Annals of Agricultural and Environmental Medicine 2022, Vol 29, No 3, 358–364
www.aaem.pl

ORIGINAL ARTICLE

Cathelicidin influence on pathological activation of Wnt pathway in murine model of hypersensitivity pneumonitis

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Lemieszek MK, Golec M, Zwoliński J, Dutkiewicz J, Milanowski J. Cathelicidin influence on pathological activation of Wnt pathway in murine model of hypersensitivity pneumonitis. Ann Agric Environ Med. 2022; 29(3): 358–364. doi: 10.26444/aaem/153082

Abstract

Introduction and Objective. Cathelicidin (CRAMP) is a defence peptide with a wide range of biological responses including antimicrobial, immunomodulatory and wound healing. Furthermore, our previous studies suggested the possibility of using cathelicidin in the prevention and treatment of pulmonary fibrosis in the course of hypersensitivity pneumonitis (HP). The molecular mechanism of CRAMP action, however, was not fully explained. Due to the fact that several studies indicated the Wnt signals pathways as a key player in wound healing and fibrosis, studies focused on this pathway in order to explain the above-mentioned therapeutic potential of cathelicidin in HP development.

Materials and method. The study was conducted in a murine model of HP, wherein lung fibrosis is induced in mice strain C57BL/6J by chronic exposure to saline extract of P. agglomerans (SE-PA). Cathelicidin was administered in the an form of aerosol during HP development. Changes in the expression of genes and proteins involved in signals transduction in the Wnt/β-Catenin pathway were examined in lung tissue homogenates by RealTime PCR and Western Blotting, respectively.

Results. The study revealed that cathelicidin decreased the elevated level of components of the Wnt/β-Catenin pathway (Ctnnd1/β-Catenin, Wnt1/WNt1, Wnt3a/Wnt3a, Wnt5a/Wnt5a) in the murine model of HP. Furthermore, CRAMP administered together with SE-PA inhibited the transcription function of β-catenin, leading to a decrease in abnormal expression of profibrotic molecules: Cyclin D1, c-Myc, MMP-7. Nevertheless, cathelicidin was not able to completely neutralize the negative changes induced by SE-PA.

Conclusions. The study demonstrated the beneficial effect of exogenous cathelicidin on signals transduction in the Wnt/β-Catenin pathway, which may prevent fibrosis development in HP.

Key words

extrinsic allergic alveolitis, Pantoea agglomerans, lung fibrosis, defence peptides, immune peptides, Wnt/ β-Catenin pathway

INTRODUCTION

Hypersensitivity pneumonitis (HP), also known as extrinsic allergic alveolitis (EAA), is a heterogenic group of interstitial lung diseases in which the chronic inhalation of a wide variety of antigens provokes a hypersensitivity reaction in susceptible subjects with inflammation in the terminal bronchioles, the pulmonary interstitium, and the alveolar tree. The inflammation often organizes into granulomas and may progress to pulmonary fibrosis [1, 2]. HP can be provoked by a wide range of antigens derived frombacteria (including mycobacteria), fungi, plant and animals, as well as some chemicals, and metals [2, 3]. Dependent on the source and the type of antigens, several varieties of HP have been distinguished; however, all of them show a similar clinical course [2]. One of the HP causative agents worthy of note is Gram-negative bacteria *Pantoea agglomerans* widely distributed in nature, especially on the surface of plants, including herbs, grain and other plant materials used in agriculture and industry [4, 5]. Because of the great variety and distribution of HP-induced antigens, millions of individuals are exposed to them as part of their occupational, home, or recreational environments. Thus, HP is estimated to be one of the most frequent reasons for pulmonary fibrosis worldwide [6, 7]. The above-mentioned state of knowledge allowed us to create an HP animal model for use in the current study, wherby lung fibrosis was induced in the prone to fibrosis mice strain C57BL/6J by chronic exposure to defined saline extract of *P. agglomerans* (SE-PA) [8, 9, 10, 11].

As the main mechanism of pulmonary fibrosis is a pathology of repair of the wounded pulmonary epithelium [1, 12, 13, 14], it was assumed that improvement of its regeneration by application of the natural enhancer of this process – cathelicidin [15, 16] – should prevent or inhibit the disease development. Cathelicidin is an endogenous antibiotic, described two decades ago, which demonstrated antimicrobial and LPS neutralizing properties as well as significant immunomodulatory activities, including the promotion of wound healing [15, 16]. Furthermore, our earlier studies have shown significant changes in cathelicidin

