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Increased pulmonary Wnt (wingless/integrated)-signaling in patients with sarcoidosis

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Summary
Background: Sarcoidosis is an inflammatory multisystemic granulomatous disease of unknown aetiology commonly affecting the lungs, and pulmonary fibrosis often develops in chronic sarcoidosis. It has been suggested that Wnt (Wingless/integrated)-signaling has a role in inflammatory and fibrotic processes in the lungs, but its role in sarcoidosis has not been investigated. We hypothesised that Wnts secreted from T cells or other inflammatory cells have a role in the pathogenesis of sarcoidosis.
Methods: Brush biopsies and bronchoalveolar lavage (BAL) were obtained through bronchoscopy from healthy controls ($n = 18$) and patients with sarcoidosis ($n = 48$). Semi-quantitative RT-PCR, electrophoretic mobility shift assay (EMSA) and immunocytochemistry were performed to analyse Wnt expression and activation of the Wnt-signal transducer β-catenin.
Results: Altered expression of Wnt5A, Wnt7A and Wnt7B mRNA in BAL cells was observed, as well as an increased activation of β-catenin, measured by EMSA and confirmed with immunocytochemistry, in resident lung cells from patients with sarcoidosis. More pronounced changes in Wnt expression were seen with advancing disease stage. Thus, by three independent methods, we have found evidence of increased pulmonary Wnt-activation in sarcoidosis.
Conclusions: In the lungs of patients with sarcoidosis there is a previously unappreciated increased Wnt-signal activation that could contribute to the inflammatory processes.

Abbreviations: BAL, bronchoalveolar lavage; Cox-2, cyclooxygenase-2; EMSA, electrophoretic mobility shift assay; fra-1, fos-related antigen 1; Fz, frizzled; IPF, idiopathic pulmonary fibrosis; MMP, matrix metalloproteinases; mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction; TCF/Lef, T cell factor/leucocyte enhancing factor; uPAR, urokinase-type plasminogen activator receptor; VEGFR, vascular endothelial growth factor receptor; Wnt, wingless/integrated; DVL1, dishevelled dsh homolog 1; SFRP2, secreted frizzled-related protein 2.

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Introduction

Sarcoidosis is a multisystemic granulomatous disease of unknown aetiology, most commonly affecting the lungs.[1] A typical accumulation of activated T helper ($CD4^+$) cells, found through the use of bronchoscopy and bronchoalveolar lavage (BAL) in patients with sarcoidosis, was described already in the 1980-ies.[2] These lung-accumulated CD4+ T cells have a Th1 cytokine profile[3,4] and are considered to be of central importance for the inflammation. The infiltrating inflammatory cells contribute to the formation of non-caseating epithelioid cell granulomas. The majority of patients recover, but some develop chronic disease with fibrosis, and in some cases respiratory failure. Löfgren's syndrome is a clinically separate subset of sarcoidosis patients, often associated with a good prognosis, distinguished by an acute onset of disease, bilateral hilar lymphadenopathy, fever, erythema nodosum and ankle arthritis or periarticular inflammation.[5]

Wnts are secreted glycoproteins involved in cell proliferation, differentiation and migration. They act on cell surface receptors to activate intracellular pathways. The dominating canonical pathway includes the signal transducing molecule β-catenin. When activated, β-catenin translocates to the nucleus, where it binds to and activates TCF/Lef (T cell factor/Leukocyte enhancing factor) transcription factors, leading to specific gene transcription. Several of the inflammatory target genes[6] of the Wnt/β-catenin pathway have been shown to be up-regulated in patients with sarcoidosis.[7]

Based on this, we hypothesised that Wnts secreted from T cells, or other inflammatory cells, are involved in the inflammatory processes in sarcoidosis. We therefore used RT-PCR to measure mRNA levels of selected Wnts in BAL cells. In the absence of a well-established method to directly measure Wnt-protein levels in biological fluids, we measured activation of Wnt-signaling in biopsies by electrophoretic mobility shift assays (EMSAs) for activated β-catenin, and by immunohistochemistry for nuclear β-catenin, both well-established and robust methods to monitor Wnt-signaling.[8–10]

Materials and methods

Subjects

The total number of subjects included in this study was 66. Sarcoidosis patients ($n = 48$; Table 1) were recruited when referred to the Division of Respiratory Medicine (Karolinska University Hospital, Stockholm, Sweden) for initial diagnostic investigation. Eighteen were non-smoking healthy controls. All patients had clinical features of sarcoidosis according to criteria established by WASOG,[11] evaluated by chest radiography, symptoms and pulmonary function tests. None of the patients received corticosteroid treatment at the time of the study. Detailed patient characteristics are given in Table 1 and Table 2. The study was approved by the local ethics committee.

