectiveness of LT in preventing disease progression in these organs, because transplantation only suppresses TTR production of liver, not affecting the other sites of production (Ando, 2005a; Ando et al., 2004).

Gene therapy

Advances in gene therapy have led to the development of gene silencing approaches to suppress the endogenous synthesis by the liver of mutated TTR in ATTRV30M patients. These techniques can be implemented independently from the underlying disease-causing mutation and suppress both mutant and wild-type TTR protein synthesis (Hanna, 2014). Two types of gene silencing therapies are currently being evaluated in phase 2/3 clinical trials: antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs) (Benson et al., 2006; Love et al., 2010). ASOs are short synthetic RNA sequences that bind to target mRNA in a sequence-specific manner, thereby specifically inhibiting its translation. Their potential to suppress TTR synthesis has been demonstrated in transgenic mouse models (Benson et al., 2006). The leading ASO being evaluated in clinical trials is Inotersen (formerly IONIS TTR-Rx). It has been shown to reduce TTR protein plasma levels in a dose-dependent manner by up to 80 % in various animal models (Ackermann et al., 2012). Similar results were obtained in healthy volunteers during a phase 1 study (Ackermann et al., 2012). Clinical trials with ATTRV30M patients are currently ongoing (Mathew & Wang, 2019).
siRNAs are double-stranded RNAs that knock down target mRNA in a sequence-specific manner by means of enzymatic degradation. The therapeutic use of siRNAs requires that they are delivered to the intracellular compartment, a problem that was solved by their formulation as lipid nanoparticles (Love et al., 2010). A lipid nanoparticle named Patisiran (ALN-TTR02), a siRNA that specifically knocks down both mutant and WT *TTR* gene

expression, has been shown to greatly reduce TTR synthesis by the liver, although it does not appear to reduce TTR synthesis by the eye and central nervous system (Coelho et al., 2013; Suhr et al., 2015). Animal models, healthy volunteers and ATTRV30M patients were already treated with Patisiran that was generally safe and well tolerated. In addition, sustained suppression of TTR plasma levels was observed (Adams et al., 2018; Coelho et al., 2020).

Currently, a novel liver-directed siRNA conjugate, Vutrisiran, has been formulated. Vutrisiran is a N-acetylgalactosamine siRNA (GalNAc-siRNA) conjugate with improved pharmacokinetic and pharmacodynamic properties, which permits a strong and sustained TTR reduction and, a satisfactory safety profile in a phase I clinical trial enrolling healthy individuals (Habtemariam et al., 2021).

Gene therapy is considered a promising future therapeutic strategy for TTR amyloidosis in substitution of liver transplantation, however many questions remain to be addressed, including delivery, suppression efficiency, safety, ethics and cost issues.

Gene editing

More recently, a novel therapeutic agent (NTLA-2001) was developed designed to treat ATTR amyloidosis by reducing the concentration of TTR in serum through gene editing. NTLA-2001 is a new CRISPR-Cas9–based in vivo gene-editing therapy, that is intended to edit *TTR* in hepatocytes, leading to a decrease in the production of both WT and mutant TTR. NTLA-2001 consists of a proprietary lipid nanoparticle delivery system with liver tropism, carrying a single guide RNA that targets human *TTR*. Results of clinical trials revealed that administration of NTLA-2001 was associated with only mild adverse events. Regarding to the group that received the lower dose, the mean reduction from baseline in serum TTR protein concentration was 52% and was 87% in the group that received the higher dose (Gillmore et al., 2021).

Anakinra

Anakinra is the recombinant form of the endogenous IL-1 receptor antagonist (IL-1Ra) and is a US Food and Drug Administration (FDA) approved drug with successful results in rheumatoid arthritis (Botsios et al., 2007) and type 2 diabetes (Larsen et al., 2007). In an

animal model of ATTRV30M, Anakinra have proved beneficial when administered prior to TTR deposition, preventing activation of toxic cascades associated with the disease (Goncalves, Vieira, et al., 2014). Other studies with the animal model indicate an increased regeneration after neuronal injury in V30M mice that received Anakinra (Goncalves et al., 2015). Results of clinical trials revealed that administration of anakinra was shown to be safe and effective in patients with amyloidosis (Lane et al., 2017).

TTR stabilizers

Pathogenic mutations increase the propensity of the TTR protein tetramers to dissociate into monomers, which in turn aggregate as soluble oligomers and then as insoluble amyloid fibrils. Therefore, stabilizing the TTR tetramer was posed as a possible method to prevent amyloidosis (Hammarstrom, Wiseman, et al., 2003). As with LT, these approaches are meant to stop disease progression and not to reverse established deficits.

Diflunisal

Diflunisal, a non-steroidal anti-inflammatory drug (NSAID), developed in the 1970s, was shown to be a strong inhibitor of TTR amyloid fibril formation in vitro (Miller et al., 2004). Subsequent phase 1 and 2 studies confirmed the ability of diflunisal to stabilize TTR tetramers in healthy volunteers and ATTRV30M patients, through interaction with thyroxin binding sites (Sekijima et al., 2006; Tojo et al., 2006). The pivotal phase 3 study was a randomized clinical trial that included 130 patients. Diflunisal was generally well tolerated, with no difference in serious adverse effects between treatment groups. The study confirmed that diflunisal could significantly slow down ATTRV30M disease and in patients at different stages of neuropathy. Nevertheless, this compound is associated with side effects, that include gastrointestinal bleeding and acute kidney injury (Berk et al., 2013). The benefit of diflunisal seems to be maintained after 2 years of treatment (Sekijima et al., 2015). Diflunisal has the advantage of being cheap and easily available worldwide, although it is currently not available in many European countries.

Tafamidis

Tafamidis is another pharmacological chaperone that can be administered orally and binds to the thyroxin binding site of TTR tetramers, thereby preventing their dissociation in monomers and the formation of soluble oligomers. Initial studies showed that tafamidis is well tolerated and treated patients displayed less neurological deterioration and slower disease progression (Bulawa et al., 2012). One of the major concerns about the use of tafamidis in ATTRV30M patients is related to potential metabolic side effects, since it could interfere with T4 delivery throughout the body. However, clinical trials have showed that thyroxine binding globulin TBG, rather than TTR, transports the majority of the circulating T4 (Coelho et al., 2012). In addition, this compound is associated with side effects, that include urinary tract infections and diarrhea. A more recent study was conducted in order to determine the safety and tolerability of tafamidis and to assess the efficacy of the drug on slowing disease progression. Overall, the results indicated that the beneficial effects of tafamidis were sustained over a 30-month period and that it is more advantageous to start treatment as early as possible (Waddington Cruz & Benson, 2015). This stabilizer was approved in Europe and Japan for the treatment of ATTRV30M patients in the early stage of disease (Ueda & Ando, 2014). More recently, tafamidis was approved by the US FDA for the treatment of ATTR amyloidosis patients with cardiac amyloid deposition of ATTR WT. In a clinical trial with 441 patients, classified by the New York Heart Association (NYHA) as stage I-III heart failure, were assigned to tafamidis vs placebo for 30 months (Maurer et al., 2018). Patients with NYHA class III were more frequently hospitalized due to cardiac issues than the NYHA class I and II. Overall, the mortality and cardiovascular-related hospitalizations were inferior in the tafamidis group when compared to the placebo (29.5% vs 42.9%), which highlights the importance of early diagnosis and treatment.

AG10

More recently, an innovative high-throughput screening methodology for the identification of compounds binding to the T4 pocket under physiological conditions was developed, allowing the selection of a group of kinetic stabilizers that display effective inhibition of TTR amyloidogenesis (Alhamadsheh et al., 2011). Some analogues were further developed, and a novel TTR stabilizer, named AG10, was identified, which presents, at physiological pH, higher TTR binding affinity, increased kinetic stability and better binding selectivity compared to tafamidis and diflunisal. Interestingly, structural studies revealed that AG10 is

unique in its capacity to form hydrogen bonds with the same serine residues at position 117 that stabilize the non-amyloidogenic TTR T119M variant, resulting in additional electrostatic interactions at the periphery of the T4 pocket to enhance stabilization (Miller et al., 2018). Recent results from phase II clinical trials revealed nearly complete stabilization of TTR by AG10 while being well tolerated and effective for the treatment of patients either carrying mutant or TTR WT. Phase III clinical trials with AG10 are ongoing (Judge et al., 2019).

Polyphenols

Some plant polyphenols have also been reported as TTR tetrameric stabilizers, such as epigallocatechin-3-gallate (EGCG) or curcumin, the major components of green tea and turmeric, respectively. While curcumin interacts with TTR in the T4 binding pocket, EGCG binds at the protein surface, in particular at two binding sites at the dimer-dimer interface exerting an effect similar to a cross-linker. It was found that curcumin strongly suppressed TTR amyloid fibril formation by generating small aggregates, while EGCG maintained most of the protein in a non-aggregated soluble form (Ferreira et al., 2013; Ferreira et al., 2012). Low bioavailability and low specificity of binding seem to be relevant conditioning factors of their effects in vivo in humans (aus dem Siepen et al., 2015; Cappelli et al., 2018).

Fibril disrupters

These experimental therapeutic approaches target the late stages of amyloid formation, acting as fibril disruptors for the modulation of cellular stress and toxicity.

IDOX

4'-iodo-4'-deoxydoxorubicin (IDOX) was described as an agent that could efficiently bind to TTR amyloid fibrils, disaggregating them into non-toxic species (Merlini et al., 1995). However, due to cardiotoxicity associated with this drug, less toxic compounds, such as doxycycline were evaluated (Cardoso et al., 2003).

Doxycycline/TUDCA

Doxycycline, a member of tetracycline antibiotics family, structurally homologous to the anthracyclines, was found to be particularly effective on the disruption of TTR amyloid fibrils *in vitro* (Cardoso et al., 2003). Administration of doxycycline to old transgenic mice reveled a decrease in several markers associated with TTR amyloid deposition (Cardoso & Saraiva, 2006). Since doxycycline has effect only in advanced phases of the amyloidogenic cascade, transgenic V30M mouse models have helped establish the synergistic effects of doxycycline and tauroursodeoxycholic Acid (TUDCA) (a biliary acid), that targets an earlier phase of the amyloid fibrils assembly. TUDCA administration decrease toxic pre-fibrillar TTR oligomers deposition and reduce the expression of several apoptotic and oxidative biomarkers associated with ATTR amyloid deposition in transgenic murine models (Cardoso et al., 2010; Cardoso & Saraiva, 2006). The authors showed a synergistic effect on lowering TTR deposits and associated biomarkers than either of the compounds alone. Based on this animal data, a Phase II open label study was conducted over a 12-month period. Preliminary results indicate that the combination of doxycycline with TUDCA stabilizes disease progression for at least 1 year (Obici et al., 2012).

Antibody-based therapies

Antibody-based therapies are another therapeutic strategy for the treatment of ATTR amyloidosis, which still remains under investigation. Specific antibodies targeting TTR monomers, oligomers or amyloid aggregates may prevent TTR fibrillogenesis. A mouse monoclonal antibody, named T24 (or PRX004) that recognizes and clear the non-native TTR aggregates associated with disease pathology, without affecting the native or normal tetrameric form of the protein has been developed (Hosoi et al., 2016). In a rat model that expressed V30M TTR, T24 inhibited TTR accumulation. Additionally, in the same rat model, humanized T24 inhibited TTR fibrillation and promoted macrophage phagocytosis of aggregated TTR. This humanized antibody did not bind to WT TTR that functioned normally in the blood (Hosoi et al., 2016). On the basis of these findings, this antibody has already entered into phase I clinical trials in ATTRV30M patients (Teng et al., 2020). Results of clinical trials revealed that PRX004 has been shown to inhibit amyloid fibril formation, neutralize soluble aggregate forms of misfolded TTR and promote clearance of insoluble

amyloid fibrils through antibody-mediated phagocytosis. In addition, the antibody was found to be safe and well tolerated (Teng et al., 2020).

Furthermore, a novel monoclonal antibody designated as NI301A was developed and selectively targets TTR amyloid aggregates (Michalon et al., 2021). NI301A binds TTR amyloid deposits in cardiac tissues obtained at autopsy from patients with cardiomyopathy and triggers TTR amyloid removal from the post-mortem tissues ex vivo via phagocytosis through added human macrophages. In WT mice grafted with patient-derived TTR amyloid fibrils, systemically administered NI301A accumulates on the grafts and rapidly removes them via Fcγ mediated phagocytosis (Michalon et al., 2021). NI301A is currently evaluated in a phase 1 clinical trial in ATTRv patients with cardiomyopathy.

SAP depletion

SAP is a universal element of amyloid fibrils, retarding their breakdown and clearance (Pepys, 2001). Data from animal models receiving anti-human-SAP antibodies indicate that antibody mediate removal of this component, facilitating endogenous clearance of amyloid fibrils, without apparent adverse side effects (Bodin et al., 2010). This approach was recently tested in a phase 1, open-label trial that included 16 patients with systemic amyloidosis. Some patients showed reduced liver amyloid load and treatment was generally well tolerated (Richards et al., 2015).

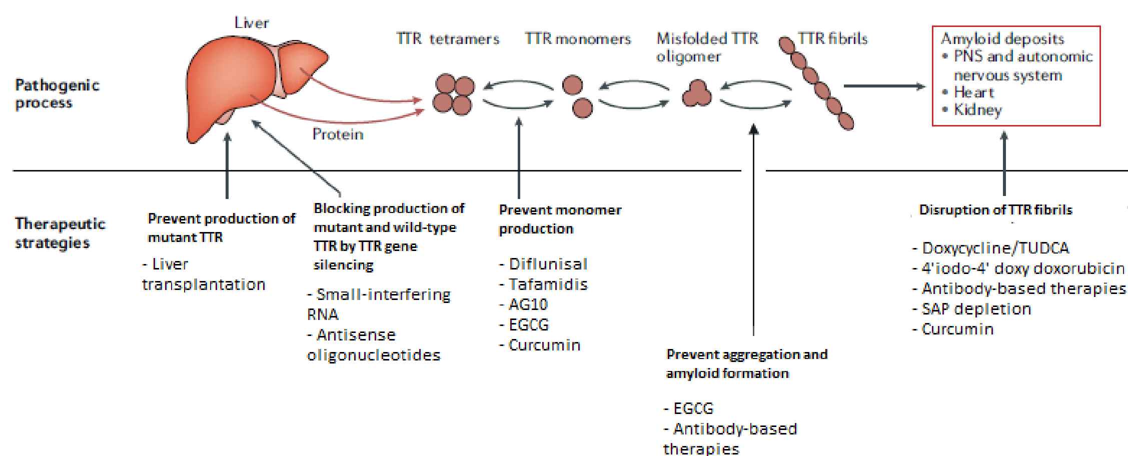


Figure 6. Therapeutic approaches to ATTRV30M. Adapted from (Adams et al., 2019).

The cellular impairment in ATTRV30M disease

As previously mentioned, the striking feature of ATTRV30M disease is the absence of an immune/inflammatory cellular infiltrate, despite the production of pro-inflammatory cytokines by axons (Misu et al., 1999; Sousa, Du Yan, et al., 2001). SCs, the myelinating glial cells of the peripheral nervous system, play a crucial role in the generation, function and maintenance of peripheral nerves. In fact, SCs act as the nerve sentinel cells and have the ability to regulate the PNS immune response by secreting cytokines and chemokines, thus playing a central role for nerve repair (Scholz & Woolf, 2007; Ydens et al., 2013). There are two types of SCs in adult peripheral nerve: myelinating and ensheathing (non-myelinating) SCs (Griffin & Thompson, 2008). Myelinating SCs form a multi-layered, membranous myelin sheath around large-calibre axons. Their myelin provides insulation that allows axons to conduct action potentials much more rapidly than they could in the absence of myelination. In contrast, ensheathing SCs, or Remak cells, loosely ensheath multiple small-diameter unmyelinated axons (Jessen & Mirsky, 2005).

Nerve injury is a complex multi-cellular response that might be triggered by traumatic or inflammatory stimulus. Wallerian degeneration comprises the rapid disintegration of the distal axons and the subsequent influx of immune cells, allowing the clearance of resulting debris and the formation of a favorable environment for regeneration to occur (Gaudet et al., 2011). Almost immediately after peripheral nerve injury, SCs of the distal stump have the ability to revert back to a nondifferentiated, proliferative phenotype. This dedifferentiation stage involves transcriptional changes with downregulation of genes related to myelination, such as myelin-associated glycoprotein, with concomitant increased expression of trophic factors and regeneration-associated genes (like brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) or growth associated protein 43 (GAP-43)) (Jessen & Mirsky, 2008). Both myelinating and ensheathing SCs begin to proliferate around 4 days after injury and align along their basal lamina to form bands of Büngner, which provide support and guide regenerating sprouts for axon growth (Scheib & Hoke, 2013; Stoll et al., 1989).

Several studies have shed light on how Schwann cells and immune cells initially sense injury to nearby axons. SCs express a broad range of toll-like receptors (TLRs) and induce NF-κB activation in response to all TLR ligands. While in basal conditions, TLR3 and TLR4 are the must abundantly present, SCs appear to be most sensitive to treatment with TLR2 and TLR1 ligands (Goethals et al., 2010). Moreover, SCs also express some of the more classical pattern recognition members, such as RAGE and LRP1 (Sousa, Du Yan, et al., 2001). The activated SCs assist in initiating a local immune response by producing

proinflammatory cytokines such as interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6), chemokines such as leukemia inhibitory factor (LIF) and monocyte chemoattractant protein (MCP)-1, as well as nitric oxide and adhesion molecules (Shamash et al., 2002; Tofaris et al., 2002). The chemoattractants induce the recruitment of immune cells to the site of injury and amplify the immune cascade (Dubovy, 2011). The best characterized axonal signals prompting phenotypic changes in SCs, upon injury, is c-Jun. c-Jun is a transcription factor required for efficient axonal regeneration. Recent studies demonstrate that c-Jun reprograms the Schwann cell transcriptional profile to generate a repair cell, looking forward correct reinnervation and functional recovery after nerve insult (Arthur-Farraj et al., 2012). Circulating monocytes are attracted to the injured nerve where they differentiate into macrophages and play a critical role in ensuring complete Wallerian degeneration (Rotshenker, 2011). Indeed, successful regeneration depends on tightly controlled cellular and molecular programs and is only achieved in the presence of a permissive environment.

In a mouse model of ATTRV30M, upon sciatic nerve injury there is an increase in the expression of local V30M TTR by SCs, leading to increased deposition as compared with WT mice (Murakami et al., 2010). Previous studies also demonstrated that an inflammatory stimulus in the peripheral nerve of TTR V30M transgenic mice is able to trigger and precede TTR V30M deposition contributing for the positive feedback loop of toxicity in ATTRV30M progression (DeFrancesco-Lisowitz et al., 2015; Goncalves, Teixeira-Coelho, et al., 2014). In line with what was observed in biopsies of ATTRV30M patients, V30M TTR mice display decreased expression of cytokines and chemokines important for the recruitment and infiltration of immune cells, known to be important for the tissue regenerative process comparatively to WT mice. There is a possibility that a non-optimal activation of SCs occurred, leading to a decreased expression of pro-inflammatory cytokines and chemokines, thus culminating in a diminished immune cellular activation and infiltration in V30M nerves (Goncalves, Teixeira-Coelho, et al., 2014).

Among the cells of the immune system, macrophages are phagocytes defined as movable cells with the capacity to engulf and digest pathogens, particles and dead cells (Gordon, 2008). Thus, phagocytes have dual functions, as immune sentinels but also as regulators of tissue homeostasis (Tauber, 2003). Prior evidence suggest that macrophages interact with SCs, including the regulation of migration, mitosis, and differentiation (Cattin et al., 2015; Chen et al., 2015; Martini et al., 2008). In ATTRv patients, macrophages and fibroblasts play a central role in the degradation of aggregated TTR (Misumi et al., 2013). However, $CD14^{+}$ monocytes from ATTRV30M patients were shown to have lower intracellular TTR immunoreactivity compared with healthy donors, suggesting that $CD14^{+}$ monocytes may affect TTR clearance, with a decreased ability in patients (Suenaga et al.,

2016). Furthermore, ATTRV30M patients display qualitative abnormalities in tissue-resident macrophages, especially inhibitory macrophages, and these abnormalities may accelerate TTR-derived amyloid deposition in some organs (Suenaga et al., 2016). Regarding to the mouse model expressing the V30M TTR, was also observed an impaired macrophage recruitment related with increased amyloid load and expression of apoptotic cell markers (Panayiotou et al., 2017).

More recently, a whole-mouse genome microarray analysis of V30M nerves as compared with those from WT mice revealed signalling pathways differentially upregulated in V30M nerve. These pathways belonged to a diverse set of categories including cell adhesion, regulation of mRNA transcription, and cell communication (Goncalves et al., 2016). In contrast, the pattern of downregulated genes was mostly associated with immunity and defence.

Circulatory chemokines in ATTRV30M patients

Regarding to ATTRV30M patients, a study was undertaken to generate accurate proteomic profiles in serum from patients with ATTRv, and to investigate the presence of phenotype-specific biomarkers unique to ATTRV30M and cardiac amyloid deposition (Chan et al., 2017). The results showed evidence that the circulating levels of more than 100 serum proteins were significantly different in patients and control samples. The levels of several proteins were found to be disease-specific, 18 in cardiac amyloidosis and 12 in ATTRV30M. By comparison, the differentially measured proteins unique to ATTV30M were mostly related to cellular process, immune system process, biological adhesion and response to stimulus. On the other hand, proteins unique to cardiac amyloidosis were related to metabolic and cellular process, localization and immune system process (Chan et al., 2017).

Interestingly, a different study that investigate the gene expression profile of peripheral blood cells from asymptomatic V30M carriers, symptomatic ATTRV30M patients and age- and sex-matched local healthy controls, identified three different signatures: a signature for differentiating symptomatic vs. asymptomatic ATTRV30M male subjects, a different signature for distinguishing symptomatic vs. asymptomatic ATTRV30M female subjects, and a third sex-independent signature for differentiating symptomatic vs. asymptomatic ATTRV30M (Kurian et al., 2016). The correlation of upregulated immune and inflammatory genes in symptomatic ATTRV30M males, but not in symptomatic females, suggests different roles for immune/inflammatory pathways in men vs. women, and may offer the

potential to explain why male patients generally progress faster in the disease (Kurian et al., 2016). Additionally, an inflammatory profile of ATTRV30M patients showed altered levels of several cytokines in the serum, such as IL-1β, TNF-α or IL-8 (Azevedo et al., 2019).

Concluding remarks

In summary, ATTRV30M is an autosomal dominant neurodegenerative disorder with extracellular deposition of mutant TTR aggregates and fibrils, particularly in nerves and ganglia of the peripheral nervous system. The mice model expressing human TTR V30M has remarkable similarities to human patients regarding TTR V30M deposition pattern and biomarkers associated with the pathology and allowed studies in transducing signaling mechanisms, particularly in the peripheral nervous system.

Moreover, it is fundamental to continue dissecting the molecular mechanism underlying the lack of inflammatory cellular infiltrates in the nerves of both ATTRV30M patients and V30M TTR animals, because elucidating how these different mediators interact in the context of ATTRV30M is of greatest importance to explain the immunological impairment observed in peripheral nerves of ATTRV30M patients and for the enhancement of new treatment approaches.

Research Project

Research objectives

This PhD work aims to study the molecular mechanisms underlying the lack of inflammatory response observed in PNS of ATTRV30M patients, particularly studying the influence and interaction of V30M TTR species in the process of DAMP's recognition by pattern recognition receptors and therefore their ability to initiate the expression of inflammatory mediators.

For that, the following specific aims will be addressed:

1. Investigate the role of S100A8/9 proteins in the development of the inflammatory process in ATTRV30M disease.
2. Understand the impact of V30M TTR variant in the reduced chemoattraction observed in nerves from ATTRV30M patients and in the mouse model of the disease.
3. Characterize the activation of signaling pathways and chemokine expression in bone marrow derived macrophages from V30M and WT animals.

Chapter I

Decreased S100A8/A9 in V30M related familial amyloid polyneuropathy: a possible pathway in misregulation of Schwann cell chemotaxis

Introduction

Inflammation is a basic defense mechanism in the human body. The inflammatory response has been shown to be important for the development of peripheral neuropathies either by leading to deterioration or to amelioration of the disease process (Ydens et al., 2013). ATTRV30M amyloidosis is a rare, progressive and life-threatening neurodegenerative disorder, caused by a mutation in the TTR protein. The mutation results in an abnormal TTR protein that is unstable and readily misfolds, forming aggregates which deposit as amyloid in various organs and tissues in the body. Previous work with nerve biopsies from patients suggest a prominent role for inflammatory pathways in the progress of the disease, since the production of inflammatory mediators were associated with the deposition of TTR non-fibrillar aggregates or amyloid fibrils (Inoue et al., 1998; Sousa, Du Yan, et al., 2001). Interestingly, despite cytokine production by axons, no influx of immune cells was found in ATTRV30M nerve biopsies, suggesting that mechanisms must operate to limit or inhibit the appropriate innate immune response (Misu et al., 1999; Sousa, Du Yan, et al., 2001).