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Received: 25.05.2022; accepted: 29.08.2022; first published: 06.09.2022

concentration in the course of respiratory pathologies [17, 18] and due to exposure to organic dust [19]. Our recent research revealed that cathelicidin therapy inhibits the development of pulmonary fibrosis in the murine model of HP. The beneficial impact of cathelicidin was associated with its ability to restore the balance in the number of immune cells (NK cells, macrophages, lymphocytes: Tc, Th, Treg, B), cytokines production (IFNγ, TNFα, TGFβ1, IL1β, IL4, IL5, IL10, IL12α, IL13) and synthesis of extracellular matrix components (collagens, hydroxyproline) disturbed by chronic exposure of mice to *P. agglomerans* [11]. Despite the discovery of the usefulness cathelicidin in the prevention of lung fibrosis development in the course of HP, the molecular mechanism of peptide action has not been fully explained. Because of the fact that several studies indicated the Wnt signaling pathway as a key player in wound healing and fibrosis [20], the current study focused on this pathway in order to explain the above-mentioned therapeutic potential of cathelicidin in HP development.

MATERIALS AND METHOD

Reagents. Unless otherwise indicated, the chemicals used in the study were purchased from Sigma-Aldrich Co. LLC.

Cathelicidin. Murine cathelicidin (ISRLAGLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPE); with a purity of 95.53%, was purchased from the Novozym Polska s.c. (Science and Technology Park, Poznań, Poland). Lyophilized peptides were resuspended in sterile distilled water with 1% BSA to a final concentration of 1 μg/ml and divided into smaller portions (10 μl/tube). Stock solutions of CRAMP were stored at -80 °C prior to use. Working solutions of CRAMP were prepared by dissolving stock solution in phosphate-buffered saline (PBS) just before use.

Saline extract of *Pantoea agglomerans*. *Pantoea agglomerans* strain M-10–3 was first isolated from the air of a grain mill by the Dutkiewicz research team [21, 22]. *P. agglomerans* was inoculated on enriched nutrient agar medium (BTL, Łódź, Poland) supplemented with peptides (Proteobak, BTL, Łódź, Poland) and incubated in Roux bottles for 72 h at 37 °C. The bacterial mass was then harvested, homogenized with a glass homogenizer and extracted in saline in the proportion of 1:2 for 48 h at 4°C, with intermittent disruption of cells by 10-fold freezing and thawing. Afterward, the supernatant was separated by centrifugation at 10,000 rpm at 4°C, and finally lyophilized. The studies were performed using saline extract of the *P. agglomerans* (SE-PA), obtained by dissolving *P. agglomerans* cells lyophilizate in PBS just before use. SE-PA contains proteins, sugars, DNA and RNA (42.3%, 15.2%, 0.018%, and 0.014%, respectively, as determined by spectrophotometric analysis), and a relatively small quantity of active endotoxin (1% as assessed by *Limulus* test; Pyroquant Diagnostik GmbH, Germany) [5].

Animal inhalation. Three-month-old female C57BL/6J mice were purchased from Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland. The mice were kept in colony cages with free access to food and tap water *ad libitum*, under standardized housing conditions (natural light-dark cycle, temperature – 22–24 °C). Proper examinations were preceded by 7 days of adaptation to laboratory conditions, followed by 7 days of adaptation to inhalation procedures. Mice were exposed to a finely dispersed aerosol of the saline extract of *P. agglomerans* (SE-PA; 10 mg/ml; 5 ml/single inhalation) or cathelicidin (CRAMP; 1.44 μg/ml; 5 ml/single inhalation) or phosphate-buffered saline (PBS; 5 ml/single inhalation), administered separately or in combinations (Tab. 1). The mice were treated with each investigated factor for 1 h for 14 or 28 days. Inhalations were carried out using the Buxco Inhalation Tower under the following conditions: pressure 0.5 cm H_2O; airflow 2.5 l/min; nebulization rate 0.3 ml/min, room temperature. Each research group contained 8 mice: 6 treated and 2 untreated animals. Both untreated (control) and treated mice were sacrificed by cervical dislocation with spinal cord rupture, and lung samples were collected. The samples were frozen in liquid nitrogen and stored at -80 °C until evaluation. The experimental protocols were approved by the Local Ethics Committee for Animal Experimentation in Lublin, Poland (Resolution Nos. 39/2016 and 2/2017).

