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Immunomodulatory role of thymulin in lung diseases

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Importance of the field: Inflammation is a hallmark of lung diseases. The available treatment options are unsatisfactory because they are not efficacious or induce major side effects. Alternative approaches need to be developed. Thymulin is a peptide exclusively produced in the thymus with several anti-inflammatory properties.

Areas covered in this review: The physiological features of thymulin and data that support its potential as an anti-inflammatory treatment for lung diseases are reviewed.

What the reader will gain: Thymulin has consistent beneficial effects in experimental models of lung diseases. It has a broad inhibitory effect on pro-inflammatory cytokines, suppresses p38 (a MAPK family member) and inhibits the activation of the NF-κB signal pathway. It is an attractive peptide for lung gene therapy because has no toxicity even at high doses and when expressed by adenoviral vectors reduces immune response against viral proteins.

Take home message: Thymulin has a selective immunomodulatory effect, enhancing anti-inflammatory and inhibiting pro-inflammatory cytokines. It suppresses p38 (implicated in glucocorticoid-resistance) and inhibits NF-κB activation, which has an important pathogenic role in several lung diseases. The broad spectrum of anti-inflammatory effects of this peptide in several animal models of lung disease makes thymulin a good candidate for future clinical trials.

Keywords: immunomodulator, inflammation, lung disease, thymulin.

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1. Introduction

Inflammation is a hallmark of a vast majority of lung diseases. Chronic obstructive pulmonary disease (COPD), asthma, interstitial lung diseases (ILDs) and pulmonary arterial hypertension (PAH) have an important underlying inflammatory process in their pathogenesis. Despite the marked differences in the pattern of inflammation between these different lung diseases, there are some similar features in the underlying pathophysiological mechanism. The evidence shows that infiltration of lung tissue with inflammatory cells (T cells, macrophages, neutrophils, eosinophils or mast cells) orchestrate the chronic inflammatory process, establishing a complex network of cytokines. These molecules can be classified as lymphokines (cytokines that are secreted by T cells), pro-inflammatory (which amplify and perpetuate the inflammatory process), growth factors (which promote cell survival and result in structural changes in the lung), chemokines (cytokines that are chemotactic for inflammatory cells) and anti-inflammatory cytokines (which negatively modulate inflammation) [1]. The current available therapeutic options directed to inflammation are unsatisfactory since available drugs like corticosteroids are in many circumstances ineffective or induce major side effects when chronically administered. These limitations constitute a serious problem in the management of

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Article highlights.

- Overview of the physiology of thymulin.
- The thymulin molecular signaling pathway includes cAMP, p38 MAPK and NF-κB.
- The anti-inflammatory effect of thymulin in animal models of lung disease.
- Thymulin's potential advantages in lung gene therapy.
- The scope of thymulin for human lung disease.

This box summarises key points contained in the article.

these patients. Thus, new and alternative anti-inflammatory approaches are desirable.

Thymulin is a peptide exclusively produced in the thymus, discovered in the seventies, which has shown appealing anti-inflammatory properties [2]. This peptide influences the cytokine secretion profile in several animal models, notably those with lung inflammation. In this review, we summarize the evidence regarding the beneficial immunomodulatory role of thymulin in lung diseases.

