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# Title: Deep-phenotyping detects a pathological $CD4^+$ T cell complosome signature in systemic sclerosis

**Running Title:** A novel pathogenic T cell signature in scleroderma

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CD4$^+$ T helper 1 cells (Th1) function is closely regulated by an intrinsic developmental program in which activation/induction and pro-inflammatory interferon (IFN)-γ secretion is followed by a deactivation/contraction period characterized by a switch into co-secretion of immunoregulatory interleukin (IL)-10. Autocrine intracellular complement (complosome) activity plays a vital role in Th1 initiation and contraction: T cell receptor (TCR) stimulation induces intracellular activation of the complement key components C3 (through cathepsin L (CTSL) cleavage) and C5 which leads to intrinsic engagement of CD46 by C3b, of the C3a receptor (C3aR) by C3a, and of the C5aR by C5a[1,2]. These events mediate the metabolic programming required for IFN-γ production and Th1 induction[3]. CD46-mediated signals also support subsequent IL-10 switching and Th1 contraction by increasing oxidative phosphorylation vs. glycolysis ratio, while autocrine C5aR2 engagement by secreted, des-Arginated C5a (C5a-desArg), suppresses intracellular C5aR1 activity (Supplementary Figure 1A depicts a model summarizing the role of the complosome in Th1 induction and contraction).

Diminished or augmented complosome activation and function is associated with recurrent infections or hyperactive Th1 responses in rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), respectively[4]. This raises the possibility that T cell complosome dysregulation may operate in other immune-mediated rheumatic diseases, such as systemic sclerosis (scleroderma, SSc)[5]. SSc is a serious connective tissue disease of unknown etiology characterized by autoimmunity, vasculopathy and progressive fibrotic changes to major internal organs (skin, lungs, heart, kidneys, gastrointestinal and musculoskeletal systems).[6] Hyperactive T helper cells, often of the Th2 subtype, and increases in IL-6 and/or IL-17-producing CD4$^+$ T cells in the blood and skin of patients have been described conclusively.[6,7,8] However, the evidence for a distinct Th1 involvement is less clear as some researchers noted augmented Th1 activity while others have failed to observe this. A method to comprehensively and rapidly monitor complosome activity in cells, however, is currently unavailable: traditional FACS-based assays generally do not permit measurement of sufficient markers to assess complosome activity and cellular effector function on a single cell-level. Similarly, RNA-seq or gene array analyses fail to inform on the intra- or extracellular localization of complement components and on their protein activation states. Here, we addressed this need for advanced complosome/complement technologies and generated the first complement-compatible antibody panel suitable to analyze the complosome signature of cells comprehensively by mass cytometry (MC, CyTOF®) technology. We further utilized this novel MC complosome panel to evaluate CD4$^+$ T cells isolated from a well-characterised cohort of early-stage treatment-naïve diffuse cutaneous

systemic sclerosis (dcSSc) for complosome perturbations. This strategy focused on detection of dysregulation in Th1 induction or contraction in SSc and our results indicate potential biological coupling of dysregulated complosome activity in a broader range of immune-mediated rheumatic disease states.

To assess for a potential defect in Th1 contraction in SSc, we measured cytokine expression from resting and activated $CD4^+$ T cells isolated from the blood of six dcSSc patients (Patients 1 to 6; Supplementary Table 1) and matched healthy donors (HDs). Indeed, T cells from these patients not only displayed significantly increased IL-6 and IL-17 secretion upon CD3+CD46 activation, they also produced proportionally significantly larger amounts of IFN-γ compared to IL-10 with increased IFN-γ:IL-10 ratio without affecting cell viability (Figure 1a and Supplementary Figure 1b and c).

To test our hypothesis that aberrant intracellular complement activity may underpin the reduced capacity for CD46-mediated Th1 contraction in SSc, we generated and validated a novel mass cytometry biomarker panel to evaluate complement protein expression and activation states in unprecedented depth. This panel simultaneously detects a combination of 18 complosome components (extra- and intracellularly), seven selected T cell markers including those for Th1 and Th17 activity, four cytokines/effector molecules, and two relevant transcription factors (Supplementary Table 2). Importantly, this novel antibody panel detects all respective (complement) antigens in resting or activated T cells in a similar pattern when compared to their 'conventional' and previously published detection patterns via FACS analysis (Supplementary Table 3a-b)[1,2]. We next assessed freshly blood-purified and not further activated or CD3+CD46-stimulated $CD4^+$ T cells isolated from five dcSSc patients (Patients 5 to 9; Supplementary Table 1) utilizing our bespoke MC panel for complosome activity and functional markers. Data were analyzed using automated dimension reduction including Uniform Manifold Approximation and Projection (UMAP) or Stochastic Neighbor Embedding (SNE) in combination with spanning-tree progression analysis of density-normalized events (SPADE) for clustering[9] as well as deep phenotyping of immune cells[10]. We further delineated newly identified relevant cell clusters using our in-house pipeline for cell clustering (CytoClustr (published[8] and available here).