Real-Time PCR. Lung samples were placed in Lysing Matrix M tubes (MP Biomedicals). Next, Lysis/Binding Buffer (High Pure RNA Tissue Kit; Roche Diagnostics GmbH) was added, and tissues were homogenized mechanically using FastPrep-24 5G homogeniser (MP Biomedicals) under the following conditions: 6 m/s, 40 s, 20 °C. The homogenates were further incubated on the rotor (15 min., 20 °C), then passed through a 70-μm nylon mesh and centrifuged (2,400 × G, 5 min., 20 °C). Supernatants were collected in new tubes. The total RNA from lung homogenates was extracted using High Pure RNA Tissue Kit (Roche Diagnostics GmbH, Mannheim, Germany), and 0.5 μg RNA was reverse-transcribed using

Table 1. Description of research groups

Name of research group		Time of exposure			Factors administration sequence
		PBS	SE-PA	CRAMP	
Untreated (n=12)		-	-	-	-
CRAMP (n=12)	(n=6)	1 hour a day for 14 days	-	1 hour a day for 14 days	one-by-one on the same day
	(n=6)	1 hour a day for 28 days	-	1 hour a day for 28 days	one-by-one on the same day
SE-PA (n=12)	(n=6)	1 hour a day for 14 days	1 hour a day for 14 days	-	one-by-one on the same day
	(n=6)	1 hour a day for 28 days	1 hour a day for 28 days	-	one-by-one on the same day
SE-PA+CRAMP (n=12)	(n=6)	-	1 hour a day for 14 days	1 hour a day for 14 days	one-by-one on the same day
	(n=6)	-	1 hour a day for 28 days	1 hour a day for 28 days	one-by-one on the same day

PBS – phosphate buffered saline; SE-PA – saline extract of Pantoea agglomerans; CRAMP – cathelicidin; n – number of animals in research group

High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instruction. Real Time PCR analysis was carried out using TaqMan Gene Expression Assays (Mm02619580_g1 for *Actb*; Mm01334599_m1 for *Ctnnd1*; Mm01300555_g1 for *Wnt1*; Mm00437337_m1 for *Wnt3a*; Mm00437347_m1 for *Wnt5a*) and TaqMan Fast Universal PCR MasterMix (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. The reaction was incubated in 96-well optical plates at 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s using a 7500 Fast Real-Time PCR System (Applied Biosystems). Relative expression was calculated using the efficiency method (relative advanced quantification) and normalized to the expression of *Actb* (SDS 1.4 Software for the 7500 Fast System, Applied Biosystems).

Western Blotting. Lung samples were placed in Lysing Matrix M tubes (MP Biomedicals). Cell Disruption Buffer (PARIS Kit; Life Technologies) was added and tissues homogenized mechanically using FastPrep-24 5G homogeniser (MP Biomedicals, Warsaw, Poland) under the following conditions: 6 m/s, 40 s, 20 °C. The homogenates were further incubated on ice for 20 min, then passed through a 70 µm nylon mesh and centrifuged (10,000 × G, 5 min., 4 °C). Supernatants were collected in new tubes. Samples collected from the untreated mice, and samples obtained from animals belonging to the common research group, were mixed in equal volumes and their protein content determined by BCA Protein Assay Kit (Pierce Biotechnology, Waltham, MA, USA). Collected homogenates were solubilized in sample Laemmli buffer and boiled for 5 min. The samples were adjusted for equal protein loading before being electrophoresed by 12% SDS-PAGE, and then transferred to a PVDF membrane. The membranes were blocked for 1 h at room temperature with 5% non-fat dry milk in TBS-0.1% Tween 20 (TBS-T) before being probed with primary antibodies directed against β-Catenin, P-β-Catenin (Ser 552), P-β-Catenin (Ser 675), Cyclin D1, c-Myc MMP-7 (Cell Signaling Technology, Danvers, MA, USA), Wnt1, Wnt3a, Wnt5a (abcam) at 4 °C overnight, followed by incubation with horseradish peroxidase (HRP) conjugated secondary antibodies (Cell Signaling Technology) for 1 h at room temperature, and visualized by using enhanced chemiluminescence (Pierce Biotechnology,) using a Kodak BioMax Light film (Eastman Kodak Company, Rochester, MA, USA). In order to confirm equal amounts of protein in the gel, after removal of the antibodies, the membranes were re-incubated with antibodies against β-actin (Cell Signaling Technology). The amount of protein was densitometrically determined using ImageJ software.

Statistical analysis. The data were presented as the mean value and standard error of the mean (SEM). Statistical analysis was performed using linear regression analysis, as well as the one way-ANOVA with Tukey *post-hoc* test; column statistics were used for comparisons. Significance was accepted at $p < 0.05$.