The S100 protein family consists of over twenty members, most of which have been associated with various medical conditions (Schafer & Heizmann, 1996). S100A8 and S100A9, also known as calgranulins A and B or myeloid-related proteins (MRP) 8 and 14, have already been confirmed to play a decisive role in the development of inflammation (Mizobuchi et al., 2014; Tsai et al., 2014). More recently, Chernov *et al.* identified S100A8 and S100A9 among the top induced genes in peripheral nerve post-injury (Chernov et al., 2015). In fact, they described that up-regulation of S100A8/A9 in SCs shortly post-injury contributes to the activation of chemokine-cytokine network and the initial chemotactic gradient that guides immune cells towards the injury site (Chernov et al., 2015).

S100A8 and S100A9 are constitutively expressed in neutrophils, monocytes (Edgeworth et al., 1991), and dendritic cells (Averill et al., 2011) but can also be induced upon activation in other cell types such as mature macrophages (Xu & Geczy, 2000; Xu et al., 2001), vascular endothelial cells (McCormick et al., 2005; Yen et al., 1997), fibroblasts (Rahimi et al., 2005) and SCs (Kim et al., 2012). Intracellularly, S100A8 and S100A9 mediate the rapid rearrangement of cytoskeleton, which is a prerequisite for successful cell migration, phagocytosis, and exocytosis. S100A8/A9 complex plays a significant role in microtubule polymerization and stabilization in resting phagocytes (Nacken et al., 2003; Roth et al., 1993; van den Bos et al., 1996). Leukocyte migration in S100A9 KO mice is deficient, and there is a reduction in polymerized microtubulin in S100A9 KO mice neutrophils, in which impaired activation of small GTPases is detected (Vogl et al., 2004). In addition, the extracellular release of S100A8 and S100A9, individually or in a heterodimeric form, can induce the secretion of multiple cytokines in inflammatory cells to sustain and exacerbate

inflammation (Devery et al., 1994; Ma et al., 2017; Nishikawa et al., 2017). Although much attention has been paid to the pro-inflammatory functions of S100A8/A9, the complex also exhibits anti-inflammatory properties under specific conditions to avoid tissue damage caused by overwhelming inflammation, suggesting that these proteins contribute to homeostasis during inflammation (De Lorenzo et al., 2010; Sun et al., 2013). TLR4 and RAGE have so far been suggested as innate immune receptors for S100A8/A9 (Harja et al., 2008; Vogl et al., 2007).

Overall, the regenerating properties of ATTRV30M peripheral nerves remains not well understood. Such knowledge is needed to improve axonal regeneration or prevent neurodegeneration caused by the deposition of TTR aggregates and associated toxic signaling cascades. Therefore, taking into consideration the prominent role of S100A8/A9 in the activation of chemokine-cytokine network, study the expression of these molecules in ATTRV30M patients and in a mouse model of the disease could be interesting to understand the immune impairment observed in this disease.

Materials and Methods

Animal Procedures

All animal experiments were carried out in accordance with National and European Union guidelines for the care and handling of laboratory animals and were performed in compliance with the institutional guidelines and recommendations of the Federation for Laboratory Animal Science Association (FELASA) and approved by the National Authority for Animal Health (DGAV; Lisbon, Portugal). Transgenic mice for human TTR V30M, in the Sv/129 and endogenous TTR-null background, with heterozygous deletion of the gene encoding transcription factor HSF-1 (labeled as HSF/V30M) (Santos et al., 2010) were analyzed at 6 and 20 months of age (n=6 of each age). WT animals heterozygous for HSF-1 deletion, in the Sv/129 background, were used as controls (n=6 of each age).
Animals were housed in a controlled temperature room, maintained under a 12-h light/dark period, with water and food ad libitum and then sacrificed with a lethal injection of a premixed solution containing ketamine (75 mg/kg) plus medetomidine (1 mg/kg). Plasma, sciatic nerve, DRG and other tissues typical of TTR deposition in ATTRv were collected. They were frozen at −80°C or fixed in formalin.

Recombinant transthyretin

Recombinant WT TTR and V30M TTR variant were produced in a bacterial expression system and purified as previously described (Furuya et al., 1991).

Recombinant transthyretin purification

Prior to use, endotoxins were removed from WT TTR and V30M TTR mutation. Briefly, Detoxi-Gel endotoxin resin (Thermo Scientific, Waltham, MA, catalog#20339) was packed appropriately in a chromatography column and was allowed to settle for 30 minutes. Detoxi-Gel Resin was regenerated by washing with five resin-bed volumes of 1% sodium deoxycholate (Sigma-Aldrich, Germany, catalog#D6750-10G), followed by 3-5 resin-bed volumes of pyrogen-free buffer to equilibrate the resin. Then, the samples were applied to the column and for greater efficiency the samples were incubated with the resin for 1 hour at room temperature. Pyrogen-free buffer was used to collect the flow-through. The protein quantification after elution was assessed by Bradford method (Bradford, 1976).

Dot blot for detecting protein aggregation

Aliquots corresponding to equal amounts of WT TTR and V30M TTR mutation (500 ng) were blotted onto a 0.2-μm pore cellulose acetate membrane and it was left to dry at room temperature for few hours. TTR was immunodetected using an antibody targeting TTR aggregates produced in our lab, mouse CE11 antibody (1:20) followed by anti-mouse horseradish peroxidase antibody (1:5000) and enhanced chemiluminescence visualization (BioRad). No aggregates were detected in all TTR samples.

Schwann cell primary culture

Mouse Schwann cell primary cultures were obtained from ScienCell Research Laboratories™ (San Diego, CA, catalog #M1700) and were cultured according to the manufacturer's instructions. Briefly, cells were cultured in Schwann cell medium, supplemented with 5% fetal bovine serum, 1% Schwann cell growth supplement and 1% penicillin/ streptomycin (all from ScienCell Research Laboratories). After monolayer propagation in T-75 flasks at 37°C in a 5% CO2 humidified chamber, 5×10^5 cells were seeded in 12-well plates and incubated with WT or V30M TTR, both at 2,5 μM in triplicates. In a different experiment Schwann cells were previously incubated with Cli-095 (10μM /well) (InvivoGen, San Diego, CA, catalog #tlrl-cli95) and sRage (3μg/mL /well) which was isolated from bacteria inclusion bodies (Monteiro, Cardoso, et al., 2006), antagonists for TLR4 and Rage respectively, and, after that stimulated with WT or V30M TTR. Twenty-four hours after stimulation, supernatants were collected, and cell lysates were prepared in trizol (Invitrogen, Waltham, MA, catalog #15596026) and assayed for the expression of proinflammatory cytokines by real-time polymerase chain reaction (RT-PCR). Unstimulated cells were also used as controls.

Bone Marrow Derived Macrophages

For cell culture experiments, cRPMI medium was prepared by supplementing RPMI 1640 with L-glutamine (Lonza, Switzerland, catalog#BE12-702F)) with 10% heat-inactivated fetal bovine serum (FBS), 1% sodium pyruvate, 1% HEPES and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Germany, catalog#M7522). Bone marrow cells from WT and V30M TTR mice were flushed from the femurs and tibia of mice and plated at $0,5 \times 10^6$ cells/mL per

petri dish (bacterial plates, Sterilin), in 8 mL of culture medium supplemented with 20% L929 cell conditioned media (LCCM). By day four, the cells were fed with 10 mL of the same media, and at day six, macrophages were harvested and seeded into 12-well tissue culture plates. BMDM from WT and V30M TTR mice were incubated with the TLR4 agonist lipopolysaccharide (LPS) (250ng/mL /well) in the absence or presence of the TLR4 receptor antagonist (CLI-095) (InvivoGen, San Diego, CA, catalog#tlrl-cli95); or incubated with the TLR2 agonist Pam3Cys (2µg/mL /well) (InvivoGen, San Diego, CA, catalog#tlrl-pms) for different time points. In all experiments, the supernatants were collected, and cell lysates were prepared in trizol (Invitrogen).

RNA extraction, cDNA conversion and RT-PCR

RNA from sciatic nerves (n=6 per group) was isolated with RNeasy Mini columns, following the manufacturer′s instructions (Qiagen, Hilden, Germany, catalog #74804). RNA from WT and V30M BMDM and mouse Schwann cells were isolated by phenol extraction (Trizol, Invitrogen). First-strand complementary DNA (cDNA) was synthesized using the SuperScript double-stranded cDNA Kit (Invitrogen) and quantitative real-time PCR performed using the iQ Syber Green Super Mix (Bio-Rad). Samples were run in duplicate and results analyzed by the Bio-Rad iQ5 software. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as reference gene. For the quantification of mRNA expression levels, the reaction was performed in a final volume of 20 µL containing 0.5 µL of each specific primer: mouse Gapdh forward: GCCTTCCGTGTTCCTACC, mouse Gapdh reverse: AGAGTGGGAGTTGCTGTTG; mouse S100A8 forward: AACCTCATTGATGTCTAC, mouse S100A8 reverse: CATTGTCACTATTGATGTC; mouse S100A9 forward: ACCTTCCATCAATACTCT, mouse S100A9 reverse: CATCAGCATCATACACTC; (all from Sigma), and 18 µL of Mix plus 1 µL of the newly synthesized cDNA. Primer sequences were designed using Beacon Designer 8 (Premier Biosoft) for all genes. Analysis of real-time PCR data was made by the comparative CT method. Individual relative gene expression values were calculated using the following formula: $2^{-(\text{Ct gene of interest} - \text{Ct constitutive gene})}$ (Schmittgen & Livak, 2008).

Immunohistochemistry (IHC) and double immunofluorescence

Sciatic nerve and DRG from HSF WT (*n*=6) and HSF V30M mice (*n*=6), were excised, post-fixed in 10% formalin, embedded in paraffin blocks and cut longitudinally at 3 μm. Histoclear (National Diagnostics) was used to deparaffinate the sections that were thereafter hydrated in a descent alcohol series. Endogenous peroxidase activity was inhibited by 3% hydrogen peroxide in methanol and sections blocked with 4% fetal bovine serum and 1% bovine serum albumin in PBS. Primary antibodies used were: S100A8/A9 heterodimer (1:100) (Novus Biologicals, Centennial, CO, catalog#MAC3157R). Secondary antibodies were biotinylated anti-rabbit (1:200, Vector). ABC kit (Vector) with extravidin-peroxidase was used and labeling was performed with 3.3´-diaminobenzidine for antigen visualization. For densitometry analysis of the immunostained sections, 5 pictures at 20 X magnification were taken from different areas and quantified using the Image pro-plus 5.1 software. Results shown represent the area occupied by pixels corresponding to the substrate reaction color that is normalized relatively to the total image area, with the corresponding standard error of the mean (SEM).

Enzyme-linked immunosorbent assay

The levels of mouse S100A8 (MyBioSource, San Diego, CA, catalog# MBS2504318), human S100A8 (R&D Systems, Minneapolis, MN, catalog# DY4570-05), human S100A9 (R&D Systems, Minneapolis, MN, catalog# DY5578) in the supernatants of Schwann cells incubated with WT and V30M TTR, in plasma from HSF WT and HSF V30M mice and in the plasma from ATTRV30M patients, asymptomatic carriers and control individuals were quantitatively determined by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer′s instructions.

Statistical analysis

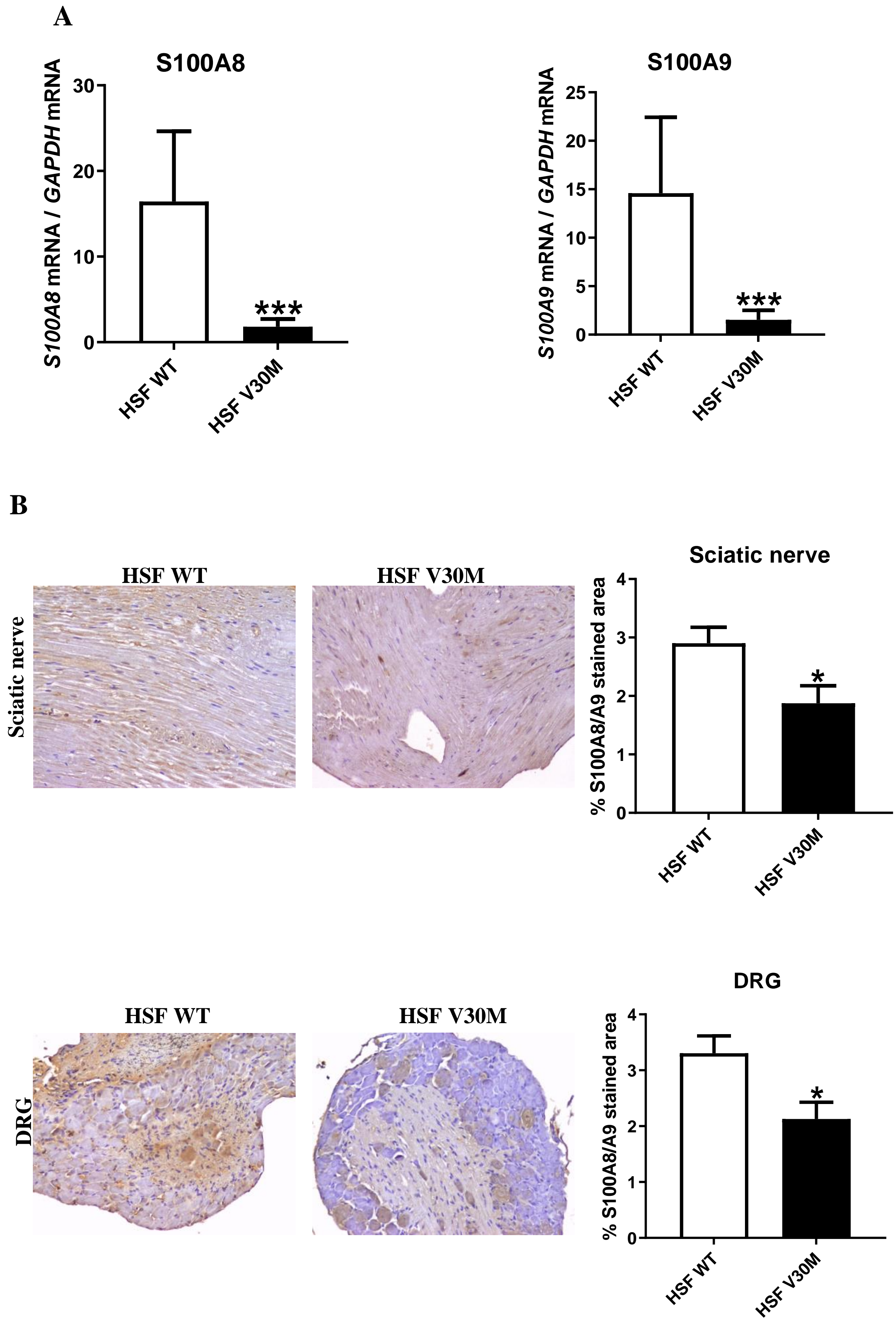
Statistical comparison of data was performed using the Student t test, one-way or two-way ANOVA followed by Bonferroni test as post-hoc analysis with Graph Pad Prism software. Quantitative data are expressed as mean ± SEM. Statistical significance was established for $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$ and $p^{****} < 0.0001$.

Results

The expression of S100A8/A9 proteins was downregulated in the peripheral nervous system of HSF V30M TTR mice

Previous work in our laboratory showed that in response to nerve injury, ATTRV30M mouse model display a downregulated innate immune response when compared to WT mice. More specifically, the expression of cytokines and chemokines important for the recruitment of immune cells like macrophages and neutrophils, known to be important for the tissue regenerative process were found decreased in V30M TTR mice (Goncalves, Teixeira-Coelho, et al., 2014).

Considering the central role of S100A8/A9 as potent initiators of the immune response in stimulated SCs of mouse injured peripheral nerves (Chernov et al., 2015), we questioned if the expression of S100A8/A9 proteins was altered in an ATTRV30M animal model. For that, the expression of these genes was assessed by RT-PCR in sciatic nerve from a mouse model of the disease (HSF V30M TTR mice) and the respective control group (HSF WT TTR mice) at 6 months of age. This ATTRV30M mouse model carries the V30M mutation in a heterozygous background for the heat shock factor 1 (HSF-1) and deposits TTR non-fibrillar species in the peripheral nervous system (Santos et al., 2010). S100A8 and S100A9 mRNA levels were highly decreased in HSF V30M mice, as compared with HSF WT mice (Figure 1A). Additionally, the protein levels of the heterodimer S100A8/A9 were assessed by immunohistochemistry in the sciatic nerve of HSF WT TTR and HSF V30M TTR mice at the same age. In accordance with the mRNA result, protein levels of S100A8/A9 were found decreased in HSF V30M mice, as compared with HSF WT mice (Figure 1B). Moreover, we assessed by immunohistochemistry the expression of the heterodimer S100A8/A9 in the DRG of the same animals. Similarly, to what we observed in sciatic nerve, the protein levels were found decreased in HSF V30M mice, as compared with HSF WT mice (Figure 1B). Finally, the protein levels of S100A8 were measured by ELISA in the serum of HSF WT and HSF V30M TTR mice at 6 and 20 months of age. No significant differences were detected in S100A8 protein levels in both mice strains, a finding also observed in older animals (Figure 1C).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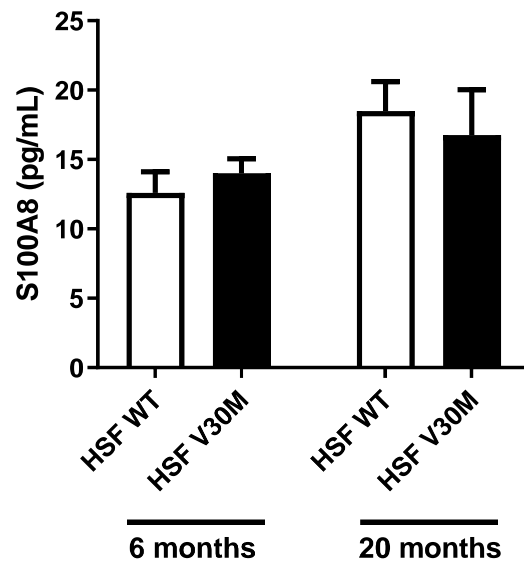
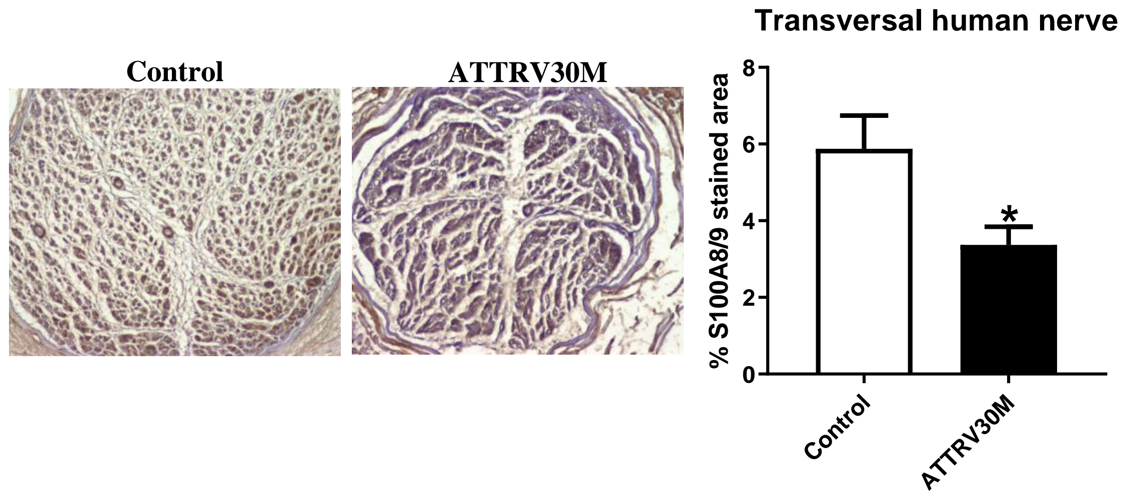


Figure 1. S100A8/A9 expression was downregulated in the peripheral nervous system of HSF V30M TTR mice. (A) RNA was extracted from sciatic nerve from HSF WT and HSF V30M TTR mice and the expression of *S100A8/A9* assessed by RT-PCR. (B) Histological examination of S100A8/9 heterodimer in sciatic nerve and DRG from HSF WT and HSF V30M TTR mice. (C) S100A8 protein levels were measured by ELISA (n=6, each group). Data were analyzed using two-way ANOVA or t test, followed by Bonferroni post-test and represented as mean ± s.e.m (*P < 0.05; ***P < 0.001).

S100A8/A9 expression is downregulated in transversal nerve biopsies from ATTRV30M patients

Impelled by the previous results that established a downregulated expression of S100A8/A9 in ATTRV30M mouse model, we next questioned if the expression of these molecules was also decreased in ATTRV30M patients. For that, we measured by immunohistochemistry the protein levels of heterodimer S100A8/A9 in transversal nerve biopsies from ATTRV30M patients and the respective control group. A significant downregulation in the expression of these proteins was observed in biopsies from ATTRV30M patients when compared to the control group (Figure 2A).

Moreover, we analyzed S100A8 concentrations in plasma samples from ATTRV30M patients, asymptomatic carriers and normal individuals. We did not notice a significant difference in plasma levels between normal and asymptomatic carriers. However, and differently to what we observed in HSF V30M TTR mice, S100A8 levels were significantly downregulated in ATTRV30M patients comparatively to control individuals (Figure 2B).



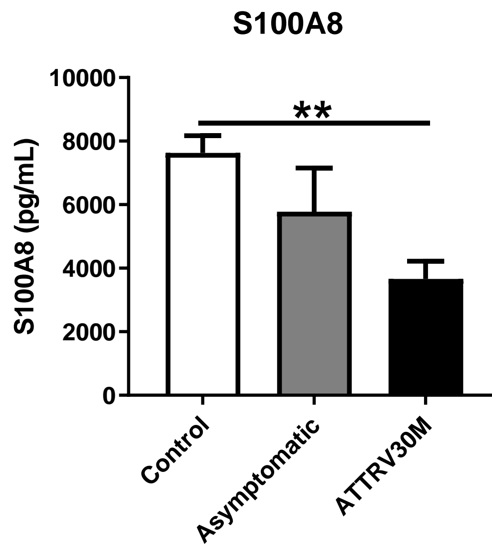


Figure 2. Decreased levels of S100A8/A9 are detected in ATTRV30M patients. (A) Histological examination of S100A8/9 heterodimer in transversal human nerve biopsies from ATTRV30M patients and the respective control group. (B) Decreased levels of S100A8 protein in ATTRV30M plasma samples, as compared to controls (n=6, each group). Data were analyzed using one-way ANOVA or t test, followed by Bonferroni post-test and represented as mean ± s.e.m (*$p < 0.05$; **$p < 0.01$).

The expression of S100A8/A9 proteins was downregulated in Schwann Cells incubated with V30M TTR

SCs are the major glial cell type in the peripheral nervous system. They play essential roles in the development, maintenance, function, and regeneration of peripheral nerves (Chen et al., 2007). Nonetheless, in sural nerve biopsies from ATTRV30M patients, SCs abnormalities have been reported, including multimembraneous bodies, vacuoles, and fibrous materials in the cytoplasm (Coimbra & Andrade, 1971b; Takahashi et al., 1974). Additionally, deposits of an amorphous material and amyloid fibrils were observed in close contact with SCs of the sural nerves (Coimbra & Andrade, 1971a; Inoue et al., 1998). Moreover, after injury, was also observed in V30M TTR mice a non-optimal activation of SCs, with decreased expression of pro-inflammatory cytokines and chemokines (Goncalves, Teixeira-Coelho, et al., 2014).

Therefore, we decided to evaluate the expression of S100A8/A9 proteins in SCs incubated with WT or V30M TTR. For that, mouse SCs were incubated with human WT or V30M TTR for 24 hours and the expression levels of these chemokines were assessed by RT-PCR. We observed a significant decrease in the expression of both S100A8 and S100A9 in Schwann cells incubated with V30M TTR comparatively with those incubated with WT TTR (Figure 3).

