CORRESPONDENCE

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

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Cellular & Molecular Immunology (2020) 17:1010–1013; https://doi.org/10.1038/s41423-019-0360-8

CD4$^+$ T-helper 1 cell (Th1) function is closely regulated by an intrinsic developmental program in which activation/induction and proinflammatory interferon (IFN)-γ secretion are followed by a deactivation/contraction period characterized by a switch into cosecretion 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 key complement 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 C5a receptors (C5aR1 and C5aR2) 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 the oxidative phosphorylation to glycolysis ratio, while autocrine C5aR2 engagement by secreted des-arginated C5a (C5a-desArg) suppresses intracellular C5aR1 activity (Supplementary Fig. 1a depicts a model summarizing the role of the complosome in Th1 induction and contraction).

Diminished or augmented complosome activation and function are 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] Designated as an orphan disease with a high unmet medical need, SSc is characterized by autoimmunity, vasculopathy and progressive fibrotic changes to major internal organs.[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.[7,8] However, the evidence for distinct Th1 involvement is less clear, as some researchers have 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 at a single-cell level. Similarly, RNA-seq and gene array analyses fail to inform on the intracellular 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 for analyzing 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-characterized cohort of early-stage treatment-naïve diffuse cutaneous systemic sclerosis (dcSSc) patients for complosome perturbations. This strategy focused on the 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 screen for a potential defect in Th1 contraction in SSc, we measured cytokine expression in resting and activated CD4$^+$ T cells isolated from the blood of six dcSSc patients (patients 1−6; 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 but also produced proportionally and significantly larger amounts of IFN-γ than IL-10, with an increased IFN-γ:IL-10 ratio, without affecting cell viability (Fig. 1a and Supplementary Fig. 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 at an unprecedented depth. This panel simultaneously detects a combination of 18 complosome components (extracellularly 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 way to "conventional" and previously published methods using FACS analysis (Supplementary Table 3a, b).[1,2] We next assessed freshly purified and not further activated or CD3 +

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Received: 17 December 2019 Accepted: 18 December 2019
Published online: 23 January 2020