Cells and samples

Brush biopsies were obtained during fiberoptic bronchoscopy under local anaesthesia. Three consecutive endobronchial brushings were collected, yielding approximately 1×10^6 cells/brush, pooled in 0.5 ml phosphate-buffered saline (PBS) and pelleted before storage at −80 °C until analysis. BAL was performed with sterile 37 °C PBS, instilled in five 50 mL aliquots in a segmental middle lobe bronchus and immediately re-aspirated. Fluid was collected in a siliconised plastic bottle and stored on ice. The recovered fluid was centrifuged at $400 \times g$ for 10 min at 4 °C, to separate BAL cells. The cell pellet was resuspended in RPMI1640 medium (Sigma–Aldrich, Irvin, UK) and viability determined by trypan blue exclusion. After Giemsa-staining, a total of 500 cells were counted and differentiated into cell types macrophages, lymphocytes, eosinophils, basophils and neutrophils. Aliquots of 1×10^6 cells were pelleted and stored at −20 °C until further use.

RT-PCR

RNA was extracted from total BAL cells using the RNeasy®MiniKit (Qiagen) according to the manufacturers' instructions. RNA concentration was measured using Nanodrop™ (Thermo Fisher Scientific, Wilmington, DE). Aliquots of total RNA were converted to cDNA with ReactionReady™ First strand cDNA synthesis kit (SABiosciences, Frederick, MD). Primers for Wnt5A (#PPH02410A), Wnt7B (#PPH02464A), Wnt7A (#PPH02465A) and the housekeeping gene PSMB2 (#PPH20109A),[12] as well as RT^{2}Real-Time™SYBRGreen PCR-Master Mix were purchased from SABiosciences. The reactions, with sample cDNA, primer sets and PCR-master mix, were subjected to PCR conditions of 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min, for 40 cycles in an ABI PRISM Sequence Detector 7700 (Applied Biosystems, Foster City, CA). PCR products were resolved by electrophoresis on 2% agarose gels to verify that the primer sets amplified a product of predicted size. In addition to agarose electrophoresis, the specificity of the PCR product was ensured by melting curve analysis.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from brush biopsy-cells as previously described.[13] The purity of the obtained cells were determined by differential counting of brush biopsy-cells cytocentrifuged onto glass slides, see below. 94% of the obtained cells were epithelial, in line with previous publications.[13] β-catenin binding activity was investigated by EMSA using an oligonucleotide harbouring a TCF/Lef consensus binding site (5′- TGT TGT TAA GCA AAG ATC AAA GCC CGG CAG AG- 3′), end-labeled with [γ32-P] ATP, as probe. Protein concentration was measured by the Bradford method[14] and equal amounts was incubated with probe before loading onto non-denaturing 5% polyacrylamide gels. Gels were fixed and vacuum dried before visualisation of resulting complexes by autoradiography. The intensity of the active TCF/β-catenin shift was measured using the freeware ImageJ.[15] The images were inverted and the shift intensity measured at the lowest point (Supplemental Fig. 1A and B). Then, the intensity of each shift was calculated in respect to exposure intensity of the entire image, by subtracting the background intensity (noise) from the total shift intensity (S) in each respective gelshift, to account for the manual film processing. S–N intensities were used in the statistical calculations.

Table 1 Characteristics of the study population.

	qRT-PCR		EMSA		Immunocytochemistry	
	Healthy	Sarcoidosis	Healthy	Sarcoidosis	Healthy	Sarcoidosis
Subjects n	8	37	5	7	5	4
Gender M/F	5/3	20/17	1/4	3/4	0/5	1/3
Age	27 (25—49)	42 (29—74) ***	22(20—35)	32 (26—46) *	24 (20—33)	34 (28—65)
Smoking NS/Ex-SM/SM	7/1/0	19/9/9	5/0/0	4/2/1	5/0/0	1/3/0
Radiographic Stage 0/I/II/III/IV	0	0/15/11/8/3	0	0/6/1/0/0	0	1/3/0/0/0
BAL Analysis						
Recovery %	76.0 (67.0—94.0)	68.0 (44.0—84.0)*	74.0 (59.0—76.0)	70.5 (57.6—76.0)	79.0 (59.0—84.0)	64.0 (53.0—74.0)
Viability %	92.0 (86.0—94.0)	94.0 (82.0—98.0)	92.0 (87.0—95.0)	92.3 (82.0—98.0)	86.0 (73.0—95.0)	93.5 (80.0—97.0)
Cell Concentration x1.0E+06*L-1	91.3 (53.6—139.4)	153.9 (53.6—521.1)*	62.0 (42.0—99.5)	216.4 (49.1—303.2)	75.0 (62.8—88.6)	204.4 (94.0—337.0)*
Löfgren's Syndrome Yes/No	—	12/21	—	0/7	—	0/4
Differential Cell Counts						
Macrophages %	77.0 (62.6—92.5)	79.5 (28.0—94.8)	86.6 (75.5—94.8)	77.7 (46.6—85.8)	90.1 (67.5—94.8)	81.3 (69—85.7)
Lymphocytes %	16.3 (5.5—34.6)	18.9 (4.0—69.0)	12.4 (3—20.5)	20.2 (13.5—52.2)	6.4 (3—30.2)	16.0 (12—29.8)
Neutrophils %	1.4 (0.3—14.8)	1.2 (0.0—5.6)	2.2 (1—3.7)	0.8 (0—4.8)	2.2 (2.1—3.4)	1.725 (0.6—3.4)
Eosinophils %	0.2 (0—1.2)	0.2 (0.0—6.2)	0.0 (0—0.3)	0.0 (0—0.8)	0 (0—0.8)	0.1 (0—1.3)
Basophils %	0.0 (0—0.2)	0.0 (0.0—0.8)	0	0.0 (0—0.2)	0	0
CD4/CD8 ratio	3.45 (1.8—5.1)	4.7 (0.7—17.0)	—	8.9 (4—13)	—	4.7 (2.3—7.6)
Pulmonary function tests						
VC%	119.0 (105.0—123.0)	90.0 (33.0—114.0)**	—	94.0 (85.0—112.0)	—	83.5 (82.0—85.0)
FVC%	113.5 (105.0—132.0)	91.5 (28.0—117.0)***	—	94.0 (85.0—111.0)	113.0 (104.0—116.0)	84.0 (83.0—85.0)
TLC%	—	91.5 (69.0—120.0)	—	93.0 (86.0—112.0)	—	86.0 (86.0—86.0)
FEV_1%	111.5 (91.0—119.0)	88.5 (23.0—120.0)**	—	89.0 (80.0—104.0)	105.0 (100.0—107.0)	82.0 (80.0—84.0)