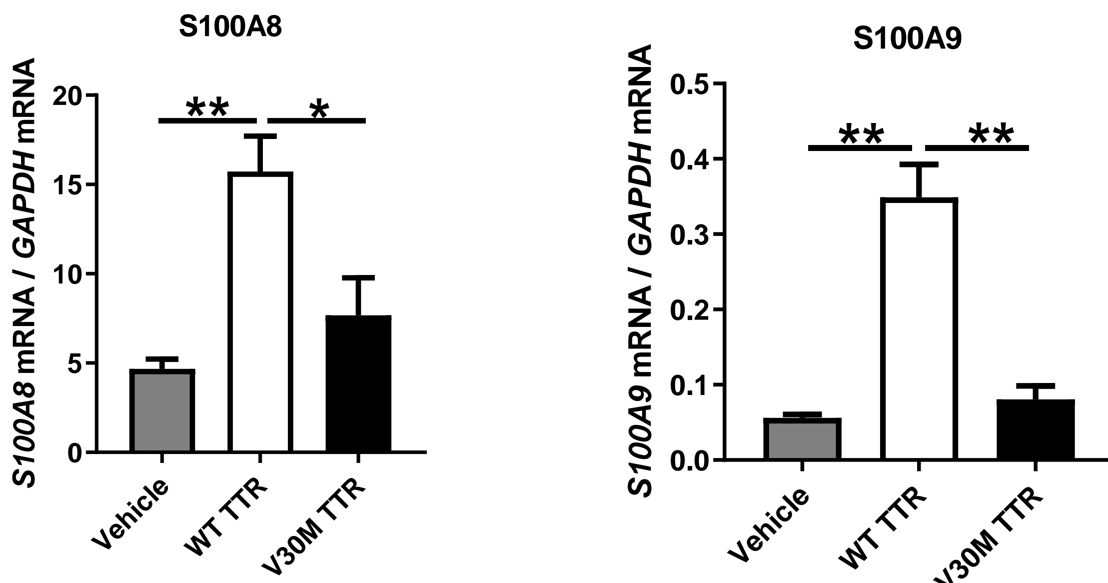


Figure 3. The expression of *S100A8/A9* genes was downregulated in SCs incubated with V30M TTR. RNA was extracted from SCs incubated with WT and V30M TTR and the levels of S100A8/A9 chemokines were found downregulated with V30M TTR incubation. Data were analyzed using one-

way ANOVA, followed by Bonferroni post-test and represented as mean ± s.e.m (* $p < 0.05$; ** $p < 0.01$).

S100A8/A9 proteins expression is mediated by TLR4 signaling pathway in SCs

The extracellular release of S100A8/A9 proteins, which are secreted as heterodimers by activated immune cells, serves as an alarmin, triggering immune responses by engaging with pattern-recognition receptors such as RAGE and TLR4 receptors (Harja et al., 2008; Vogl et al., 2004).

Considering the downregulated expression of *S100A8/A9* genes in SCs incubated with mutated TTR, we next decided to investigate which receptors were responsible to induce the expression of these proteins in SCs after incubation with WT or V30M TTR. For that, we decided to evaluate the role of TLR4 and RAGE receptors, which are both constitutively expressed in SCs (Goethals et al., 2010; Sousa, Du Yan, et al., 2001).

TLR4 inhibition with the antagonist CLI-095 highly reduced the expression of S100A8 in SCs incubated with WT TTR (fold decrease of 3,40). Regarding to SCs incubated with V30M TTR, we also observed a downregulated expression of S100A8 in the presence of CLI-095, but this variation was lower when compared with the WT TTR (fold decrease of 2,96) (Figure 4A).

Inhibition of the RAGE receptor with an antagonist (sRAGE) also led to the downregulation of S100A8 in SCs incubated with both WT (fold decrease of 1,68) and V30M TTR (fold decrease of 2,87). However, in SCs incubated with WT TTR, the differences obtained were not so prominent, when compared to TLR4 inhibition (Figure 4B). Collectively, our findings indicate that SCs activation by TTR triggers preferentially TLR4 signaling pathway leading to the expression of S100A8. Once again, lower expression levels of S100A8 were observed in SCs incubated with V30M TTR.

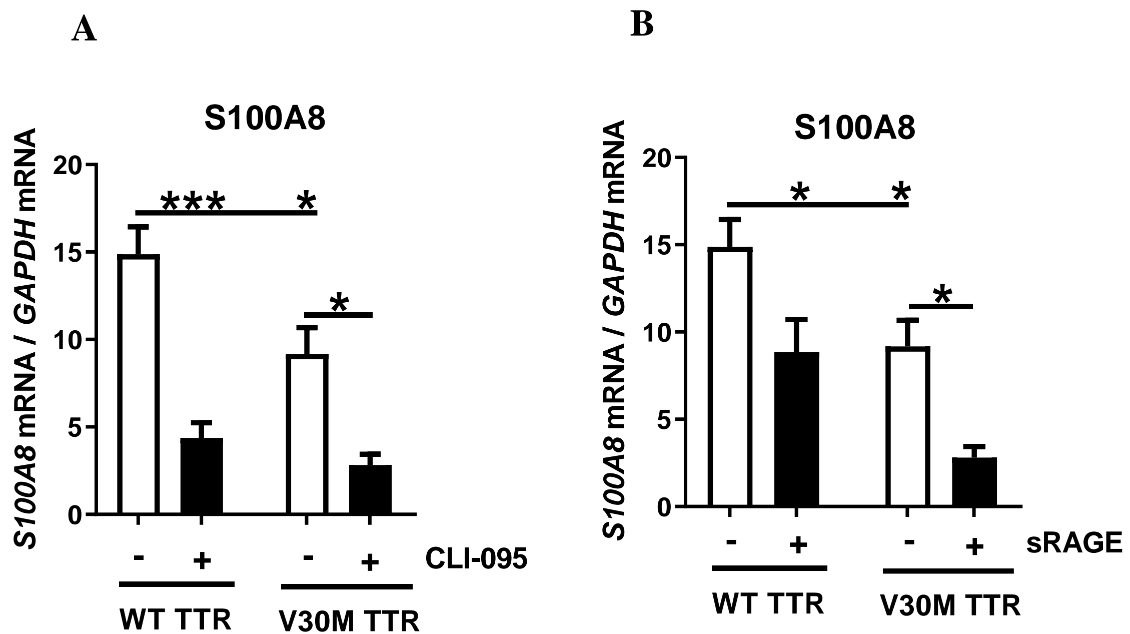


Figure 4. The expression of S100A8 was mainly mediated via TLR4 signaling pathway in SCs. RNA was extracted from SCs incubated with WT and V30M TTR in the presence of TLR4 (A) and RAGE (B) antagonists and a more pronounced downregulation in the expression of S100A8 was denoted in SCs incubated with CLI-095. Data were analyzed using two-way ANOVA, followed by Bonferroni post-test and represented as mean ± s.e.m (*p < 0.05; ***p < 0.001).

The expression of S100A8 in BMDM derived from WT and V30M TTR animals was different in response to stimulation with LPS

The early expression of S100 proteins during infection-induced inflammation suggests that S100A8 and S100A9 participate in innate immunity and mediate the inflammatory response (Mizobuchi et al., 2014). We next questioned if the TTR genetic background of innate immune cells as macrophages impacted the S100 expression in response to PRR stimulation. For that, BMDM of either genetic background were incubated with LPS and the expression of S100A8 was measured at different time points. Overall, BMDM from V30M TTR mice stimulated with TLR4 agonist displayed less expression of S100A8 throughout time than BMDM derived from WT animals (Figure 5A). We next questioned if the differences observed upon stimulation of the TLR4 pathway, were extended to other TLRs. To address this question, we stimulated BMDM of either genetic background via TLR2 which shares with TLR4 the same signaling pathway, via MyD88 adaptor (Yamamoto, Sato, Hemmi, Uematsu, et al., 2003). Once again, a downregulated expression of S100A8 was

observed in V30M TTR BMDM upon TLR2 stimulation, similarly to what happened in the case of TLR4 activation (Figure 5B). Collectively, these observations suggest that macrophages derived from V30M TTR animals may present a compromised response to TLR4 and TLR2 stimulation, with lower expression of S100A8 protein.

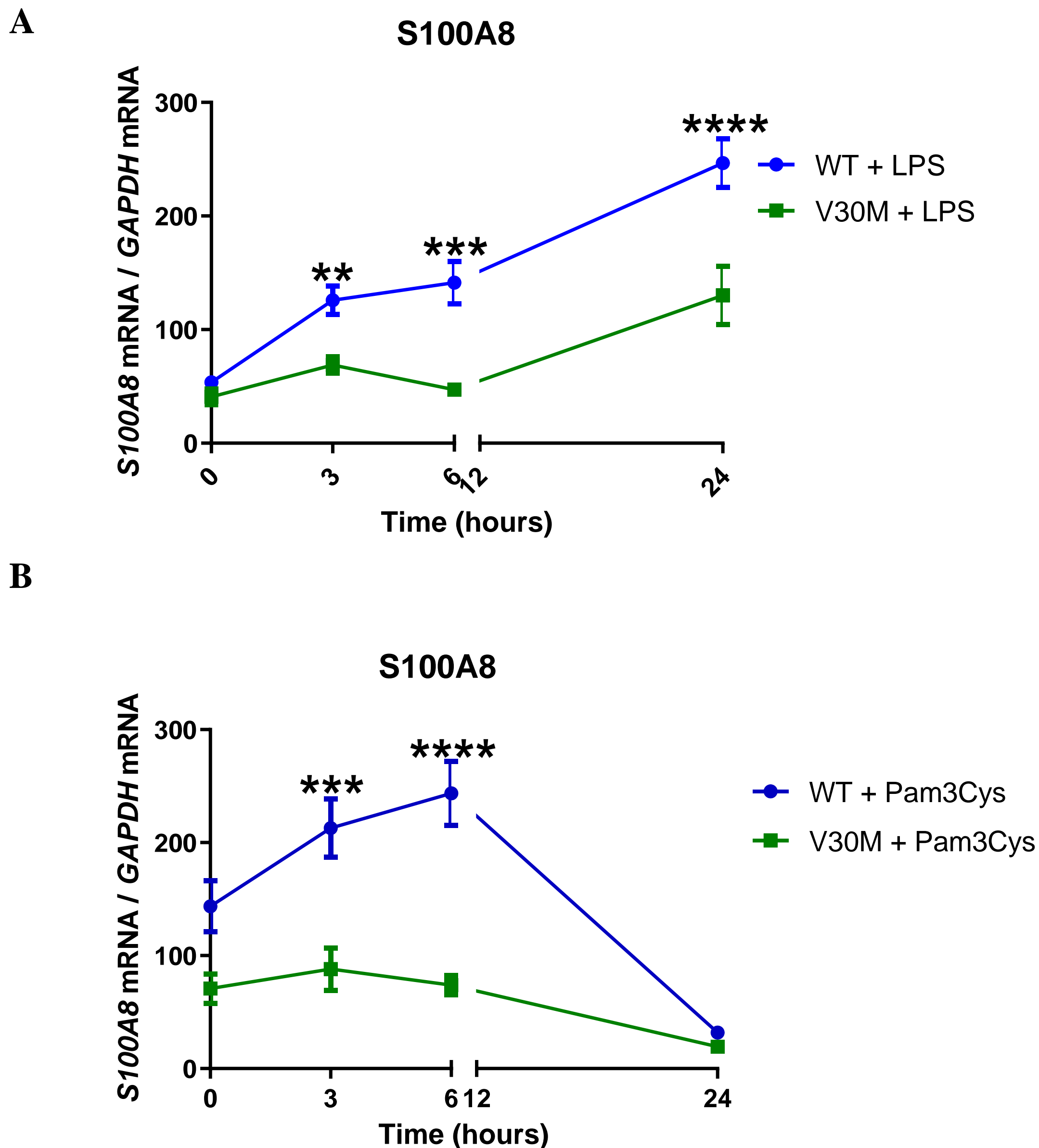


Figure 5. BMDM derived from WT and V30M TTR animals responds differently to stimulation with different TLR agonists. BMDM from WT and V30M TTR mice were stimulated with (A) LPS and (B) Pam3Cys at different time (3 and 6 hours) points and the expression of chemokines were assessed by RT-PCR. Data were analyzed using two-way ANOVA, followed by Bonferroni post-test and represented as mean ± s.e.m (**p < 0.01; ***p < 0.001; p**** < 0.0001).

Discussion

In this chapter, we showed a significant downregulation in the expresson of S100A8/A9 proteins, in the nerve biopsies from ATTV30M patients and in the peripheral nerve from HSF V30M TTR mice. Additionally, we showed a downregulated expression of these molecules in mouse SCs incubated with mutant V30M TTR, as well as, in BMDM derived from the animal model of the disease.

The importance of S100A8/A9 for the immune response, chemotaxis and cytokine expression is extensively studied and these proteins play several functions in different immune cell types, like SCs, neutrophils or macrophages (Chernov et al., 2015; Lackmann et al., 1993; Simard et al., 2013). Previous studies in ATTRV30M disease have described the presence of inflammatory markers in *ex vivo* biopsies from patients (Goncalves, Vieira, et al., 2014; Sousa et al., 2005; Sousa, Du Yan, et al., 2001). However, around TTR aggregates/amyloid deposits no immune cellular infiltrate was observed, which possibly contributes to slow down TTR clearance and aggravating the pathophysiology of the disease (Sousa, Du Yan, et al., 2001). Therefore, we decided to evaluate the expression of S100A8/A9 in ATTRV30M amyloidosys and observed a significant downregulation in the expression of these proteins in the sciatic nerve from HSF V30M mice, as compared with the control group. The next obvious step was to evaluate the S100A8/A9 expression in human patients and we observed a significant downregulation in the expression of S100A8/A9 proteins in biopsies from ATTRV30M patients when compared to the control group. Since S100A8/A9 have already been confirmed to play a decisive role in the development of inflammation, the lower expression of S100A8/A9 proteins could help to explain the lack of inflammatory cellular infiltrates observed in ATTRV30M nerves. Adittionally, the levels of S100A8 were also significantly downregulated in the serum of ATTRV30M patients comparatively to control individuals, indicating that S100A8 downregulation was not only restricted to peripheral nerve in ATTRV30M patients. However, further studies on a greater number of ATTRV30M blood samples are required to strengthen the results obtained so far and, ultimately, to evaluate whether blood levels of S100A8 can be used as a possible biomarker for ATTRV30M. Indeed, S100A8/A9 is already used as a biomarker in many inflammatory diseases, such as inflammatory arthritis disease (Kane et al., 2003), inflammatory bowel disease (Pruenster et al., 2016), cystic fibrosis (Cohen & Larson, 2005), and autoimmune diseases such as juvenile dermatomyositis (Nistala et al., 2013). In fact, it would be interesting to analyse the expression of S100A8/A9 in samples from patients undergoing different treatments resulting in inhibition or clearance of deposits to shed further light on the role of these proteins in

ATTRV30M pathogenesis. Therefore, the application of S100A8/9 can lead to new possibilities for diagnosis in clinical practice.

SCs have the ability to regulate local immune responses by secreting cytokines and chemokines thus amplifying the local immune response (Ydens et al., 2013). Regarding to ATTRV30M disease, several studies have indicated an impairment in SCs ability to express chemokines and neurotrophic factors important to drive tissue regeneration (Coimbra & Andrade, 1971b; Inoue et al., 1998; Sousa, Du Yan, et al., 2001). Consequently, we decided to evaluate the expression of S100A8/A9 in *in vitro* SCs. In line with the previous results, a significant decrease was observed in the expression of both S100A8 and S100A9 in SCs incubated with V30M TTR comparatively with incubation with WT TTR. The motive why V30M TTR variant fails to activate SCs expression of S100A8/A9 remains unknown and further investigation is required to unravel and understand the molecular pathways underlying this finding. However, it is tempted to speculate that V30M variant might gain a molecular or conformational specificity that impairs SCs activation and consequently S100A8/A9 expression. Taking into consideration that recent studies showed that SCs present in the PNS or cultured SCs are potent sources of S100A8/A9 after stimulation (Chernov et al., 2015), the impairment observed in the expression of these immune mediators could help to explain the lack of immune cells observed in the nerves of ATTRV30M patients, contributing this way for the development of the disease.

S100A8/A9 binding triggers MyD88-mediated TLR4 signaling, leading to NF-kB activation and secretion of pro-inflammatory cytokines such as TNF-α and IL-17 (Loser et al., 2010; Vogl et al., 2007). Similarly, S100A8/A9 binding to RAGE leads to MAP kinase phosphorylation and NF-kB activation, promoting leukocyte production (Boyd et al., 2008; Eggers et al., 2011). Thus, we next decided to investigate which receptors were involved in the expression of these proteins in SCs after incubation with WT or V30M TTR. We implicate TLR4 as the main receptor being triggered by TTR incubation in SCs, since TLR4 inhibition with the antagonist CLI-095 highly reduced the expression of S100A8 in SCs induced by WT TTR. Regarding to RAGE receptor, the presence of RAGE inhibitor also downregulates the expression of S100A8 in SCs incubated with WT TTR, but, in this case, the decreased expression was not so marked. As before, SCs incubated with V30M TTR induced a decreased expression of S100A8, with lower activation of both receptors. However, in this case, the inhibition of both receptors results in similar S100A8 fold decrease. The reason why TLR4 receptor is the mainly receptor involved in S100A8/A9 mediated SCs activation remains unclear and additional studies are required to fully address this question. However, recent studies have reported that in some particular circumstances, S100A8/A9 proteins preferentially activates one specific receptor, instead of both. For instance, in the context of acute lung injury, TLR4 is exclusively responsible for S100A8 mediated effects on alveolar

epithelial cells and RAGE receptor plays no relevant role, despite the dominant expression of RAGE receptor on these cells (Chakraborty et al., 2017). Consequently, these results are in line with the literature that point out that S100A8/A9 interaction with RAGE is more associated with inflammation-mediated carcinogenesis whereas its interaction with TLR4 is associated with inflammatory processes in infection and autoimmune disorders (Schelbergen et al., 2012; van Lent et al., 2008).

S100A8 and S100A9 are not only derived from SCs but also from several cell populations, such as neutrophils and macrophages and participate in inflammatory process (Altwegg et al., 2007). Macrophages are the main phagocytic cells and expected to infiltrate surrounding pre-fibrillar and TTR amyloid deposits to help on their clearance. However, recent *in vitro* studies showed that primary macrophages derived from HSF V30M transgenic mice have lower internalization and degradation rates when stimulated with TTR-aggregated toxic species (Ferreira et al., 2016). Accordingly, our results showed that BMDM from V30M TTR mice stimulated with LPS displayed less expression of S100A8 throughout time than BMDM derived from WT animals.

Altogether, in this work we suggest a link between the TTR microenvironment and the expression of S100s. We show that SC respond with enhanced S100A8/A9 expression to the presence of WT but not mutated TTR. Additionally, BMDM with mutated TTR genotype fail to induce S100A8 expression in response to LPS or Pam3Cys. Taken together, these findings implicate variant TTR microenvironments with limited innate responses which in turn likely hamper immune cell recruitment to the site of disease. It will be of importance and interest to in the future continue to investigate which reprogramming alterations occur in different cells and how mutated TTR promotes them.

Acknowledgements

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Chapter II

The expression of chemokines is downregulated in a pre-clinical model of TTR V30M amyloidosis

The expression of chemokines is downregulated in a pre-clinical model of TTR V30M amyloidosis

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Key words: transthyretin; familial amyloidotic polyneuropathy; chemokines; macrophages; Schwann cell; immune regulation.

Running title: chemokines in ATTRv

Abstract

Inflammation is a hallmark of several neurodegenerative disorders including hereditary amyloidogenic transthyretin amyloidosis (ATTRv). ATTRv is an autosomal dominant neurodegenerative disorder with extracellular deposition of mutant transthyretin (TTR) aggregates and fibrils, particularly in nerves and ganglia of the peripheral nervous system. Nerve biopsies from ATTRv patients show increased cytokine production, but interestingly no immune inflammatory cellular infiltrate is observed around TTR aggregates.

Here we show that as compared to wild-type (WT) animals, the expression of several chemokines is highly downregulated in the peripheral nervous system of a mouse model of the disease. Interestingly, we found that stimulation of mouse Schwann cells (SCs) with WT TTR results in the secretion of several chemokines, a process that is mediated by toll-like receptor 4 (TLR4). In contrast, the secretion of all tested chemokines is compromised upon stimulation of SCs with mutant TTR (V30M), suggesting that V30M TTR fails to activate TLR4 signaling.

Altogether, our data shed light into a previously unappreciated mechanism linking TTR activation of SCs and possibly underlying the lack of inflammatory response observed in the peripheral nervous system of ATTRv patients.

Introduction

Amyloid disorders are a heterogeneous group of diseases that can either be focal or systemic. They are due to the extracellular deposition of insoluble misfolded proteins, ultimately disrupting normal tissue function (Merlini & Bellotti, 2003). Among them, transthyretin (TTR) amyloidosis is the most common form of hereditary autosomic dominant systemic amyloidosis, in which TTR point mutations result in deposition of amyloidogenic species in different tissues (Benson & Kincaid, 2007). Recently this disease has been designated as ATTRv (Benson et al., 2018). Over 150 mutations in the primary sequence of TTR have been identified, most of which are associated with peripheral neuropathy as the main clinical manifestation. Often, cardiomyopathy and carpal tunnel syndrome occur as well (Jacobson et al., 1992; Saraiva, 2001). The most common TTR mutation in ATTRv results from an exchange of a methionine for a valine at position 30 (TTR V30M) (Saraiva et al., 1984). The expression of TTR was recently reported in Schwann cells (SCs) of the sciatic nerve (Murakami et al., 2010), but this protein is mainly synthesized in the liver and choroid plexus of the brain being secreted into the blood stream and cerebrospinal fluid, respectively (Saraiva et al., 2012; Soprano et al., 1985).

V30M related ATTRv was originally described as FAP (Familial Amyloidotic Polyneuropathy) by Corino de Andrade in northern Portugal and, despite being rare, the disease is distributed worldwide (Ando, 2005b; Andrade, 1952). It is a fatal neurodegenerative disorder, characterized by the extracellular deposition of aggregates and fibrils of mutant forms of TTR, particularly in the nerves and ganglia of the peripheral nervous system (PNS) (Benson & Kincaid, 2007). Pro-inflammatory mechanisms are upregulated in ATTRv, especially in endoneurial axons, with increased expression of TNF-α and IL-1β since earlier stages of disease, increasing with the ongoing neurodegenerative process (Sousa, Du Yan, et al., 2001). Therefore, infiltration of inflammatory cells in response to TTR deposition in tissues of ATTRv patients would be expected. Surprisingly, despite local increased cytokine production, such as IL-1β and TNF-α, by axons, as well as local activation of NF-kB, no immune inflammatory cellular infiltrate is observed around TTR aggregates in nerves from V30M patients, which might contribute to disease progression and suggests that mechanisms must operate to prevent or inhibit the correct innate immune response (Misu et al., 1999; Sousa, Du Yan, et al., 2001). Furthermore, immunochemical studies of patient's nerves suggest an impairment in the ability to express chemokines and neurotrophic factors important to drive tissue regeneration (Sousa, Du Yan, et al., 2001; Ydens et al., 2013). Additionally, after nerve injury in animal models carrying the V30M mutation (Kohno et al., 1997; Santos et al., 2010) a downregulated innate immune response

was observed when compared to WT mice. More specifically, a decreased expression of several cytokines and chemokines important for the recruitment of immune cells like macrophages was found (Goncalves, Teixeira-Coelho, et al., 2014).

All these observations correlated with the downregulated expression of chemokines, including CCL20 found in previous microarrays (Goncalves et al., 2016) in the peripheral nerve of a TTR V30M transgenic mouse model, prompted us to investigate the chemokine response activation in ATTRv pathogenesis.

Material and Methods

Animals

All animal experiments were carried out in accordance with National and European Union guidelines for the care and handling of laboratory animals and were performed in compliance with the institutional guidelines and recommendations of the Federation for Laboratory Animal Science Association (FELASA) and approved by the National Authority for Animal Health (DGAV; Lisbon, Portugal). Transgenic mice for human TTR V30M, in the Sv/129 and endogenous TTR-null background, with heterozygous deletion of the gene encoding transcription factor Hsf-1 (labeled as HSF/V30M) (Santos et al., 2010) were analyzed at 6 and 20 months of age (n=6 of each age). WT animals heterozygous for Hsf-1 deletion, in the Sv/129 background, were used as controls (n=6 of each age).
Animals were housed in a controlled temperature room, maintained under a 12-h light/dark period, with water and food ad libitum and then sacrificed with a lethal injection of a premixed solution containing ketamine (75 mg/kg) plus medetomidine (1 mg/kg). Plasma, sciatic nerve, DRG and other tissues typical of TTR deposition in ATTRv, such as the gastrointestinal tract, kidney and heart, were collected. They were frozen at −80°C, or fixed in formalin.

Recombinant transthyretin

Recombinant WT TTR and TTR variants, namely V30M, I68L, T119M and I84S TTR were produced in a bacterial expression system and purified as previously described (Furuya et al., 1991).

Recombinant transthyretin purification

Prior to use, endotoxins were removed from WT TTR and V30M, I68L, T119M and I84S TTR mutations. Briefly, Detoxi-Gel endotoxin resin (Thermo Scientific, Waltham, MA, catalog#20339) was packed appropriately in a chromatography column and was allowed to settle for 30 minutes. Detoxi-Gel Resin was regenerated by washing with five resin-bed volumes of 1% sodium deoxycholate (Sigma-Aldrich, Germany, catalog#D6750-10G), followed by 3-5 resin-bed volumes of pyrogen-free buffer to equilibrate the resin. Then, the

samples were applied to the column and for greater efficiency the samples were incubated with the resin for 1 hour at room temperature. Pyrogen-free buffer was used to collect the flow-through. The protein quantification after elution was assessed by Bradford method (Bradford, 1976).