RESULTS

The beneficial effect of cathelicidin on the expression of the Wnt gene family altered in a murine model of hypersensitivity pneumonitis. Real-Time PCR (Fig. 1, Tab. 2) revealed that chronic exposure of mice to cathelicidin did not cause any changes in the expression of the Wnt gene family (*Wnt1, Wnt3a, Wnt5a, Ctnnd1*). On the contrary, *Pantoea agglomerans* treatment induced evident alterations in the expression of all investigated genes, and the observed effect intensified with the increasing time of mice exposure. The most significant changes were observed in the case of *Wnt5a,* the expression of which increased by 29.0% and 69.8% after 14 and 28 days of SE-PA exposure, respectively. The most sensitive to *P. agglomerans* treatment was the expression of *Ctnnd1*, which reached the levels of 1.245 (14 days of inhalations) and 1.422 (28 days of inhalations). Cathelicidin administration, together with the antigen of *P. agglomerans,* significantly decreased the expression of all investigated genes elevated by SE-PA; however, CRAMP was not able to completely eliminate negative changes. Average differences in the expression of the examined gene in mice treated with both SE-PA and CRAMP vs. animals exposed to SE-PA were 14.5% (after 2-weeks of inhalations) and 27.2% (after 4-weeks of inhalations). The strongest beneficial effect of cathelicidin on the gene expression altered by *P. agglomerans* antigen was observed in *Wnt5a* after 14 days of mice exposure to SE-PA+CRAMP (mRNA level decreased by 16.0%) and in *Wnt3* after 28 days of mice treatment with the same agents (mRNA level decreased by 32.1%).

Cathelicidin eliminated negative changes in the expression of proteins associated with signals transduction in the Wnt/β-Catenin pathway in the murine model of hypersensitivity pneumonitis. Western blots (Fig. 2, Tab. 3) revealed that cathelicidin used alone did not impact on the expression of proteins involved in signals transduction in the Wnt/β-Catenin pathway: Wnt1, Wnt3a, Wnt5a, β-Catenin, Phospho-β-Catenin (Ser 552), Phospho-β-Catenin (Ser 675). On the contrary, chronic exposure of mice to saline extract of *P. agglomerans* distinctly increased the expression of all above-mentioned proteins. Among the components of

Table 2. Expression of Wnt gene family in response to cathelicidin (CRAMP) and/or saline extract of *Pantoea agglomerans* (SE-PA) treatment. Results presented as the mean of relative mRNA amount ± SEM

	Wnt1	*Wnt3a*	*Wnt5a*	*Ctnnd1*
Untreated	1.001 ± 0.027	1.003 ± 0.024	1.002 ± 0.017	1.003 ± 0.010
CRAMP (14 days of exposure)	1.010 ± 0.024	1.038 ± 0.022	1.027 ± 0.015	1.003 ± 0.025
CRAMP (28 days of exposure)	1.000 ± 0.016	1.012 ± 0.016	1.023 ± 0.033	1.008 ± 0.020
SE-PA (14 days of exposure)	1.255 ± 0.008	1.285 ± 0.017	1.290 ± 0.012	1.245 ± 0.012
SE-PA (28 days of exposure)	1.565 ± 0.008	1.553 ± 0.013	1.698 ± 0.015	1.422 ± 0.021
SE-PA+CRAMP (14 days of exposure)	1.123 ± 0.004	1.148 ± 0.010	1.130 ± 0.009	1.093 ± 0.004
SE-PA+CRAMP (28 days of exposure)	1.317 ± 0.008	1.232 ± 0.011	1.398 ± 0.010	1.203 ± 0.012