2. Thymulin overview

2.1 Structure and production of thymulin

Facteur Thymique Serique (FTS, which is an acronym for serum thymic factor in French) was first described by Bach *et al.* in 1977 [3] and it is an inactive nonapeptide exclusively secreted by the thymic epithelial cells. Its amino acid sequence was determined to be Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn [4]. Thymulin is a metallopeptide formed by FTS coupled in an equimolar ratio to cationic zinc, which confers biological activity to the molecule [5,6]. The circulating levels of thymulin follow a circadian rhythm and peaks at night, coinciding with the activity of the hypothalamus-pituitary axis [7]. It achieves maximal values early in postnatal life and declines with age [2]. Physiological level of thymulin in the blood of young healthy individuals was defined as 10^{-5}mol/l [6]. Its level is decreased in healthy aged populations [8]. Thymulin blood level has already been studied in specific populations such as malnourished children [9] and individuals with diabetes [10], growth hormone deficiency [11], HIV infection [12], dementia [8] and other conditions [13-15]. However, there is a lack of knowledge about its blood level in inflammatory diseases, particularly, in lung inflammatory diseases. The control of thymulin secretion is dependent of a complex network of events. It was demonstrated early that it displayed a negative-feedback effect induced by its own secretion [16,17]. Furthermore, its production and secretion is influenced by the neuroendocrine system. Growth hormone can increase the synthesis and secretion of thymulin, directly or indirectly through IGF-1 [18]. The thyroid axis also influences its secretion; a positive effect of thyroxine and triiodothyronine on thymulin secretion has been documented [19]. Prolactin, glucocorticoids and gonadal steroids also seem to modulate this hormone secretion by thymic epithelial cells [20-22]. Hypothalamic and pituitary extracts stimulate thymulin secretion, a stimulus that is more effective with biological extracts from young mice [23,24]. This interdependence suggests that thymulin is a player in this homeostatic bidirectional communication between the immune and endocrine systems.

There is little knowledge about thymulin receptors. Two receptor types were found in tumor-derived human T cell lines that showed different affinities for thymulin [25,26]. More recently, Brown *et al.* used anterior pituitary cells to demonstrate the prolactin and thyroid-stimulating hormone releasing effect of thymulin [27]. The response was specific and dose-dependent to thymulin, which suggested the existence of receptor sites on pituitary cells.

2.2 Function of thymulin

Thymulin has been implicated in several aspects of intra- and extra-thymic T-cell differentiation [2]. It is able to enhance thymocyte proliferation and to induce the expression of several T cell differentiation markers [28,29]. In addition to the several influences exerted by multiple hormones in thymulin secretion, there is accumulating evidence that indicates a hypophysiotropic activity of thymulin (**Figure 1**). It could have either a direct influence as a secretagogue of some pituitary hormones and act as a facilitator substance for the other physiologic secretagogues [30]. This cross-talk between the neuroendocrine and immune systems was demonstrated by the positive effect of thymulin in the release of growth hormone, prolactin, thyrotropin and gonadotropin [30], an effect that is reduced with aging [27,31]. Further evidence that supports this important physiological effect is the functional impairment of the hypothalamo–adrenal axis in congenitally athymic mice [32]. Thymulin also interacts with the nervous system to modulate pain [33]. Curiously, there seems to be a dual effect. It was first demonstrated that low doses of locally (plantar) or systemically (intraperitoneal) injected thymulin had a hyperalgesic effect, probably mediated by prostanglandin-E2 interaction with afferent nerve terminals [34,35]. Later, supraphysiological levels of systemic thymulin reduced the hyperalgesia induced by endotoxin injection, an effect mediated by the inhibition of some pro-inflammatory mediators [33]. These analgesic and anti-inflammatory actions were also documented in a model of intracerebroventricular (i.c.v.) endotoxin injection [36].

3. Thymulin and inflammation

3.1 Thymulin in inflammatory diseases

In addition to its action as a thymic hormone regulating several aspects of thymus physiology and to its hypophysiotropic and analgesic properties, thymulin has a potent immunomodulatory action. The mechanisms responsible for this role are largely unknown. It influences the activity of numerous immunological cells like T cells [37-40], B cells [41] and NK cells [40,42]. It also influences the cytokine secretion profile,

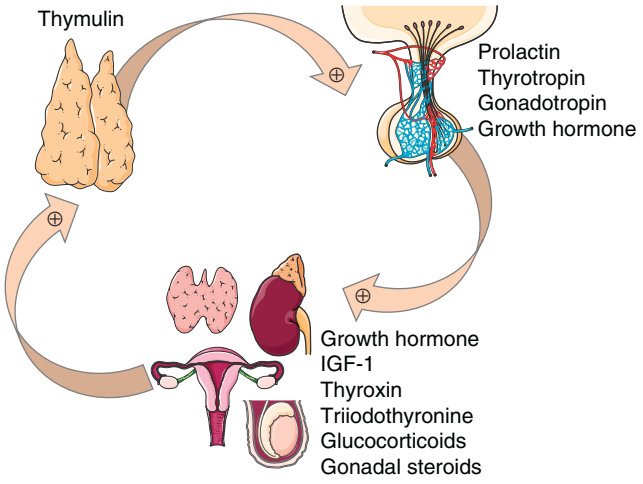

Figure 1. Role of thymulin in neuroendocrine system.

enhancing an anti-inflammatory cytoprotective response and depressing inflammatory cascades [43].