Dot blot for detecting protein aggregation

Aliquots corresponding to equal amounts of WT TTR and V30M, I68L, T119M and I84S TTR mutations (500 ng) were blotted onto a 0.2-µm pore cellulose acetate membrane and it was left to dry at room temperature for few hours. TTR was immunodetected using an antibody targeting TTR aggregates produced in our lab, mouse CE11 antibody (1:20) followed by anti-mouse horseradish peroxidase antibody (1:5000) and enhanced chemiluminescence visualization (BioRad). No aggregates were detected in all TTR samples.

Schwann cell primary culture

Mouse Schwann cell primary cultures were obtained from ScienCell Research Laboratories™ (San Diego, CA, catalog #M1700) and were cultured according to the manufacturer's instructions. Briefly, cells were cultured in Schwann cell medium, supplemented with 5% fetal bovine serum, 1% Schwann cell growth supplement and 1% penicillin/ streptomycin (all from ScienCell Research Laboratories). After monolayer propagation in T-75 flasks at 37°C in a 5% CO2 humidified chamber, 5×10^5 cells were seeded in 12-well plates and incubated with WT or V30M TTR, both at 5 µM in triplicates. In a different experiment Schwann cells were incubated with Cli-095 (InvivoGen, San Diego, CA, catalog #tlrl-cli95) and sRage which was isolated from bacteria inclusion bodies (Monteiro, Cardoso, et al., 2006), antagonists for TLR4 and Rage respectively, before a second incubation with WT or V30M TTR. Twenty-four hours after stimulation, supernatants were collected and cell lysates were prepared in trizol (Invitrogen, Waltham, MA, catalog #15596026) and assayed for the expression of proinflammatory cytokines by real-time polymerase chain reaction (RT-PCR). Unstimulated cells were also used as controls.

RNA extraction, cDNA conversion and RT-PCR

RNA from sciatic nerves (n=6 per group) was isolated with RNeasy Mini columns, following the manufacturer's instructions (Qiagen, Hilden, Germany, catalog #74804). RNA from mouse Schwann cells culture were isolated by phenol extraction (Trizol, Invitrogen). First-strand complementary DNA (cDNA) was synthesized using the SuperScript double-stranded cDNA Kit (Invitrogen) and quantitative real-time PCR performed using the iQ Syber Green Super Mix (Bio-Rad). Samples were run in duplicate and results analyzed by the Bio-Rad iQ5 software. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as reference gene. For the quantification of mRNA expression levels, the reaction was performed in a final volume of 20 µL containing 0.5 µL of each specific primer: mouse Gapdh forward: GCCTTCCGTGTTCCTACC, mouse Gapdh reverse: AGAGTGGGAGTTGCTGTTG; mouse CCL20 forward: TACAGACGCCTCTTCCTT, mouse CCL20 reverse: TCGTGTGAAAGATGATAGCA; mouse CCL5 forward: CAATCTTGCAGTCGTGTT, mouse CCL5 reverse: AATAGTTGATGTATTCTTGAACC; mouse CXCL5 forward: CTACGGTGGAAGTCATAG, mouse CXCL5 reverse: TTCTTTATCACAGGAGCTT; mouse CCL2 forward: GATGATCCCAATGAGTAG, mouse CCL2 reverse: AAATAAAGTTGTAGGTTCTG; mouse CCL8 forward: AGAGAATCAACAATATCCA, mouse CCL8 reverse: CTACACAGAGAGACATAC; mouse CXCL3 forward: CCAACCACCAGGCTACAG, mouse CXCL3 reverse: AACTTCTTGACCATCCTTGA; mouse CXCL2 forward: CCAACCACCAGGCTACAG, mouse CXCL2 reverse: CTTCAGGGTCAAGGCAAAC; mouse IL-1β forward: AAGATGAAGGGCTGCTTCCA, mouse IL-1β reverse: AAGGTCCACGGGAAAGACAC; mouse IFN-β forward: GCACTGGGTGGAATGAGACT, mouse IFN-β reverse: AGTGGAGAGCAGTTGAGGACA; mouse IL-6 forward: CTGTCTATACCACTTCAC, mouse IL-6 reverse: GCTTATCTGTTAGGAGAG; (all from Sigma), and 18 µL of Mix plus 1 µL of the newly synthesized cDNA. Primer sequences were designed using Beacon Designer 8 (Premier Biosoft) for all genes. Analysis of real-time PCR data was made by the comparative CT method. Individual relative gene expression values were calculated using the following formula: $2^{-(\text{Ct gene of interest} - \text{Ct constitutive gene})}$ (Schmittgen & Livak, 2008).

RNA sequencing

RNA from sciatic nerves (n=4) was isolated from HSF WT and HSF V30M mice with RNeasy Mini columns and first-strand complementary DNA (cDNA) was synthesized using the SuperScript double-stranded cDNA Kit as abovementioned. Subsequently, the cDNA

samples were delivered to Bioinf2Bio Company for RNA sequence analysis. All reads were aligned to the Ensembl genome of *Mus musculus* reference genome GRCm38. Bioinf2Bio used the reference mouse genome, based on the strain C57BL/6J, for read alignment. The differential expression between the different groups of samples were identified by the tool *Cuffdiff* to find significant changes in gene expression across conditions. All data used in the present study are available in NCBI GEO database with the access number GSE165126.

Western blot analysis

Protein was isolated from Schwann cells by homogenization with lysis buffer containing 10 µL phenylmethylsulfonyl fluoride (PMSF), 10 µL sodium orthovanadate solution and 10 µL protease inhibitor cocktail solution per mL of 1x RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, catalog# sc-24948). Protein concentration was determined using the Bradford protein assay (Bio-Rad, Hercules, CA, catalog# 5000006). Subsequently, samples were run on a 12% polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on nitrocellulose WhatmanTM membrane (GE Healthcare). Primary antibodies against Phospho-p38 (Cell Signaling, Netherlands, catalog# 4631), and α-Tubulin (Sigma, Germany, catalog# T8203) were diluted in 5% bovine serum albumin in phosphate buffer saline (PBS) supplemented with 0.1% Tween 20 (PBS-T), overnight at 4 °C. In the next day, blots were washed with PBS-T and incubated with anti-rabbit or anti-mouse conjugated to horseradish peroxidase (1:5000; The Binding Site) for 45 min at room temperature. After three washes in PBS-T, bands were visualized with enhanced chemiluminescence using the LuminataTM Crescendo (Millipore). Protein bands were quantified by densitometry using Quantity One software (Bio-Rad) and α-Tubulin quantification was used to correct for total protein loading variation.

Enzyme-linked immunosorbent assay

The levels of CCL20 (R&D Systems, Minneapolis, MN, catalog# MCC200) and CXCL2 (R&D Systems, Minneapolis, MN, catalog# MM200) in the supernatants of Schwann cells incubated with WT and V30M TTR and in plasma from HSF WT and HSF V30M mice were quantitatively determined by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer′s instructions.

Statistical analysis

Statistical comparison of data was performed using the Student t test or one-way ANOVA with Graph Pad Prism software. Quantitative data are expressed as mean ± SEM. Statistical significance was established for $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$ and $p^{****} < 0.0001$.

Results

The expression of several chemokines is downregulated in the peripheral nervous system of HSF V30M TTR mice

Despite local increased cytokine production, the recruitment of immune cells like macrophages and neutrophils, known to be important for the tissue regenerative process, is reduced in ATTRv nerve biopsies (Misu et al., 1999). We questioned if the impaired immune cell recruitment was due to a compromised chemokine response. To address this hypothesis, a RNA sequence analysis was performed in sciatic nerve from a mouse model of the disease (HSF V30M TTR mice) and the respective control group (HSF WT TTR mice) with 6 months of age. This ATTRv mouse model carries the V30M mutation in a heterozygous background for the heat shock factor 1 (HSF-1) and deposits TTR non-fibrillar species in the peripheral nervous system (Santos et al., 2010). The expression of most chemokines was significantly downregulated in the sciatic nerve of HSF V30M TTR mice when compared to HSF WT control at 6 months of age (Table 1), a finding that we validated by RT-PCR (Figure 1A). In older animals (22 months), a similar and possibly more marked profile revealing the downregulation of chemokine expression was observed (Figure 1B).
To further validate the RNA data, we measured the levels of CCL20 and CXCL2 by ELISA in the serum of HSF WT and HSF V30M TTR mice at 6 and 20 months of age. A significant decrease in CCL20 levels from HSF V30M mice with 6 months of age comparatively with HSF WT mice was observed, but no significant differences were detected in HSF V30M mice with older age (Figure 1C). In contrast, a significant decrease in CXCL2 levels was observed in the serum of older, but not young, HSF V30MTTR mice as compared to HSF WT mice (Figure 1C). These data are in line with the RNA profiles obtained.
Collectively, our data point to a decreased expression of chemokines in a mouse model of ATTRv.

Table 1

Gene Name	Symbol	Fold change
Chemokine (C-C motif) ligand 8	CCL8	2,56
Chemokine (C-C motif) ligand 5	CCL5	2,4
Chemokine (C-X-C Motif) ligand 3	CXCL3	Not detected in V30M TTR mice
Chemokine (C-C Motif) ligand 20	CCL20	Not detected in V30M TTR mice

Table 1. Chemokines found downregulated in V30M sciatic nerve compared with sciatic nerve from WT mice. Genes were considered up-regulated with fold-change >1.5 after class comparison, assuming significances $P < 0.05$.

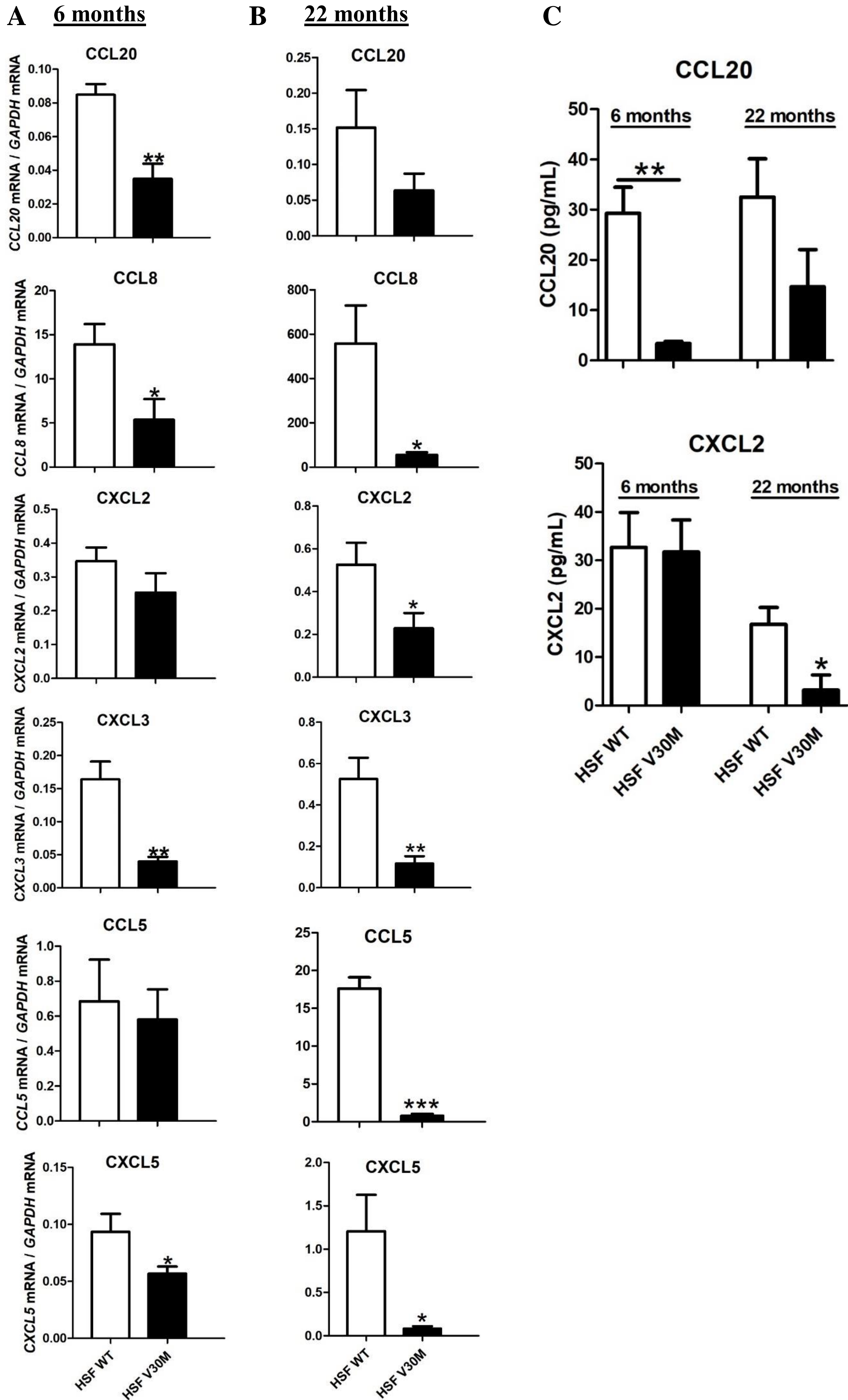

A
6 months
B
22 months
C
CCL20
CCL20 mRNA / GAPDH mRNA
**
CCL8
CCL8 mRNA / GAPDH mRNA
*
CXCL2
CXCL2 mRNA / GAPDH mRNA
CXCL3
CXCL3 mRNA / GAPDH mRNA
***
CCL5
CCL5 mRNA / GAPDH mRNA
CXCL5
CXCL5 mRNA / GAPDH mRNA
HSF WT
HSF V30M
CCL20 (pg/mL)
CXCL2 (pg/mL)
6 months
22 months

Figure 1. The levels of several chemokines were downregulated in an ATTRv mouse model of the disease. RNA was extracted from sciatic nerve of HSF WT and HSF V30M mice with 6 (A) and 20 (B) months of age and the levels of several chemokines were found highly downregulated in the sciatic nerve of HSF V30M mice at both ages. (C) Decreased plasma levels of CCL20 and CXCL2 were measured by ELISA in the serum of HSF V30M mice with 6 and 20 months of age. Data were analyzed using one-way ANOVA followed by Bonferroni post-test and represented as mean±s.e.m (*p<0.05; **p<0.01; ***p<0.001).

Schwann cells are activated by WT TTR, but not by V30M TTR

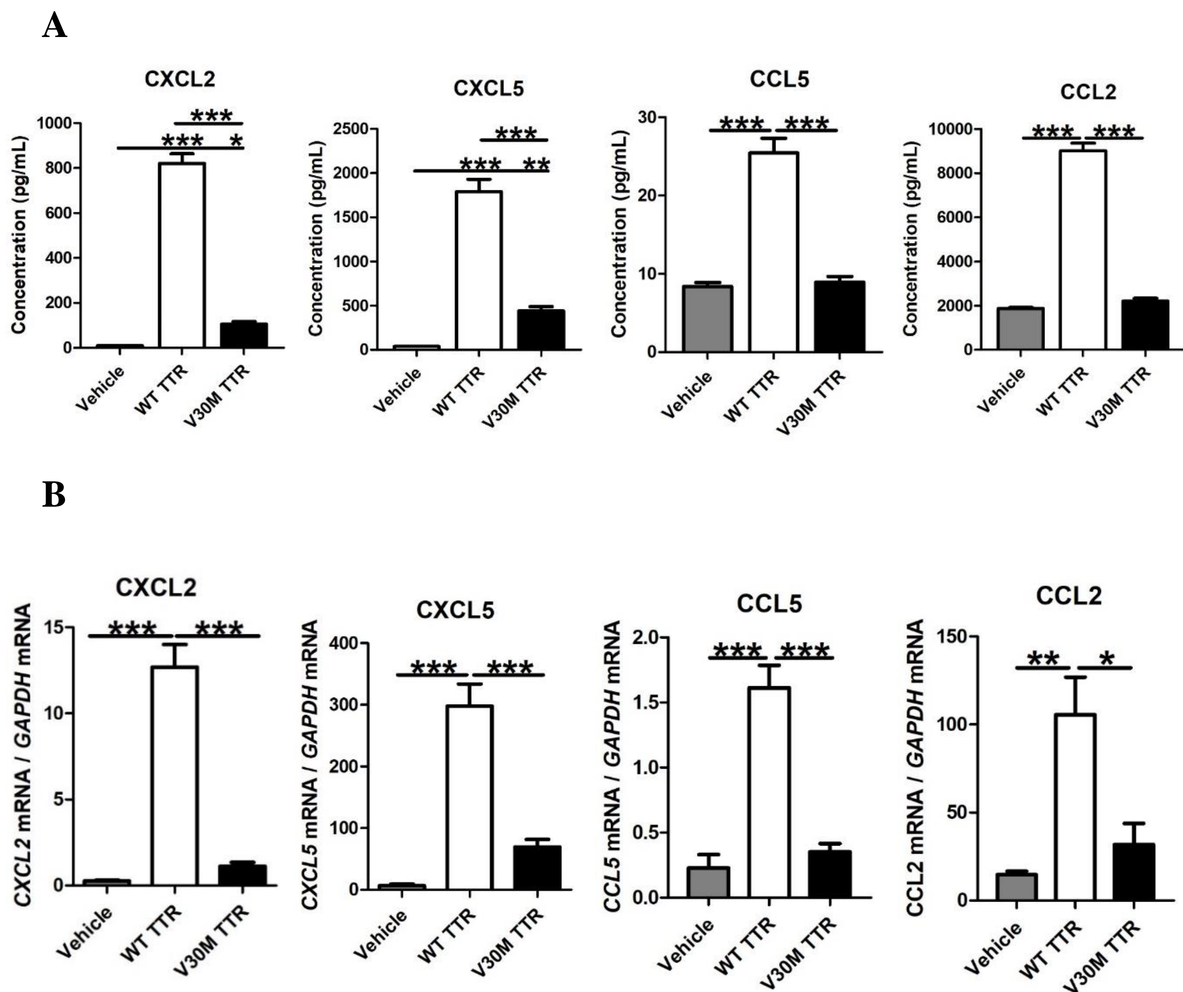
SCs, the myelinating glial cells of the peripheral nervous system, play a crucial role in the generation, function and maintenance of peripheral nerves (Chen et al., 2007). In fact, SCs act as the nerve sentinel cells and have the ability to regulate the PNS immune response by secreting cytokines and chemokines, thus playing a central role in nerve repair (Scholz & Woolf, 2007; Ydens et al., 2013). Because the expression of chemokines is limited in the nerve of ATTRv mouse models and because TTR accumulates in the nerves of ATTRv, we next studied the inflammatory profile of SCs in response to WT and V30M TTR.

For that, mouse SCs were incubated with human WT or V30M TTR for 24 hours and the secretion of a series of chemokines measured by luminex. Whereas WT TTR activated SC leading to the secretion of all tested chemokines, V30M TTR failed to do so (Figure 2A). To understand if the observed differences resulted from a transcriptional alteration, SCs were stimulated as above, RNA was extracted and the expression levels of the chemokines were assessed by real-time PCR. In accordance with the protein results, the expression of all tested chemokines was upregulated in SCs incubated with WT TTR, but not with V30M TTR (Figure 2B). Additionally, an upregulation of the transcription of CCL20, CXCL3 and CCL8, which were altered in the mouse model, was detected in SCs stimulated with WT TTR, but not with V30M TTR (Figure 2C). These results indicate that WT TTR, but not mutated TTR, activates SCs leading to the production of proinflammatory chemokines. Furthermore, we incubated SCs with mouse TTR as a control, measured the expression of chemokines and observed similar results between SCs incubated with mouse or human WT TTR (data not shown).

To address how broad the response of SCs to TTR is, we also measured the expression of several cytokines upon SC stimulation. The expression of IL-6 induced in SCs by WT TTR

was significantly higher than that induced by V30M TTR (Figure 2D). Both TNF and IFN-β were poorly induced by either form of TTR (Figure 2D).

Lastly, we evaluated if incubation of SCs with WT or V30M TTR induced signal transduction pathways by measuring the phosphorylation of the MAP kinase p38 over time post-stimulation, by western blot analysis. MAP kinase p38 regulates multiple cellular functions, including cell proliferation, differentiation, stress response, apoptosis, and cell migration and survival, among others, by interacting with a plethora of substrates (Cuenda & Sanz-Ezquerro, 2017; Gupta & Nebreda, 2015). p38 is activated by a wide range of environmental stimuli, inflammatory cytokines, PAMPs (pathogen-associated molecular patterns), and DAMPs (danger-associated molecular patterns) (Kyriakis & Avruch, 2012). Whereas an increase of phosphorylated p38 was observed upon activation of SCs with WT TTR, the very opposite was observed in SCs incubated with V30M TTR (Figure 2E). These observations suggest that WT TTR activates SCs to produce several immune mediators, while V30M TTR does not.



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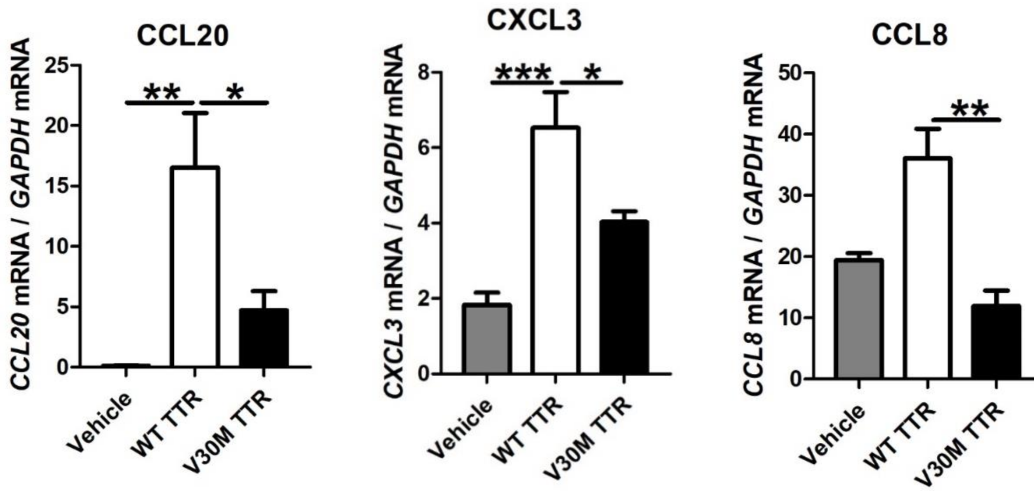

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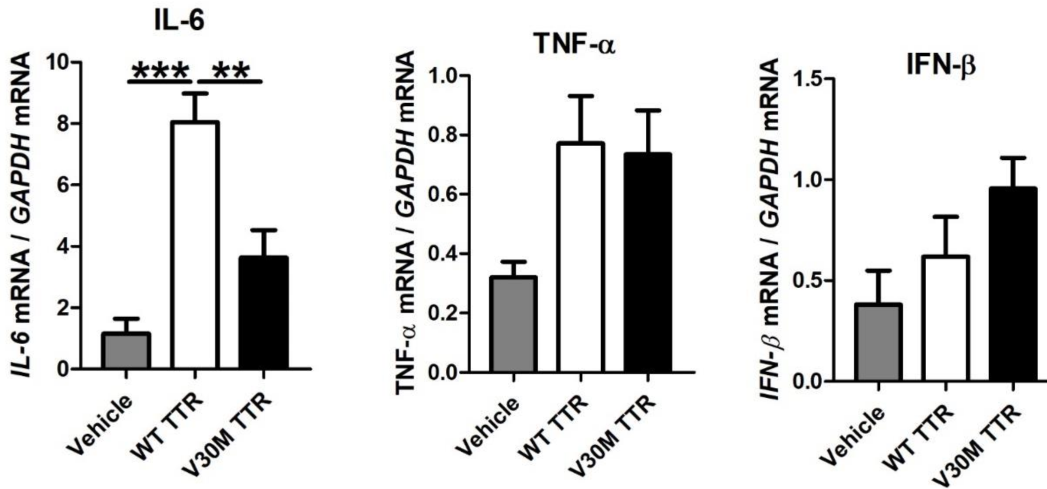

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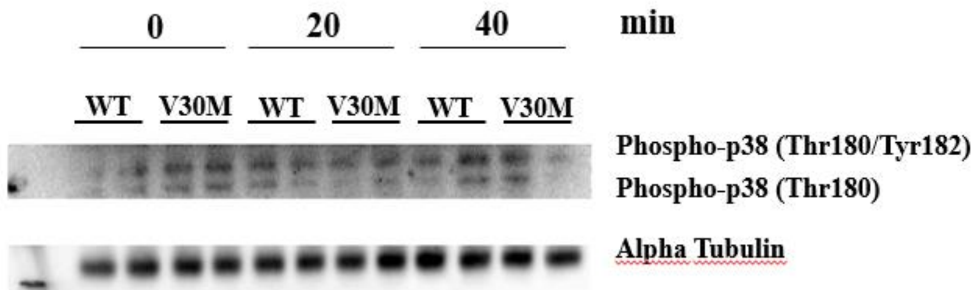


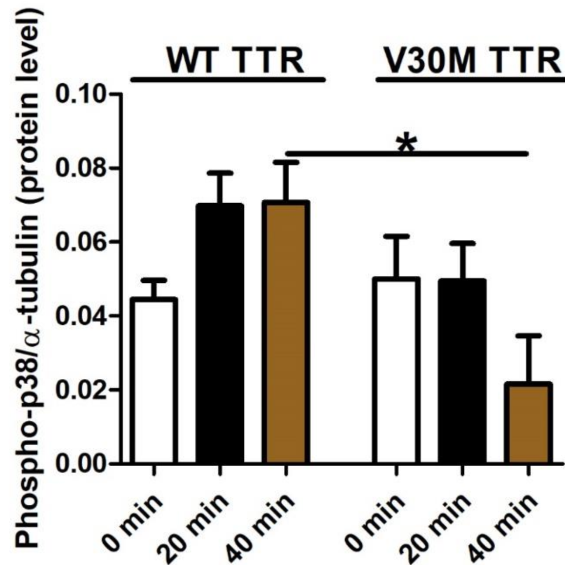


Figure 2. The expression of chemokines were downregulated in Schwann cells incubated with V30M TTR. (A) A Luminex analysis showed a decreased expression of chemokines in SCs incubated with V30M TTR when compared with SCs incubated with WT TTR for 24 hours. (B) RNA was extracted from SCs incubated with WT and V30M TTR and the levels of chemokines were found highly downregulated with V30M TTR incubation. (C) Reduced levels of CCL20, CCL8 and CXCL3 were determined by RT-PCR in SCs incubated with V30M TTR when compared to WT TTR. (D) The expression of cytokines were assessed by RT-PCR in SCs incubated with WT or V30M TTR. (E) Western blot analysis for the phosphorylation of p-38 in SCs incubated with WT or V30M TTR at different time points. Were observed two forms of Phospho-p38 corresponding to Phospho-p38 MAP Kinase dually phosphorylated at threonine 180 and tyrosine182, and with Phospho-p38 singly phosphorlyated at Thr180. Data were analyzed using one-way ANOVA followed by Bonferroni post-test and represented as mean±s.e.m (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Schwann cells are activated by other mutated TTR variants

So far, over 150 mutations in the primary sequence of TTR have been identified, most of which are associated with amyloidosis (Saraiva, 2001; Sipe et al., 2016). Therefore, we questioned if other mutated TTR variants besides V30M also failed to activate SCs. For that, SCs were incubated with WT TTR as a control, I68L TTR (a cardiomyopathy mutation), T119M TTR (a non-pathological mutation) and I84S (a mutation that leads to carpal tunnel syndrome and affects heart and eye) for 24 hours and assessed the expression levels of several chemokines by real-time PCR, as before. The I84S mutation also serves as a control since it has lower affinity for the TTR-thyroxine and TTR-RBP complexes (Pappa et

al., 2015) serving to rule out the effect of these ligands in the assays. TTR molecules carrying other pathological mutations (I68L and I84S) or the non-pathological mutation T119M activated SCs, leading to an expression of chemokines globally similar to that induced by WT TTR (Figure 3). The expression of IL-6 was also similar between SCs incubated with WT or mutant TTRs (Figure 3). Altogether, these results indicate that in contrast with V30M TTR, other mutations in the TTR protein did not impair SCs activation to induce chemokine expression.