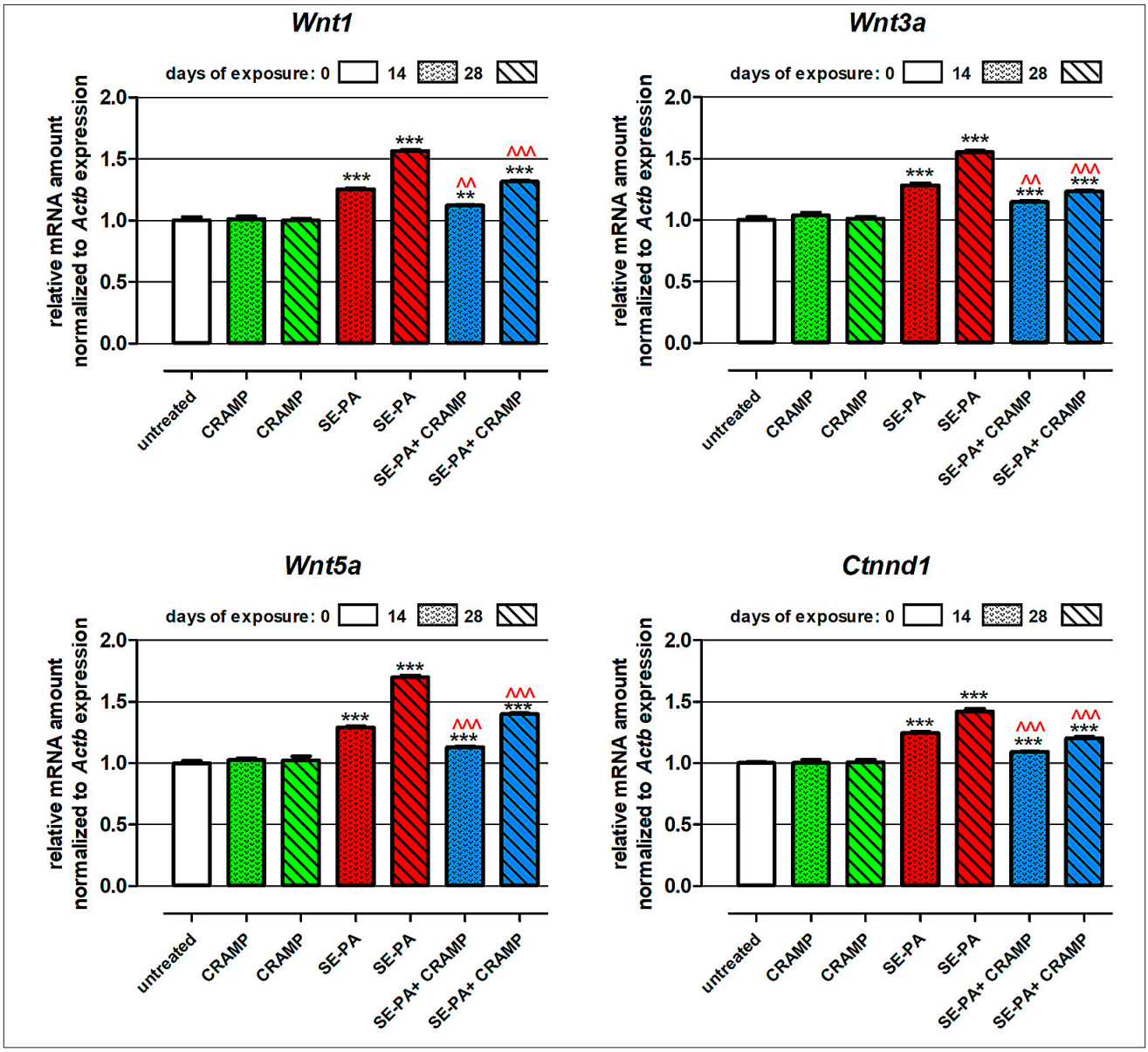


Figure 1. Cathelicidin influence on the pathological expression of Wnt gene family in the murine model of hypersensitivity pneumonitis. Gene expression was investigated in homogenates of lungs collected from untreated mice (control) and animals exposed to investigated compounds for 14 or 28 days using the Real-Time PCR method. Results are presented as the mean of relative mRNA amount ± SEM.
Statistically significant differences compared to the control (untreated mice) at $p< 0.01$ (**); $p< 0.001$ (***). Statistically significant differences compared to SE-PA+CRAMP vs. SE-PA (comparison within corresponding time points) at $p< 0.01$ (^^); $p< 0.001$ (^^^).
One-way ANOVA test; *post-hoc* Tukey test

Table 3. Expression of components of Wnt/β-Catenin pathway in response to cathelicidin (CRAMP) and/or saline extract of *Pantoea agglomerans* (SE-PA) treatment. Results presented as the mean of protein expression ± SEM

	Untreated	CRAMP (14 days exposure)	CRAMP (28 days exposure)	SE-PA (14 days exposure)	SE-PA (28 days exposure)	SE-PA+CRAMP (14 days exposure)	SE-PA+CRAMP (28 days exposure)
Wnt1	100.0 ± 2.2	114.8 ± 1.4	108.1 ± 3.1	299.4 ± 7.9	568.1 ± 12.4	227.7 ± 4.8	246.0 ± 7.3
Wnt3a	100.0 ± 1.5	111.7 ± 4.2	109.7 ± 3.6	378.2 ± 9.8	491.4 ± 11.3	196.2 ± 3.5	223.9 ± 4.2
Wnt5a	100.0 ± 4.3	110.3 ± 1.6	104.7 ± 3.4	190.7 ± 1.9	231.9 ± 1.9	121.4 ± 4.4	124.7 ± 3.7
β-Catenin	100.0 ± 2.1	100.5 ± 2.8	98.9 ± 3.4	186.8 ± 3.9	231.4 ± 4.8	129.6 ± 2.7	163.2 ± 3.4
Phospho-β-Catenin (Ser 552)	100.0 ± 4.9	97.8 ± 1.9	109.4 ± 1.3	187.8 ± 3.3	197.1 ± 3.4	102.2 ± 2.8	101.6 ± 2.9
Phospho-β-Catenin (Ser 675)	100.0 ± 0.7	111.9 ± 2.5	105.5 ± 4.2	213.3 ± 2.2	271.9 ± 3.0	175.3 ± 4.2	237.7 ± 4.4
c-Myc	100.0 ± 1.7	97.9 ± 4.6	117.3 ± 2.1	409.9 ± 7.1	452.6 ± 8.1	162.5 ± 3.1	204.9 ± 3.6
Cyclin D1	100.0 ± 1.7	99.5 ± 1.9	111.8 ± 2.0	355.7 ± 3.5	434.5 ± 3.9	180.3 ± 3.3	283.8 ± 3.5
MMP-7	100.0 ± 2.3	99.4 ± 3.3	109.2 ± 3.7	355.3 ± 6.0	512.7 ± 8.4	207.9 ± 2.1	303.9 ± 5.7