With respect to signaling pathways, an experimental study with anterior pituitary cells showed that the hormone-releasing effect of thymulin was dependent, in part, on calcium, cAMP and inositol phosphates [31]. In some inflammatory disease models thymulin actions were attributed to the modulation of MAPK family members such as extracellular signal-regulator kinase (ERK) and p38 [44-46]. The p38 MAPK pathway is involved in expression of inflammatory cytokines and chemokines [47] and seems to be inhibited by thymulin [46.] Furthermore, NF-κB is a transcription factor that plays an important role in inflammatory diseases [48,49] and therefore has been a major target for drug development. The pathogenic role of NF-κB in lung diseases with inflammatory component has been thoroughly investigated [50]. The potential of NF-κB as a therapeutic target in lung diseases lead to development of NF-κB inhibitors studied in animal models, with some of them already being tested in clinical trials [50]. Recently, Novoselova *et al.* demonstrated that thymulin is able to repress the NF-κB signaling pathway in an endotoxin-induced sepsis model [51]. Furthermore, using thymulin in an *in vitro* model of fetal alveolar type II epithelial cells, Haddad [52] has shown a cAMP-dependent downregulation of endotoxin-induced production of pro-inflammatory cytokines and inhibition of NF-κB nuclear translocation and activation. These and other studies [53,54] indicate that the immunomodulatory potential of thymulin involves the adenylyl cyclase–cAMP system that influences NF-κB activation, which could be mediated by MAPK members, notably, p38 MAPK. Consequently, the inhibition of NF-κB reduces the expression of pro-inflammatory cytokines (**Figure 2**).

There is a growing body of evidence, either experimental or clinical, supporting these beneficial effects of thymulin in inflammatory disorders (**Table 1**). In a mouse model of acute systemic inflammation induced by an injection of Gram-negative bacteria lypopolysaccharide, Lunin *et al.* [55] used a synthetic analogue of thymulin. This peptide prevented the accumulation of pro-inflammatory cytokines in plasma, as well as the production of those by spleen lymphocytes and peritoneal macrophages. These results were consistent with those from other systemic inflammation animal model induced by endotoxin [56]. This anti-inflammatory effect was also documented in organ-specific inflammation animal models such as alloxan- and streptozotocin-induced pancreatitis and diabetes [57], myocarditis caused by encephalomyocarditis virus [58], nephrotoxicity induced with cephaloridine [44] and cisplatin [45], thyroiditis caused by reovirus [59] and acute experimental allergic encephalomyelitis [60] and CNS inflammation induced by i.c.v. endotoxin injection [36]. The immunomodulatory role of thymulin was also documented in human immunological cells. Thymulin was able to enhance CD3, CD4 and CD8 expression in lymphocytes from immunodeficient children [38] and increased IgA and IgE in patients with ataxia telangiectasia [61]. Thymulin enhanced T cell proliferation of bone marrow transplantation patients cells [62] and induced mature T cell markers in circulating immature T lymphocytes of malnourished children [39]. Using peripheral blood mononuclear cells, *in vitro* exposure to thymulin increased IL-1 and decreased IL-6 and TNF-α production from cells of healthy volunteers and decreased IL-1, IL-2 and TNF-α in those from patients with systemic lupus erythemathosus [63].

4. Lung diseases: role of inflammation

The pathophysiology of the most common lung diseases is multifactorial and it is characterized by an interplay of genetic predisposition and environmental factors that culminates in an extended and chronic inflammation.