Data are presented as median (minimum-maximum), unless otherwise stated. EMSA: Electrophoretic mobility shift assay; VC: Vital capacity; FVC: forced vital capcity; TLC: total lung capacity; FEV_1: forced expiratory volume in 1 s; NS: Never smoker; Ex-SM: Ex-smoker; SM: Smoker; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ between Healthy and Sarcoidosis within one study group.

Table 2 Characteristics of subjects in the RT-PCR experiments and BAL-findings.

Pat#	Gender	Age	Smoking	Sarcoidosis chest radioqraphic staqe	Lofgren's syndrome	Macrophages %	Lymphocytes %	Neutrophils %	Eosinophils %	Basophils %	vc%	FVC %	TLC %	FEV_1%	CD4/CD8
HI	M	27	NS	0	—	88,7	11,0	0,3	0,0	0,0	—	114	—	119	—
H2	F	32	NS	0	—	76,0	23,0	0,6	0,4	0,0	119	132	—	111	1,8
H3	M	27	NS	0	—	87,6	10,0	2,0	0,4	0,0		114	—	115	—
H4	M	25	NS	0	—	92,5	55	2,0	0,0	0,0		105	—	103	—
H5	F	26	Ex-SM	0	—	73,0	25,8	1,2	0,0	0,0	105	108	—	101	—
H6	M	25	NS	0	—	77,0	8,0	14,8	0,0	0,2	—	113	—	91	—
H7	M	27	NS	0	—	77,0	21,6	0,6	0,8	0,0	—	111	—	113	—
H8	F	49	NS	0	—	62,6	34,6	1,6	1,2	0,0	123	123	—	112	4,3
S01	F	43	NS	I	—	86,2	8,8	4,4	0,6	0,0	78	105	—	99	5,1
S02	M	43	NS	I	No	79,0	19,0	1,8	0,2	0,0	84	88	—	89	6,6
S03	F	31	NS	II	No	61,6	34,8	2,6	0,8	0,2	—	—	—	—	1,0
S04	F	52	NS	I	Yes	88,0	10,4	1,6	0,0	0,0	80	83	—	90	7,3
SO5	M	41	Ex-SM	III	No	72,0	28,0	0,0	0,0	0,0	94	95	96	97	5,3
SO6	F	37	NS	I	No	81,0	15,6	3,4	0,0	0,0	97	98	106	93	3,4
SO7	M	47	NS	IV	No	84,8	13,8	1,2	—	0,2	63	63	69	68	4,4
SO8	M	33	NS	II	No	80,8	18,7	0,3	0,2	0,0	93	92	92	94	3,4
SO9	F	74	NS	IV	No	50,0	38,2	5,6	6,2	0,0	65	64	—	80	4,3
S10	M	44	NS	III	No	83,6	13,4	1,6	1,2	0,2	70	67	81	57	2,3
S11	F	48	NS	III	No	91,0	8,2	0,2	0,6	0,0	76	74	83	88	8,3
S12	M	51	NS	II	No	86,8	10,8	2,0	0,4	0,0	90	91	79	80	2,5
S13	F	51	NS	II	No	63,7	34,5	0,0	1,0	0,8	89	85	97	95	5,7
514	M	34	Ex-SM	I	No	64,2	34,2	1,2	0,4	0,0	75	74	76	74	0,7
S15	M	38	NS	IV	No	71,2	28,4	0,4	0,0	0,0	69	68	75	46	2,5
S16	M	39	NS	II	No	71,8	27,4	0,8	0,0	0,0	—	95	—	83	17,0
S17	M	39	NS	I	Yes	86,6	12,8	0,6	0,0	0,0	87	83	84	87	6,0
S18	M	72	SM	III	—	92,7	6,8	0,3	0,2	0,0	96	99	89	102	4,7
S19	M	32	SM	I	Yes	71,2	26,6	1,8	0,2	0,0	97	94	102	92	14,0
S20	M	63	Ex-SM	II	No	74,6	23,0	1,8	0,2	0,2	33	28	—	23	1,4
S21	M	49	SM	I	No	89,0	9,4	1,6	0,0	0,0	90	93	—	100	0,9
S22	F	44	Ex-SM	I	Yes	90,8	7,6	1,4	0,2	0,0	99	100	120	92	5,2
S23	F	44	Ex-SM	III	No	85,0	13,0	1,0	0,8	0,2	83	84	99	81	4,2
S24	M	35	Ex-SM	I	Yes	55,0	43,4	1,4	0,2	0,0	112	113	106	120	7,9
S25	F	44	NS	II	No	85,2	14,2	0,4	0,2	0,0	114	117	—	111	3,7
S26	F	32	Ex-SM	I	Yes	94,8	4,0	1,0	0,2	0,0	—	—	—	—	2,7
S27	F	69	SM	III	No	28,0	69,0	0,6	2,2	0,2	—	83	82	80	9,3
S28	F	39	SM	I	Yes	—	—	—	—	—	95	88	88	86	—
S29	F	58	NS	III	No	86,6	13,0	0,2	0,0	0,2	71	72	72	75	3,1
S30	M	31	SM	II	Yes	88,2	10,0	1,6	0,0	0,2	103	104	101	78	2,6
S31	F	37	Ex-SM	I	Yes	70,0	29,3	0,8	0,0	0,0	93	94	91	82	13,0