Firstly, UMAP analysis of non-activated T cells isolated from three dcSSc patients (Patient 6, 8 and 9) and three matched HDs revealed a strikingly different single cell complosome expression/activation landscape between patients and HDs and further a highly complement-enriched

island in patients which was absent in HDs (Figure 1b). The identified island was particularly enriched in C3/C3b, C5/C5b and C5aR1; the three key complosome components that we previously associated with Th1 (hyper)activity[1,2] (Figure 1b). To next assess these complement-enriched cells observed in the data set in relation to the additional activation, cytokine and transcription factor markers, normalized FCS expression was Z-scaled, and cells expressing each of C3/C3b, C5/C5b, and C5aR1 at $Z>1.96$ ($p<0.05$) were retained and regarded as 'hi' (high in these components). All other cells were regarded as 'normal'. The expression of all panel markers across these two cell groups, and across HDs and patients, was cross-analyzed via box and whisker plots (Figure 1c and Supplementary Figure 2a). This analysis confirmed the presence of a distinct cluster of complement-enriched cells, almost exclusively in patients but not in HDs (Figure 1c) and further showed that these cells were enriched for the presence of activated Factor B (Bb Neo), intracellular CD46 and C3aR expression, the canonical Th1 lineage transcription factor T-bet, and IL-17 (Supplementary Figure 2a). Subsequent calculation of average expression of markers following viSNE and SPADE, further supported a substantially altered complosome signature in circulating T cells from these patients (Figure 1d), with the increased levels of of intracellular C3a and C5a in patient T cells denoting augmented intracellular C3 and C5 activation. Patient T cells also express higher intracellular levels of the activating complement receptors C3aR and C5aR1 whilst the inhibitory receptor C5aR2 is decreased (Figure 1d). Expression of the complement regulator decay accelerating factor (DAF, CD55) is also augmented, in line with DAF upregulation generally observed on activated T cells, while CD46 shows a dysregulated isoform expression pattern with a reduction of surface protein expression and an increase in intracellular presence of the CYT-1-bearing isoform of CD46 (Figure 1d). The latter indicates likely ongoing autocrine activation of CD46 as CD46 is normally lost on the cell surface upon stimulation due to metalloprotease-mediated cleavage. A receiver operating curve performed with pROC package in R and based on markers in Supplementary Figure 2a showed that this specific complosome signature was able to discriminate patients from HDs (AUC 0.879) (Supplementary Fig. 2b).

We next performed a similar analysis of the patients' T cells after CD3+CD46 activation and observed that perturbed complosome activity is further augmented. SPADE analysis to group phenotypically related cells into clusters using both resting and activated cells confirmed marked differences between the dcSSc and the HD groups: although $CD4^+$ T cells are evenly distributed within the SPADE tree prior to stimulation in both dcSSc and HDs cells, cell cluster formation itself is visibly distinct in

resting cells from dcSSc patients when compared to HDs. CD3+CD46 activation of HD and patient T cells induced extensive remodeling in both donor groups, and further confirmed that T cells from patients displayed sustained discrete and more dynamic changes that designate the majority of their cells into a distinctive area of the SPADE tree (yellow underlayed area) (Figure 1e). A heatmap depiction of data derived from activated T cells from HDs and patients (Supplementary Figure 2c) showed, for example, that the levels of C3a and the activating receptors C3aR and C5aR1 remained increased, whilst expression of the inhibitory receptor C5aR2 was further reduced when compared to activated HD T cells (Figure 1e). C5a levels are now reduced in comparison to HD cells, which could reflect C5a consumption/usage during T cell activation. The negative regulator CD55 showed an 'ambivalent' pattern with a clear intracellular decrease cell surface increase on patients' T cells. Importantly, the patients' T cells respond normally to general TCR activation denoted by the expected increase in CD25, CD28, and CD95 expression, and the concurrent down-regulation of the IL-7 receptor.

Our MC analysis of resting and CD3+CD46 activated T cells from five dcSSc patients indicated that a shared common feature of their perturbed complosome signature includes (at minimum) augmented C3 and C5 activation and C5aR1 expression with concurrent reduction in C5aR2 expression (Figure 1b-e). Excitingly, we confirmed via 'conventional' FACS analysis that these markers indeed followed this distinctive pattern in resting $CD4^+$ T cells from two additional dcSSc patients (Patients 10 and 11) (Figure 1f). This indicates that presence of our MC-identified specific complosome signature may be extended to dcSSc patients across key SSc-hallmark autoantibody specificities. We had previously shown that reducing CTSL-mediated activation of C3 within T cells through a cell-permeable CTSL inhibitor normalizes hyperactive Th1 activity in T cells from the synovial fluid of RA patients *in vitro*[1]. CD3+CD46 stimulation of T cells from dcSSc patients in presence of the CTSL inhibitor not only normalized the IFN-γ:IL-10 ratio (Figure 1g) but also significantly reduced IL-6 production without affecting cell viability (Supplementary Figure 3a and b). In contrast, only C5aR2 agonism significantly reduced IL-17 expression (Supplementary Figure 3a). TNF-α or IL-4 production in cultures remained unaltered in HDs and patients' T cells under any condition assessed, in line with our previous observations that the complosome is not required for TNF production or Th2 induction in human $CD4^+$ T cells (Supplementary Figure 3a).

