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## Brief Communication

# The magnitude of cytokine production by stimulated CD56<sup>+</sup> cells is associated with early stages of systemic sclerosis



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## ABSTRACT

Immune activation is a hallmark of systemic sclerosis (SSc). However, the immunological alterations that occur in preclinical and non-fibrotic SSc and that differentiate these subjects from those with primary Raynaud's phenomenon (PRP) or healthy controls (HC) are poorly defined. We isolated CD56<sup>+</sup> (NK/NKT-like) cells from HC, patients with PRP, early SSc (EaSSc) and definite SSc without skin or lung fibrosis. Cytokine production upon different activating stimuli was measured via a multiplex immuno assay. Clearly discriminative patterns among the different stages of SSc were most markedly observed after TLR1/2 stimulation, with increased IL-6, TNF- $\alpha$  and MIP-1 $\alpha$ /CCL3 production in definite SSc patients as compared to HC and/or PRP. Initial alterations were observed in EaSSc patients with an intermediate secretion pattern between HC/PRP and definite SSc. CD56<sup>+</sup> cells from patients at different stages of SSc differentially respond to TLR stimulation, highlighting the relevance of natural immunity in the developmental and pre-fibrotic SSc.

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## 1. Introduction

Systemic Sclerosis (SSc) is a multisystem disease characterized by activation of the immune system and widespread vasculopathy culminating in fibrosis of the skin and internal organs [1]. Whereas Raynaud's phenomenon (RP) usually is the first manifestation of disease, both vasculopathy as well as activation of the immune system could precede the diagnosis of definite SSc by years. Indeed, subjects with RP who test positive for SSc-specific autoantibodies and/or display typical alterations at the nailfold videocapillaroscopy are at risk for SSc [2–4]; this condition is therefore defined as undifferentiated connective tissue disease at risk for SSc (UCTD/SSc) [5] or early SSc (EaSSc) [6].

Innate immune system alterations have extensively been described in established SSc with overt skin fibrosis [7,8], yet little is known about changes in natural immunity that may occur in EaSSc or in pre-fibrotic scleroderma as compared to subjects with primary Raynaud's phenomenon (PRP). Recently, we have demonstrated that interferon

type I signatures in whole blood are a prominent feature of SSc in its developmental phase [9] and that EaSSc distinctly have increased plasma levels of dendritic cells (DC)-derived C-X-C motif chemokine 4 (CXCL4) [10].

Other components of the natural immunity, such as natural killer (NK, CD56<sup>+</sup> CD3<sup>-</sup>) and NKT-like (CD56<sup>+</sup> CD3<sup>+</sup>) cells, may also have a role in the development and progression of SSc. NK are able to release a wide variety of cytokines upon different activating triggers and to tune the maturation of other cells of the immune system, such as DC and T cells, skewing them into a pro-inflammatory or tolerogenic status or into different Th patterns of activation [11]. NKT-like cells are a broad, diverse group of T cells co-expressing T cell receptor and NK receptors, which cluster very close to NK cells in terms of multiparameter surface-molecule expression, but exhibit a relative transcriptional quiescence, requiring a more pro-inflammatory milieu to exert similar immunoregulatory properties [12]. The role of NK and NKT-like cells in SSc has not been fully clarified yet, as some authors claimed a reduced number and function of NK and NKT-like cells, whereas others suggested a normal or increased number of NK cells in the disease along with an activated phenotype but defective killing capability, all reviewed in [13]. The current knowledge about NK and NKT-like cells in SSc comes from studies conducted on established SSc; only one recent report studied NK and NKT-like cells also in a small group of preclinical SSc, finding no differences in

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the flow cytometry absolute count of both NK and NKT-like cells as compared to healthy subjects, but no further functional analyses were performed [13].

To assess whether functional alterations in NK and NKT-like cells can be detected in the earliest and non-fibrotic phases of SSc to differentiate developing scleroderma from PRP, we investigated the response of CD56<sup>+</sup> cells to different activating stimuli in patients with long-standing PRP, EaSSc and definite SSc yet lacking any cutaneous or pulmonary fibrotic involvement.

