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CLINICAL SCIENCE

Characterising the autoantibody repertoire in systemic sclerosis following myeloablative haematopoietic stem cell transplantation

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ABSTRACT

Objectives Results from the SCOT (Scleroderma: Cyclophosphamide Or Transplantation) clinical trial demonstrated significant benefits of haematopoietic stem cell transplant (HSCT) versus cyclophosphamide (CTX) in patients with systemic sclerosis. The objective of this study was to test the hypothesis that transplantation stabilises the autoantibody repertoire in patients with favourable clinical outcomes.

Methods We used a bead-based array containing 221 protein antigens to profile serum IgG autoantibodies in participants of the SCOT trial.

Results Comparison of autoantibody profiles at month 26 (n=23 HSCT; n=22 CTX) revealed antibodies against two viral antigens and six self-proteins (SSB/La, CX3CL1, glycyl-tRNA synthetase (EJ), parietal cell antigen, bactericidal permeability-increasing protein and epidermal growth factor receptor (EGFR)) that were significantly different between treatment groups. Linear mixed model analysis identified temporal increases in antibody levels for hepatitis B surface antigen, CCL3 and EGFR in HSCT-treated patients. Eight of 32 HSCT-treated participants and one of 31 CTX-treated participants had temporally varying serum antibody profiles for one or more of 14 antigens. Baseline autoantibody levels against 20 unique antigens, including 9 secreted proteins (interleukins, IL-18, IL-22, IL-23 and IL-27), interferon-α2A, stem cell factor, transforming growth factor-β, macrophage colony-stimulating factor and macrophage migration inhibitory factor were significantly higher in patients who survived event-free to month 54.

Conclusions Our results suggest that HSCT favourably alters the autoantibody repertoire, which remains virtually unchanged in CTX-treated patients. Although antibodies recognising secreted proteins are generally thought to be pathogenic, our results suggest a subset could potentially modulate HSCT in scleroderma.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Autoantibodies are highly prevalent in systemic sclerosis (SSc), but it is unknown if they are pathogenic or epiphenomena.
⇒ Haematopoietic stem cell transplant (HSCT) appears to be clinically beneficial in a significant subset of patients.
⇒ Antic-cytokine antibodies (ACAs) have been described in SLE and other disorders.

WHAT THIS STUDY ADDS

⇒ This study identified new markers that can be used to monitor transplanted SSc patients.
⇒ Surprisingly, the autoantibody profile in an individual patient changes very little over time, particularly in patients treated with Cytoxan.
⇒ ACAs are newly identified in SSc and correlate with clinical parameters.
⇒ EGF signalling pathways may be important in SSc and autoantibodies were identified that bind to EGFR.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ New ACA may identify targets for future therapeutic interventions.
⇒ High prevalence of autoantibodies specific for thyroid antigens in HSCT-treated patients suggests clinicians should actively monitor for thyroid dysfunction.
⇒ Surprisingly, some patients treated with HSCT develop SSc-specific autoantibodies such as anti-CENP-B that suggest patients with diffuse cutaneous SSc have the capacity to develop antibodies more commonly found in limited SSc.

INTRODUCTION

Systemic sclerosis (SSc) is a severe autoimmune condition with one of the highest morbidity and mortality rates among autoimmune disorders.[1 2] Although its aetiology is poorly understood, extensive evidence implicates a key role for defects in components of the adaptive immune system in disease pathogenesis. The vast majority of patients with SSc have class-switched, serum IgG anti-nuclear antibodies directed against a variety of ribonucleoprotein and DNA-associated complexes, including topoisomerase I (Scl-70), centromere proteins (CENP-A and CENP-B), Th/To and RNA polymerases I/III.[3–5] Moreover, B-cells and T-cells are prevalent in skin biopsies of subsets of SSc patients, particularly those with early diffuse