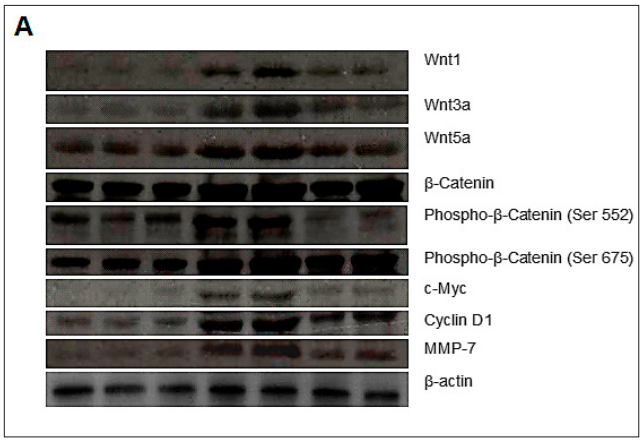
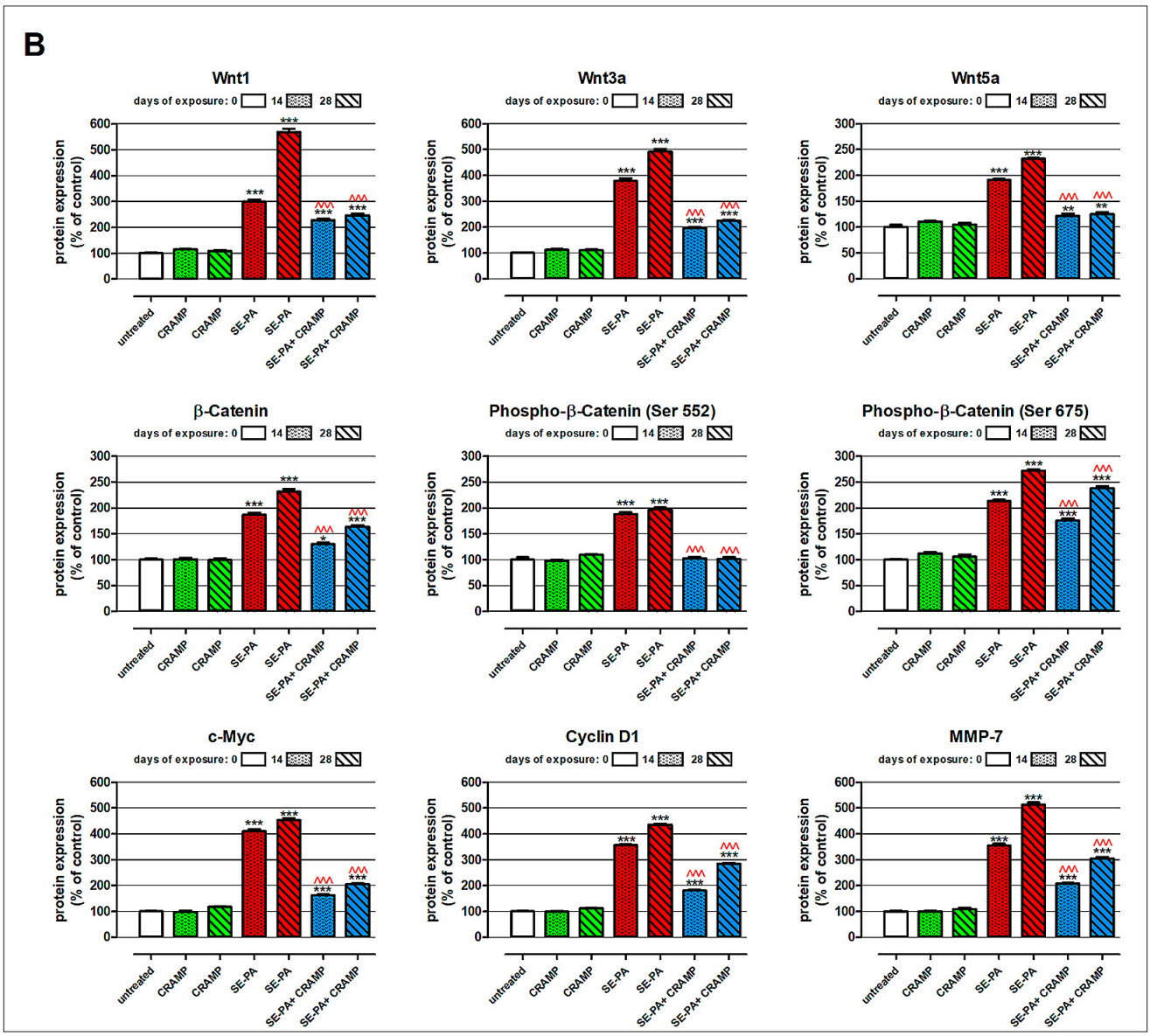