The pulmonary inflammatory response in patients with COPD demonstrates an activation of both innate and acquired immune processes [64]. It involves the migration of leukocytes, the production of inflammatory mediators and the release of proinflammatory cytokines and proteases that contributes to lung injury. Many of these mediators such as IL-6, IL-8, TNF-α and IL-10, appear to be useful biomarkers to evaluate the intensity of the disease process [65-67].

Asthma is defined as a chronic inflammatory disorder with airway inflammation in response to inhaled stimuli that originates not only an adaptative allergen-dependent but also, as recent work suggests, an innate non-antigen-dependent response [68].

ILDs are a heterogeneous group of complex disorders with similar clinical, radiographic and physiological manifestations. Although frequently unknown, occupational and environmental exposures can cause ILDs. The pathobiology is variable and still uncharacterized yet it appears to be a chronic inflammatory process that eventually leads to fibrosis induced by fibroblast activation [69].

PAH is characterized by a sustained elevation of pulmonary arterial pressure and is associated with several inflammatory

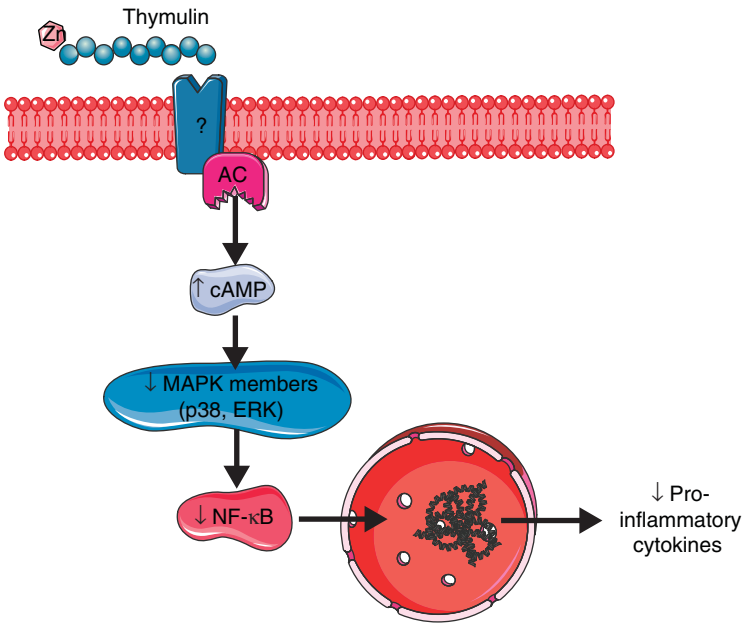

Figure 2. Molecular signaling pathway of thymulin.
AC: Adenylyl cyclase; ERK: Extracellular-signal-regulated kinase.

Table 1. Effect of thymulin in experimental inflammatory animal models.

Model	Stimulus	Thymulin effect
Systemic inflammation [55]	*Escherichia coli* LPS (i.p.)	↓ IL-1, IL-2, IL-6, IL-10, TNF-α, IFN-γ
Systemic inflammation [56]	*Salmonella typhosa* endotoxin (i.p.)	↓ IL-1, IL-6, TNF-α, PGE2
Diabetes and pancreatitis [57]	Alloxan Streptozotocin	Prevented pancreatic β-cells inflammation and destruction
Myocarditis [58]	Encephalomyocarditis virus	Prevented myocardial inflammation
Nephrotoxicity [44,45]	Cephaloridine Cisplatin	Attenuated renal dysfunction ↓ p38 MAPK activation
Thyroiditis [59]	Reovirus	Supression of autoantibodies
Acute allergic encephalomyelitis [60]	Vaccinia virus	↓ Inflammatory cells infiltration
CNS inflammation [36]	Salmonella typhosa endotoxin (i.c.v.)	↓ IL-1, IL-6

i.c.v.: Intracerebroventricular; i.p.: Intraperitoneal.

conditions (e.g., HIV infection and connective tissue diseases) [70]. Several types of inflammatory cells, including activated T cells, B cells and macrophages, have been documented to infiltrate the pulmonary arteries in patients with PAH. Also, several pro-inflammatory cytokines and chemokines such as IL-6, stromal derived factor 1 (SDF-1) and monocyte chemoattractant protein 1 (MCP-1) Have been demonstrated to be increased in serum of PAH patients [71-73].