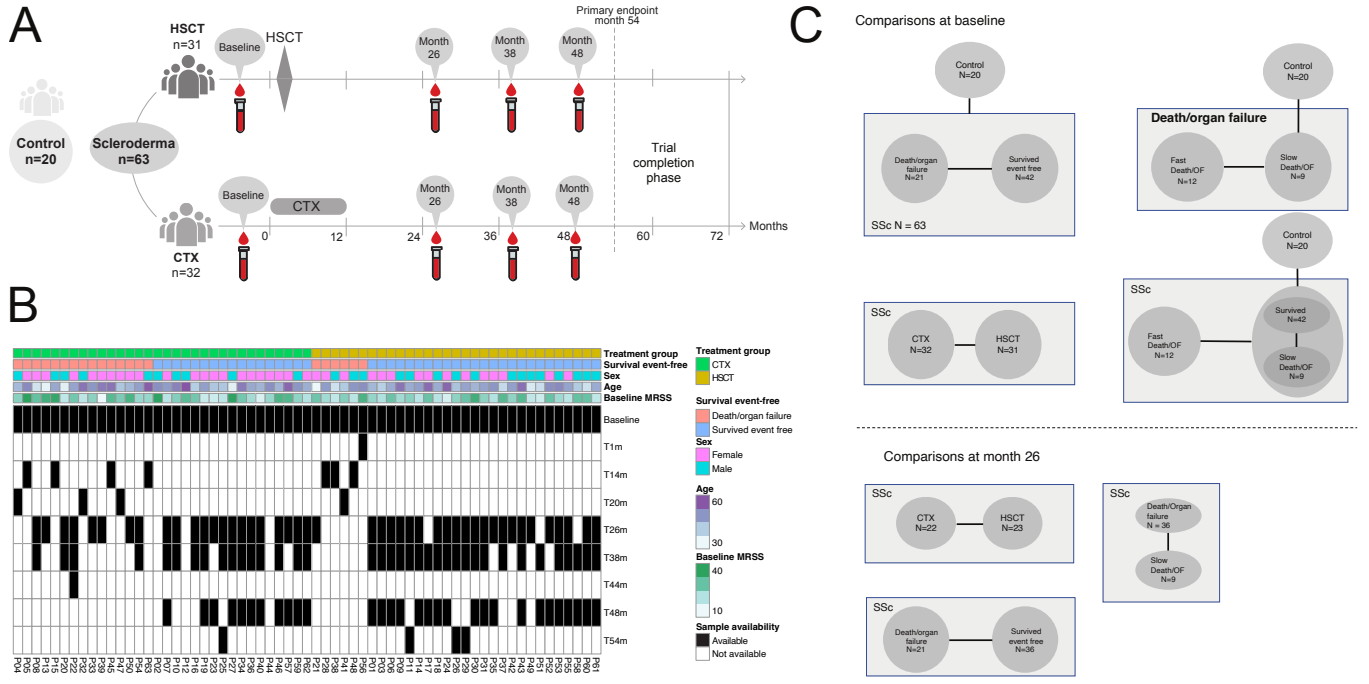

Figure 1 Study design, cohort description and overview of comparative analyses. (A) In the SCOT trial, 75 participants with systemic sclerosis (SSc) were randomly assigned to treatment with haematopoietic stem-cell transplantation (HSCT) or high-dose monthly cyclophosphamide (CTX). Serum samples in each treatment group were collected before treatment (baseline) and at various time points, including months 26, 38 and 48. The primary end point, assessed at month 54, was a global rank composite score. The trial ended when the last subject completed the month 54 evaluation, with a maximum follow-up of 72 months. Of the 75 randomised participants, baseline samples were available from 63 (HSCT n=31; CTX n=32). In addition to the sera from SCOT participants, serum samples from healthy controls (HC, n=20) were included in the study design for characterisation of serum autoantibodies. (B) Each black-filled cell represents availability of a sample from a corresponding SCOT subject (numbered P01–P63). Baseline serum samples were available for 63 of 75 SCOT participants. For a majority of the participants (n=45), serum was available at month 26, followed by month 38 (n=37) and month 48 (n=30). For other time points, serum was available for a small portion of the participants, ranging between 1 and 7 individuals. For each of the 63 SCOT participants, colour annotations indicate treatment arm (HSCT or CTX), treatment response evaluated at month 54 (survival event-free vs death or organ damage), sex, age and modified Rodnan skin score (MRSS) at baseline. (C) Using a multiplexed antigen suspension bead array platform, all available sera from SCOT participants as well as HC were analysed for their autoantibody reactivity profiles. Several group comparisons in terms of treatment arm and event-free survival status were conducted for autoantibody profiles obtained for serum samples at baseline and month 26. SCOT, Scleroderma: Cyclophosphamide Or Transplantation.

cutaneous disease.[6 7] Clonal expansion of T-cells has been described in early onset SSc but not in stable SSc, supporting the notion that antigen-specific immune responses are important to SSc pathogenesis.[8–10] Therefore, most SSc therapies used in standard practice (eg, methotrexate, mycophenolate mofetil (MMF) and cyclophosphamide (Cytoxan, CTX)) target either the underlying immune dysregulation hypothesised to drive the disease, or the fibrotic end organ damage (eg, nintedanib)[11] and vascular abnormalities.[12–14]

Although patients with SSc are often treated for specific manifestations with immunosuppressive drugs such as CTX or MMF, treatment strategies vary widely among physicians. Moreover, treatment algorithms for severe, multiorgan SSc remain controversial. In a randomised, open-label, phase II clinical trial called SCOT (Scleroderma: Cyclophosphamide Or Transplantation),[15] patients with SSc were randomly assigned to treatment with myeloablative CD34$^+$ selected autologous haematopoietic stem-cell transplantation (HSCT) or with high-dose, monthly CTX. The global rank composite scores at 54 months revealed significant clinical benefits with HSCT compared with CTX treatment. Beyond evaluating the safety and clinical efficacy of HSCT, SCOT was designed to incorporate a variety of mechanistic studies, including whole blood global transcriptome profiling,[16 17] flow cytometry analyses, serum protein profiling[18] and B-cell receptor repertoire analysis (see companion paper by Adamska *et al* in press in *ARD*). Collectively, the goals of these analyses were to (1) further define the pathophysiology of SSc; (2) identify biomarkers for disease activity and treatment response and (3) understand how the immune repertoire is reshaped when an abnormal adaptive immune system is ablated and reconstituted.

Here, we created a 280-plex, bead-based, custom SSc autoantigen array composed of common connective tissue disease antigens, a small number of vaccine and control antigens, and a large panel of secreted proteins. We then used the array to comprehensively profile IgG autoantibodies in sera from healthy controls (HC) and participants enrolled in the SCOT trial to characterise longitudinal serum autoantibody profiles of participants.

METHODS

Detailed methods are provided in online supplemental methods.

RESULTS

Characterisation of serum autoantibody profiles across SSc participants and HC

We analysed IgG autoantibody reactivities with a 280-plex bead-based antigen array in 212 serum samples in a blinded manner.

Table 1 Overview of sample numbers in each subject group, treatment arm, sampling time point and response group

	CTX participants			HSCT participants			All SSc
	Death/organ failure	Survived event-free	Total CTX	Death/organ failure	Survived event-free	Total HSCT	Total
No of participants	15	17	32	6	25	31	63
	CTX time points			HSCT time points			All SSc time points
Time point	Death/organ failure	Survived event-free	Total CTX	Death/Organ failure	Survived event-free	Total HSCT	Total (per time point)
Baseline	15	17	32	6	25	31	63
T1m	0	0	0	1	0	1	1
T14m	4	0	4	3	0	3	7
T20m	3	0	3	1	0	1	4
T26m	8	14	22	1	22	23	45
T38m	4	12	16	0	21	21	37
T44m	1	0	1	0	0	0	1
T48m	0	11	11	0	19	19	30
T54m	0	1	1	0	3	3	4
Total	35	55	90	12	90	102	192

A total of 192 serum samples from 63 SCOT participants were available. The table provides a detailed overview of sample numbers per time point for each treatment arm.
CTX, cyclophosphamide; HSCT, haematopoietic stem cell transplant; SCOT, haematopoietic stem cell transplant; SSc, systemic sclerosis.

This included 192 serum samples from treated SCOT subjects (who underwent transplant or received at least 9 of 12 CTX doses) with specimens at baseline (n=63), at least one postbaseline specimen at months 26, 38, 48 (or month 54 if missing) or at the last time point prior to death or organ failure, as well as 20 specimens from HC with age and sex distributions similar to the SCOT participants (online supplemental table 1). The number of samples in each subject group, treatment arm, sampling time point and outcome group (defined as surviving event-free vs deceased or had organ failure) are summarised in figure 1A,B and table 1.[15]

Baseline samples (HSCT, n=31; CTX, n=32) were compared with samples at month 26 (HSCT, n=23; CTX, n=22; figure 1C). We selected this time point because transplanted participants would have been fully engrafted by month 26, and fewer samples (≤36 samples) were available for analysis at later time points (figure 1B and table 1).