## 2. Methods

### 2.1. Patients and controls

Forty-six patients referring to the Scleroderma Unit of the Fondazione IRCCS Ca' Granda Policlinico di Milano were considered. The study group included 10 PRP subjects according to the criteria proposed in [14], 24 EaSSc patients according to LeRoy and Medsger criteria [6] without any signs and symptoms indicative of definite disease, 12 SSc according to the 2013 ACR/EULAR criteria [2] without skin fibrosis (modified Rodnan skin score = 0) [15] and without any sign of lung fibrosis defined as a typical involvement of the lung parenchyma >5% on high resolution computed tomography [16] accompanied by a reduced forced vital capacity or diffusing capacity for carbon monoxide <80% of predicted values. Nine healthy controls (HC) matched for sex and age were also included as a comparison group. Clinical characteristics of the study groups are shown in Table 1.

All the subjects gave their written consent to have their laboratory and epidemiological data anonymously used for the study, which was approved by the local ethic committee (comitato etico Area B Milano, protocol 425/2014) and performed in accordance with the Declaration of Helsinki.

### 2.2. Samples collection and analysis

Peripheral Blood Mononuclear Cells (PBMC) were isolated by density-gradient centrifugation over ficoll-Paque from heparinized venous blood. Subsequent positive selection (Miltenyi Biotec) was used to isolate CD56<sup>+</sup> cells from the PBMC fraction (purity >95%).  $5 \times 10^4$  CD56<sup>+</sup> cells/well were seeded in a 96-well plate in a final volume of 100  $\mu$ l and stimulated for 24 h in culture medium (Gibco® RPMI 1640 medium with 10% Fetal Calf Serum, 1% Penicillin-Streptomycin, 1% Glutamine) with TLR agonists (5  $\mu$ g/ml TLR1/2 Pam3CSK4, EMC microcollections; 10  $\mu$ g/ml TLR3 Poly(I:C) and 2  $\mu$ g/ml TLR7/8 R848; InvivoGen); additionally, CD56<sup>+</sup> cells were stimulated with human

recombinant IL-2 (60 U/ml, R&D Systems) and IFN- $\alpha$  (1000 U/ml, Cell Sciences). Cell-free supernatants were stored at  $-80^\circ\text{C}$  until analysis.

Levels of IL-6, TNF- $\alpha$ , IL-10, IFN- $\gamma$ , IL-4, IL-13, IL-17, IL-22, IL-8/CXCL8, GM-CSF, RANTES, MIP-1 $\alpha$ /CCL3 were measured in cell-free supernatants of CD56<sup>+</sup> cells by the MultiPlex Core Facility of the Laboratory of Translational Immunology (LTI) with in-house-developed bead sets as previously described [17]. For statistical analysis, concentrations below the detection limit were converted to half of the lower limit of detection. When >10% of measurements were below detection limit, the mediator was excluded for further analysis, unless >90% was detected in one specific subgroup; IFN- $\gamma$ , IL-4, IL-13, IL-17 didn't respect these prerequisites and were therefore removed before performing statistical analysis. All the concentrations are expressed in pg/mL throughout the paper.

### 2.3. Statistical analysis

All the analyses were conducted via the SPSS ver 22.0 software (IBM Corp, Armonk NY). Continuous variables are expressed as mean  $\pm$  standard deviation (SD). Comparisons were performed via one-way ANOVA; the Levene's test was used to test the equality of variances; when the test was not significant (>0.05) the F-statistic was used to establish the significance of the model, the Welch's test was used otherwise. When ANOVA results were declared significant at the 0.00625 level ( $\alpha = 0.05/8$  comparisons) post-hoc tests were used to evaluate the significance of pairwise comparisons, namely, the Tukey's test or the Games-Howell test in relation to Levene's test significance. Post-hoc tests were then considered significant at the 0.05 threshold. The ANOVA polynomial linear test was used to evaluate trends for continuous variables; polynomial tests were declared significant at the 0.00625 level. To provide more robust estimates, univariate outliers, as determined by the boxplot method, were removed.

To characterize and to provide a better visualization of trends among groups, subjects' data were also categorized: individuals whose analyte concentrations were <1st quartile were classified as low producers; individuals with concentrations in the 1st–3rd quartile as intermediate producers; individuals with concentrations >3rd quartile as high producer. Categorical trends were evaluated via the Somer's D test with a nominal significance level as above; during categorization outliers were not removed.