(*continued on next page*)

Table 2 (*continued*)

Pat#	Gender	Age	Smoking	Sarcoidosis chest radiographic staqe	Lofgren's syndrome	Macrophages %	Lymphocytes %	Neutrophils %	Eosinophils %	Basophils %	vc%	FVC %	TLC %	FEV_1%	CD4/CD8
S32	F	35	NS	II	No	35,0	64,0	0,2	0,4	0,4	–	108	–	115	6,3
S33	M	29	NS	II	Yes	80,0	18,0	0,8	0,4	0,1	99	100	98	102	11,0
S34	M	67	SM	III	–	35,8	64,0	0,2	0,0	0,0	–	–	–	–	–
S35	M	32	SM	II	Yes	59,6	38,3	1,5	0,6	0,0	113	114	110	119	11,5
S36	F	42	Ex-SM	I	–	77,8	20,0	2,2	0,0	0,0	110	103	98	107	3,2
S37	M	33	SM	I	Yes	44,2	54,2	1,4	0,2	00	82	83	–	72	5,4

H = Healthy, S = Sarcoidosis, M = Male, F = Female, NS = Never-smoker, Ex-SM = Ex-Smoker, SM = Smoker.

Immunocytochemistry

An aliquot of bronchial brush biopsy-cells (60,000 cells/slide) were cytocentrifuged onto SuperFrost®Plus glass slides (Menzel-Gläser, Braunschweig, Germany) for differential counts and immunocytochemistry. A monoclonal β-catenin antibody was used as primary antibody (1:100, BD Biosciences, San Jose, CA) and biotin-conjugated horse anti-mouse serum (1:300, Vector Laboratories, Burlingame, CA) as secondary. Briefly, slides were fixed in 4% PFA, washed in PBS before antigen-retrieval by boiling in citrate buffer for 15 min and permeabilisation by ice-cold MeOH for 10 min. Endogenous peroxidase activity was blocked by 0.3% H_2O_2. 5% horse serum in PBS was used to block unspecific binding. Samples were incubated at 4 °C overnight with primary antibody, and after washing incubated 1h with secondary antibody. Antigen-antibody complexes were visualised using the Vectastain ABC peroxidase kit (Vector Laboratories) and DAB-tablets (Sigma–Aldrich) according to the manufacturers' instructions. Slides were briefly counter-stained with hematoxylin before mounting. Cultured Cos-7 cells overexpressing β-catenin were used as positive controls. Slides were viewed for positive staining by a manual visual determination by a double blind evaluation completed by two independent observers.

Statistical methods

The results from the electrophoretic mobility shift assays and RT-PCR data were evaluated by Mann–Whitney test. Correlations between different parameters were determined with Spearman's rank correlation test. *P* values ≤ 0.05 were considered to be significant.

Results

We performed semi-quantitative RT-PCR on BAL cells for selected Wnts, and combined this with EMSA and immunocytochemistry to measure Wnt-signal activation in epithelial cells.

Wnt expression in BAL cells

Expression of Wnt3, -4, -5A, -7A, -7B, -10B and -11 has been described in lung and lung cell lines.[16–20] The lung of patients with sarcoidosis exhibit accumulation of of T-lymphocytes and macrophages, that are considered to have a role in the pathogenesis, and both T cells and macrophages are a known source of Wnts.[21,22] Based on this, we performed semi-quantitative RT-PCR to investigate the mRNA expression of Wnt3, Wnt5A, Wnt7A, Wnt7B and Wnt11 in BAL cells. The results showed significant increases in Wnt5A ($p = 0.03$) and decreases in Wnt7A ($p = 0.04$) expression in patients with sarcoidosis compared to healthy control subjects (Fig. 1). Changes in Wnt7B expression did not reach significant levels, but a trend towards an increased expression could be seen ($p = 0.13$). The change in Wnt7B approached significance when we only included never-smokers in the comparison ($p = 0.06$, data not shown). There were no detectable levels of Wnt3 and Wnt11 (data not shown). Subgrouping the sarcoidosis

patients according to their chest radiography stage (9), revealed changes in expression of all three genes Wnt5A, Wnt7A and Wnt7B with advancing radiographic stage (Fig. 2), all reaching significance at more severe disease stages. All three genes exhibited higher levels of significance when only non-smokers were included in the statistical analysis (data not shown). Twelve of the sarcoidosis patients had Löfgren's syndrome, and showed a significantly lower expression of Wnt7B ($p = 0.02$, Fig. 3).