Online supplemental figure 1 shows a heat map displaying all IgG antibody reactivities for all 63 SCOT participants only at baseline, as well as 20 HC participants. Collectively, this represents 20 169 data points. Online supplemental figure 2 shows antibody reactivities for all 63 SCOT participants at all time points that were studied, including baseline (online supplemental figure 2, bottom panel, 192 SSc samples total) and for all 20 HC participants (online supplemental figure 2, top panel), representing 46 899 data points.

Using unsupervised clustering, we observed that all individual participants clustered with themselves across multiple time points, demonstrating that the IgG antibody repertoire is stable in an individual subject over time, even when the immune system is ablated and reconstituted with HSCT (online supplemental figure 2). HC participants had lower reactivity to traditional autoantigens and to secreted proteins than SSc participants. Approximately 30% of antigens were unreactive in all samples tested. Finally, we observed strong, scattered reactivities among SSc participants. Many of these represent the discovery of potentially biologically important autoantibodies specific for secreted proteins in individual patients.

Significance analysis of microarrays (SAM) algorithm[19] identified 60 statistically significant autoantigens between SSc participants (n=63), irrespective of the treatment arm, and HC participants (n=20) at baseline (q value ≤10% and |log2 fold-change|≥1; figure 2A; online supplemental figure 1 and online supplemental table 2). Among these 60 antigens, we identified autoantibodies specific for many antigens within the 'Standard SSc Antigen' panel (see online supplemental methods) known to be relatively specific for, and associated with, clinical phenotypes of SSc including Scl-70, CENP-B, U1-snRNP, histone proteins, POLR3H and B23/nucleophosmin (figure 2A,B).

Interestingly, autoantibodies specific for antigens from the 'Traditional Autoantigen' subpanel (see online supplemental methods) associated with other chronic autoimmune diseases[20] were more prevalent at low levels in baseline sera of SSc participants than HC. These include antigens such as DNA, Ro52/TRIM21, Ro60, La, Ku, Smith proteins, RNP proteins, thyroglobulin, sp100, annexin V, β2GPI, cardiolipin, azurocidin and SRP54. Further, we found statistically significant autoantibodies against 10 of the 80 serum factors included in the 'Discovery Autoantigen' array subpanel (cytokines IL-1β, IL-2, IL-3, IL-8, IL-13 and TNFα; growth factors (granulocyte colony stimulating factor, GCSF and BMP4) and chemokines (CCL2 and CXCL4)). Collectively, our analysis identified autoantibodies known to be specific for SSc, low levels of traditional autoantibodies associated with other systemic autoimmune rheumatic diseases and novel autoantibodies directed against cytokines, chemokines and growth factors.

Comparison of serum autoantibody profiles between SCOT participants treated with HSCT or CTX

We hypothesised that a subset of serum autoantibodies would change in HSCT-treated compared with CTX-treated participants. No differences were detected in baseline samples between SSc participants treated with HSCT (n=31) or high-dose monthly CTX (n=32, data not shown). Next, we compared the HSCT and CTX treatment arms at 26 months after treatment initiation (HSCT, n=23; CTX, n=22, table 1 and figure 1B), and identified antibodies (q value≤10% and |log2 fold-change|≥1) against six self-antigens and two viral antigens, hepatitis B surface antigen (HBSAg) and Epstein-Barr virus (EBV) antigen p-18 (figure 3A and online supplemental table 3). Participants in the HSCT arm (but not CTX arm) were vaccinated for Hepatitis