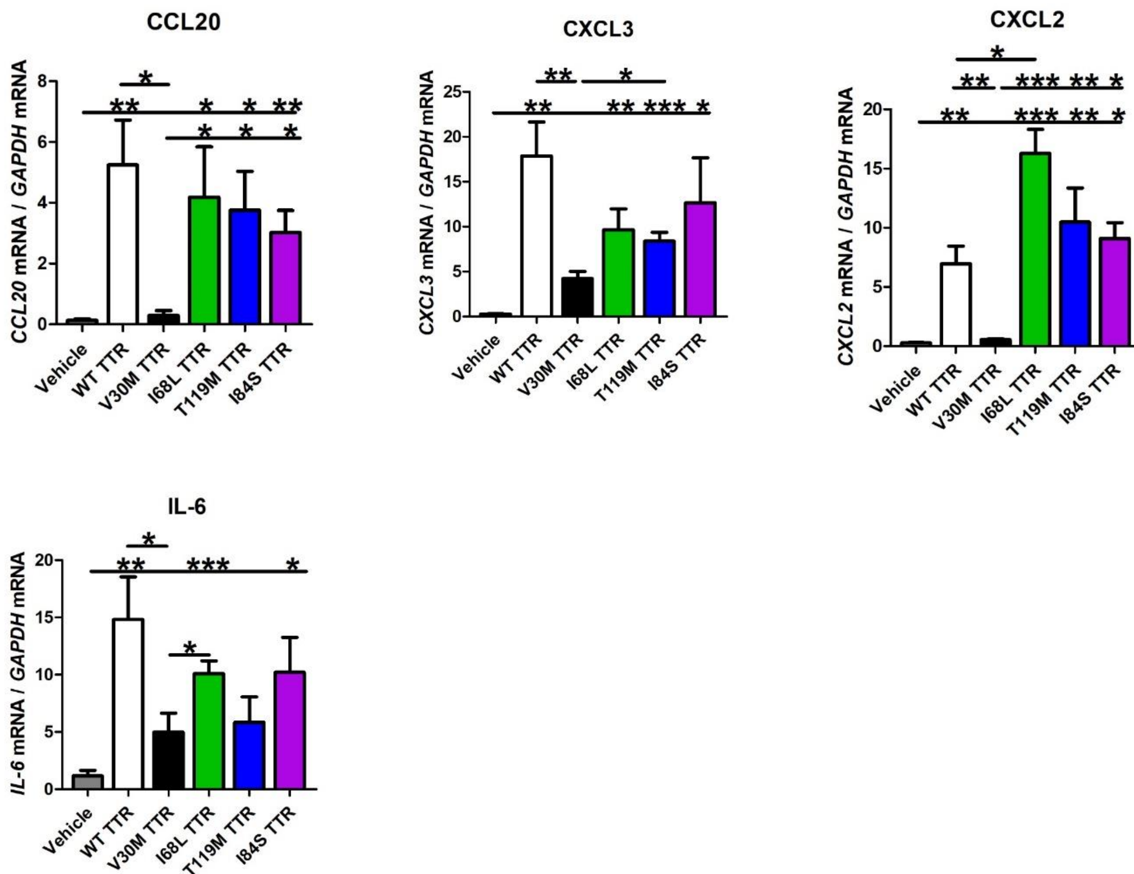
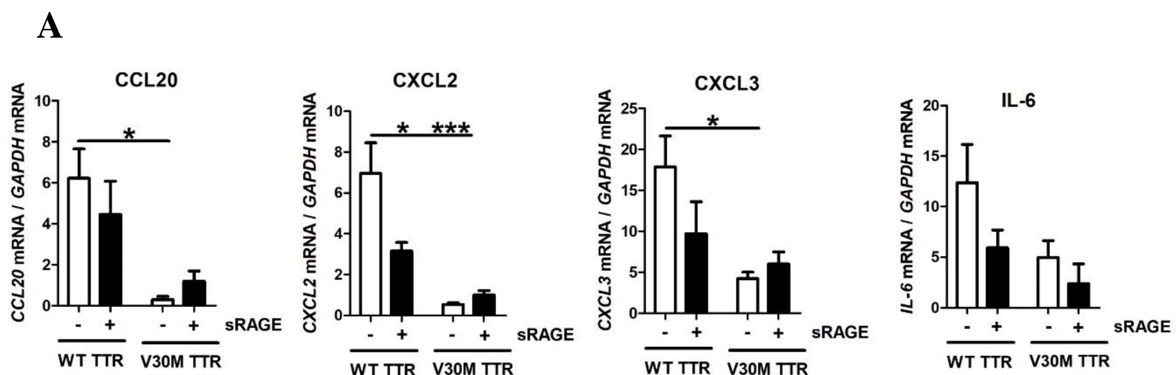


Figure 3. Schwann cells are activated by other TTR variants. (A) Schwann cells were activated by different TTR pathological or non-pathological mutations, leading to an expression of chemokines globally similar to that induced by WT TTR. IL-6 expression was also similar between SCs incubated with WT or mutant TTRs. Data were analyzed using one-way ANOVA followed by Bonferroni post-test and represented as mean±s.e.m (*p<0.05; **p<0.01; ***p<0.001).

TLR4 mediates the expression of chemokines in Schwann cells incubated with WT TTR

Several studies suggest a role for RAGE receptor in mediating TTR signaling mechanisms (Sousa, Du Yan, et al., 2001; Sousa, Yan, et al., 2000). Thus, we investigated the role of RAGE in mediating the activation of SCs by TTR. Inhibition of the RAGE receptor with an antagonist (sRAGE) did not significantly impact the expression of chemokines or of IL-6 by SCs incubated with WT TTR (Figure 4A). Since RAGE interacts with TLR4 (van Zoelen et al., 2009; Zhong et al., 2020), we next questioned whether TLR4 may itself play a role in the induction of chemokines by SCs incubated with TTR. TLR4 inhibition with the antagonist CLI-095 highly reduced the expression of chemokines and of IL-6 induced by WT TTR stimulation of SCs (Figure 4B). As before, V30M TTR only induced residual expression of the tested genes, which was not altered by the presence of the TLR4 inhibitor (Figure 4B). The expression data for CCL20 and CXCL2 were further validated at the protein level (Figure 4C). Collectively, our findings indicate that WT TTR triggers TLR4 signaling in SCs thus leading to the expression and secretion of several chemokines.

Given these findings, we then questioned if TLR4 also mediated the activation of SCs by other TTR mutations. For that, we incubated SCs with WT, I68L, T119M or I84S in the presence of antagonists for TLR4 and RAGE as above. Overall, the TLR4 inhibition by CLI-095 reduced the expression of all tested chemokines in SCs incubated with all mutant forms of TTR tested (Figure 4D). We also measured the cytokine IL-6 with similar results (Figure 4D).



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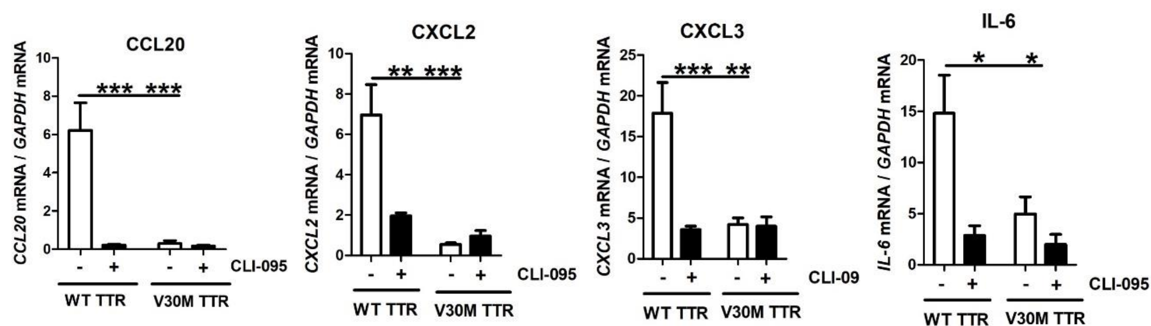

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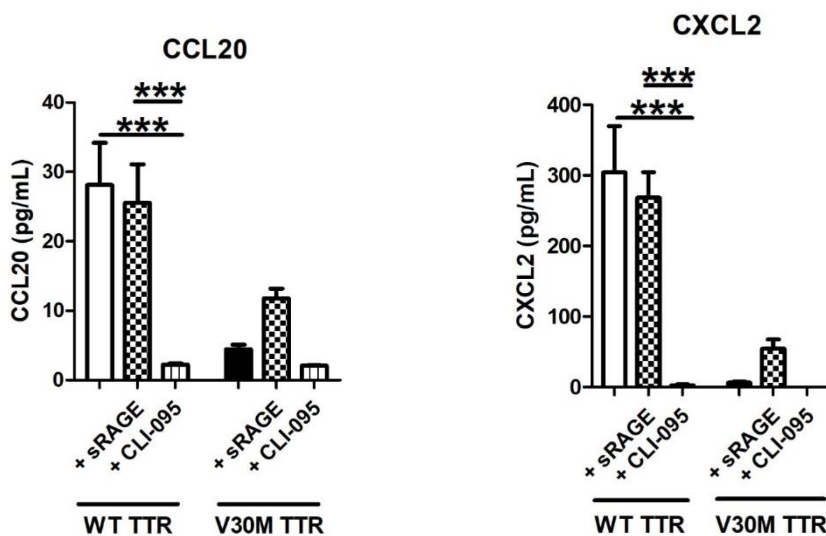

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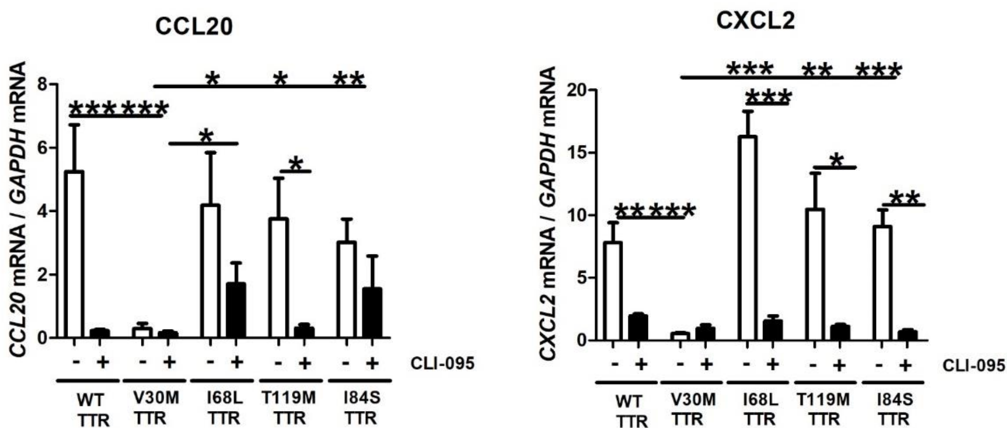

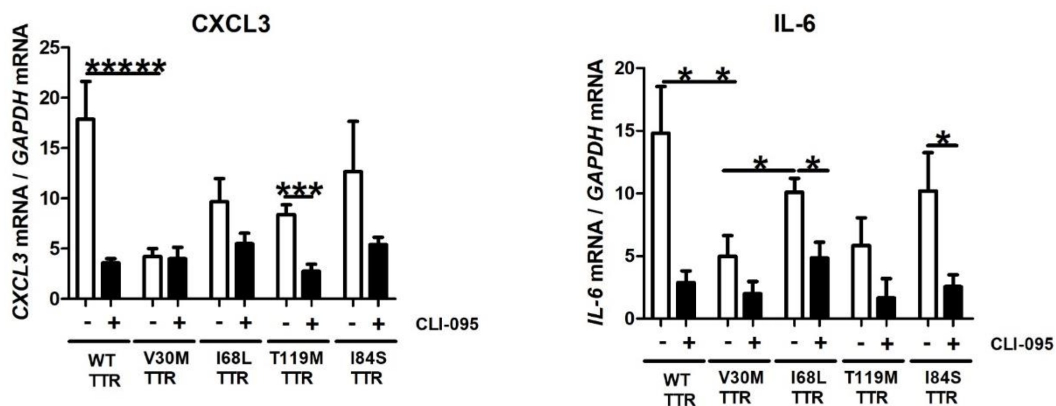

Figure 4. WT TTR activates TLR4 signaling pathway and V30M TTR fail to do so. RNA was extracted from SCs incubated with WT and V30M TTR in the presence of TLR4 (A) and RAGE (B) antagonist and denoted a downregulation in the expression of several chemokines in SCs incubated with WT TTR but not in SCs incubated with V30M TTR. (C) Decreased concentration levels of CCL20 and CXCL2 in the supernatant of SCs incubated with WT TTR in the presence of TLR4 antagonist. (D) SCs were incubated with pathological and non-pathological mutations in the presence of TLR4 antagonist and the inhibition of this receptor reduces the expression of some chemokines.
Data were analyzed using one-way ANOVA followed by Bonferroni post-test and represented as mean±s.e.m (*p<0.05; **p<0.01; ***p<0.001).

Discussion

In the present study, we showed an impairment in the production of several chemokines in the peripheral nervous system of a pre-clinical model of TTR V30M amyloidosis. Additionally, we demonstrate that WT TTR activates the TLR4 receptor in cultured SCs contrariwise to V30M TTR. We consequently hypothesize that SCs are compromised in their function in ATTRv patients, which may explain the downregulated expression of several chemokines and the lack of inflammatory infiltrates observed in peripheral nerves.

The inflammatory response has been shown to be important for several neurodegenerative disorders. (Amor et al., 2010). Immune responses are typically mediated by immune cells such as antigen-presenting cells, macrophages or T-cells. However, also non-immune cells such as SCs in the PNS might play a key role in innate and adaptive immune responses (Meyer zu Horste et al., 2008; Ydens et al., 2013). Regarding to ATTRv, despite cytokine production by axons, no influx of inflammatory cells is found in patients nerve biopsies, suggesting that mechanisms must operate to prevent the correct innate immune response (Misu et al., 1999; Nyhlin et al., 2000). Consistent with this observation, our laboratory previously showed that in V30M mouse model, 7 days post sciatic nerve injury, neutrophil and macrophage infiltration is lower when compared to WT mice, which is indicative of a specific phenotype associated with the V30M mutation. We hypothesized that a non-optimal activation of SCs occurred since, after injury, decreased expression of pro-inflammatory cytokines and chemokines was observed, thus culminating in a diminished immune cellular activation and infiltration in V30M nerves (Goncalves, Vieira, et al., 2014). Likewise, in nerves of ATTRv patients, SCs are impaired in their ability to express chemokines and neurotrophic factors important to drive tissue regeneration, contributing this way for neuronal dysfunction present in the disease (Sousa, Du Yan, et al., 2001). In line with previous findings, our data demonstrate that several chemokines such as CCL20, CCL8, CCL5, CXCL5, CCL2, CXCL2 and CXCL3 were highly downregulated in HSF V30M TTR mice in both peripheral nerves and plasma samples, pointing towards a close interface between the absence of chemokine response and cellular infiltration with the development of the disease. Chemokines are also produced by other cells than SC. The fact that lower levels of chemokines were observed in circulation may hint at differences in the response of other cell types (in addition to SC) to WT or V30M TTR. Future studies addressing this hypothesis are needed.

It will be important to address the differential chemokine production in human patients. Our preliminary data obtained for different chemokines in the human plasma suggested only minor differences between a small group of ATTRv patients and controls (data not shown).

However, this apparent contradiction with the animal data may be explained by several reasons. Firstly, further studies on a greater number of ATTRv blood samples from carriers of different TTR mutations are required to strengthen the results obtained so far and, ultimately, to evaluate whether blood levels of CCL20 or other chemokine can be used as a possible biomarker for ATTRv. Secondly, by measuring the total levels of circulating chemokines, we may be overcoming a possible local downregulation observed in the sciatic nerve. Additionally, in contrast to transgenic mice and cultured SCs, the vast majority of ATTRv patients are heterozygous for the mutation and, therefore lower levels of WT TTR are present in circulation. Even lower than normal levels of WT TTR may be sufficient to stimulate the TLR4 receptor correctly. Therefore, in future studies, it will be interesting to understand if the addition of WT TTR can rescue the V30M TTR phenotype. Recent studies suggest that ATTRv patients have altered levels of several cytokines in the serum, some of them involved in pro- and anti-inflammatory response (Azevedo et al., 2019; Goncalves, Vieira, et al., 2014). In fact besides neuropathic condition, patients with ATTRv also present gastrointestinal symptoms, cachexia, malnutrition, diarrhea and others symptoms (Plante-Bordeneuve & Said, 2011).

SCs play an important role in immune surveillance and have the capacity to detect pathogens and to orchestrate an ensuing immune response (Stratton et al., 2016). Charcot–Marie–Tooth disease, Guillain–Barré syndrome, schwannomatosis and chronic inflammatory demyelinating polyneuropathy are all neuropathies involving SCs (Campana, 2007). It is well known that SCs differentiation and migration plays a critical role to guide and support axonal growth (Jessen & Mirsky, 2005; Richner et al., 2014) and, in ATTRv patients, SCs are impaired in their ability to express chemokines and neurotrophic factors important to drive tissue regeneration, contributing in this way for neuronal dysfunction present in the disease (Sousa, Du Yan, et al., 2001). Taken together the fact that TTR gene is expressed in SCs suggesting a specific role of TTR in the peripheral nervous system, such as SC–neuron interaction (Murakami et al., 2010; Samara et al., 2013) and the compromised nerve regeneration after injury in the transgenic V30M mouse model (Goncalves, Teixeira-Coelho, et al., 2014), we decided to investigate the role of SCs in ATTRv immune response. Interestingly, we found that SCs are activated by incubation with WT TTR contrariwise to incubation with V30M TTR and respond with the production of several chemokines and cytokines. Additionally, we also observed an increase in the phosphorylation of MAP kinase p38 in SCs incubated with WT TTR. p38 mediates inflammatory responses partly through activating gene expression. Proteins phosphorylated by a mechanism dependent on MAP kinase p38 activity include sequence-specific transcription factors, transcriptional regulators, nucleosomal proteins, and regulators of mRNA translation (Zarubin & Han, 2005). The lower phosphorylation of p38 in SCs

incubated with V30M TTR could help to explain the lack of inflammatory cells observed in ATTRv patient's nerve biopsies. However, additional studies are required to further investigate this finding.

Moreover, we implicate TLR4 as the receptor being triggered by TTR in SCs. It was previously shown that neurite outgrowth was impaired in DRG neurons from TTR-knockout mice, and the addition of WT TTR could rescue that condition (Fleming et al., 2007). Thus, WT TTR secreted from SCs might play important roles in maintaining the microenvironment of the peripheral nervous system necessary for nerve regeneration (Fleming et al., 2009; Fleming et al., 2007). Contrariwise, SCs incubated with V30M TTR do not respond with the expression of several chemokines important for the chemoattraction of macrophages, neutrophils and other immune cells. This may be the reason underlying the lack of significant inflammatory infiltrates in the nerves of animal models and ATTRv patients.
The reason why only V30M TTR variant fails to activate SCs remains unclear and additional studies are required to unveil and understand the molecular pathways underlying this observation. However, it is tempted to speculate that only the neuropathic form of TTR fails to activate SCs. To further understand the activation of SCs by TTR variants, we incubate these cells with other pathological and non-pathological mutated forms of TTR. Interestingly, all tested variants triggered the expression of chemokines in SCs via TLR4 globally similar to what was observed for WT TTR. It is possible that the V30M mutation might acquire a molecular or conformational specificity that impairs the activation of SCs, leading to disease progression. Independently of the mechanism, the dissection of the cellular pathways involved in this disease is of greatest importance for the enhancement of new treatment approaches, which as our findings suggest may need to be adjusted to the mutation present in TTR.

Taking into consideration the role of WT TTR in the activation of SCs and consequential expression of chemokines, it would be interesting in the future to investigate if TTR may also trigger a chemokine response in other innate immune cells, such as macrophages, the main phagocytic cells of the immune system. In fact, ATTRv patients display quantitative and qualitative abnormalities in macrophages with defects in cell adhesion and chemotaxis (Tsukuba et al., 2009). Additionally, it was recently demonstrated that the number of heart resident macrophages is significantly decreased in ATTRv patients as compared with healthy donors (Suenaga et al., 2016). Therefore, the possible role of TTR in inflammation and immune response, as well as the underlying mechanisms, needs to be further elucidated.

Overall, we show that WT TTR activates SCs via TLR4 receptor leading to the expression of several chemokines. Critically, V30M TTR does not activate SCs thus failing to induce the expression of chemokines. How these mediators interact in the context of ATTRv may be crucial to explain the immunological impairment observed in peripheral nerves of ATTRv patients, because all of these observations suggest that V30M TTR mutation may be responsible for modify the function of certain signaling pathways.

Acknowledgements

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Chapter III

TLR2/4 signaling pathways are altered in macrophages from V30M TTR mice with downregulated expression of chemokines

TLR2/4 signaling pathways are altered in macrophages from V30M TTR mice with downregulated expression of chemokines

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Key words: transthyretin; familial amyloidotic polyneuropathy; macrophages; tool-like receptors, chemokines; immune regulation.

Abstract

Hereditary transthyretin amyloidosis (ATTRv) is a fatal neurodegenerative disorder, first identified in Portugal, characterized by the extracellular deposition of aggregates and fibrils of mutant forms of transthyretin (TTR), particularly in the nerves and ganglia of the peripheral nervous system. The most common TTR mutation in ATTRv results from an exchange of a methionine for a valine at position 30 (V30M). Macrophages promote homeostasis by responding to changes within the body, not only as phagocytes in defense against microbes and in clearance of dead cells, but also through trophic, regulatory and repair functions. Macrophages are engaged in removal of TTR deposits in ATTRv patients.