5. Thymulin and lung diseases

Some research groups have shown promising beneficial effects of thymulin in lung disease (**Figure 3** and **Table 2**). An *in vitro* model of fetal alveolar type II epithelial cells exposed to endotoxin was used to investigate the anti-inflammatory properties of thymulin in this tissue. It inhibited the release of IL-1 and TNF-α and enhanced the production of IL-10, an anti-inflammatory cytokine [43]. This effect was synergistically amplified by zinc. These results documented the anti-inflammatory potential of thymulin in lung epithelium and also established thymulin bioactivity in the fetal lung close to term. Recently, in the same model, it was established that these anti-inflammatory potentials were mediated by cAMP and were NF-κB-dependent [52].

The intrapulmonary instillation of bleomycin releases a variety of cytokines that induces the transmigration and

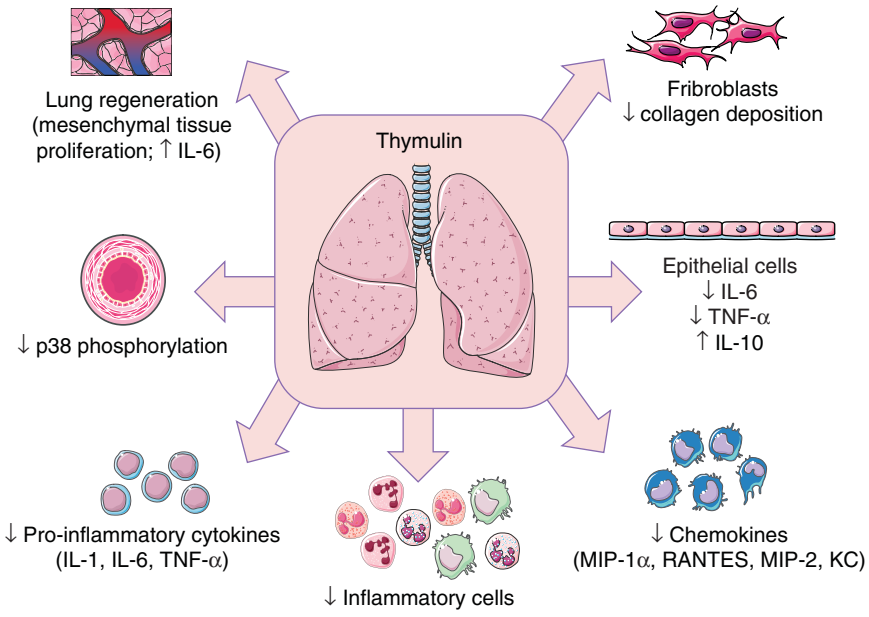

Figure 3. Effects of thymulin in lung inflammation.

KC: Keratinocyte-derived chemokine; MIP-1α: Macrophage inflammatory protein-1 alpha; MIP-2: Macrophage inflammatory protein-2; RANTES: Regulated on activation normal T-cell expressed and secreted.

Table 2. Thymulin effect in lung disease animal models.

Model	Stimulus	Thymulin effect
Fetal alveolar type II cells inflammation (*in vitro*) [43]	Gram-negative bacteria LPS	↓ IL-1, TNF-α ↑ IL-10
Pulmonary fibrosis [75]	Bleomycin	↓ Inflammatory cells accumulation ↓ Fibrosis ↓ Proinflammatory cytokines (TNF-α, IL-1) ↓ Chemokines (MIP-1α, RANTES, MIP-2, KC)
Perinatal infection (aberrant lung development) [78]	Gram negative bacteria LPS	↓ TNF-α ↑ IL-6 ↑ Lung mesenchyme tissue proliferation
Pulmonary arterial hypertension [46]	Monocrotaline	↓ IL-6 Prevented morphological and hemodynamic PAH features ↓ p38 MAPK activation
Lung metastases [79]	FSA-1 fibrosarcoma	↓ growth rate of pulmonary metastases