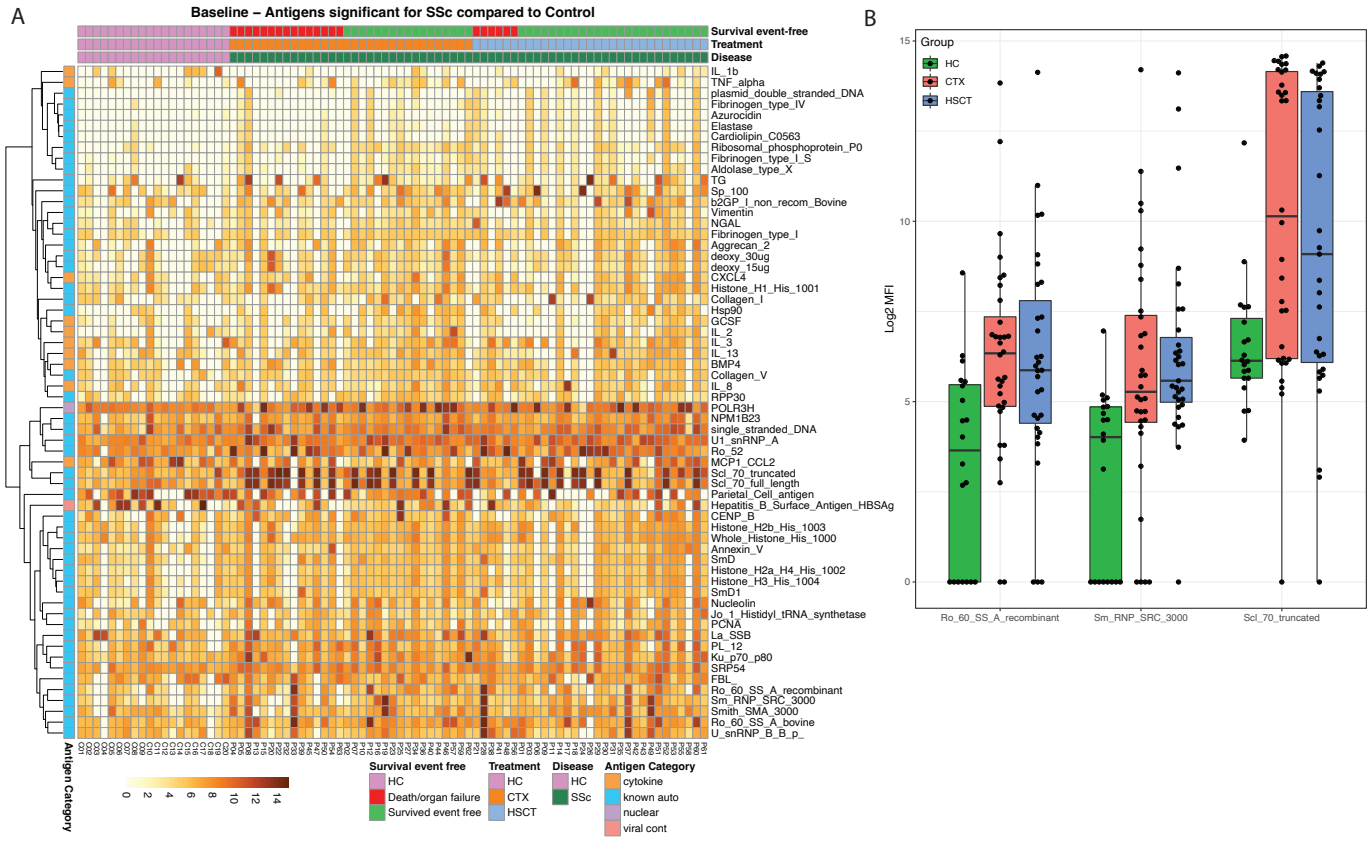

Figure 2 Comparison of serum autoantibody profiles of SCOT participants at baseline and HC. (A) A heat map showing log2 transformed MFI values for serum autoantibody reactivity in baseline SCOT participants (n=63) and HC (n=20) against 62 antigens identified to be significantly different between groups (q<10% and absolute value of log2 fold-change>1). Heat map columns represent serum samples and rows represent statistically significant antigens. For each sample, their sample group, treatment arm and treatment response are colour-annotated. IL-1b, interleukin-1 beta; TNF-alpha, tumour necrosis factor alpha; TG, thyroglobulin; SP100, interferon-inducible autoantigen associate with primary biliary cirrhosis; B2GP1, beta 2 glycoprotein one non-recombinant protein from bovine source; NGAL, neutrophil gelatinase associate lipocalin; deoxy, DNA/DNA; CXCL4, CXC chemokine ligand 4/platelet factor-4; HSP90, heat shock protein 90; GCSF, granulocyte colony stimulating factor; IL, interleukin (2, 3, 8, 13); BMP4, bone morphogenetic protein 4; RPP30, ribonuclease P protein subunit p30; PolR3H, RNA polymerase III; NPM1B23, nucleophosmin B23; U1-snRNP-A, U1-small nuclear ribonucleoprotein A; MCP1/CCL2, monocyte chemoattractant protein 1/chemokine ligand 2; Scl70, scleroderma autoantigen 70; CENPB, centromere protein autoantigen B; SmD, Smith protein D; PCNA, proliferating cell nuclear antigen; PL-12, alanyl t-RNA synthetase; Ku, Ku protein p70/80; SRP54, signal recognition particle 54; FBL, fibrillarin; Sm/RNP C, Smith/U1-small nuclear ribonucleoprotein C; Smith/SMA, Smith protein A; U snRNP B_B, U1-small nuclear ribonucleoprotein Smith proteins B/B′. (B) A box and whisker plot showing the top three antigens ranked by the q-value from the SAM analysis comparing the autoantibody levels in the baseline SCOT participants with the levels in healthy controls. For each sample group, the box and whisker plot represents log2 transformed MFI values within lower and upper quantile (box), the median (horizontal line within box), 5th and 95th percentiles (whiskers), and outliers (dots). MFI, median fluorescence intensity; SCOT, Scleroderma: Cyclophosphamide Or Transplantation.

B at post-transplantation months 14, 16 and 26. Serum antibody profiling confirmed that all vaccinated participants had vaccine responses, although these responses varied among those vaccinated. Conversely, we identified slightly higher serum antibody reactivity in CTX participants against EBV p-18, the significance of which is unclear.[21]

Among the six statistically significant self-antigens at month 26, HSCT-treated participants had higher serum autoantibody levels against bactericidal permeability-increasing and epidermal growth factor receptor (EGFR), whereas CTX-treated participants had higher autoantibody levels against SSB/La, parietal cell antigen, glycyl-tRNA synthetase (EJ) and CX3CL1 (figure 3A,B).

Comparison of temporal changes in serum autoantibody profiles between SCOT CTX and HSCT arms

We applied a linear mixed model to each antigen to compare the serum autoantibody repertoire between each treatment arm. Random effects due to individual subject differences were included. Autoantibodies specific for three antigens (HBSAg, CCL3 and EGFR) showed statistically significant increases in the HSCT arm (false discovery rate (FDR) <20%) compared with the CTX arm, in which trends were largely unchanged (figure 4A).

We then asked if there were subject-specific antigen trends by fitting a standard linear mixed model to each subject-antigen intensity profile (online supplemental tables 4−7). Based on the distribution of the slopes of the regression lines, subject-antigen pairs with slopes >2 SD were identified. Eight of 32 HSCT-treated and one of 31 CTX-treated participants had temporally varying serum antibody profiles for one or more of 14 antigens with statistical significance (p<0.05; figure 4B). This analysis revealed several clinically relevant changes in the autoantibody repertoire in a subset of HSCT-treated participants that were not observed among CTX-treated participants. Six participants were