Here, we show a significant downregulation in the expression of several chemokines by bone marrow derived macrophages (BMDM) from V30M TTR mice upon stimulation with TLR4 and TLR2 receptors agonists. Additionally, the phosphorylation of p38 MAP kinase, important for TLR4 and TLR2 signaling pathways, was also downregulated in V30M macrophages.

Overall, our data contribute with new insights to unravel the molecular mechanisms underlying the lack of inflammatory immune responses observed in ATTRv patients, more specifically to pattern recognition receptors signaling and chemokines/cytokines network, which is fundamental for the development of new immune therapeutic strategies for the disease.

Introduction

Amyloidosis is a term used to define a wide-ranging group of progressive and fatal protein folding disorders pointing up the aggregation and tissue deposition of one of more than 40 distinct proteins (Benson et al., 2020). Soluble amyloidogenic oligomers are believed to have cytotoxic effects, and the formation of amyloid deposits causes disruption of normal cellular function with alarming consequences (Merlini & Westermark, 2004; Sousa, Cardoso, et al., 2001). One group of amyloid diseases is associated with transthyretin (TTR), a carrier protein for thyroxin and retinol (Kanai et al., 1968; Woeber & Ingbar, 1968), mainly synthesized in the liver and choroid plexus of the brain, being secreted into the blood stream and cerebrospinal fluid, respectively (Soprano et al., 1985). Over 150 mutations in the primary sequence of TTR have been identified, most of which are associated with amyloidosis (Rowczenio et al., 2014).

Hereditary amyloid transthyretin (ATTRv) amyloidosis is a fatal neurodegenerative disorder characterized by the extracellular deposition of aggregates and fibrils of mutant forms of TTR, particularly in the nerves and ganglia of the peripheral nervous system (PNS) (Sousa & Saraiva, 2003). The most common TTR mutation in ATTRv results from an exchange of a methionine for a valine at position 30 (TTR V30M) (Saraiva et al., 1984). ATTRv initially presents with symptoms that are associated with sensory and autonomic nervous system dysfunction. These include loss of pain and temperature sensation in the distal limbs, gastrointestinal disturbances and bladder dysfunction (Sousa & Saraiva, 2003). A striking feature of ATTRv is that, despite the presence of cytokine-driven neuroinflammation, no immune cellular infiltration is usually found near TTR deposits (Sousa, Cardoso, et al., 2001; Sousa, Du Yan, et al., 2001), suggesting that mechanisms must operate to prevent or inhibit the correct innate immune response. Moreover, nerves from ATTRv patients present impaired production of agents that mediate regeneration such as neurotrophins and chemokines (Sousa, Du Yan, et al., 2001). In line with that, we recently demonstrated that Schwann cells (SCs) are activated by WT TTR via TLR4 receptor leading to the expression of several chemokines. On the other hand, V30M TTR does not activate SCs thus failing to induce the expression of chemokines (Moreira et al., 2021). Regarding animal models, V30M mice display a downregulated innate immune response in response to nerve injury when compared to WT mice (Goncalves, Teixeira-Coelho, et al., 2014).

Among the cells of the immune system, macrophages are phagocytes defined as movable cells with the capacity to engulf and digest pathogens, particles and dead cells (Gordon, 2008). Thus, phagocytes have dual functions, as immune sentinels but also as regulators of tissue homeostasis (Tauber, 2003). In ATTRv patients, macrophages and fibroblasts play

a central role in the degradation of aggregated TTR (Misumi et al., 2013). However, recent studies suggest that ATTRv patients display quantitative and qualitative abnormalities in tissue resident macrophages, and these abnormalities may accelerate TTR amyloid deposition in some organs (Suenaga et al., 2016). All these observations associated with the fact that the mechanism of decreased tissue-resident macrophage activity in ATTRv patients is still unknown, prompted us to investigate the response of bone marrow derived macrophages (BMDM) from wild-type (WT) and V30M mice to several TLR agonists and the respective expression of chemokines. Elucidating how these mediators interact in the context of ATTRv may be crucial to explain the immunological impairment observed in peripheral nerves of ATTRv patients.

Material and Methods

Animals

All animal experiments were carried out in accordance with National and European Union guidelines for the care and handling of laboratory animals and were performed in compliance with the institutional guidelines and recommendations of the Federation for Laboratory Animal Science Association (FELASA) and approved by the National Authority for Animal Health (DGAV; Lisbon, Portugal). A transgenic mouse model for human TTR V30M in an endogenous TTR null background (labeled as V30M) (Kohno et al., 1997) and backcrossed for 10 generations into the Sv/129 background, was used. V30M TTR mice and the respective Sv/129 WT control were used for BMDM preparation at 6 months of age. Animals were housed in a controlled temperature room, maintained under a 12-h light/dark period, with water and food ad libitum and then sacrificed with a lethal injection of a premixed solution containing ketamine (75 mg/kg) plus medetomidine (1 mg/kg).

Recombinant transthyretin

Recombinant WT and V30M TTR were produced in a bacterial expression system and purified as previously described (Furuya et al., 1991).

Recombinant transthyretin purification

Prior to use, endotoxins were removed from WT and V30M TTR. Briefly, Detoxi-Gel endotoxin resin (Thermo Fisher Scientific, Waltham, MA, catalog#20339) was packed appropriately in a chromatography column and was allowed to settle for 30 minutes. Detoxi-Gel Resin was regenerated by washing with five resin-bed volumes of 1% sodium deoxycholate (Sigma-Aldrich, Germany, catalog#D6750-10G), followed by 3-5 resin-bed volumes of pyrogen-free buffer to equilibrate the resin. Then, the samples were applied to the column and for greater efficiency the samples were incubated with the resin for 1 hour at room temperature. Pyrogen-free buffer was used to collect the flow-through. The protein quantification after elution was assessed by Bradford method (Bradford, 1976).

Dot blot for detecting protein aggregation

Aliquots corresponding to equal amounts of WT and V30M TTR (500 ng) were blotted onto a 0.2-μm pore cellulose acetate membrane and it was left to dry at room temperature for few hours. TTR was immunodetected using an antibody targeting TTR aggregates produced in our lab, mouse CE11 antibody (1:20) followed by anti-mouse horseradish peroxidase antibody (1:5000) and enhanced chemiluminescence visualization (BioRad). No aggregates were detected in all TTR samples.

Bone Marrow Derived Macrophages

For cell culture experiments, cRPMI medium was prepared by supplementing RPMI 1640 with L-glutamine (Lonza, Switzerland, catalog#BE12-702F)) with 10% heat-inactivated fetal bovine serum (FBS), 1% sodium pyruvate, 1% HEPES and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Germany, catalog#M7522). Bone marrow cells from WT and V30M TTR mice were flushed from the femurs and tibia of mice and plated at 0.5×10^6 cells/mL per petri dish (bacterial plates, Sterilin), in 8 mL of culture medium supplemented with 20% L929 cell conditioned media (LCCM). By day four, the cells were fed with 10 mL of the same media, and at day six, macrophages were harvested and seeded into 6 or 12-well tissue culture plates. BMDM from WT and V30M TTR mice were incubated with the toll-like receptor (TLR) 4 agonist lipopolysaccharide (LPS) (250ng/mL /well) in the absence or presence of the TLR4 receptor antagonist (CLI-095) (10μM /well) (InvivoGen, San Diego, CA, catalog#tlrl-cli95); or incubated with the TLR2 agonist Pam3Cys (2μg/mL /well) (InvivoGen, San Diego, CA, catalog#tlrl-pms) or the TLR3 agonist Poly IC (2μg/mL /well) (InvivoGen, San Diego, CA, catalog#tlrl-pic) for different time points. In all experiments, the supernatants were collected, and cell lysates were prepared in trizol (Invitrogen).

Confocal microscopy

BMDM from WT and V30M TTR mice were plated over a glass lipopolysaccharide-free coverslip, in a 24-well plate, and fixed with 4% paraformaldehyde. After fixation, cells were blocked with phosphate buffer supplemented with 10% fetal bovine serum, 1% bovine serum albumin and 0.1% saponin for 1 hour at room temperature and incubated overnight at 4 °C with mouse monoclonal antibody against TLR4 (1:100) (Abcam, United Kingdom, catalog#ab22048). Secondary antibodies included goat anti-mouse Alexa Fluor 488

(1:1000) (Molecular Probes, Eugene, OR, catalog#A11029). Sections were washed and cover-slipped in Vectashield, including DAPI as a nuclear dye (Vector Laboratories, Burlingame, CA, catalog#h-1200) and visualized under a Laser Scanning Confocal microscope Leica SP5.

THP-1 cells

Human THP-1 monocytic cells were grown in RPMI 1640 media containing 10% of FBS. Cells were maintained in a humidified atmosphere with 5% CO2 at 37ºC.
Differentiation into macrophages was achieved by resuspending and plating the cells at 0.5 x 10^6 cells/mL in 12-well tissue culture treated plates in growth medium with the addition of phorbol 12-myristate 13-acetate (PMA) at 100nM for 24 hours. PMA was prepared from a stock solution at 2.5mg/mL in dimethyl sulfoxide. Stock was diluted with medium to obtain the final concentration. After 24 hours, the PMA was removed, and the cells were incubated with normal medium and rested for 48 hours. After this period, the macrophages were able for the experiments.

RNA extraction, cDNA conversion and Real-Time PCR

RNA was extracted from BMDM from WT and V30M TTR mice with 6 months of age and from human THP1 monocytic cells by phenol extraction (Trizol) (Invitrogen, Waltham, MA, catalog#15596026). First-strand complementary DNA (cDNA) was synthesized using the SuperScript double-stranded cDNA Kit (Invitrogen, Waltham, MA) and quantitative real-time PCR performed using the iQ Syber Green Super Mix (Bio-Rad). Samples were run in duplicate and results analyzed by the Bio-Rad CFX96 software. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as reference gene. For the quantification of mRNA expression levels, the reaction was performed in a final volume of 20µL containing 0.5µL of each specific primer: mouse Gapdh forward: GCCTTCCGTGTTCCTACC, mouse Gapdh reverse: AGAGTGGGAGTTGCTGTTG; mouse CCL20 forward: TACAGACGCCTCTTCCTT, mouse CCL20 reverse: TCGTGTGAAAGATGATAGCA; mouse CCL5 forward: CAATCTTGCAGTCGTGTT, mouse CCL5 reverse: AATAGTTGATGTATTCTTGAACC; mouse CXCL5 forward: CTACGGTGGAAGTCATAG, mouse CXCL5 reverse: TTCTTTATCACAGGAGCTT; mouse IRF3 forward: ATGTGAACAACTTCCTAAA, mouse IRF3 reverse: CACCTCGAACTCCCATTG; mouse CXCL3 forward: CCAACCACCAGGCTACAG, mouse CXCL3 reverse:

AACTTCTTGACCATCCTTGA; mouse CXCL2 forward: CCAACCACCAGGCTACAG, mouse CXCL2 reverse: CTTCAGGGTCAAGGCAAAC; mouse IFN-β forward: GCACTGGGTGGAATGAGACT, mouse IFN-β reverse: AGTGGAGAGCAGTTGAGGACA; mouse TNF-α forward: ACAAGGCTGCCCCGACTAC, mouse TNF-α reverse: TGGAAGACTCCTCCCAGGTAT; human Gapdh forward: GAGTCCACTGGCGTCTTC, human Gapdh reverse: GATGATCTTGAGGCTGTTGTC; human TNF-α forward: GGGACCTCTCTCTAATCA, human TNF-α reverse: CTACAACATGGGCTACAG; human CCL20 forward: GTGACATCAATGCTATCATCTT, human CCL20 reverse: CTGAGGAGACGCACAATA; human CXCL2 forward: AAGTGTGAAGGTGAAGTC, human CXCL2 reverse: TTTCTGCCCATTCTTGAG; human CXCL3 forward: AACATCCAAAGTGTGAAT, human CXCL3 reverse: TTTCTTCCCATTCTTGAG; human CCL5 forward: GCCCACATCAAGGAGTAT, human CCL5 reverse: GTTCTTTCGGGTGACAAA; human CXCL5 forward: ATCAGTAATCTGCAAGTGT, human CXCL5 reverse: ATTTCCTTCCCGTTCTTC; (all from Sigma), and 18μL of Mix plus 1μL of the newly synthesized cDNA. Primer sequences were designed using Beacon Designer 8 (Premier Biosoft) for all genes. Analysis of real-time PCR data was made by the comparative CT method. Individual relative gene expression values were calculated using the following formula: $2^{-(\text{Ct gene of interest} - \text{Ct constitutive gene})}$ (Schmittgen & Livak, 2008). Primer efficiency is available in Supplementary Table 1.

Gene	Primer efficiency
Mouse Gapdh	98,2
Mouse TNF-α	95,3
Mouse CCL20	92,4
Mouse CXCL2	90,1
Mouse CXCL3	96,3
Mouse CCL5	97,6
Mouse CXCL5	92,7
Mouse IFN-β	107,1
Mouse IRF-3	101,5
Human Gapdh	91,8
Human TNF-α	94,0
Human CCL20	101,5
Human CXCL2	106,5
Human CXCL3	108,3
Human CCL5	105,5
Human CXCL5	91,1

Supplementary Table 1. Primer efficiency (%).

Western blot analysis

Protein was isolated from BMDM from WT and V30M TTR mice with 6 months of age with lysis buffer containing 10μL phenylmethylsulfonyl fluoride (PMSF), 10 μL sodium orthovanadate solution and 10μL protease inhibitor cocktail solution per mL of 1x RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, catalog#sc-24948). Protein concentration was determined using the Bradford protein assay (Bio-Rad, Hercules, CA, catalog#5000006). Subsequently, samples were run on a 10% polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on nitrocellulose WhatmanTM membrane (GE Healthcare). Primary antibodies against p38 MAPK (1:1000) (Cell Signaling, Netherlands, catalog#9212), Phospho-p38 MAPK (1:1000) (Cell Signaling, Netherlands, catalog#4631), Phospho-IRF3 (1:1000) (Cell Signaling, Netherlands, catalog#29047), IkB-α (1:1000) (Cell Signaling, Netherlands, catalog#9242) and Calnexin (1:1000) (Merck, Germany, catalog#AB2301) were diluted in 5% bovine serum albumin in TBS supplemented with 0.1% Tween 20 (TBS-T), overnight at 4 °C. Next day, blots were washed with TBS-T and incubated with anti-rabbit conjugated to horseradish peroxidase (1:5000; The Binding Site) for 45 min at room temperature. After three washes in TBS-T, bands were visualized with enhanced chemiluminescence using the LuminataTM Crescendo (Millipore). Protein bands were quantified by densitometry using Quantity One software (Bio-Rad) and Calnexin quantification was used to correct for total protein loading variation.

Enzyme-linked immunosorbent assay

The levels of CCL20 (R&D Systems, Minneapolis, MN, catalog#MCC200) and TNF-α (R&D Systems, Minneapolis, MN, catalog#MTA00B) in the supernatants of BMDM from WT and V30M TTR mice with 6 months of age were quantitatively determined by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions.

Statistical analysis

Statistical comparison of data was performed using the two-way ANOVA or three-way ANOVA, followed by Bonferroni test as post-hoc analysis with Graph Pad Prism software. Quantitative data are expressed as mean ± SEM. Statistical significance was established for $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$ and $p^{****} < 0.0001$.

Results

Both WT and V30M TTR activate macrophages via TLR4

Recently, we demonstrated that SCs secreted several chemokines in response to WT TTR, a process that was mediated by the TLR4 signaling pathway. In contrast, the secretion of all tested chemokines was compromised upon stimulation of SCs with mutated V30M TTR, suggesting that V30M TTR fails to activate TLR4 signaling pathway in that cell population (Moreira et al., 2021). We now questioned if macrophages may also respond differently to WT or mutated TTR. For that, WT mouse BMDM were stimulated with WT or V30M TTR, both at 2.5μM, and the expression of several chemokines assessed by RT-PCR 6h later. Interestingly, BMDM from WT mice were highly activated by both forms of TTR, with no significant differences observed regarding their chemokine production (Figure 1A). Moreover, incubation with the TLR4 antagonist CLI-095 reduced the expression of chemokines by BMDM incubated with WT or V30M TTR, indicating that both forms of TTR activate the macrophages through TLR4 (Figure 1A). A similar pattern of response is observed for TNF-α (Figure 1B)

Impelled by the previous results, and given the difference in relation to SCs, we wondered how human macrophages responded after incubation with WT or V30M TTR, both at 2.5μM. For that, we used THP1 cells, a human monocytic cell line, that differentiate into macrophages upon PMA incubation. Similarly to what we observed in mouse macrophages, the expression levels of all tested chemokines remained identical in THP1 cells incubated with WT or V30M TTR protein (Figure 1C). Again, a similar pattern was observed in the case of TNF-α (Figure 1D). Furthermore, incubation with TLR4 antagonist CLI-095 reduced the expression of all tested chemokines in THP1 cells incubated with WT or V30M TTR, suggesting that THP1 cells are also activated via TLR4 (Figure 1C and 1D).

Collectively, our findings suggest that BMDM from WT mice and human THP1 macrophages recognize and respond equally to both WT and V30M TTR, upregulating their expression of several chemokines and of TNF-α.

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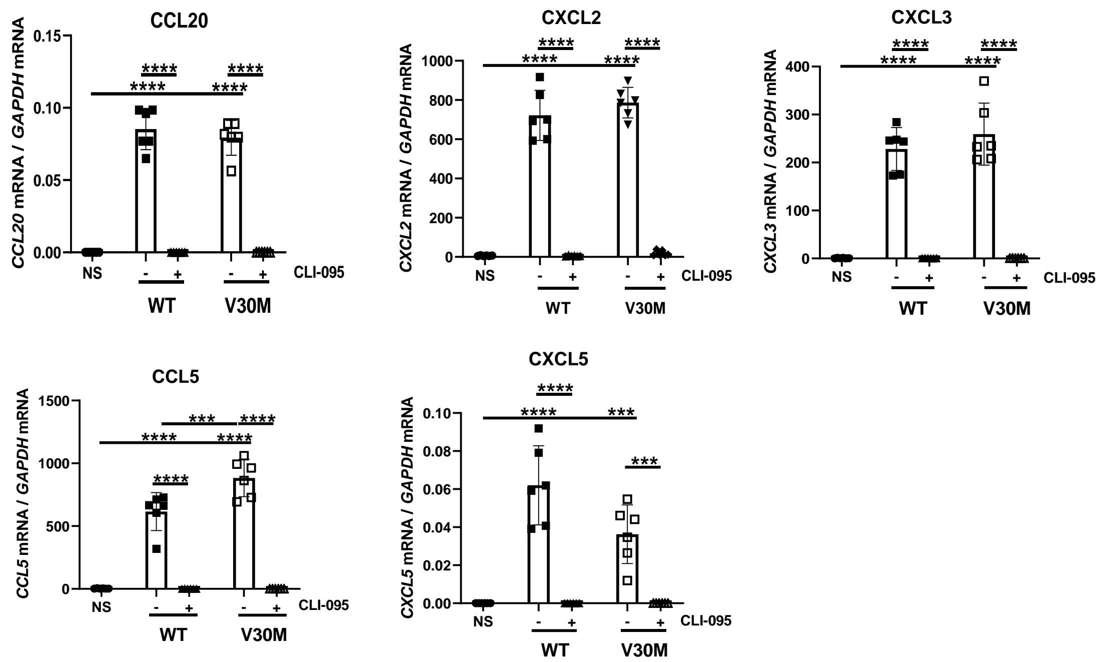

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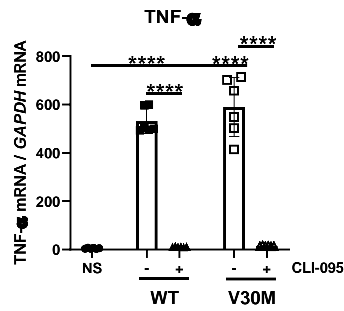

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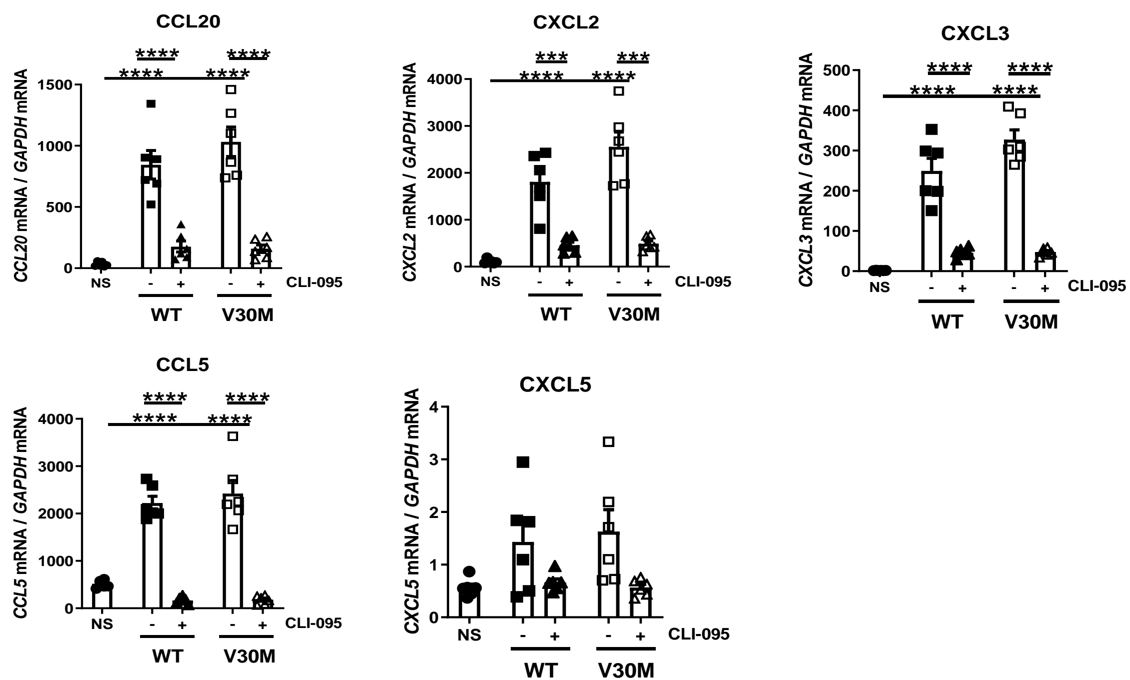

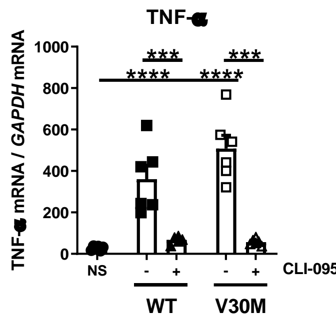

Figure 1. Macrophages respond to both WT and V30M TTR via TLR4. (A) Chemokine expression was assessed in WT BMDM after incubation with WT or V30M TTR in the presence of TLR4 antagonist (Cli-095). (B) The expression of TNF-α was also assessed in WT BMDM after incubation with WT or V30M TTR. (C) RNA was extracted from human THP1 monocytic cells after WT or V30M TTR incubation. (D) The expression of TNF-α was evaluated in human THP1 monocytic cells after incubation with WT or V30M TTR. The results were normalized against Gapdh. Three independent experiments were performed. Data were analysed using two-way ANOVA followed by Bonferroni post-test and represented as mean±s.e.m (***p<0.001, ****p<0.0001).

BMDM derived from WT and V30M TTR animals respond differently to stimulation with different TLR agonists

We showed previously that 7 days post sciatic nerve injury, the neutrophil and macrophage infiltration to the nerve of V30M TTR mouse model was lower when compared to that of WT TTR mice (Goncalves, Teixeira-Coelho, et al., 2014). Considering that macrophages appear to respond to both WT and mutated V30M TTR forms (Figure 1), we next questioned if macrophages derived from V30M TTR mice responded differently to TLR stimulation comparatively to control animals. To address this question, we compare the response to LPS (a TLR4 agonist) in BMDM of different genetic backgrounds: WT TTR vs V30M TTR and measured the expression of several chemokines 6 hours later. Overall, BMDM from V30M TTR mice displayed less expression of chemokines than BMDM derived from WT animals, which is particularly visible for CCL20 (Figure 2A).

As expected a significant downregulation of chemokine expression was observed in the presence of CLI-095 (Figure 2A). To further validate these results, the protein levels of CCL20 were assessed by ELISA in the culture supernatants. Consistent with the mRNA data, a significant decrease in the amount of CCL20 was observed for V30M TTR BMDM as compared to WT TTR BMDM, upon LPS stimulation (Figure 2B). Finally, we also

measured the transcription and secretion of TNF-α. In this case, although the mRNA levels of TNF were not significantly different upon BMDM stimulation of either TTR background, at the protein level a reduction in TNF was observed in the case of LPS-stimulated V30M BMDM (Figure 2C). Overall, these observations suggest that macrophages derived from V30M TTR animals may present a compromised response to LPS stimulation.