Figure 2. Cathelicidin influence on the pathological signals transduction in the Wnt/β-Catenin pathway in the murine model of hypersensitivity pneumonitis. Protein expression was investigated in homogenates of lungs collected from untreated mice (control) and animals exposed to investigated compounds for 14 or 28 days using the Western Blotting method. Examination of ß-actin expression level was used as the internal control. A) Representative Western blots from 3 independent experiments. B) Results of the densitometric analysis are presented as the mean of protein expression ± SEM.
Statistically significant differences compared to control (untreated mice) at $p<0.05$ (*1); $p<0.01$ (**), $p<0.001$ (***). Statistically significant differences compared to the SE-PA+CRAMP vs. SE-PA (comparison within corresponding time points) at $p<0.001$ (^^^). One-way ANOVA test; *post-hoc* Tukey test.



the Wnt/β-Catenin pathway, the most significant changes were shown in the expression of Wnt1 and Wnt3a whose levels in response to 14 and 28 days of SE-PA exposure increased from 299.4% and 378.2% to 568.1% and 491.4%, respectively. Noteworthy was also induced by *P. agglomerans* enhancement of β-Catenin phosphorylation at Ser 552 and Ser 675; expression of the mentioned molecules reached 187.8% and 213.3% of control after 2-weeks of exposure, as well as 197.1% and 271.9% of control after 4-weeks of inhalations with antigen. Cathelicidin administered together with SE-PA significantly decreased the expression of the selected components of the Wnt/β-Catenin pathway elevated

by inhalations with *P. agglomerans;* the most significant improvement was observed in the case of Phospho-β-Catenin (Ser 552), the expression of which after 14 and 28 days of CRAPM+SE-PA exposure approached the level of control. Nevertheless, a comparison of the results obtained in the 'SE-PA' and 'SE-PA+CRAMP' research groups at the corresponding time points revealed the most significant changes in the expression of Wnt1 and Wnt3a, the expression of which elevated by *P. agglomerans* decreased after 28 days of cathelicidin treatment by 322.1% and 267.5%, respectively. On the contrary, the most resistant to the beneficial effect of cathelicidin treatment was Phospho-β-Catenin (Ser 675), the expression levels of which were recorded in 'SE-PA' and 'SE-PA+CRAMP' differed only by 38.0% (day 14 of experiment) and 34.2% (day 28 of the experiment).

In the next step of the study, changes in the level of profibrotic proteins (c-Myc, Cyclin D1, MMP-7), the expression of which is regulated by β-Catenin, were investigated and revealed that chronic exposure of mice to CRAMP did not affect the expression of the mentioned β-Catenin molecular targets. On the contrary, animals inhalations with SE-PA significantly increased the expression of c-Myc, Cyclin D1 and MMP-7, and the observed effect intensified with the time of exposure, reaching the following levels on day 28 of the experiment: 452.6%, 434.5% and 512.7%, respectively. Cathelicidin administration together with *P. agglomerans* evidently lowered the abnormal expression of the mentioned proteins; however, CRAMP treatment was not able to completely neutralize the negative effect of SE-PA. The strongest beneficial effect of cathelicidin was observed in the case of c-Myc, the expression of which after 2-weeks and 4-weeks of mice exposure to both SE-PA and CRAMP, decreased by 247.4% and 247.7%, respectively.

DISCUSSION

The Wnt signalling pathway is an evolutionarily conserved pathway involved in several crucial biological processes in both embryonic developments, as well as adult cell maintenance and regeneration. The extra-cellular Wnt signal stimulates 3 intra-cellular signals transduction pathways, the canonical Wnt/β-Catenin pathway, the non-canonical Wnt/calcium pathway and the non-canonical planar cell polarity pathway [23]. Among these signals transduction pathways the first one has been the most extensively investigated as its deregulation is linked to a wide range of diseases, including fibrotic diseases [24, 25, 26, 27]. In the absence of Wnt ligands, the β-Catenin destruction complex coordinates a series of sequential β-Catenin phosphorylation, which finally labels the protein for degradation in the proteasome. On the contrary, the Wnt/β-Catenin pathway is activated when extracellular Wnt ligands bind to a specific transmembrane receptor, and induce subsequent changes causing the dissociation of the destruction complex, stabilization of β-Catenin (phosphorylation at Ser 552 and Ser 675), its translocated into the nucleus wherein after binding with to members of the TCF/LEF (T-cell factor/lymphoid enhancing factor) family of transcription factors, induces expression of hundreds of genes, including genes coding pro-fibrotic molecules like c-Myc, Cyclin D1, the matrix metalloproteinase MMP-7 [23].