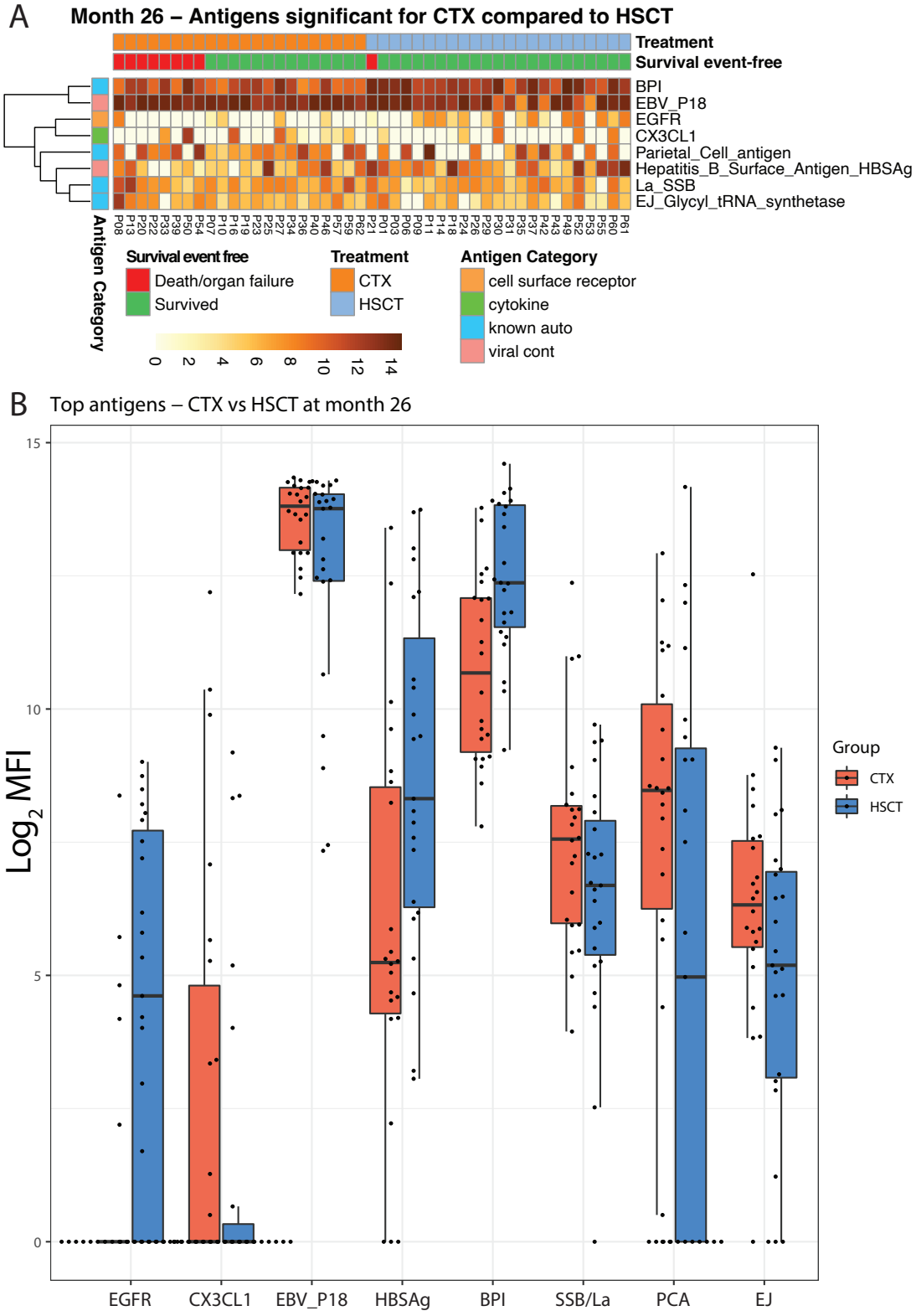

Figure 3 Comparison of serum autoantibody profiles of SCOT participants between the HSCT and CTX arm at month 26. (A) A heat map showing log2-transformed MFI values for serum autoantibody reactivity in SCOT participants treated with HSCT (n=23) or CTX (n=22) against 27 antigens. Antigens identified to be statistically significantly different (q<10% and absolute log2 fold-change >1) at month 26 are shown. Heat map columns represent serum samples and rows represent statistically significant antigens. For each sample, treatment arm and treatment response are colour-annotated. BPI, bactericidal permeability inducing protein; PCA, parietal cell antigen; EJ, EJJ glycyl-tRNA synthetase; CX3CL1, CX3C motif chemokine ligand 1/fractalkine; EBV-p18, Epstein-Barr Virus protein p18. (B) A box and whisker plot showing the top six self-antigens and two viral antigens ranked by the q-value from the SAM analysis comparing the antibody level in the SCOT participants treated with CTX with the level in participants treated with HSCT. For each sample group, the box and whisker plot represents log2-transformed MFI values within lower and upper quantile (box), the median (horizontal line within box), 5th and 95th percentiles (whiskers) and outliers (dots). HSCT, haematopoietic stem cell transplant; SCOT, Scleroderma: Cyclophosphamide Or Transplantation. MFI, median fluorescence intensity.