We next questioned if the differences observed upon stimulation of the TLR4 pathway, were extended to other TLRs. To address this question, we stimulated BMDM of either genetic background with Pam3Cys (a TLR2 agonist) or Poly IC (a TLR3 agonist). Of note, whereas TLR4 signals through both the MyD88 and the TRIF pathways, TLR2 only depends on the MyD88 adaptor, and TLR3 on the TRIF one (Yamamoto, Sato, Hemmi, Hoshino, et al., 2003; Yamamoto, Sato, Hemmi, Uematsu, et al., 2003). We observed a downregulated expression of several chemokines and of TNF in V30M TTR BMDM upon TLR2 stimulation, similarly to what happened in the case of TLR4 activation (Figure 2D). Chemokine expression in response to TLR3 stimulation was mostly undetectable independent of the genetic background of the BMDM (Figure 2E). To better investigate the TRAM-TRIF signaling pathway, we assessed the expression of interferon regulatory factor 3 (IRF3) and of IFN-β. We denoted a significant downregulation in the expression of this transcription factor in BMDM from V30M TTR mice comparatively to BMDM from WT TTR mice (Figure 2F). The expression of IFN-β was only detected upon TLR4 stimulation and was also downregulated in the case of V30M BMDM (Figure 2F).

Given the impaired TLR response observed for V30M TTR macrophages, we hypothesized that BMDMs of the mutated background might express different levels of TLRs in steady state, which would explain a compromised response to the respective agonists. Thus, we assessed the expression of TLR4, TLR2 and TLR3 in resting WT and V30M BMDM, by RT-PCR. Interestingly, no significant differences were observed regarding TLR2, TLR3 or TLR4 expression in BMDM from either genetic background (see Supplementary Figure 1A). These data were further supported by the immunofluorescence detection and quantification of TLR4, which revealed no differences in WT or V30M BMDM (see Supplementary Figure 1B).

Altogether, our findings indicate that V30M BMDM are impaired in their function, with decreased capacity to respond to TLR stimulus and, consequently, with lower production of chemokines. However, the TLR expression were very similar in both BMDMs.

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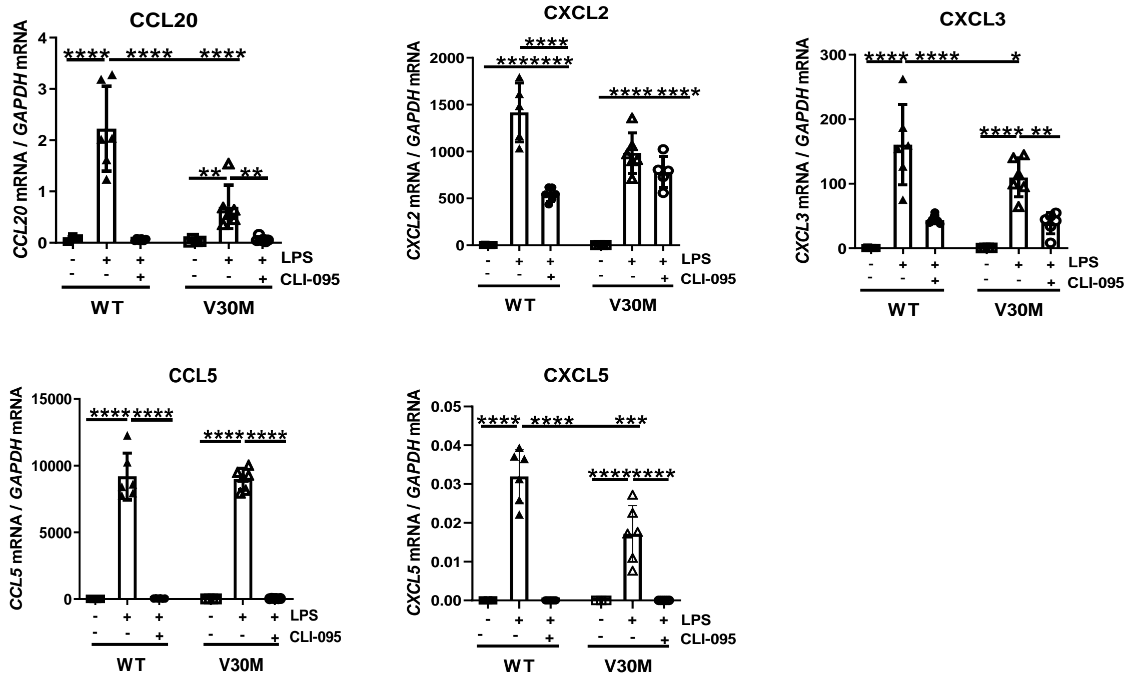

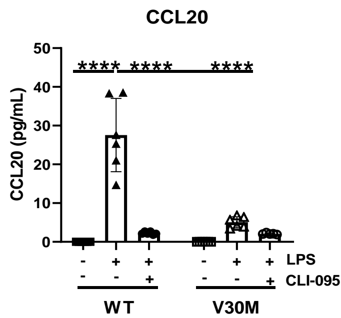

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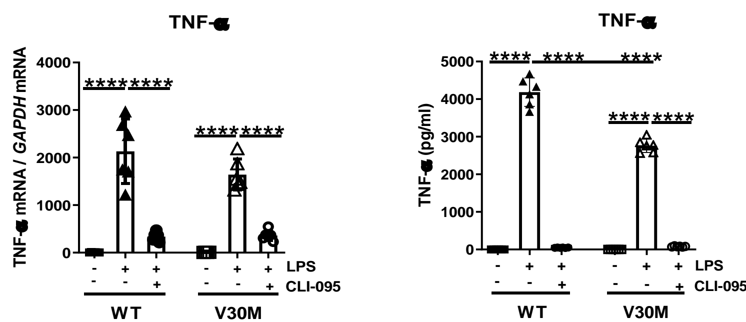

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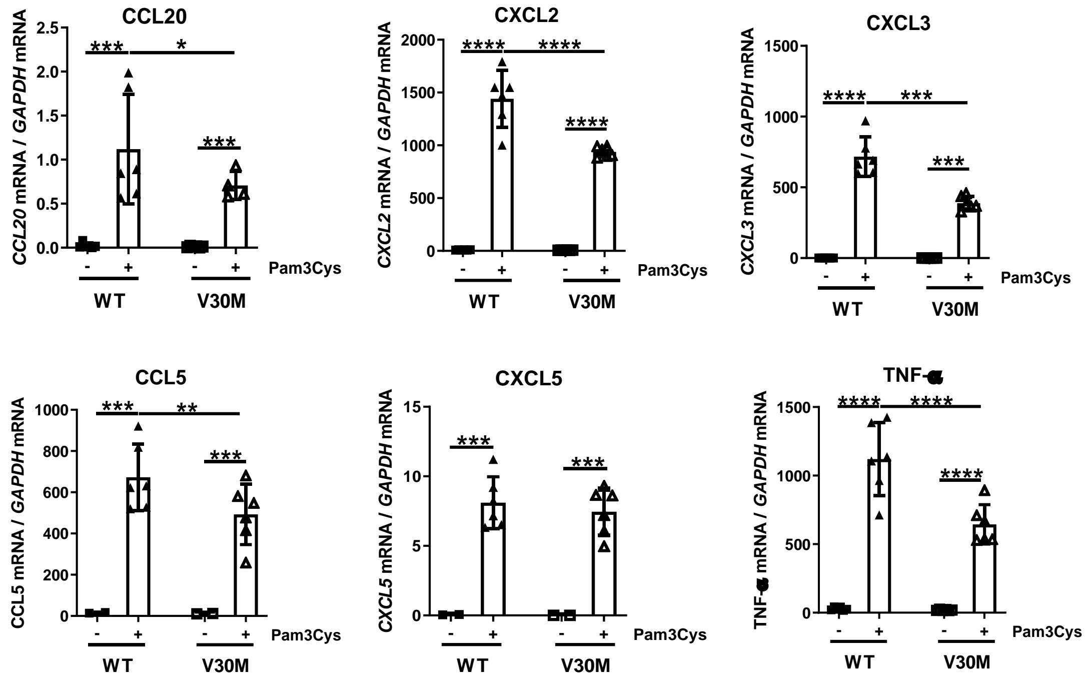

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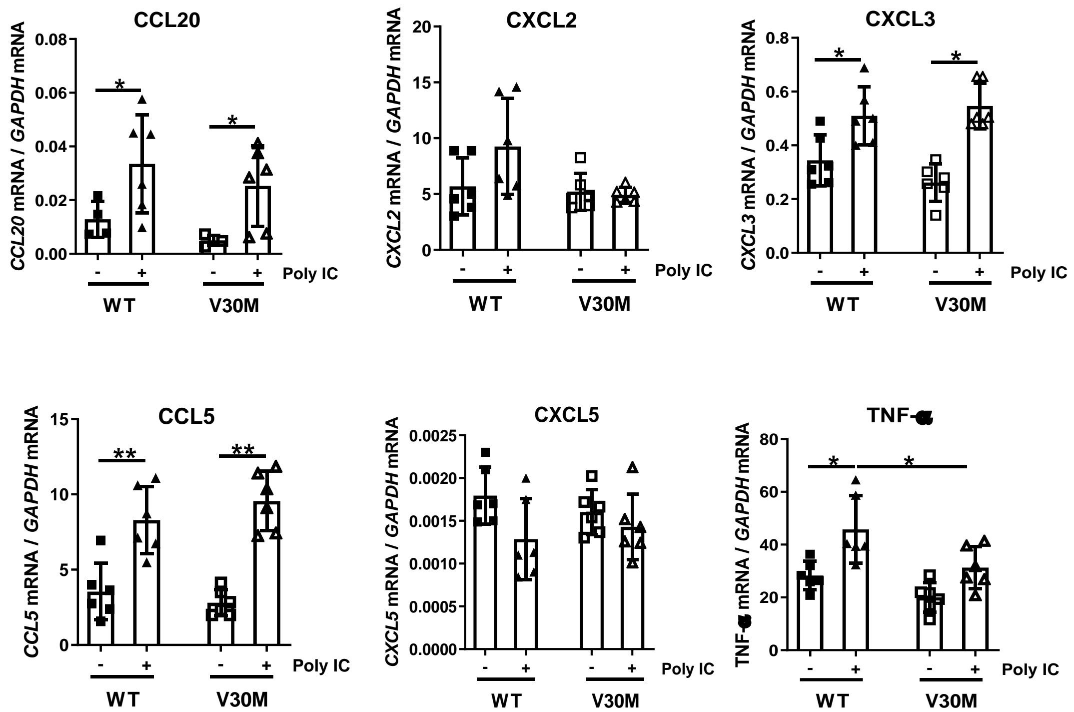

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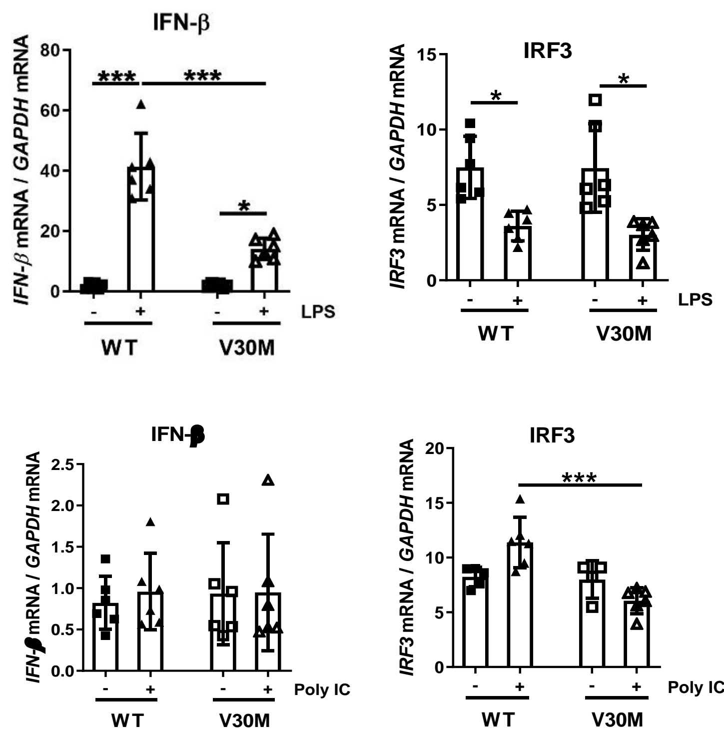
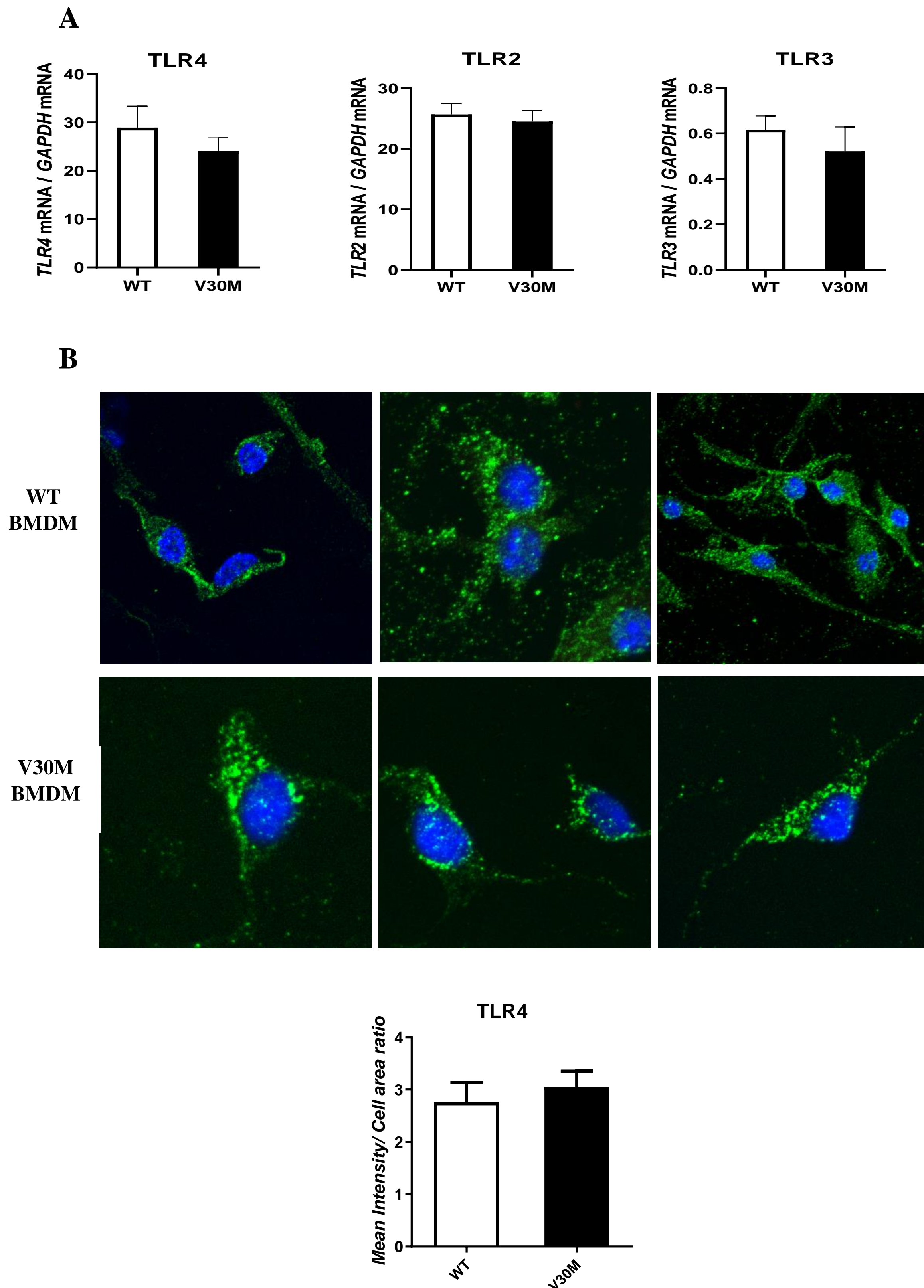


Figure 2. BMDM derived from WT and V30M TTR animals responds differently to stimulation with different TLR agonists. (A) The expression of chemokines was assessed in BMDM from WT and V30M mice after LPS incubation in the presence of CLI-095. BMDM from V30M TTR mice displayed a downregulated expression of chemokines. (B) CCL20 levels were assessed by ELISA in the supernatant of WT and V30M TTR BMDM incubated with LPS in the presence of CLI-095. (C) TNF-α levels were also assessed by RT-PCR and ELISA from WT and V30M TTR BMDM incubated with LPS in the presence of CLI-095. BMDM from WT and V30M TTR mice were stimulated with Pam3Cys (D) and Poly IC (E) for 6 hours and the expression of chemokines were assessed by RT-PCR. (F) IFN-β and IRF3 expression from WT and V30M BMDM after stimulation with LPS and Poly IC for 6 hours. Three independent experiments were performed. We observed a decreased expression of several chemokines in V30M TTR BMDM. Data were analysed using two-way ANOVA or three-way ANOVA followed by Bonferroni post-test and represented as mean±s.e.m (*p<0.05; **p<0.01; ***p<0.001, ****p<0.0001).



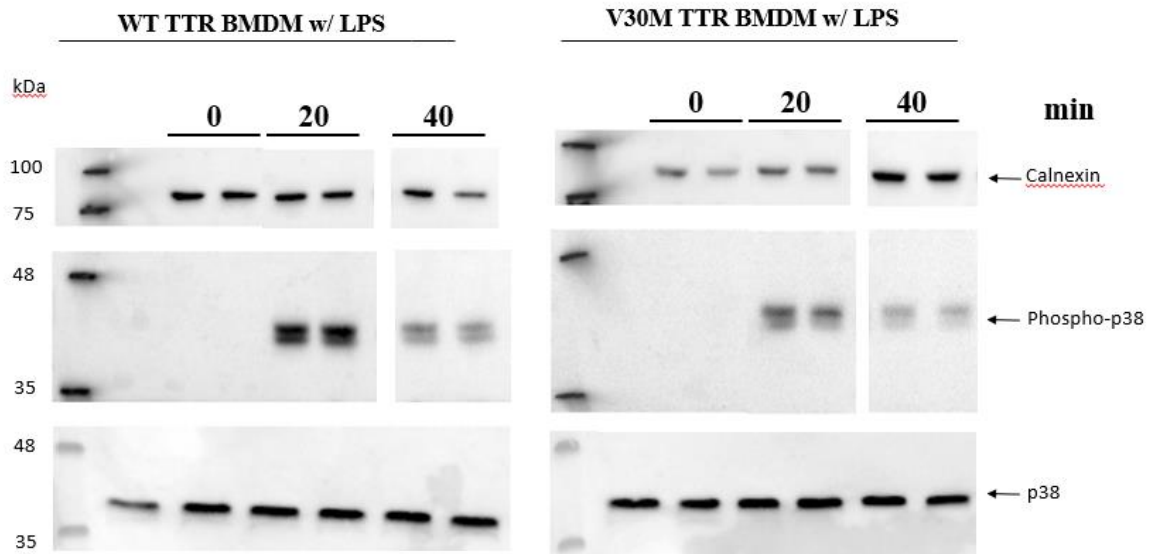
Supplementary Figure 1. TLR4, TLR2 and TLR3 expression was assessed in both WT and V30M TTR BMDM. (A) The expression of TLR4, TLR2 and TLR3 receptors was assessed by RT-PCR in BMDM from WT and V30M TTR mice before stimulation with the different TLR agonists. (B) Representative images of TLR4 immunofluorescence (green) in WT and V30M BMDM without any

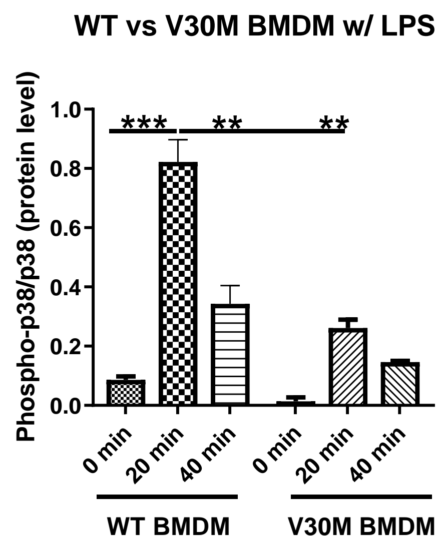
stimulation. For quantification, macrophage area was delimited by bright field. Three independent experiments were performed. Data were analysed using one-way ANOVA followed by Bonferroni post-test and represented as mean±s.e.m.

BMDM from V30M TTR mice fail to activate the MAPK p38 in response to TLR2 and TLR4 stimulation

Considering the previous results pointing out to an impaired TLR response in V30M TTR macrophages, despite normal levels of TLR expression, we decided to investigate whether the activation of signal transduction pathways downstream of TLR activation might be compromised in V30M BMDM. For that, we stimulated WT or V30M TTR BMDM through TLR4, TLR2 or TLR3, and measured the protein levels of total and phosphorylated MAP kinase p38 over time, by western blot analysis. Although both WT and V30M mutant BMDM upregulated the phosphorylation of p38 in response to LPS or Pam3Cys, this upregulation was significantly lower in the case of V30M BMDM (Figure 3A and B). No significant differences were observed in the protein levels of phosphorylated p38 between WT or V30M TTR BMDM after Poly IC incubation (Figure 3C). However, the levels of p38 phosphorylation in the case of TLR3 stimulation were markedly lower than those observed upon TLR4 or TLR2 activation, which is in line with the low profile of gene expression in the case of TLR3 stimulation (Figure 2D).
Taking into consideration the previous findings, that showed no significant variations in phosphorylated p38 after Poly IC incubation, we decided to further investigate the activation of TRAM-TRIF signaling pathway by measuring the phosphorylation of IRF3, characteristically activated by this signaling pathway. Of note, we had observed an upregulation of IRF3 expression in WT BMDM upon TLR3 activation (Figure 2E). In contrast to p38 phosphorylation, TLR3 stimulation did not yield a noticeable activation of phosphorylated IRF3 and furthermore no significant differences in the protein levels of phosphorylated IRF3 between WT or V30M TTR BMDM were observed after Poly IC incubation (see Supplementary Figure 2). Finally, we decided to investigate the NK-kB signalling pathway, since both TLR4 and TLR2 can induce the activation of this transcription factor. For that, we measured the expression of IkB-α, a regulatory protein that inhibits the activity of NF-kB complex, in both WT and V30M BMDM after LPS stimulation, at different time points. No significant differences in the protein levels of IkB-α were observed after LPS stimulation over time (see Supplementary Figure 3).

Altogether, these observations suggest an impaired response in V30M TTR BMDM after stimulation with TLR4 and TLR2 agonists, characterized by a lower upregulation of phosphorylated p38 comparatively to WT BMDM.