The current study shows that the chronic exposure of mice to cathelicidin did not impact on the expression of the components of Wnt signaling pathways as well as β-Catenin targets. On the contrary, saline extract of *P. agglomerans* (SE-PA) up-regulated the expression of *Wnt1*, *Wnt3a*, *Wnt5a* and *Ctnnd1* and also increased the expression of their translation products. It should be emphasized that SE-PA elevated the phosphorylation of β-Catenin at Ser 675 and Ser 552, which induces protein accumulation in the nucleus and increases its transcriptional activity [23]. It needs to be highlighted that activation of the Wnt signalling pathways intensified with the time of mice exposure to the antigen of *P. agglomerans,* and the observed changes correspond with previously reported development of lung fibrosis (accumulation of extracellular matrix components, as well as reduction of respiratory capacity) [11]. Presented results are consistent with several scientific reports which also demonstrated a significant accumulation of β-Catenin in the nucleus, as well as over-expression of Wnt target genes in lung fibrotic tissue [28, 29, 30, 31]. Additionally induced by SE-PA changes in components of the Wnt pathway were similar to data obtained by Konigshoff et al., who reported increased expression of *WNT1*, *WNT3A*, *WNT7B*, *WNT10B*, *FZD2*, *FZD3* and *LEF1* in patients with idiopathic pulmonary fibrosis, and also demonstrated that Wnt1, Wnt3a, β-Catenin, and GSK-3b are localized in the alveolar and bronchial epithelium [29].

To further support the functional significance of β-Catenin, the expression of 3 target proteins (Cyclin D1, c-Myc, MMP-7) was examined. It needs to be highlighted that over-expression of Cyclin D1 and MMP-7 was reported by Chilosi et al. in lung tissue samples obtained from idiopathic pulmonary fibrosis/usual interstitial pneumonia (IPF/UIP), and the changes discovered were connected with abnormal β-Catenin activation [28]. Zuo et al. also indicated MMP-7 as the molecules most highly expressed in IPF [32]. At the same time, over-expression of c-Myc, as well as its pivotal role in IPF, have been shown in both *in vivo* and clinical studies [33, 34], which revealed that chronic exposure of mice to SE-PA significantly increased the level of the above-mentioned molecules relevant for pulmonary fibrosis [28, 32, 33, 34, 35]. Consequently, presented data as well as disorders in both respiratory function and lung tissue morphology, previously reported by our research team [11], revealed a connection between activation of the Wnt/β-Catenin pathway and development of fibrosis in the course of HP.

A observed in the murine HP model, accelerated activation of the Wnt/β-Catenin pathway was effectively inhibited by cathelicidin, which administered together with SE-PA significantly down-regulated the expression of *Wnt1*, *Wnt3a*, *Wnt5a* and *Ctnnd1*, decreased the level of proteins coding by the mentioned genes, and also inhibited phosphorylation of β-Catenin at Ser 675 and Ser 552. Furthermore, altered expression of β-Catenin targets (Cyclin D1, c-Myc and MMP-7) was also restored by cathelicidin treatment and the observed effects depended on the time of exposure. Nevertheless, changes induced by cathelicidin in the HP model were significant, but the investigated peptide was not able to completely neutralize the negative influence of *P. agglomerans* antigen on the signals transduction in the Wnt/β-Catenin pathway. This observation corresponds with our earlier studies [11], which also revealed the great potential of cathelicidin use in the prevention of fibrosis in the course of HP, at the same time indicating the necessity to increase the effectiveness of restoration of cathelicidin physiological concentration in order to improve the therapeutic properties of the investigated peptide.

CONCLUSIONS

The presented study has revealed the beneficial impact of cathelicidin treatment on the abnormal expression of genes and proteins involved in signals transduction in the Wnt/β-Catenin pathway in the murine model of hypersensitivity pneumonitis. Despite the fact that exogenous cathelicidin was not able to completely neutralize the upregulated components of the Wnt/β-Catenin pathway, as well as β-Catenin molecular targets, the peptide effectively decreased undesirable changes. Furthermore, cathelicidin used alone did not affect the expression of the examined genes and proteins. Nevertheless, it needs to be highlighted that for the first time this study has demonstrated the beneficial effect of exogenous cathelicidin on signals transduction in the Wnt/β-Catenin pathway, which may prevent fibrosis development in HP.

Acknowledgements
The studies were supported by the National Science Centre, Poland [Grant No, 2015/19/D/NZ7/02952, 2016].

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