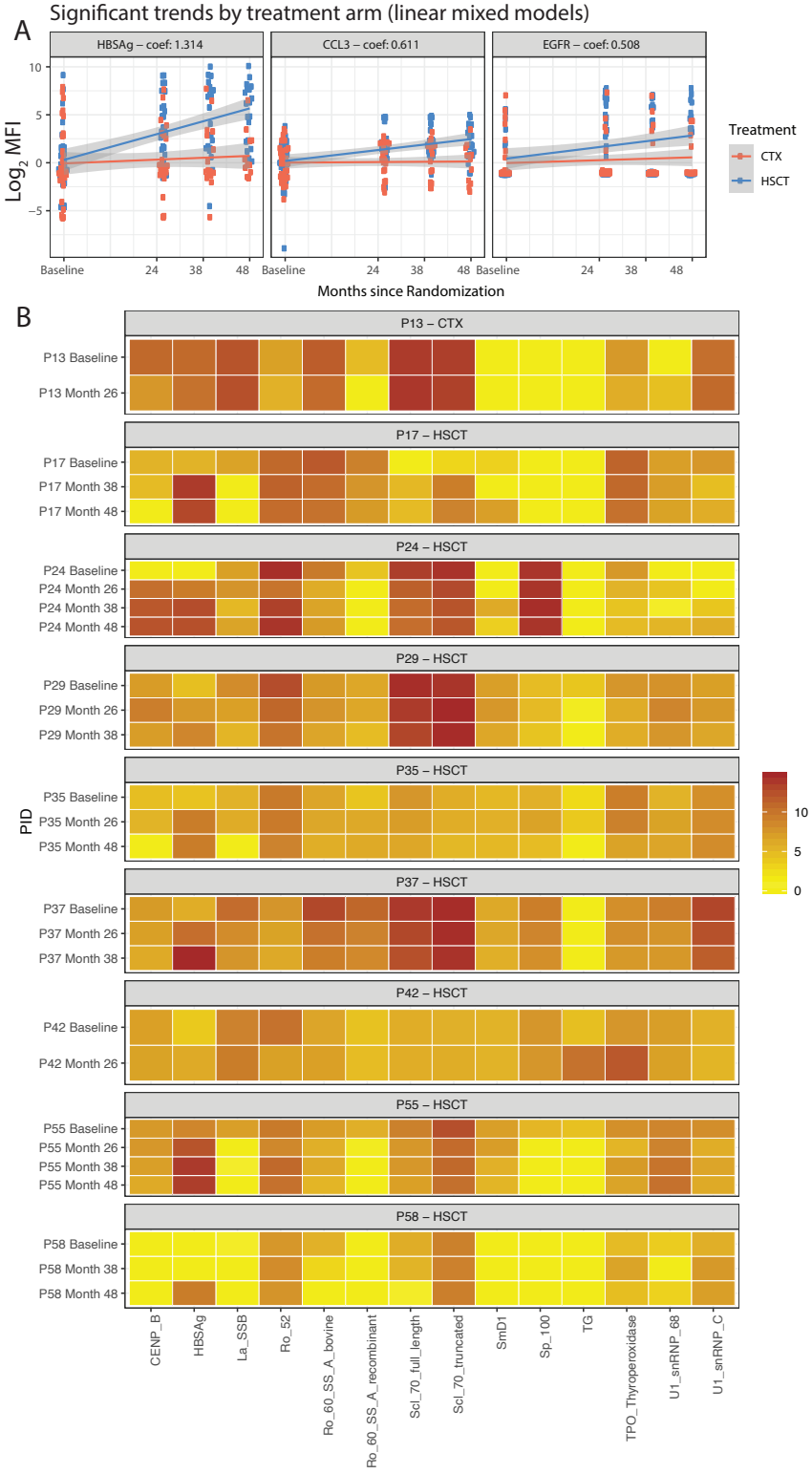

Figure 4 Comparison of temporal changes in serum autoantibody profiles between the CTX and HSCT treatment arms. (A) Linear mixed model analysis was used to identify antigens showing significantly different temporal trends between the two treatment arms. In each model, random effect due to individual subject differences was included. The line plots show three antigens with significantly different trends between treatment arms (FDR p<0.2). The lines represent the mean trend over time within each treatment arm. Coefficients are reported in each panel label. A positive coefficient indicates a higher slope for HSCT participants compared with CTX participants, which indicates that reactivity to the antigen increases more, over time, in HSCT-treated subjects compared with CTX-treated participants. (B) Subject-specific antigen trends were obtained by fitting a standard linear model to each subject-antigen intensity profile. Based on the distribution of the slopes of the regression lines, subject-antigen pairs with slopes >2 SD from the distribution mean were identified for 8 of 32 HSCT-treated participants and only 1 of 31 CTX-treated participants. The panel shows log2-transformed serum autoantibody profiles for 14 temporally-varying antigens in all nine participants across their available sampling time points. CCL3, chemokine ligand 3; CENP, centromere protein autoantigen; EGFR, epidermal growth factor receptor; HBSAg, hepatitis B surface antigen; HSCT, haematopoietic stem cell transplant; TPO, thyroperoxidase; TG, thyroglobulin; Scl70, scleroderma autoantigen 70.

Scl-70 positive at baseline (5 HCST and 1 CTX). Scl-70 autoantibody titres decreased in two participants (participants #55 and #58), while one subject (subject #17) was initially Scl-70 negative at baseline and seroconverted to Scl-70 positive at month 38 and remained Scl-70 positive at month 48 (figure 4B, columns 7 and 8). Autoantibodies specific for both of the 52kD and 60kD Ro proteins and the SSB/La protein were more variable, and tended to decrease in titre following HSCT (figure 4B, columns 3–6). Although only subject #42 developed new reactivity to TPO and TG, analysis of all participants in the SCOT trial demonstrated that 17 of 31 HSCT-treated participants developed varying levels of new-onset TPO or TG antibodies during the course of the trial, while only 3 of 32 CTX-treated participants developed antithyroid antibodies (p=0.003; online supplemental figure 2). Subject #24 was Scl-70 positive at baseline but then developed persistent, high-titre anti-CENP antibodies (associated with limited SSc) at 26 months following HSCT (figure 4B, column 1).

Comparison of baseline serum autoantibody profiles and clinical outcomes

We investigated whether baseline levels of specific autoantibodies were associated with clinical outcomes, irrespective of whether a patient was treated with CTX or HSCT. We compared autoantibody profiles obtained at baseline in those participants who survived event-free (n=42) vs deceased or had organ failure (n=21). Autoantibodies directed against 20 unique antigens were significantly different in patients who survived event-free compared with those who were deceased or had organ failure at 54 months (q value <10%, |log2 fold-change|>1 (online supplemental table 8, 9). Surprisingly, levels of these autoantibodies were higher in participants who survived event-free (n=20 antigens) than those who deceased or had organ failure (figure 5A). Nine proteins were secreted factors including interleukins (IL-18, IL-22, IL-23 and IL-27), Interferon-α2A, stem cell factor (SCF), TGF-β, macrophage CSF (M-CSF) and migration inhibitory factor (MIF). Of these, we identified the top three autoantigen targets as IL-27, interferon-α2A and PDC-E2 in those who survived event-free (figure 5B). These autoantibodies may serve as biomarkers in future studies to determine whether they can predict successful clinical responses to HSCT.

DISCUSSION

A fundamental question in treating severe autoimmunity with transplantation is whether the adaptive immune system 'reboots' the B cell repertoire, leading to antigen-specific tolerance and clinical improvement. We demonstrate that patients with SSc at baseline have serum autoantibodies that recognise a plethora of autoantigens commonly targeted in SSc and other CTDs, while HC have significantly less or no reactivity to standard SSc autoantigens. Our data also show that individual SCOT participants reproducibly cluster with themselves across multiple time points, demonstrating that the bulk of the antibody repertoire is strikingly stable in the same individual over time. This was particularly the case in CTX-treated subjects, where, somewhat surprisingly, no overt changes were observed over the 54-month trial.

Few studies have described autologous HSCT to treat severely ill patients with CTDs such as SLE and SSc. In one study, an SSc patient was reported to develop multiple autoimmune diseases following HSCT.[22] Clinical manifestations were accompanied by elevated TGF-β and IL-17, and low levels of regulatory T cells. In a patient with SLE, autoantibodies and SLE disease activity improved dramatically following HSCT.[23] The patient subsequently developed factor VIII autoantibodies and haemophilia that was refractory to all treatment, but then resolved spontaneously when SLE relapsed 3 years following transplantation. In a retrospective review of 155 autologous HSCT autoimmune disease transplants, only six patients developed secondary autoimmunity, two of whom were SLE patients who developed autoimmune haemophilia.[24] Large, uncontrolled autologous HSCT trials have reported rare development of secondary autoimmunity (eg, Evans syndrome, thyroiditis and erosive osteoarthritis) in SLE HSCT transplanted patients. New-onset, clinically evident autoimmune disorders were observed in 7 subjects in the SCOT study (n=3 HSCT: RA, Sjögren's, and thyroiditis; n=4 CTX: myositis, RA, psoriasis and type 1 diabetes), despite the observation that autoantibody profiles changed most markedly in patients in the HSCT arm of the trial (figure 4).