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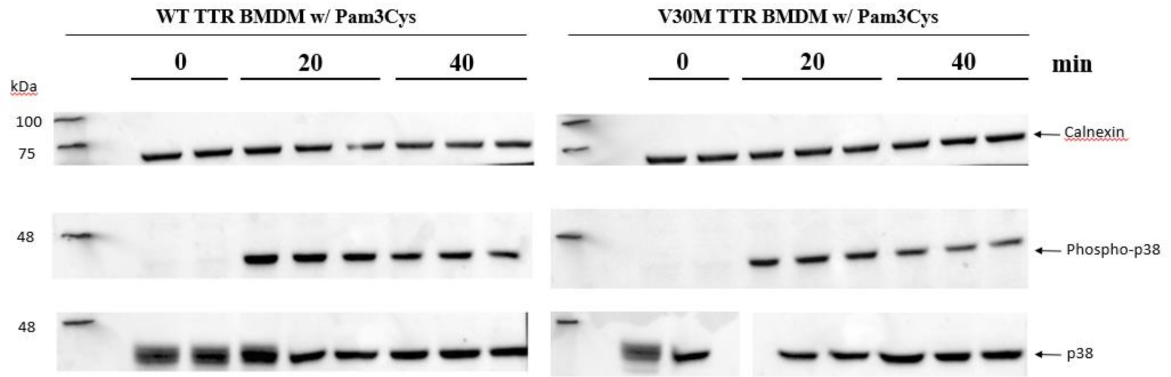

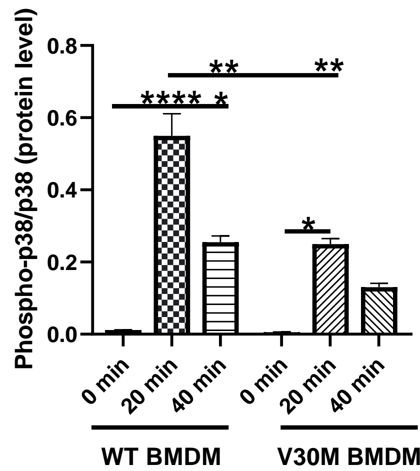

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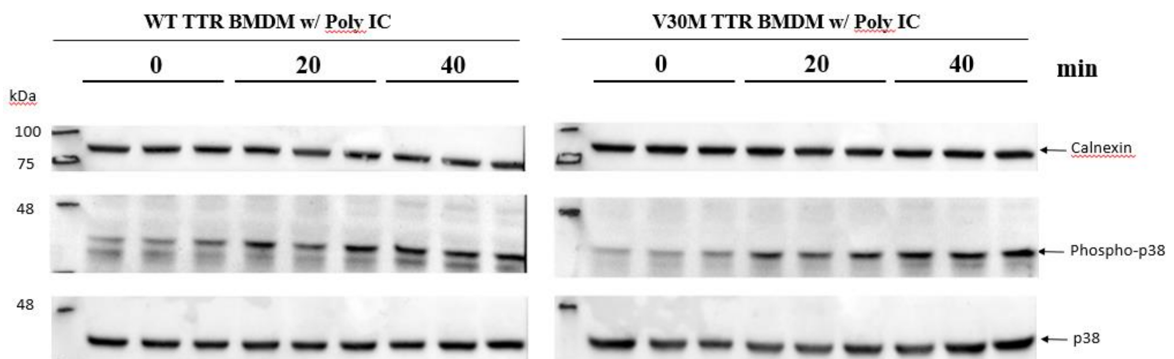


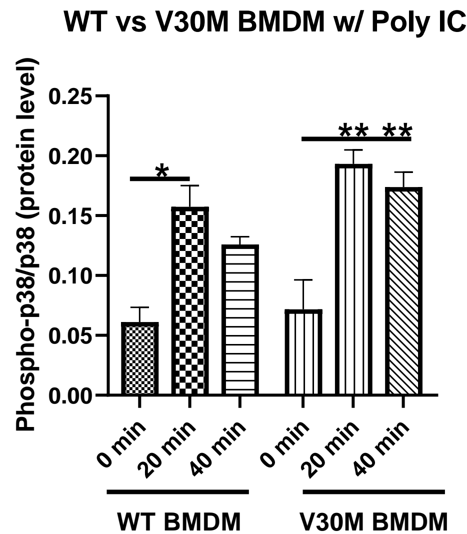
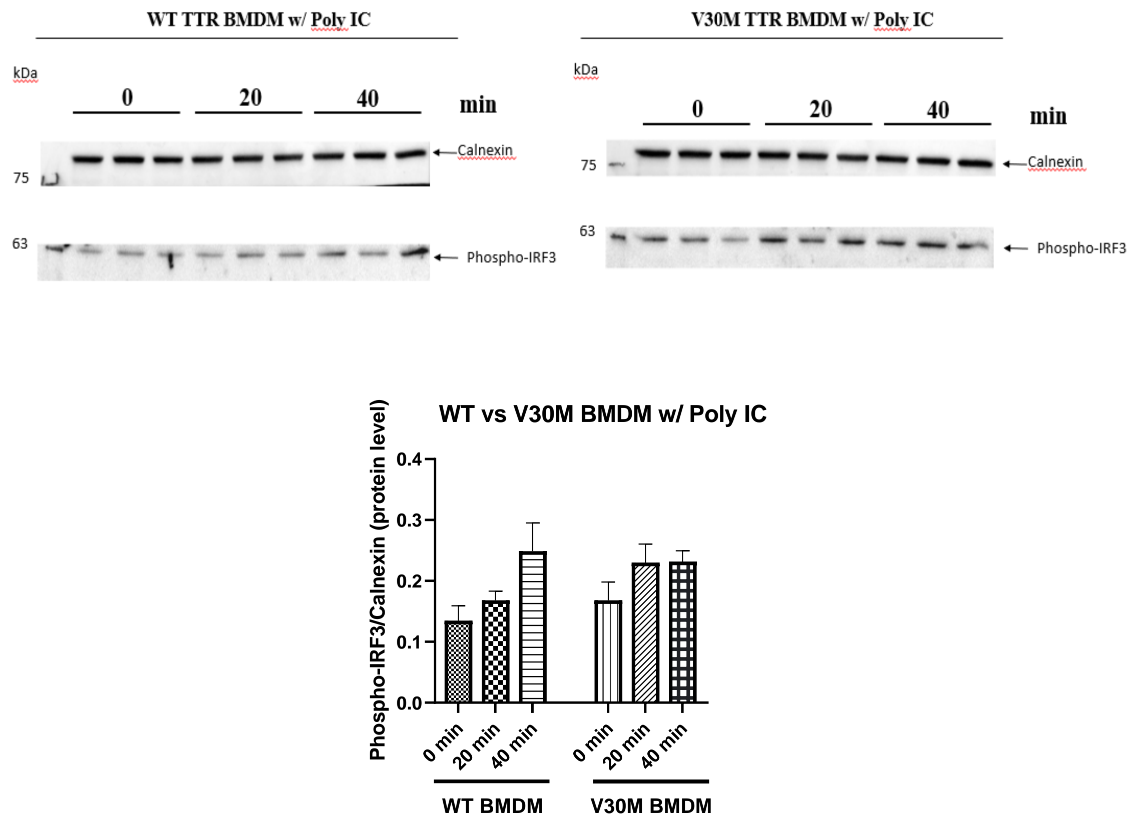
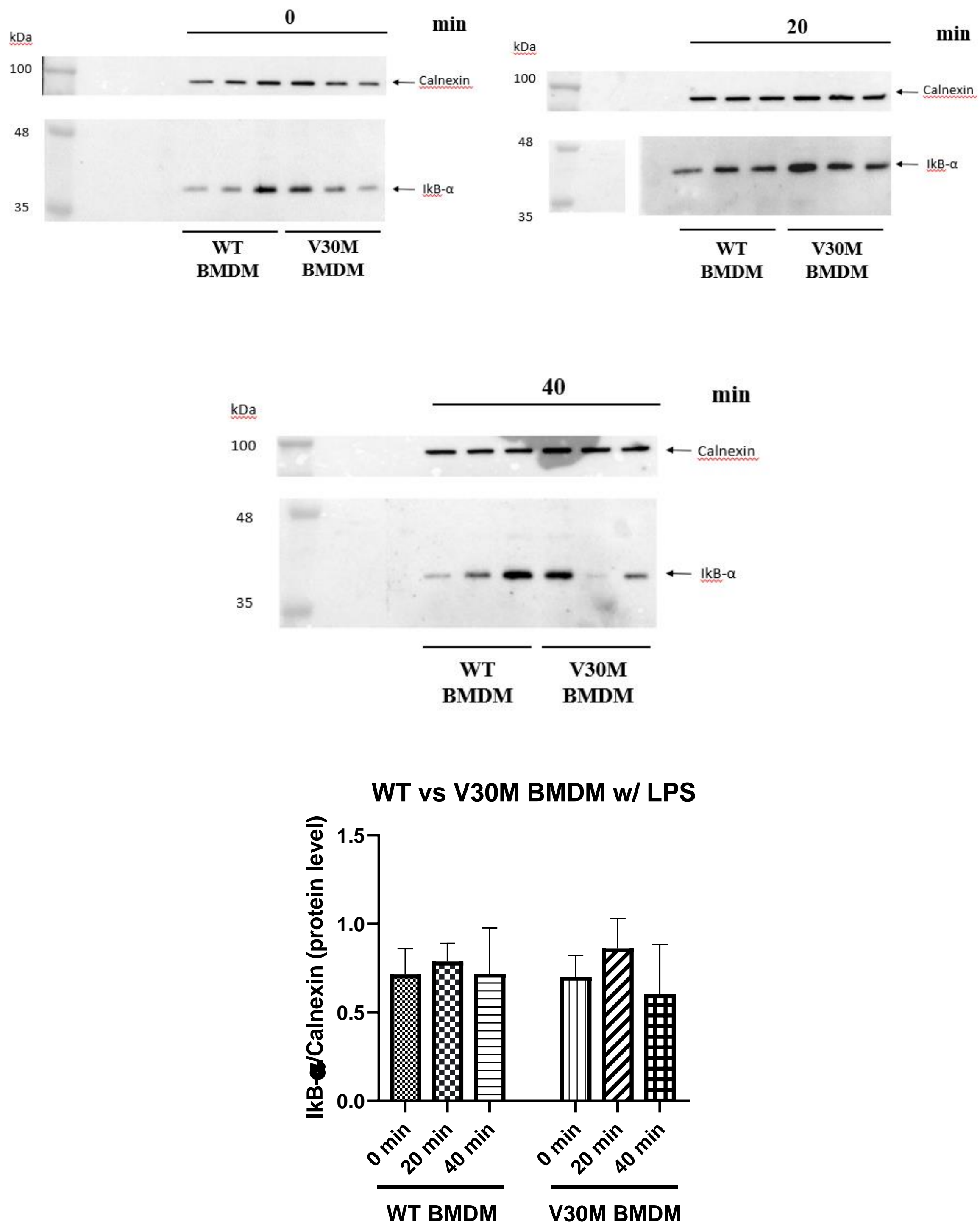


Figure 3. The induction of signalling transduction pathways was altered in BMDM from V30M TTR mice. Representative western blot analysis of total and phosphorylated p-38 in both WT and V30M BMDM incubated with LPS (A), Pam3Cys (B) and Poly IC (C). Three independent experiments were performed. Data were analysed using two-way ANOVA followed by Bonferroni post-test and represented as mean±s.e.m (*p<0.05; **p<0.01; ***p<0.001, ****p<0.0001).



Supplementary Figure 2. Western blot analysis for the phosphorylation of p-IRF3 in both WT and V30M BMDM incubated with Poly-IC. Three independent experiments were performed. Data were analysed using two-way ANOVA followed by Bonferroni post-test and represented as mean±s.e.m.



Supplementary Figure 3. Protein levels of IkB-α were measured by western blot analysis in both WT and V30M BMDM incubated with LPS at different time points. Three independent experiments were performed. Data were analysed using two-way ANOVA followed by Bonferroni post-test and represented as mean±s.e.m.

Discussion

In the present study, we demonstrated downregulated expression of several chemokines in BMDM generated from a transgenic mouse model for human V30M TTR upon stimulation with agonists for TLR4 and TLR2 receptors. Besides chemokine expression, we also described an impairment in the p38 signaling transduction pathway in these macrophages. We previously reported a downregulated expression of several chemokines and TNF-α in SCs after incubation with V30M TTR when compared to incubation with WT TTR (Moreira et al., 2021). Therefore, to further investigate this finding, we decided to study the chemokine expression in a different immune cell population. We chose macrophages since several immunochemical studies from ATTRV30M nerve biopsies suggest lower expression of chemokines and neurotrophic factors important to drive tissue regeneration. Moreover, a striking feature of the disease is the absence of an immune/inflammatory infiltrate, despite the production of pro-inflammatory cytokines by axons. A possible explanation for this lack of recruitment of effector cells may reside in impaired chemokine expression. Therefore, in this paper, rather than focusing in the phagocytic function of the macrophages, we were more interested in study the expression profile of chemokines triggered in these cells by WT or mutated TTR. In fact, recent studies have reported an important new role for the proteins of the complement with substantial phagocytic cell activation in macrophages, associated with the reduction of amyloid deposits in a mouse model of the disease (Fella et al., 2017).

Macrophages are an essential component of the innate immune system with pivotal functions in homeostasis and many physiological processes beyond innate immunity, including clearance of cellular debris, tissue repair and remodeling (Bosurgi et al., 2017; Herzog et al., 2019). Macrophages are fundamental to the induction of inflammatory responses by amyloid or protein aggregates. Macrophages are engaged in removal of TTR deposits in ATTRv patients and mouse models of the disease (Fella et al., 2017; Panayiotou et al., 2017; Sommer et al., 2018). Furthermore, ATTRv patients displayed quantitative or qualitative anomalies in tissue resident macrophages, and these anomalies may accelerate TTR amyloid deposition in some organs (Suenaga et al., 2016). To understand the interaction between macrophages and TTR, we first incubated WT BMDM with WT or V30M TTR protein. Interestingly WT macrophages recognized and responded equally to both TTR forms with increased expression of several chemokines and this interaction was mediated via TLR4 receptor. Additionally, we also evaluated the response of human macrophages to both WT or V30M TTR. In accordance with the previous results, human macrophages also

recognized both forms of TTR and induced the expression of several chemokines. Once again, this activation was mediated via TLR4 receptor.

Prior evidence suggest that macrophages interact with SCs, including the regulation of migration, mitosis, and differentiation (Cattin et al., 2015; Chen et al., 2015; Martini et al., 2008). In response to nerve injury, V30M mice displayed a downregulated innate immune response with lower expression of chemokines and cytokines such as CXCL2 and CXCL3, important for the recruitment of immune cells, when compared to WT controls (Goncalves, Teixeira-Coelho, et al., 2014). Additionally, we already showed that several chemokines such as, CCL20, CXCL2 and CXCL3 were highly downregulated in V30M TTR mice in both peripheral nerves and plasma samples, pointing towards a close interface between the absence of chemokine response and cellular infiltration with the development of the disease (Moreira et al., 2021). We thus considered the hypothesis that V30M mutated macrophages might be themselves impaired in their ability to respond to TLR activation. Therefore, we decided to generate macrophages from mutant V30M TTR mice and evaluate chemokine expression in response to TLR stimulation as compared to WT macrophages. Our data now demonstrate a downregulated expression of chemokines in BMDM from V30M TTR mice comparatively with BMDM derived from WT animals after LPS stimulation. Additionally, in response to Pam3Cys stimulation, similar results were obtained regarding to chemokine expression, indicating that the signaling pathways shared by these two TLRs might be diminished in their function. Given these findings, it will be interesting to investigate whether the recognition of TTR itself by macrophages of the V30M genetic background is also diminished in comparison with WT macrophages.

The reason why macrophages derived from a V30M genetic background respond differently remains unknown and further studies are necessary to understand this observation. Since the TTR gene was not expressed in macrophages (Goncalves et al., 2017; Hao et al., 2016), we are tempted to hypothesize that somehow the presence of mutated TTR in circulation can interfere with the biological functions or even the development of this cell population. Overall, our data is in accordance with other studies that showed that ATTRv patients display abnormalities in macrophages with defects in cell adhesion and chemotaxis (Suenaga et al., 2016).

The expression levels of TLR4, TLR2 and TLR3 were very similar in resting WT and V30M BMDM, demonstrating that the lower chemokine expression observed in V30M BMDM is likely not explained by discrepancies in TLR expression. We then investigated differential activation of signaling pathways downstream TLR activation. The MAP kinase p38 mediates inflammatory responses partly through activating gene expression. In p38γ/δ-deficient mice the production of TNF-α, IL-1β, and IL-10 is severely reduced in LPS-stimulated

macrophages (Risco & Cuenda, 2012). In addition, p38 deficient macrophages showed substantially lower MKK1-ERK1/2 activation and inflammatory cytokine production (Risco et al., 2012). In line with these observations, we found an upregulation of p38 activation upon TLR stimulation but denoted a decreased phosphorylation of p38 in BMDM from V30M TTR mice upon stimulation with both LPS and Pam3Cys. The lower phosphorylation of p38 in mutated macrophages is most likely linked to the lack of inflammatory cells and chemokine expression observed in V30M patients.

Beyond our finding that altered MAPK activation may be operating in V30M macrophages, recent studies also suggest a new role for TTR in the modulation of microtubules dynamics through regulation of α-tubulin acetylation. In fact, TTR KO mice failed to modulate microtubules dynamics in response to sciatic nerve injury (Eira et al., 2021). Microtubules play fundamental roles in facilitating the selective delivery of transport intermediates between the ER and the Golgi (Cole & Lippincott-Schwartz, 1995; Lippincott-Schwartz, 1998). Additionally, microtubules also play many important roles in cell function, such as migration, phagocytosis of pathogens, and antigen presentation (Allen & Aderem, 1996; Khandani et al., 2007; Nogales, 2000). Macrophage activation results in enhanced levels of stable cytoplasmic microtubules which potentially play a role as tracks for the secretion of vesicles loaded with cytokine/chemokine (Patel et al., 2009). Thus, evaluating the impact of mutated TTR in the modulation of microtubules dynamics may help to explain the differences observed in chemokine secretion between BMDM from WT and V30M TTR mice. Further studies are needed to validate or not this exciting possibility.

Novel insights into ATTRv are of major importance for the development of new therapeutic strategies. Recent studies also suggest that ATTRv patients have altered levels of several cytokines in the serum, such as IL-1β, TNF-α or IL-8 (Azevedo et al., 2019). Therefore, it will be important to address the differential chemokine production in human patients. Our preliminary data obtained for CXCL2 and CCL20 levels in the human plasma suggested only minor differences between a small group of ATTRv patients and controls (Moreira et al., 2021). However, further studies on a greater number of ATTRv blood samples from carriers of different TTR mutations are required to strengthen the results obtained.

Overall, in this work we demonstrated a significant downregulation in the expression of several chemokines in BMDM from V30M TTR mice upon stimulation with LPS and Pam3Cys and an impairment in the phosphorylation of p38 kinase, affecting both the TLR4 and TLR2 signaling pathways. It will be of importance and interest to in the future to investigate which reprogramming alterations occur in different cells and how mutated TTR promotes them.

Acknowledgements

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Conclusions and Future perspectives

Conclusions and Future perspectives

The inflammatory response has been shown to be important for the development of peripheral neuropathies including ATTRV30M, either by leading to deterioration or to amelioration of the disease process (Ydens et al., 2013). ATTRV30M, formerly known as familial amyloid polyneuropathy is an autosomal dominant hereditary disease characterized by the deposition of mutated TTR-derived amyloid fibrils in peripheral nerves, the gastrointestinal tract, and the heart (Benson & Kincaid, 2007). Paradoxically, several studies have described the presence of inflammatory markers in ex vivo biopsies from ATTRV30M patients, suggesting that nerves and surrounding tissue might produce pro- and anti-inflammatory molecules as a stress response or to modulate disease progression (Sousa, Du Yan, et al., 2001; Ydens et al., 2013). However, despite cytokine production, no change in leukocyte markers was found, suggesting that mechanisms must operate to block the correct immune response (Misu et al., 1999; Nyhlin et al., 2000; Sousa, Du Yan, et al., 2001).

In chapter I, we reported that the expression levels of S100A8/A9 proteins, known to play an important role in the development of inflammation, were downregulated in the nerve biopsies from ATTV30M patients and in the peripheral nerve from a mouse model of the disease. Considering that during inflammation S100A8/A9 is released actively and exerts a critical role in modulating the inflammatory response by stimulating leukocyte recruitment and inducing cytokine secretion (Chernov et al., 2015), we hypothesized that the lower expression of S100A8/A9 proteins could help to explain the lack of inflammatory cellular infiltrates observed in ATTRV30M nerves. Furthermore, we also assessed the levels of S100A8 protein in the in the serum of ATTRV30M patients. Our data indicate that the levels of S100A8 were also significantly downregulated in the serum of ATTRV30M patients comparatively to control individuals. However, further studies on a greater number of ATTRV30M blood samples are required to strengthen the results obtained and, ultimately, to evaluate whether blood levels of S100A8 can be used as a viable biomarker. Furthermore, considering the vital function of SCs and macrophages in tissue homeostasis, either by initiating the immune response in the PNS or exhibiting phagocytotic function against invading and dying cells, we decided to evaluate the expression of S100A8/A9 proteins in these immune cells. In accordance with the previous results, we detected a downregulated expression of S100A8/A9 proteins in SCs incubated with V30M TTR when compared to SCs incubated with WT TTR and in BMDM derived from V30M mice. Previous studies have reported that SCs from ATTRV30M patients are impaired on their ability to produce neurotrophins in a context of neuronal stress, so our findings provide more

information to help to understand the lack of immune response observed in ATTRV30M disease. The reason why the presence of V30M TTR variant impair the activation of immune cells is still unknown. We hypothesized that V30M variant might gain a molecular or conformational specificity that impairs cell activation, but in depth studies are needed in the future to clarify this issue. To further understand the role of S100A8/A9 proteins in ATTRV30M disease, additional experiments should be considered in the future to clearly understand the exact contribution of S100A8/A9 proteins in the development of the disease and how mutated TTR affects the interaction between these proteins and the respective receptors. The use of small interfering RNA (siRNA) has gained attention as a potential therapeutic reagent due to its ability to inhibit specific genes in many genetic diseases. Therefore, studying SCs transfected with the siRNA for the S100A8/A9 proteins will give more information about the role of these proteins in the inflammatory response after incubation with WT or V30M TTR. Additionally, study the interaction between TLR4 and RAGE receptors with the mutated TTR will be of great importance to try to understand how mutated TTR impairs cellular activation.

In chapter II, to further investigate the lack of inflammatory response in ATTRV30M disease, we performed an RNA sequence analysis, to disclose differentially expressed genes between the sciatic nerve from V30M mice and the sciatic nerve from WT mice. The pathways that were downregulated in V30M mice were associated with immune system process and antigen processing and presentation. Through RT-PCR validation, we detected that several chemokines responsible for the chemoattraction of immune cells, such as CCL20, CXCL2, CXCL3 and CCL8, were downregulated in the PNS of the mouse model of the disease when compared to the control group. Consequently, the lack of trophic factors, immune cells and regenerating molecules might be relevant for neuronal dysfunction, poor functional recovery and lower regenerative abilities observed in the V30M TTR transgenic mice.

SCs are the immunocompetent cells of the PNS. After peripheral nerve injury, SCs dedifferentiate and are able to phagocyte myelin debris for regeneration to occur (Jessen & Mirsky, 2005). Consequently, we decided to measure the expression levels of several chemokines in SCs incubated with WT or V30M TTR and we observed that WT TTR, but not mutated TTR, activates SCs leading to the production of proinflammatory chemokines. Moreover, we also incubated SCs with additional TTR mutated variants, such as TTR with a cardiomyopathy mutation (I68L) , a non-pathological mutation (T119M) and a mutation that leads to carpal tunnel syndrome and affects heart and eye (I84S). Interestingly, among all the TTR variants tested, only the V30M one impaired SCs activation to induce chemokine expression. Therefore, nerve deposition of toxic V30M TTR aggregates most probably impairs SCs function, hampering their role in regeneration and disrupting the crosstalk

between SCs and axons. Consequently, in future studies, it will be interesting to understand if the addition of WT TTR can rescue the V30M TTR phenotype with increased expression of chemokines. In this regard, studies with primary SCs derived from V30M mice and the respective control group stimulated either with WT or mutated TTR might give novel insights into new molecular pathways underlying ATTRV30M pathology. In chapter II we also implicated the TLR4 as the receptor being triggered by WT TTR in SCs. The dissection of the cellular pathways involved in ATTRV30M disease is of greatest importance for the enhancement of new treatment approaches

Regarding chapter III, and to further explore the findings observed in the previous chapters, we decided to study the chemokine expression triggered by TTR in a different immune cell population. We chose macrophages since several immunochemical studies from ATTRV30M nerve biopsies showed several anomalies in macrophages from ATTRV30M patients when compared to healthy donors, which may accelerate TTR amyloid deposition in some organs (Suenaga et al., 2016). First, we investigated the chemokine expression in WT BMDM and human THP1 macrophages in response to WT or V30M TTR and showed that both cell types are activated by either WT or mutated TTR with no significant differences observed in chemokine production. In both cases, this interaction was mediated by TLR4 receptor. Differently to what we observed in SCs, WT BMDM and human macrophages were able to respond with chemokine expression to WT and V30M TTR. It will be intriguing to understand why these two cell populations respond in a different way to mutated TTR. Interestingly, when we compared the response to different TLR agonists in BMDM of different genetic backgrounds, WT TTR vs V30M TTR, we observed that macrophages derived from mice with a V30M genetic background failed to activate chemokine expression in response to LPS and Pam3Cys when compared to WT macrophages. The reason why macrophages derived from a V30M genetic background respond differently remains unknown and further studies are necessary to understand this observation. However, we are tempted to hypothesize that somehow the mutated TTR can interfere with the biological functions or even the development of this cell population. Since the expression levels of the different TLR's were very similar in resting WT and V30M BMDM, we decided to investigate the differential activation of signaling pathways downstream TLR activation. The lower phosphorylation of p38 observed in mutated macrophages could be associated to the lack of inflammatory cells and chemokine expression observed in V30M patients.

Overall, our data revealed that the mutation V30M in TTR associates with an extensive downregulated expression of several chemokines produced particularly by SCs and BMDM in ATTRV30M patients and in a mouse model of the disease. This downregulation may compromise the development of an appropriate chemotactic gradient vital for the

recruitment of immune cells, needed for the removal of amyloid deposits and fibrils. It will be of importance and interest to investigate other possible reprogramming alterations in other immune cell populations and how mutated TTR promotes them. Overall, understanding the role of inflammation in the disease and the molecular mechanisms underlying the lack of inflammatory cellular infiltrates in the nerves of in ATTRV30M patients will help improving the quality of life and disease management in affected patients, contributing this way for the development of more effective and targeted therapies for the treatment of ATTR amyloidosis.

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