As expected, BAL cell populations differed between patient and control groups, with a higher number of lymphocytes in sarcoidosis (Table 2). We investigated possible correlations between the BAL cell composition and Wnt mRNA levels by Spearman's rank correlation tests. No significant correlations to BAL cell compositions could be seen (data not shown). Neither could any significant correlations be seen to age, gender, smoking history, nor to CD4/CD8 ratios. Thus we concluded that the detected differences are due to the disease stage rather than any of these secondary reasons.

Activation of β-catenin in resident lung cells

Prompted by our results of altered Wnt mRNA expression in BAL cells, we continued by investigating intracellular activation of Wnt-signaling in resident lung cells. Wnt binding to its receptors activates β-catenin, which translocates to the nucleus. There it binds to DNA together with the transcription factor TCF/Lef and promotes transcription. Lung epithelial cells are known to express several Wnt-receptors.[18,23] To monitor Wnt-signal activation, we performed EMSA on nuclear extracts of epithelial cells obtained by bronchial brush biopsies during bronchoscopy with a labeled DNA-oligonucleotide harbouring a TCF/Lef-binding site. We observed a significant increase of activated TCF/β-catenin complex in sarcoidosis (Fig. 4 A and B),

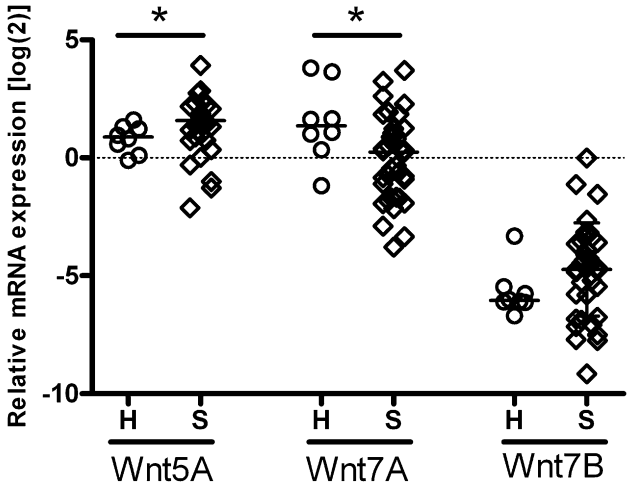

Figure 1 Wnt expression in patients with sarcoidosis compared to healthy controls. Quantitative real-time RT-PCR to assay levels of Wnt5A, Wnt7A and Wnt7B mRNA in patients with sarcoidosis ($n = 37$) compared to controls ($n = 8$). Relative mRNA expression levels are expressed as second logarithm [log (2)]. Bar indicates median. H = healthy, S = sarcoidosis. *$p < 0.05$.