## 3. Results

Cytokine levels in supernatants from unstimulated CD56<sup>+</sup> cells did not differ among groups and no statistical trends could be observed either (Table 2). CD56<sup>+</sup> cell stimulation, however, not only led to a substantial increase in the amount of produced cytokines but also showed a number of significant trends across groups as well as a few significant pairwise comparisons. The data are shown in Table 2 with the exception of the ones referring to IL-10 and IL-22 production (shown in Suppl. Table 1), whose levels were just above the detection limits in each conditions applied and in all groups of patients and HC, thus providing only arguable biological relevance. The most consistent results were observed after Pam3CSK4 (TLR1/2) stimulation with a gradual increase from HC to SSc in IL-6 (polynomial linear trend  $p = 2 * 10^{-4}$ ), in TNF- $\alpha$  ( $p = 3.47 * 10^{-4}$ ) and in MIP-1 $\alpha$  concentrations ( $p = 0.002$ ). For IL-6 and TNF- $\alpha$ , a full statistical significance was reached comparing the concentrations elicited in CD56<sup>+</sup> cells from HC vs SSc or vs EaSSc, and from PRP vs SSc subjects (ANOVA  $F = 0.001$  and post-hoc tests <0.05); substantial or exploratory significance was also reached for MIP-1 $\alpha$  for the same comparisons (ANOVA  $F < 0.05$  and post-hoc tests <0.05).

Categorical analysis (summarized in Supplemental Table 2 and detailed in Supplemental Tables 3–8) confirmed the quantitative results with significant trends (Somer's D < 0.00625) observed after TLR1/2 stimulation for IL-6, TNF- $\alpha$  and MIP-1 $\alpha$  (Fig. 1), as well as after TLR7/

**Table 1**  
Baseline clinical and demographic characteristics.

	HC	PRP	EaSSc	SSc
N	9	10	24	12
Females, n (%)	8 (88.9)	10 (100)	21 (87.5)	12 (100)
Age, yrs	47.8 $\pm$ 8.4	48.9 $\pm$ 18.7	53 $\pm$ 14.9	56.2 $\pm$ 11.3
RP duration, yrs	NA	22.5 $\pm$ 14.2	*9.8 $\pm$ 8.7	18.4 $\pm$ 13.3
Autoantibodies, n (%)				
ANA	0 (0)	0 (0)	20 (83.3)	12 (100)
ACA	0 (0)	0 (0)	13 (54.1)	9 (75)
ATA	0 (0)	0 (0)	3 (12.5)	1 (8.3)
Capillaroscopy, n (%)	0 (0)	0 (0)	18 (75)	12 (100)
Teleangiectasia, n (%)	0 (0)	0 (0)	0 (0)	5 (41.7)
Puffy fingers, n (%)	0 (0)	0 (0)	0 (0)	10 (83.3)
Digital ulcers, n (%)	0 (0)	0 (0)	0 (0)	2 (16.7)

HC, healthy controls; PRP, primary Raynaud's phenomenon; EaSSc, early systemic sclerosis; SSc, definite systemic sclerosis; N, number; yrs, years; RP, Raynaud's phenomenon; ANA, Anti-Nuclear antibodies; ACA, Anti-Centromere antibodies; ATA, Anti-Topoisomerase I antibodies; NA, not applicable. Values are expressed as mean  $\pm$  standard deviation, when not differently specified.

\*  $p < 0.05$  if compared to PRP group.