Adamska *et al* in a companion paper in *ARD* characterised the heavy and light chain immunoglobulin gene repertoire from the same SCOT subjects that we analysed in this study.[25] Their results suggest that autoimmune B cells are depleted during the conditioning regimen and that unswitched B cells continue to repopulate the repertoire after engraftment, leading to a new, 'autotolerant stabilisation' of the B cell repertoire. Our autoantigen array analysis is consistent with the above data, supporting the hypothesis that transplantation limits expansion of pathogenic clones and that a limited cadre of new antigens are instead targeted over time in transplanted patients, particularly thyroid-specific antigens. Contrary to conventional beliefs that higher levels of autoantibodies at baseline might correlate with worse clinical outcomes, our data showed that autoantibody levels were higher in participants who survived event-free than those who died or had organ failure, for all 20 baseline autoantibodies that were identified (figure 5A). In subject-specific longitudinal analysis, 9 participants had changing levels of autoantibodies against 1 or more antigens among a pool of 14 antigens. In pooled longitudinal analyses, antibodies against three antigens (HBSAg, which was expected since transplanted patients were vaccinated for hepatitis B at months 14, 16 and 26 after HSCT, EGFR and CCL3) increased in the HSCT group over time. Taken together, these data suggest that some autoantibodies (eg, anti-EGFR or anti-CCL3) may have previously unrecognised immunomodulatory functions associated with HSCT that may play a role in reconstituting the B-cell repertoire in a subset of patients.

Anti-cytokine antibodies (ACAs) have been increasingly recognised in many different immunodeficiency and infectious diseases,[26] more recently in severely ill COVID-19 patients[27–29] and in patients with ARDS caused by many different pathogens,[30] and in autoimmune diseases such as RA, SLE, autoimmune polyendocrine syndrome type I, multiple sclerosis (MS) and others.[31 32] Here, levels of ACA against multiple cytokines (IL-1β, IL-2, IL-3, IL-8, IL-13 and TNFα) were elevated in baseline SSc sera compared with sera from HC. Increases in ACA against many additional cytokines (IL-18, IL-22, IL-23, IL-27, interferon-α2A, SCF, TGFβ, M-CSF and MIF) were observed in SSc participants who survived event-free endpoint compared with those who died or developed organ failure. In contrast, antibody titres against traditional SSc autoantigens such as Scl-70 did not correlate with clinical endpoints. The most likely explanation for these

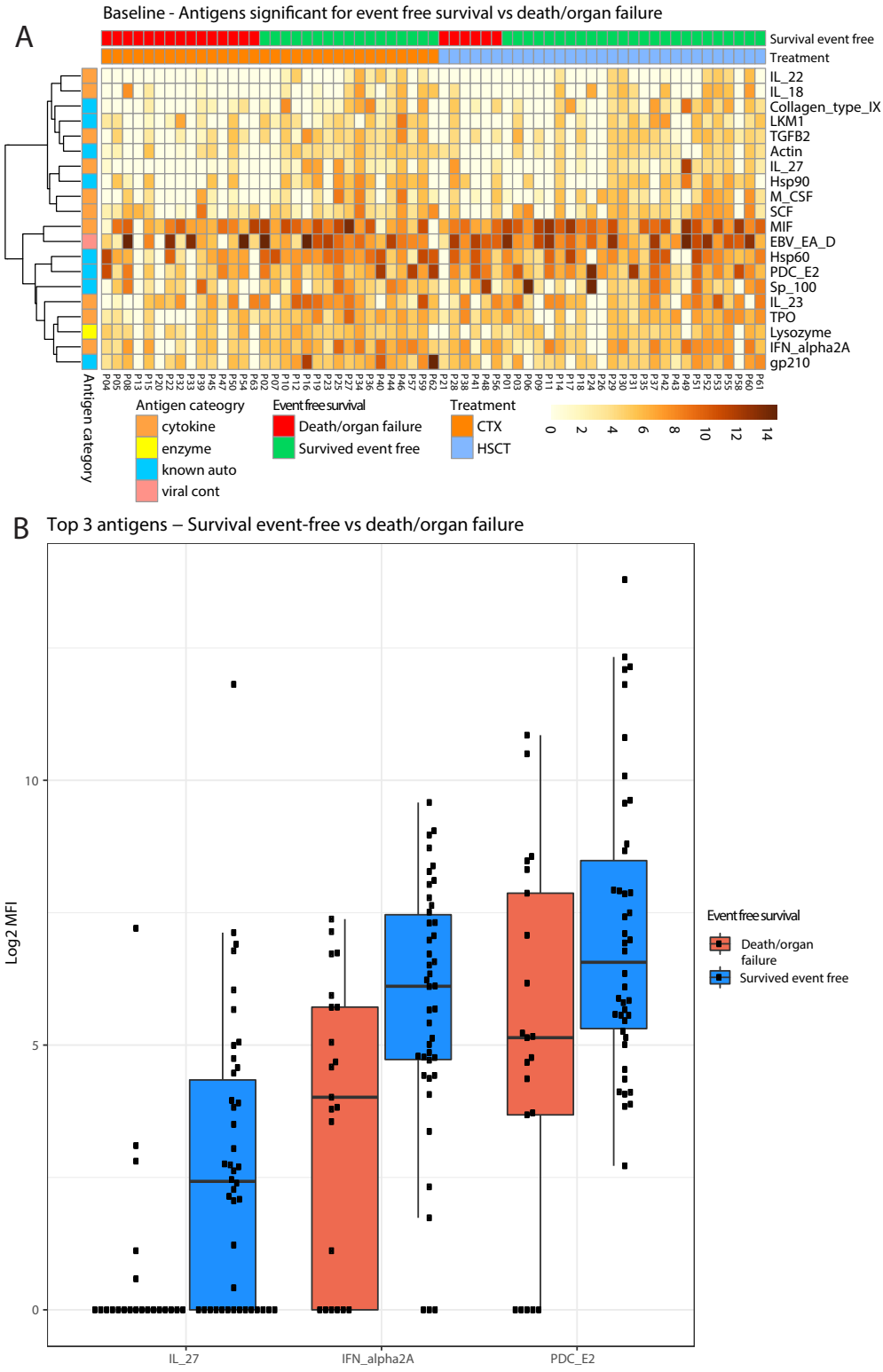

Figure 5 Comparison of baseline serum autoantibody profiles of SCOT participants who were responsive or not-responsive to their assigned treatments. (A) A heat map showing log2-transformed MFI values for baseline serum autoantibody reactivity against 20 antigens in SCOT participants who survived event-free (n=42) vs died or had organ damage (n=21). Antigens identified to be statistically significantly different (q-value<15% and absolute log2 fold-change>2) are shown. Heat map columns represent serum samples and rows represent statistically significant antigens. For each subject, treatment arm and treatment response are colour-annotated. (B) Boxplots representing the top three autoantibodies that significantly differ at baseline between SCOT participants who survived event-free versus died or had organ damage. Each dot shows log2-transformed MFI value representing baseline serum autoantibody reactivity against IL-27, IFN-α2a and PDC-E2. For each sample group, the box-and-whisker plot represents log2-transformed MFI values within lower and upper quantile (box), the median (horizontal line within box), percentiles of 5 and 95% (whiskers) and outliers (dots). HSCT, haematopoietic stem cell transplant; M-CSF, macrophage colony stimulating factor; MFI, median fluorescence intensity; MIF, macrophage migration inhibitory factor; SCF, stem cell factor; SCOT, Scleroderma: Cyclophosphamide Or Transplantation; TGF-B, transforming growth factor beta.