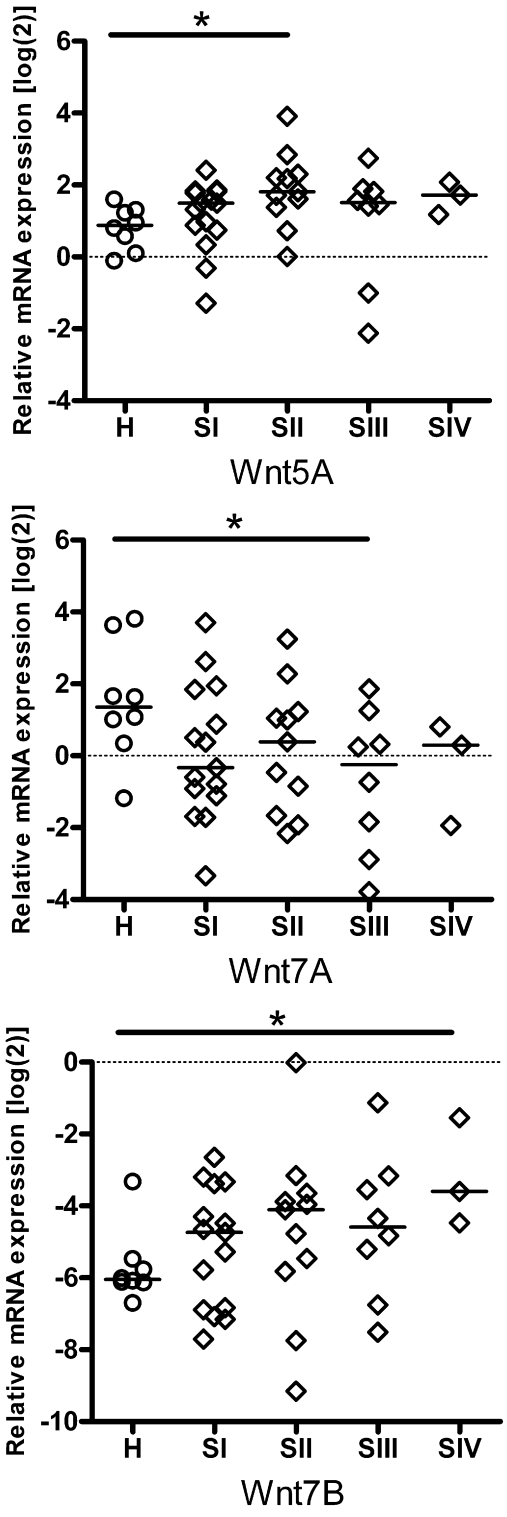

Figure 2 Correlation of Wnt expression to sarcoidosis chest radiographic stages. Quantitative real-time RT-PCR to assay levels of Wnt5A, Wnt7A and Wnt7B mRNA in patients with sarcoidosis. Relative mRNA expression levels are expressed as second logarithm [log(2)]. Bar indicates median. H = healthy, S = sarcoidosis, I-IV = radiographic stages 1–4. *$p < 0.05$ [Wnt5A $p = 0.012$ (healthy vs X-ray stage I), Wnt7A $p = 0.014$ (healthy vs stage III) and Wnt7B $p = 0.049$ (healthy vs stage IV)].

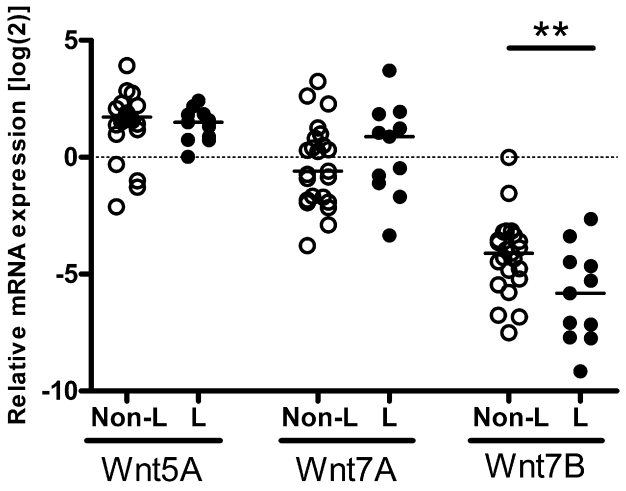

Figure 3 Correlation of Wnt expression to Löfgren's vs non-Löfgren's syndrome. Quantitative real-time RT-PCR to assay levels of Wnt5A, Wnt7A and Wnt7B mRNA in patients with sarcoidosis. Relative mRNA expression levels are expressed as second logarithm [log(2)]. Bar indicates median. L = Löfgrens syndrome, Non-L = Non-Löfgrens. $^{**}p < 0.01$.

indicating increased Wnt-signal activation in pulmonary epithelial cells of these patients in comparison with healthy never-smoking controls.

To confirm the EMSA results indicating an increased β-catenin signal activation, we performed immunocytochemistry for β-catenin on cytospins prepared from bronchial epithelial cells (Fig. 5). Unactivated β-catenin binds to the cytoplasmic domain of transmembrane cadherins at the cell membrane. In sarcoidosis, staining at the cell membrane was weaker than in the healthy controls, and evidence of nuclear accumulation was observed, together corroborating that β-catenin has been activated and translocated to the nucleus. These results thus confirm the increased levels of Wnt-signal activation in the airway epithelium of patients with sarcoidosis.

Discussion

The Wnt family of signaling molecules have been demonstrated to have profound effects on multiple cellular processes. However, not much is known about their role in sarcoidosis. We hypothesised that Wnt ligands secreted from infiltrating inflammatory cells in the pulmonary tissue could be involved in the disease processes of sarcoidosis. We thus measured mRNA levels of selected Wnts in BAL cells, and also found evidence for activation of downstream signaling in resident lung cells, together indicating increased Wnt-activation in sarcoidosis.

The so called canonical pathway is the most well-characterised of the intracellular pathways activated by Wnts. In this pathway, binding of Wnt ligands to the Frizzled (Fz) receptors allows the translocation of β-catenin to the nucleus where it activates the TCF/Lef family of transcription factors.[24] Genes stimulated by the Wnt—TCF/Lef pathway include inflammatory genes such as Cyclo-oxygenase-2 (Cox-2)[25] and Matrix metalloproteinases (MMPs),[20,26] as well as proliferative genes such as Vascular endothelial growth factor receptor (VEGFR),[27] c-myc,[28] c-jun,[29] Fos-related antigen 1 (fra-1)[29] and cyclin D1,[30,31] several of which have been demonstrated to be up-regulated in BAL from individuals with sarcoidosis. Thus, the observed activated Wnt-signaling could play a role in the inflammatory processes in sarcoidosis.