**Table 2**  
Concentrations of analytes according to the different groups.

Analyte	Stimulus	HC	PRP	EaSSc	SSc	Trend p	ANOVA p
IL-6	None	3.29 ± 2.34	12.75 ± 23.4	4.94 ± 4.05	10.12 ± 11.53	0.464	0.244
	IFN- $\alpha$	10.24 ± 9.6	10.75 ± 7.55	9.93 ± 6.53	16.05 ± 14.18	0.228	0.354
	IL-2	25.23 ± 12.47¶	79.65 ± 73.18	53.41 ± 42.9	167.8 ± 141.7	0.001	0.001
	TLR1/2	126.7 ± 80.1¶	247.7 ± 217.5	336.1 ± 269.2†	729.7 ± 574.2	0.000	0.001
	TLR3	13.47 ± 8.73	11.32 ± 8.46	17.15 ± 14.03	32.42 ± 25.2	0.014	0.022
	TLR7/8	426.1 ± 282.7	505 ± 210.6	1004.6 ± 1053.8	1202.8 ± 889.1	0.018	0.096
TNF- $\alpha$	None	3.19 ± 0.96	4.28 ± 1.53	3.26 ± 0.78	4.07 ± 0.95	0.278	0.026
	IFN- $\alpha$	3.68 ± 0.6	3.58 ± 0.24	4.66 ± 1.85	4.67 ± 2.14	0.111	0.262
	IL-2	8.48 ± 4.11	11.72 ± 8.62	10.52 ± 7.95	22.96 ± 17.47	0.006	0.007
	TLR1/2	14.16 ± 8.78¶	15.09 ± 11.6¶	26.11 ± 19.49	53.73 ± 41.06	0.000	0.001
	TLR3	5.94 ± 5.48	4.64 ± 2.29	5.1 ± 4.21	5.86 ± 5.32	0.974	0.888
	TLR7/8	34.03 ± 20.7	49.46 ± 32.49	130.3 ± 153.6	129.1 ± 125.7	0.043	0.125
MIP-1 $\alpha$	None	223.1 ± 68.65	232.4 ± 55.42	207.3 ± 44.21	226.6 ± 75.6	0.866	0.659
	IFN- $\alpha$	470.4 ± 468.4	296.2 ± 117.3	308 ± 135.9	362 ± 178	0.333	0.291
	IL-2	2184 ± 1215.1	1536.5 ± 565.3	2505.4 ± 4565.9	3485.9 ± 1903.3	0.280	0.577
	TLR1/2	1329 ± 695.5¶	1454.7 ± 942.1¶	2693.8 ± 2364.7	4123.7 ± 2928.4	0.002	0.012
	TLR3	549.7 ± 406.8	390.6 ± 183.5	330.9 ± 157.6	588.8 ± 392	0.891	0.055
	TLR7/8	2193.2 ± 1254.8	2083.8 ± 958.2	3678.9 ± 3791.1	4117.2 ± 2644.5	0.090	0.284
RANTES	None	28.93 ± 8.75	34.63 ± 16.45	23.06 ± 22.24	35.42 ± 23	0.794	0.289
	IFN- $\alpha$	40.29 ± 11.96	52.76 ± 27.5	28.89 ± 16.12	47.72 ± 25.69	0.984	0.020
	IL-2	113.9 ± 62.74	104.2 ± 45.91	62.06 ± 36.2¶¶	142.07 ± 64.48	0.548	0.001
	TLR1/2	47.04 ± 27.77	60.3 ± 47.56	37.3 ± 36.25	65.15 ± 29.89	0.540	0.163
	TLR3	64.61 ± 54.61	52.28 ± 31.05	26.11 ± 21.18	51.46 ± 37.03	0.169	0.022
	TLR7/8	42.88 ± 8.57	46.28 ± 27.69	31.29 ± 31.69	60.73 ± 37.67	0.398	0.079
GM-CSF	None	1.83 ± 0.62	1.76 ± 0.79	1.98 ± 0.53	1.78 ± 0.73	0.934	0.773
	IFN- $\alpha$	1.5 ± 0.61	2.1 ± 0.21	1.81 ± 0.5	2.24 ± 1.84	0.022	0.033
	IL-2	66.03 ± 50.51	56.13 ± 35.9	31.67 ± 25.77¶	179.3 ± 148.6	0.008	0.000
	TLR1/2	25.17 ± 21.02	33.8 ± 26.28	30.78 ± 23.54	53.2 ± 60.39	0.102	0.266
	TLR3	2.86 ± 2.41	1.76 ± 1.27	1.26 ± 1.13	2.54 ± 2.36	0.550	0.077
	TLR7/8	34.26 ± 20.86	30.73 ± 12.68	47.66 ± 46.16	96.66 ± 73	0.003	0.007
IL-8	None	118.8 ± 107.5	173.7 ± 112	202.4 ± 203.7	174.6 ± 129.4	0.415	0.670
	IFN- $\alpha$	468.7 ± 581	72.57 ± 59.82	149.1 ± 158.5	219.9 ± 273.4	0.117	0.027
	IL-2	617 ± 399.2	636.3 ± 249.6	1447.5 ± 2089.5	1281.1 ± 979	0.230	0.430
	TLR1/2	1361.1 ± 868.3	1875.6 ± 1316.9	5935.2 ± 8117.4	3624.3 ± 3963.3	0.207	0.150
	TLR3	273.8 ± 433.6	155 ± 126.9	232.8 ± 264.2	190.4 ± 97.9	0.657	0.780
	TLR7/8	1028.2 ± 705.1	1338.1 ± 952.2	1970.8 ± 2340.1	1767.4 ± 1423.5	0.278	0.566