KC: Keratinocyte-derived chemokine; MIP-1α: Macrophage inflammatory protein-1 alpha; MIP-2: Macrophage inflammatory protein-2; PAH: Pulmonary arterial hypertension; RANTES: Regulated on activation normal T-cell expressed and secreted.

accumulation of inflammatory cells in the interstitial area. It originates activation of fibroblasts, eventually resulting in diffuse alveolar damage followed by fibrosis [74]. It is used as an animal model of pulmonary fibrosis. Yara *et al.* [75] studied the effect of thymulin in bleomycin-treated animals. They documented a suppressed cellular inflammation response in the lungs, with reduced accumulation of leukocytes and reduced synthesis of pro-inflammatory cytokines (TNF-α and IL-1) and some chemokines, such as macrophage inflammatory protein-1 alpha (MIP-1α), regulated on activation normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein-2 (MIP-2) and keratinocyte-derived chemokine (KC). Thymulin also ameliorated the fibrotic changes, as evidenced by the reduced accumulation of hydroxyproline. These results suggest a potential therapeutical application of thymulin in pulmonary fibrosis.

Intra-amniotic inflammation increases the risk of preterm labor and aberrant lung development with subsequent respiratory disorders. Although not well established, those anomalies are, at least in part, caused by the accumulation

of inflammatory cells and overexpression of pro-inflammatory cytokines, namely IL-1, TNF-α and IL-6 [76,77]. Land and Darakhshan [78] studied the effect of thymulin in fetal lung exposed to a Gram-negative bacteria lipopolisaccharide. They were able to document the suppression of TNF-α release and increased IL-6 expression through a CCAAT-enhancer-binding-protein-β-dependent pathway, which is a known path for regenerative repair in other tissues. These two outcomes favor the proliferation of mesenchyme tissue, which is a necessary condition for lung tissue regenerative repair. This work suggests the therapeutic potential of thymulin to promote lung morphogenic response, thus altering the course of lung damage during perinatal infection.

Both experimental and human data supports inflammation as an important mechanism in PAH pathophysiology. PAH patients have an accumulation of inflammatory cells in pulmonary vessels and elevated levels of pro-inflammatory cytokines, notably IL-6 [70]. Our group studied the immunomodulatory properties of thymulin in an experimental model of PAH [46]. Thymulin administration to rats with monocrotaline-induced pulmonary hypertension prevented the morphological and hemodynamic features of PAH. This effect could be, at least partially, mediated through the inhibition of IL-6 expression in both right ventricle and lung and by suppression of phosphorylation of lung p38, a MAPK family member implicated in PAH.

Additionally, biologically active thymulin exerted beneficial effects against tumor growth rate of pulmonary metastases [79]. Although these studies did not implicate intrinsic lung diseases, they suggest that the anti-inflammatory effect in the lung has the potential to regulate cellular growth and differentiation of the lung.

5.1 Therapeutic application of thymulin

The efforts to test therapeutic applications of thymulin have been directed to clinical situations associated with markedly low levels of this peptide like aging [80], AIDS [81] and other immunodeficiencies [82,83]. The goal of thymulin treatment in these conditions is to restore the neuroendocrine balance disrupted by its deficiency. There are some studies that document the beneficial effects of thymulin gene therapy in congenitally athymic animals, improving some associated endocrine anomalies such as reproductive system dysfunction [30,84] and glucose and lipid homeostasis [85]. Neuroscience is another field where thymulin is being studied, in particular, pursuing its anti-inflammatory and analgesic properties in chronic brain inflammatory diseases [33,86]. Gene therapy was considered an interesting option to supplement thymulin in deficient animals since it was difficult to do it pharmacologically. Recently, a DNA sequence coding for bioactive thymulin analogue was constructed and cloned in an adenoviral vector that induced sustained supraphysiological thymulin serum levels in thymectomized animals [87]. Interestingly, when injected directly to the brain there was a longer duration of adenoviral-mediated expression of thymulin than of other proteins [88]. This suggests that the anti-inflammatory activity of thymulin analogues could prevent the immune response to viral proteins or viral-encoded proteins, a major limitation of gene therapy.