The inflammation in sarcoidosis and the associated pulmonary accumulation of T cells and macrophages, and formation of granulomas, can in the chronic form lead to fibrosis. Fibrosis usually develops in the later stages of the inflammation. A possible hypothesis for this is that the activated infiltrating inflammatory cells produce and secrete mediators that start a process of epithelial-mesenchymal signaling that once initiated becomes self-sustaining. Epithelial-mesenchymal signaling has been implicated in the pathogenesis of human idiopathic pulmonary fibrosis (IPF), and aberrant activation of the Wnt—β-catenin signaling pathway was found in fibrotic foci of patients with IPF.[6,32] Similar to our findings in sarcoidosis, they showed an increased expression of Wnt7B in IPF. Recently, by selectively blocking the β-catenin/CBP interaction, without interfering with the p300 interaction, Henderson et al. suggested an important balance between the two distinct transcriptional pathways in the development of fibrosis.[33] Emblom-Callahan et al. demonstrated, by microarray analysis of IPF patients, an alteration of 11 genes within the wnt pathway, with a downregulation of of dishevelled dsh homolog 1 (DVL1) and an upregulation of secreted frizzled-related protein 2 (SFRP2) to mention a couple known to play a role in the development of fibrosis.[34] Thus, the more pronounced changes in Wnt expression with more advanced radiographic stage, and less pronounced changes in Löfgren's syndrome, hint at a role for Wnts in the fibrosis associated with chronic sarcoidosis.

The BAL cells investigated were not separated into subpopulations prior to the RT-PCR experiments. This might affect the results, due to differing compositions of cells present in the various study groups. Thus, BAL cell differentials were determined for each patient, allowing us to take this into account in the post-experimental analysis of the results. Sarcoidosis patients tend to have raised amounts of T-lymphocytes compared to healthy control subjects. However, no correlations were observed neither when comparing levels of Wnt5A, Wnt7A and Wnt7B mRNA to the amount of lymphocytes and macrophages, nor to other factors including gender, age, or CD4/CD8 ratio. This indicates that the observed differences in Wnt expression is not merely due to differences in BAL cell composition.

Our EMSA and immunocytochemistry results indicate that the intracellular β-catenin signaling is increased in epithelial cells from patients with sarcoidosis. Even though the lung epithelium is not considered to play a central role in sarcoidosis pathology, these results, using the epithelium as a reporter, support increased Wnt-signaling in the sarcoid lung, and indicate that Wnts secreted from infiltrating inflammatory cells can activate the canonical pathway of resident lung cells. Moreover, there has been evidence of β-catenin signaling involvement in the induction of epithelial-mesenchymal transition (EMT), which is an important process during fibrotic tissue repair.[35] Even though we observed a decreased expression of Wnt7A in contrast to the increased levels of Wnt5A and Wnt7B, the