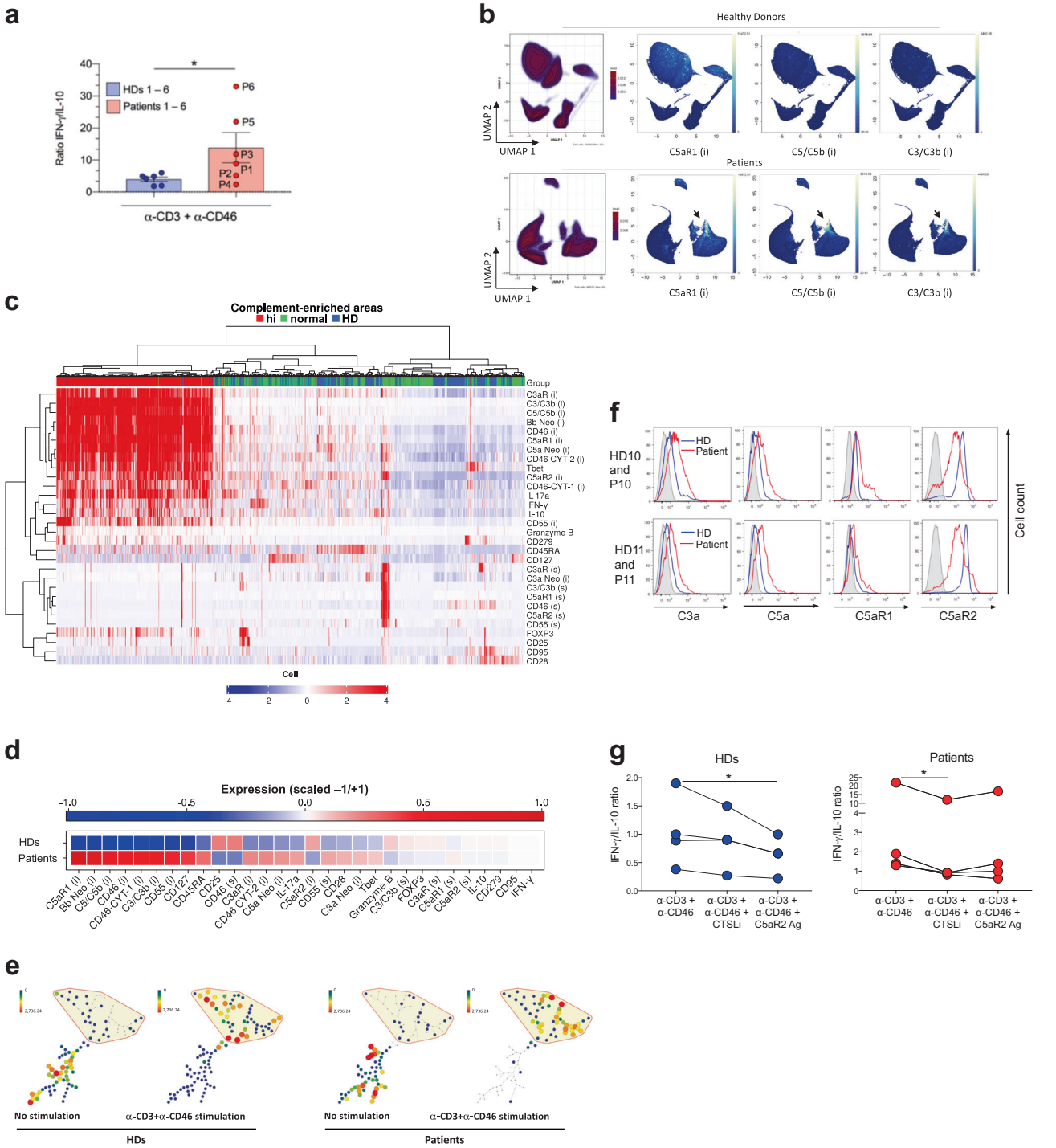

Fig. 1 T cells from patients with diffuse cutaneous scleroderma have reduced capacity for Th1 contraction and a distinct complosome signature. a Purified blood CD4$^+$ T cells from treatment-naïve patients newly diagnosed with diffuse cutaneous systemic sclerosis (dcSSc; patients 1−6) showed a perturbed IFN-γ:IL-10 ratio upon activation. **b** Resting CD4$^+$ T cells from three dcSSc patients (patients 6, 8 and 9) and three matched healthy donors (HDs) were stained using the custom MC panel. UMAP identifies 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 an equal [to 'hi'] number of random-sampled patient cells ("normal") and HD cells. Frequencies of complement "hi" cells and their correlation with other assessed markers were calculated and visualized as a bar plot. **d** Expression summary depicted as a heatmap of all intracellular and surface antigens assessed in nonactivated T cells from dcSSc patients and HDs. The 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 h). Cellular abundance is denoted by node size, and internode linkage distance indicates the degree of phenotype relatedness. The level of complosome activity is indicated by the 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 the 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 the IFN-γ:IL-10 ratio was assessed. Data are represented as the means ± SEMs. *$p < 0.05$. (i) intracellular staining, (s) surface staining.

CD46-stimulated CD4$^+$ T cells isolated from the blood of five dcSSc patients (patients 5−9; Supplementary Table 1) utilizing our custom 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[10] and available here (https://github.com/kordastilab/cytocluster))).