The evidence for the beneficial effect of thymulin in animal models of lung disease favors this hormone as a potential therapeutic option to lung conditions for which there are no current efficacious treatments. Therapeutic interest in thymulin has been present since its discovery in seventies. However, until recently its unavailability hindered its use. In the late 1980s, Calenda *et al.* achieved the production of large quantities of purified thymulin with full biological activity through a synthetic DNA sequence inserted into a bacterial expression vector [89]. More recently, in 2006, Reggiani *et al.* [87] constructed and cloned a sequence of a thymulin analogue in an adenoviral vector, disclosing an interesting opportunity for lung disease treatment. The lung is an attractive organ for gene transfer because of the accessibility of its airways and vessels. Although gene therapies have been aimed at lung diseases with single gene defects like cystic fibrosis or α1-antitrypsin deficiency, other lung diseases such as COPD, asthma, ILDs and PAH are characterized by a chronic inflammation with imbalance between anti- and pro-inflammatory mediators and could also benefit from gene therapy [90]. Even congenital lung disease could potentially be treated by gene therapy with *in utero* intrapulmonary injection as already described [91]. Apparently, thymulin could have advantages over other immunomodulator substances considering the data that suggests the reduced extent of the immune response to viral proteins. Furthermore, thymulin has no known toxic effects even at high doses [30].

6. Conclusion

Thymulin immunomodulatory properties are well studied in various systemic and organ-specific inflammation models, where this peptide has demonstrated consistent and remarkable anti-inflammatory effects. An important pathophysiological mechanism of lung diseases is inflammation. Although scarce, experimental data on animal models of lung diseases identify thymulin as a potential therapeutic option that needs to be explored.

7. Expert opinion

The currently available anti-inflammatory therapeutic strategy for lung diseases is based on glucocorticoids. The management of these patients is determined by the response to this pharmacological agent and its numerous side effects. The prevalence of glucocorticoid resistance in lung glucocorticoid-sensitive diseases is unknown because the absence of unified definition and criteria and the variations in the course of the disease. Several molecular mechanisms of glucocorticoid resistance have been identified [92,93]. One of them is the reduced glucocorticoid receptor function mediated via phosphorylation

of the receptor by p38 MAPK [94,95]. On the other hand, there are other lung diseases that are normally insensitive to glucocorticoids such as COPD, pulmonary fibrosis and PAH. Besides glucocorticoid resistance, chronic systemic administration of glucocorticoids has numerous and serious side effects representing an important barrier to effective treatment, leading to the use of steroid-sparing drugs, although all these drugs also have major side effects. This scenario makes the task of treating these patients hard and drives the need for research on other anti-inflammatory agents.

Thymulin is one possibility since it has beneficial effects in several animal models of lung disease in which inflammation is an important hallmark. Blocking specific pro-inflammatory cytokines or their receptors has so far been disappointing in clinical studies, suggesting that a broader spectrum of anti-inflammatory effects is needed. Thymulin has an anti-inflammatory effect, inhibiting the synthesis of several pro-inflammatory cytokines, chemokines and influencing the activity of inflammatory cells.

The p38 MAPK pathway is involved in expression of inflammatory cytokines and chemokines. Interestingly, thymulin also inhibits MAPK member p38, a protein involved in glucocorticoid resistance. Several p38 MAPK inhibitors are being tested in clinical trials; however there have been several problems regarding side effects and toxicity [1]. Thymulin could be a useful alternative, for the reason that it is an endogenous substance with a favorable pharmacodynamic profile – there are no known side effects even at high doses.

Recently, an adenoviral vector with a sequence of thymulin analogue was developed. With respect to lung diseases, gene therapy is not a theoretical concept, but a realistic goal, since lung airways and vessels have good accessibility. Thymulin stands at a good position for lung gene therapy because of its lack of toxicity so far and the reduced extent of immune response against viral proteins, which is one of the main hurdles for gene therapy implementation.

Further research is needed to clarify the mechanisms that mediate the anti-inflammatory effect of thymulin and to develop oral formulations of thymulin. It is also necessary to test thymulin in lung disease models through viral and non-viral vector systems.

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Declaration of interest

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