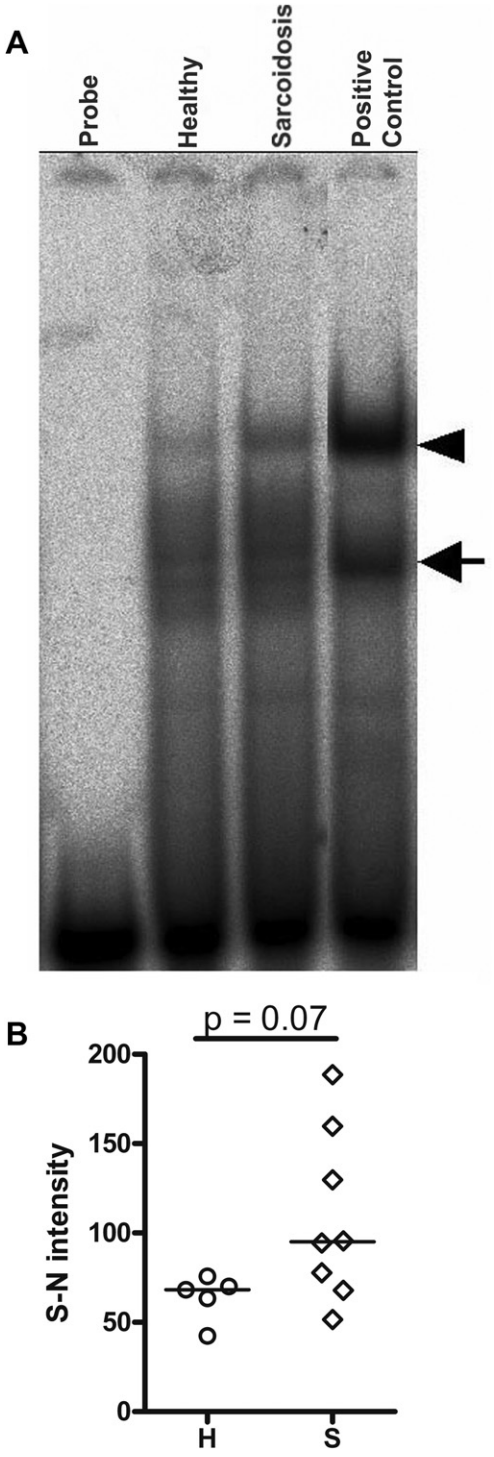

Figure 4 Electrophoretic mobility shift assay for β-catenin activation in patients with sarcoidosis. (A) Electrophoretic mobility shift assays were performed on nuclear extracts from epithelial cells obtained by fiberoptic bronchoscopy. An oligonucleotide harbouring a TCF/Lef consensus binding site was end-labeled with [γ32-P] ATP, and used as a probe. The arrow-head indicate the specific TCF/β-catenin complex and the arrow indicate the TCF shift. The TCF/β-catenin complex is the active form and the larger, stronger complex therefore indicate increased Wnt-signaling in sarcoidosis. As an internal positive control nuclear extract prepared from U937 cells was used. Representative gel of several experiments ($n = 8$). (B) Graphic representation of the Signal-Noise (S—N) of all the gels included in the study. S—N was calculated for each image separately in order to account for differences in the manual film processing and associated intensity differences (see also Supplemental Fig. 1). Bar indicates median. H = healthy, S = sarcoidosis. $p = 0.07$.

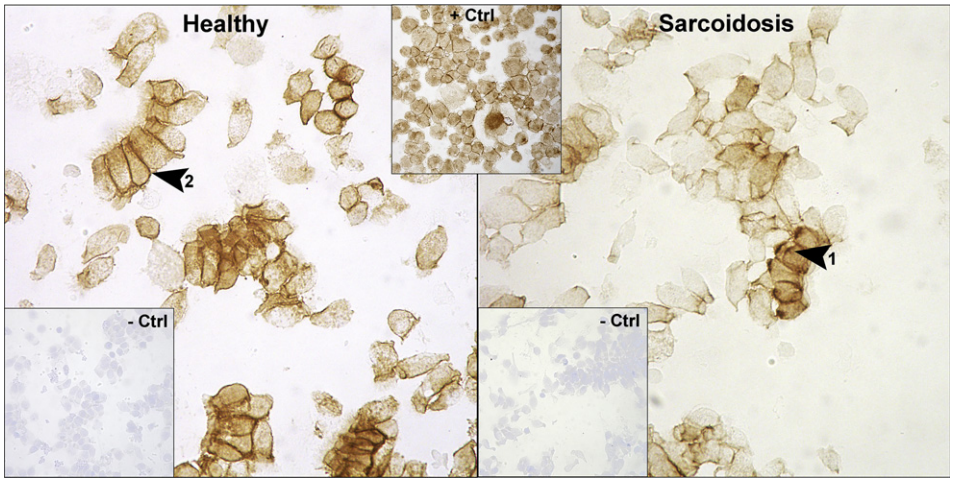

Figure 5 Nuclear accumulation of β-catenin in patients with sarcoidosis. 1) Immunocytochemistry confirmed the increased levels of active, i.e. nuclear, β-catenin in epithelial cells from patients with sarcoidosis ($n = 4$). Representative image of several slides. 2) β-catenin normally binds to the cytoplasmic domain of transmembrane cadherins and is involved in cell—cell adhesion. This can be seen on the left on the slides from the healthy controls ($n = 5$). In patients with sarcoidosis there is less staining at the cell membranes and nuclear accumulation of β-catenin is observed (indicated by arrow), which indicates that the β-catenin has translocated to the nucleus to form the active complex with the transcription factor TCF/Lef. The inserts are representative images of control staining; negative control (−Ctrl) with primary antibody omitted, positive control (+Ctrl) cultured Cos-7 cells overexpressing β-catenin.

EMSA and immunocytochemisty showed that down-stream signaling was increased. This provides evidence that activation of Wnt−β-catenin signaling is dominating, and the biological significance of the differences in Wnt5 and 7B, and Wnt7A, remains to be determined. Also, it cannot be ruled out that other source of Wnts than the BAL cells investigated here contribute to the activated β-catenin signaling. In sarcoidosis, T cells and macrophages accumulate in the lung. T cells are a known source for Wnts,[21] and it has also been shown that macrophages can produce these factors in the mouse.[22] Our findings thus provide support for the hypothesis that Wnts secreted by inflammatory cells in lungs of patients with sarcoidosis activate β-catenin to play a role in the pathogenesis of sarcoidosis.

Conclusion

In summary, we have found evidence of altered expression of Wnt5A, Wnt7A and Wnt7B mRNA in BAL-fluid cells, as well as increased activation of the canonical β-catenin signaling pathway in resident lung cells of patients with sarcoidosis. As Wnts and β-catenin are implicated in the processes of inflammation, migration and fibrosis, these correlative results between expression of signaling molecule, and activation of the down-stream pathway, suggest a role for this signaling pathway in the inflammatory processes of sarcoidosis. A possible role in the fibrosis that can develop in the chronic form of the disease warrants further investigations of these signaling molecules in sarcoidosis.

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Authors' contributions

BL conducted the experiments and wrote the first draft of the manuscript. AE performed all bronchoscopies. BL and MN analysed the data and performed the statistical analyses. MN, JG and AE conceived and designed the study, coordinated and helped to draft and revise the manuscript and contributed key concepts to the study. ÅW analysed data and revised the manuscript. All authors read and accepted the final draft of the manuscript.

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Conflict of interest

The authors declare that they have no competing interests.

Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.rmed.2010.11.018

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