findings is that traditional autoantigens such as Scl-70 are not pathogenic and serve largely as diagnostic markers, and that memory B cells specific for such antigens are spared from the lymphoablation regimen used in the SCOT protocol. Although we did not test the functions of ACA in our studies, antibodies with receptor blocking activity (such as anti-IFN-alpha) are unlikely to be induced during transplantation and are rarely observed in patients with ARDS or connective tissue diseases such as SLE.[30 33] ACAs are generally considered to be pathogenic, at least in the setting of acute viral infections; however, our results suggest that some ACA may modulate immune responses in potentially favourable ways, warranting future functional characterisation of the many ACAs identified in our experiments.

Previous studies have suggested the involvement of growth factor receptors such as EGFR and PDGFR-β in the pathogenesis of SSc, particularly SSc-associated pulmonary arterial hypertension.[34–38] Increased EGFR protein expression in fibroblasts from patients with SSc has been demonstrated.[35 36] An integrated, multicohort analysis of SSc skin transcriptomic data identified a 415-gene SSc signature whose functional characterisation revealed significant enrichment for EGFR ligands.[39] Pathway analysis of the 415-gene signature identified PI3K/Akt signalling components, supporting the involvement of EGFR and its downstream pathways in SSc pathogenesis. In another report, autoantibodies to EGFR have been found in autoimmune mice, as well as in subpopulations of human patients with SSc, and to a lesser extent in SLE patients.[40] Since HSCT was clinically beneficial in the SCOT trial, and EGFR signalling pathways are prominently activated in the 415-gene SSc signature, we postulate that EGFR autoantibodies may function as steric inhibitors of EGF ligands. Future studies are required to understand the precise roles of anti-EGFR autoantibodies in SSc, as well as the mechanisms underlying their production and potential roles in HSCT in general.

The second self-antigen target in HSCT participants at month 26 was CCL3. Increased levels of CCL2 and CCL3 have been reported in sera and peripheral blood mononuclear cells from SSc patients, and positively correlated with the presence of pulmonary fibrosis.[41 42] CCL3, CCL2 and CCL5 have been implicated in the pathogenesis of SSc through recruitment of monocytes and T helper lymphocytes, and upregulation of adhesion molecules which then enhance diapedesis.[41–45]

Several of the autoantibodies that we discovered are clinically actionable, particularly antibodies specific for thyroid tissue. Strikingly, 17/31 (55%) HSCT-treated participants developed new-onset TPO and/or TG during the trial, while only 3/32 (9%) of CTX-treated participants developed antithyroid antibodies (p=0.005). Compared with the general population, a higher prevalence of antithyroid antibodies has been reported in SSc patients,[46] particularly in limited SSc.[47] Thyroid autoimmunity is also observed in transplantation in general. TBI decreases expression of TPO in cultured thymocytes and impairs thyroid hormone synthesis.[37] Development of antithyroid autoimmunity during immune restoration is consistent with previous reports of transient autoimmune thyroiditis,[38] and suggests that HSCT in general (as opposed to HSCT in SSc or SSc itself) is associated with development of new-onset TPO and TG observed in this trial. Our data clearly suggests that TPO, TG and thyroid function should be closely monitored in HSCT-treated patients. Other autoantibodies which would be useful in identifying preclinical autoimmunity where an intervention could be considered, include antiparietal autoantibodies (associated with pernicious anaemia) and anti-PDC (associated with primary biliary cirrhosis).

Although the multiplexed arrays used here were developed solely for research purposes, bead-based arrays for measuring autoantibodies against traditional connective tissue disease antigens are available as a clinical-grade assay at some academic medical centres.[48] For our research-grade assays, we rigorously validated 31 antigens using 30 unique monoclonal antibodies and/or prototype human serum samples derived from patients who met classification criteria for scleroderma, lupus, myositis, Sjögren's and other CTDs (online supplemental figure 3). We further compared our array results for a common autoantigen (Ro60) with a clinical grade assay, demonstrating that many reactivities are 'true positives' (R=0.99, p=0.0003 for the top six most reactive samples tested (online supplemental figure 4). For ACA measurements, our lab[29] and the Casanova lab[27] have used multiplexed bead-based arrays that were directly compared with ELISAs,[27] and in previous reports we also compared ELISA and planar arrays for two different ACAs with excellent correlation (r=0.89 and 0.98).[49] These same prototype serum samples were used to develop our most recent generation Luminex bead assay.[29 30]

Autoantibodies that are detectable in research settings, but may not reach a threshold for 'positivity' in a clinical lab, may still be important for understanding evolution of autoimmunity. As just one example, a recent paper on long COVID-19[50] measured only six autoantibodies (against IFN-α2, Ro, La, U1-RNP, Jo-1 and ribosomal protein P1) using research-grade ELISAs and identified correlations between low levels of autoantibodies and specific long COVID-19 clinical manifestations.[50] We conclude that a subset of autoantibodies we identified using the SAM algorithm are present at levels that may be clinically relevant (see online supplemental figures 3 and 4). In some SCOT patients, measurements of a subset of autoantibodies are likely below what would be considered as 'positive' by practising clinicians, yet still provide meaningful clues about the underlying biology of HSCT in scleroderma.

In summary, HSCT is emerging as a promising therapy for severe diffuse SSc, and for other autoimmune diseases with unmet medical needs. However, little is understood regarding how the abnormal immune system in SSc or other autoimmune diseases is reshaped by HSCT, following which ongoing tissue damage is reduced or eliminated. Our results and those of Adamska *et al* demonstrate that the autoantibody repertoire is remarkably stable in HSCT-treated patients over time, although significant differences in autoantibody targets were identified in a subset of HSCT-treated subjects, particularly antigen targets such as soluble cytokines and chemokines, and cell surface receptors. Future studies that integrate single-cell B cell phenotyping, antibody repertoire analysis and antibody functional assays are necessary to better understand how the B cell repertoire is reshaped in autoimmune diseases following HSCT.

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