In summary, utilization of our new MC-compatible complosome-specific antibody panel allowed us to observe specific perturbations of the complosome in circulating T cells from patients with SSc.

Importantly, this complosome signature is further exaggerated upon stimulation and remains distinguishable from those of healthy donors. Thus, biological coupling of perturbed complosome activity may occur in a wide range of autoimmune rheumatic disease states, including RA, SLE and SSc. Importantly, this technique/panel can be used to quickly assess other Th1-driven pathologies for distinct changes in complosome signatures and can be adapted rapidly to probe for in-depth complosome activity in other cell populations of interest. A refined FACS analysis 'distilled' from such initial exploratory MC complosome screens can then potentially become a tool for early and easy screening of (T) cell dysregulation in selected patient groups and may provide new biomarkers for disease stratification. Our results clearly need to be validated in a larger SSc patient cohort and other rheumatic diseases and we need to gain a better understanding of the diverse activities of the complosome per se.

## Figure legend

**Figure 1. T cells from patients with diffuse cutaneous scleroderma have reduced capacity for Th1 contraction and a distinct complosome signature. a** Purified blood $CD4^+$ T cells from treatment-naïve patients newly diagnosed with diffuse cutaneous systemic sclerosis (dcSSc; P1 to 6) shown a perturbed IFN-γ:IL-10 ratio upon activation. **b** Resting $CD4^+$ T cells from three dcSSc patients (Patients 6, 8 an 9) and three matched healthy donor (HDs) were stained using the bespoke MC panel**.** UMAPs identify patient-specific cell clusters which are enriched in intracellular C5aR1, C5/C5b and C3/C3b (arrows). **c** Z-scale cross-analysis of normalized FCS expression from $C3/C3b^+$, $C5/C5b^+$, and $C5aR1^+$ patient cells ('hi') versus all other patient cells ('normal') and HD cells. Frequencies of complement 'hi' cells and correlation with other markers assessed were calculated and visualized as a barplot. **d** Expression summary depicted as heat map of all intracellular and surface antigens assessed in non-activated T cells dcSSc patients and HDs. Color range indicates relative expression levels between comparatives (markers) and not absolute values. **e** SPADE analysis of data derived from MC staining of resting and CD3+CD46-activated $CD4^+$ T cells (36 hrs). Cellular abundance is denoted by node size and internode linkage distance indicates degree of phenotype relatedness. The level of complosome activity indicated by colors in the side bar. The circumscribed area contains the population phenotypes that emerge majorly in response to *ex vivo* stimulation. **f** Freshly purified $CD4^+$ T cells from two patients with recent onset dcSSc (Patients 10 and 11) and two

matched healthy donors (HDs 10 and 11) were assessed for presence of intracellular C3a, C5a, C5aR1 and C5aR2 by FACS analysis (n = 2). **g** Purified $CD4^+$ T cells isolated from dcSSc Patients 5, 6, 10, and 11 and from matched HDs 5, 6, 10, and 11 were CD3+CD46 activated in the presence or absence of either a cell-permeable cathepsin L inhibitor or a C5aR2 agonist and IFN-γ:IL-10 ratio assessed. Data are means ± SEM. $*p < 0.05$. (i), intracellular staining; (s), surface staining.


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## Author contributions

D.E.H, C.K. and S.K. conceived and directed the study, performed experiments and wrote the manuscript. G.A., B.C., L.P., T.M.W., and C.K., designed, performed and/or analyzed the T cell activation and 'rescue' experiments. L.M., R.E., S.H., S.K., K.B., and P.N., generated and validated the heavy metal-conjugated CyTOF® compatible antibody panel and/or performed and/or analysed the CyTOF experiments. V.H.O., D.A., and C.P.D., designed and analyzed experiments and data

derived from cells isolated from the patients. All authors discussed and edited the manuscript. G.A. and V.H.O. contributed equally to the work and are shared first authors.

## Conflict of interest

T.M.W is co-inventor on a patent for C5aR2 agonists as immunomodulators for inflammatory disease. The authors have no additional financial interests.

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Figure 1: T cells from patients with diffuse cutaneous scleroderma have a perturbed complosome signature and reduced capacity for Th1 contraction.