HC, healthy controls; PRP, primary Raynaud's phenomenon; EaSSc, early systemic sclerosis; SSc, definite systemic sclerosis. Trend p, ANOVA polynomial linear trend. ANOVA, analysis of variance. Significant results after Bonferroni correction ( $\alpha = 0.00625$ ) are depicted as dark-shaded cells; results significant at the exploratory 0.01 threshold are depicted as light-shaded cells. The symbol ¶ depicts the significant pairwise comparisons,  $p < 0.05$  or ¶¶,  $p < 0.001$  as compared to the SSc group; the symbol † depicts the significant pairwise comparison,  $p < 0.05$ , as compared to the HC group. Values are expressed in pg/mL as mean ± standard deviation.

8 stimulation (R848) for the same analytes. As displayed in Fig. 1, after TLR1/2 stimulation, high production of IL-6, TNF- $\alpha$  and MIP-1 $\alpha$  by CD56<sup>+</sup> cells is mainly observed in SSc patients while in HC or PRP subjects low- and/or intermediate production dominate; EaSSc show an intermediate activation pattern.

#### 4. Discussion

Immune system activation is a cardinal feature of SSc that may antedate the onset of a full-blown fibrotic disease by years [1–4]. Yet, little is known about the ongoing autoimmune process in the preclinical stage of SSc. From a pathogenetic point of view, autoimmune diseases are thought to be the consequence of an environmental trigger in a genetically susceptible host, where a cascade of events leads to a chronic immune response and finally breakthrough of tolerance. Under this conceptual framework, cells of the innate immune system may have a fundamental role in eliciting and perpetrating autoimmunity, being the body's first line of response.

The mechanisms at the basis of the lack of self-recognition and initiation of the autoimmune response remain elusive. In particular, it is hard to distinguish which part of the inflammatory response observed in the context of autoimmune phenomena is causative and which is a mere consequence of the process, yet perpetuating inflammation and tissue damage with continued exposure of self antigens. NK cells have been considered as decisive players in many autoimmune diseases and suggested to play an important role in the earliest, pre-clinical phases (reviewed in [18]). The loss of self-recognition in NK cells could be partly due to failure to deliver inhibitory signals in the form of a MHC class I - Killer Immunoglobulin-like Receptors (KIR) mismatch, with inadequate dampening of the response to foreign triggers as consequence. More recently though, the current work on NK cells points firmly towards a disproportional, maintained activating signaling in patients with different autoimmune diseases. Therefore, hyperactivated NK cells could directly cause tissue damage by uncontrolled cytotoxic response but also modulate the immune response [11], mostly after TLR stimulation [19] in the interaction with antigen-presenting cells and T cells, via direct cell-to-cell contact or via cytokine production. In line with this premise, we found an increased activation of CD56<sup>+</sup> cells after TLR1/2 and to some extent after TLR7/8 stimulation that allow a clear differentiation and separation of definitive SSc from HC and PRP subjects. These findings are of paramount importance as in our study we considered subjects with definite SSc yet without any sign of advanced disease and that represent a unique population with early signs of evolution. Interestingly, even if statistical differences in cytokine concentrations between EaSSc and HC was only depicted with regard to IL-6 secretion, upon TLR1/2 stimulation we observed also clear increasing trends in CD56<sup>+</sup> cells activation patterns with regard