First, the UMAP analysis of nonactivated T cells isolated from three dcSSc patients (patients 6, 8 and 9) and three matched HDs revealed a strikingly different single-cell complosome expression/activation landscape between patients and HDs and a highly complement-enriched island in patients that was absent in HDs (Fig. 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] (Fig. 1b). To next assess these complement-enriched cells observed in the dataset in relation to the additional activation, cytokine and transcription factor markers, normalized FCS expression was *Z*-scaled, and cells expressing C3/C3b, C5/C5b, and C5aR1 at $Z \geq 1.96$ ($p < 0.05$) 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 clustering, heatmap and box and whisker plots (Fig. 1c and Supplementary Fig. 2a). This analysis confirmed the presence of a distinct cluster of complement-enriched cells, almost exclusively in patients but not in HDs (Fig. 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 Fig. 2a). Subsequent calculation of the average expression of markers following viSNE and SPADE further supported a substantially altered complosome signature in circulating T cells from these patients (Fig. 1d), with increased levels of intracellular C3a and C5a in patient T cells denoting augmented intracellular C3 and C5 activation. Patient T cells also expressed higher intracellular levels of the activating complement receptors C3aR and C5aR1, while the inhibitory receptor C5aR2 was decreased (Fig. 1d). Expression of the complement regulator decay accelerating factor (DAF, CD55) was also augmented, in line with the DAF upregulation generally observed on activated T cells, while CD46 showed a dysregulated isoform expression pattern with a reduction of surface protein expression and an increase in the intracellular presence of the CYT-1-bearing isoform of CD46 (Fig. 1d). The latter indicates that the cell was likely experiencing ongoing autocrine activation of CD46, as CD46 is normally lost on the cell surface upon stimulation owing to metalloprotease-mediated cleavage. A receiver operating characteristic curve generated with the pROC package in R and based on the markers in Supplementary Fig. 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 was further augmented. SPADE analysis to group phenotypically related cells into clusters using both resting and activated cells confirmed marked differences between the dcSSc and HD groups: although CD4$^+$ T cells were evenly distributed within the SPADE tree prior to stimulation in both dcSSc and HD cells, cell cluster formation itself was visibly distinct in resting cells from dcSSc patients when compared to the pattern seen in resting cells from 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 designated the majority of their cells into a distinctive area of the SPADE tree (yellow underlay area) (Fig. 1e). A heatmap depiction of data derived from activated T cells from HDs and patients (Supplementary Fig. 2c) showed, for example, that the levels of C3a and the activating receptors C3aR and C5aR1 remained increased while expression of the inhibitory receptor C5aR2 was further reduced in activated T cells from patients compared to the respective levels in activated HD T cells (Fig. 1e). C5a levels in patient cells were reduced in comparison with those in 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 and 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 downregulation 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 included (at a minimum) augmented C3 and C5 activation and C5aR1 expression with a concurrent reduction in C5aR2 expression (Fig. 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) (Fig. 1f). This indicates that the 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 the 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 the presence of the CTSL inhibitor not only normalized the IFN-γ:IL-10 ratio (Fig. 1g) but also significantly reduced IL-6 production without affecting cell viability (Supplementary Fig. 3a, b). In contrast, only C5aR2 agonism significantly reduced IL-17 expression (Supplementary Fig. 3a). TNF-α and IL-4 production in cultures remained unaltered in HD and patient T cells under all conditions 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 Fig. 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 the signature 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.

ACKNOWLEDGEMENTS

We thank the patients and the healthy donors for their support. This work was financed by MRC Centre grant MR/J006742/1, an EU-funded Innovative Medicines Initiative BTCURE (C.K.), a Wellcome Trust Investigator Award (C.K.), the King's Bioscience Institute at King's College London (G.A.), The King' College London BRC Genomics Facility, the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy's and St Thomas' NHS Foundation Trust and King's College London, National Institute Of Health grant R21 AI123789 (D.E.H.) and by the

Division of Intramural Research, National Heart, Lung, and Blood Institute, NIH (C.K.). D.E.H. and L.M. are grateful for the support of the Washington University School of Medicine Immunomonitoring Laboratory. V.H.O., C.P.D. and D.A. are grateful for funding support from Versus Arthritis, Scleroderma & Raynaud's UK, Rosetrees Trust and Royal Free Charity. T.M.W. is supported by a National Health and Medical Research Council Fellowship (1105420). SK is supported by Cancer Research UK (CRUK). The authors acknowledge financial support from the Department of Health via the National Institute for Health Research (NIHR) Biomedical Research Center awarded to Guy's & St Thomas' NHS Foundation Trust in Partnership with King's College London and King's College Hospital NHS Foundation Trust.

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 analyzed the CyTOF experiments. V.H.O., D.A., and C.P.D. designed and analyzed the 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.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41423-019-0360-8) contains supplementary material.

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

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