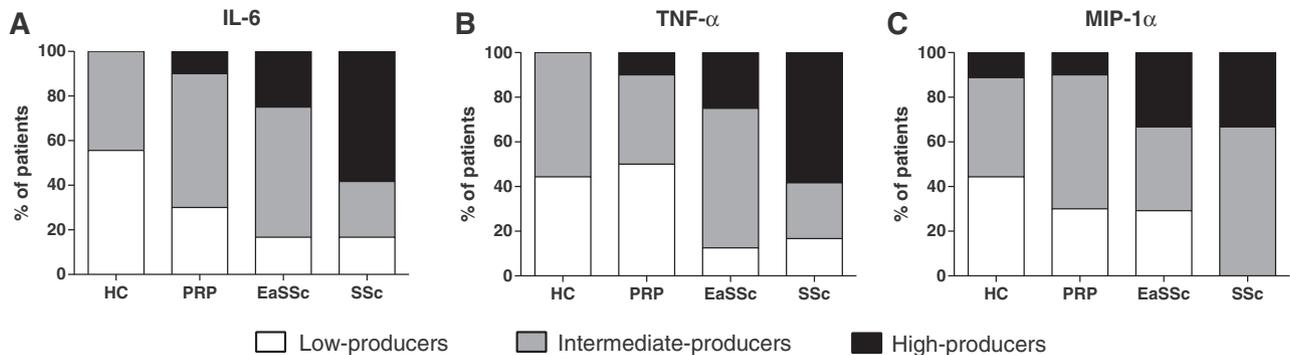
to TNF- $\alpha$  and MIP-1 $\alpha$ . These findings enforce the notion that in EaSSc subjects vascular and immune mechanisms that will eventually lead to the appraisal of a definite SSc are already active yet less preponderant than in established SSc [20]. This striking trend can primarily be observed for IL-6 but also for MIP-1 $\alpha$ /CCL3 and TNF- $\alpha$ , which is indeed consistent with previous observations correlating IL-6 and MIP-1 $\alpha$ /CCL3 levels to SSc disease progression [21]. Our group has shown that a functionally relevant TLR2 SNP may affect IL-6 and TNF- $\alpha$  production in SSc myeloid and monocyte-derived DC [22], likely through a higher surface expression and a slowed trafficking of TLR1/2. It could be hypothesized that the linearly increasing pro-inflammatory effect that we observed in CD56<sup>+</sup> cells from HC and PRP to EaSSc and ultimately to SSc patients under TLR1/2 stimulation might reflect a specific alteration in downstream signaling in CD56<sup>+</sup> cells. Alternatively, some of the lipopeptidic ligands responsible for TLR1/2 activation, as opposed to nucleic acids recognized by intracellular TLRs, might be involved into the inflammatory process.

NK cells and NKT-like cells share functional capability and they both respond to TLR triggering [23] and to the different NK receptors on the cell surface via ligation of MHC class I molecules and stress-induced molecules. NKT-like cells though, represent a very diverse subset of T cells also capable of TCR-mediated and MHC-restricted activation. CD56<sup>bright</sup> NK cells are known to be the main cytokine-producing subset and express the highest level of TLR2 [24]. Similarly, KIR- NKT-like cells have been shown to be more capable of cytokine production than their KIR<sup>+</sup> counterpart in response to different stimuli, among which TLR2 triggering [12]. The potentiated cytokine production in response to TLR1/2 triggering described in our work could mirror a preferential activation of the CD56<sup>bright</sup> NK compartment and/or of the KIR- NKT-like subset in Early and more prominently in definite SSc patients.

In our study, we were not able to phenotype and differentiate between the effect exerted by NK (CD56<sup>+</sup> CD3<sup>-</sup>) and NKT-like (CD56<sup>+</sup> CD3<sup>+</sup>) cells separately, and therefore additional investigations focusing on the influx and contribution of each population are currently underway to study this in more detail. Furthermore, it would be of great value to assess prospectively the CD56<sup>+</sup> pattern of activation after TLR stimulation in EaSSc patients who evolve into SSc.

#### 5. Conclusions

Herein we describe for the first time the unique pattern of activation of NK/NKT-like cells in the different stages of preclinical and non-fibrotic SSc. We showed that innate immune system responses can initially be altered in subjects with a diagnosis of EaSSc to be substantially modified when signs of a definite disease are found. This finding is of relevance as it may allow the identification of pathways leading to



**Fig. 1.** Distribution of low- intermediate- and high- IL-6, TNF- $\alpha$  and MIP-1 $\alpha$  producers after TLR1–2 stimulation in relation to the diagnosis. HC, healthy controls; PRP, primary Raynaud's phenomenon; EaSSc, early systemic sclerosis; SSc, definite systemic sclerosis. The stacked percent bar plots represent the percentage (%) of low- (white), intermediate- (grey) and high- (black) IL-6 (A), TNF- $\alpha$  (B), MIP-1 $\alpha$  (C) producers after TLR1/2 stimulation within the different patients groups.

full-blown SSc and may give rise to the possibility of disease interception, treating molecular aberrances before actual disease onset.

### Conflict of interest

None of the authors has any potential financial conflict of interest related to this manuscript.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.clim.2016.09.004>.

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