SUPPLEMENTARY METHODS

Bead-based antigen arrays

We generated a custom, 280-plex, bead-based antigen array designed specifically for the study of connective tissue diseases such as SSc, and modelled based on similar arrays previously used to study murine SLE repertoires[1] and cloned human monoclonal antibodies derived from patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED)[2]. A total of 221 commercial protein antigens were used to construct the array and included: 109 unique proteins known to be associated with connective tissue diseases (*e.g.* standard SSc antigens such as Scl-70, centromere proteins, and RNA polymerases); a large panel of 80 secreted proteins including cytokines, chemokines, growth factors, acute phase proteins and extra-cellular matrix proteins; five viral proteins commonly used to measure antibodies to pathogens, such as Epstein Barr Nuclear Antigen-1 (EBNA-1), and vaccine constituents such as Hepatitis B Surface Antigen (HBSAg); and an additional set of 27 proteins selected based on previous studies (11 enzymes; seven nuclear proteins; and nine cell surface receptors)[2-7] (***Supplementary Table 9***). The remaining bead IDs were conjugated to 12 different human and anti-human antibody subtypes to create three-point dilution series, and were used as positive and negative control analytes for quality control.

All antigens were coupled to carboxylated magnetic beads (MagPlex-C, Luminex Corp.) such that each antigen was linked to beads with unique barcodes, as previously described[1 8]. Briefly, 6 μg of each antigen and a three-point dilution series of control human and anti-human antibodies (0.25, 1, and 4 μg) were diluted in phosphate buffered saline (PBS) and transferred to 96-well plates. Diluted antigens and control antibodies were conjugated to 1×10^6 carboxylated magnetic beads per ID. Beads were distributed into 96-well plates (Greiner BioOne), washed and re-suspended in phosphate buffer (0.1M NaH_2PO_4, pH 6.2) using a 96-well plate washer (Biotek).

The bead surface was activated by adding 100 µl of phosphate buffer containing 0.5 mg 1-ethyl-3(3-dimethylamino-propyl)carbodiimide (Pierce) and 0.5 mg N-hydroxysuccinimide (Pierce). After 20 min incubation on a shaker, beads were washed and resuspended in activation buffer (0.05M 2-N-Morpholino EthaneSulfonic acid, MES, pH 5.0). Diluted antigens and control antibodies were incubated with beads for 2 h at room temperature. Beads were washed 3× in 100 µl PBS-Tween, re-suspended in 60 µl storage buffer (Blocking reagent for ELISA, Roche), and stored in plates at 4°C overnight.

Immobilization of antigens and control antibodies on the correct bead IDs was confirmed by analysis of the following commercially-available mouse monoclonal antibodies: anti-La/SSB and anti-Ro52 (Santa Cruz); anti-His6 tag (Invitrogen); anti-Scl-70 (ImmunoVision); anti-IL-1β, anti-IL-2, anti-IL-4, anti-IL-5, anti-IL-6, anti-IL-10, anti-IL-13, anti-IL17-A, anti-CCL2, anti-IFNβ and anti-IFNγ (eBiosciences); anti-GM-CSF (Biolegend), and a rabbit polyclonal anti-Histone H2b antibody (Abcam). All antibodies were analyzed at a concentration of 1 µg/ml. In addition, dilution series of various prototype human plasma samples derived from participants with autoimmune diseases with known reactivity patterns (*e.g.* ds-DNA, Scl-70, centromere, SSA, SSB, cardiolipin, whole histones, and RNP (ImmunoVision), as well as normal human sera (ImmunoVision), were used to test two different dilution buffers (0.05% PBS-Tween supplemented only either with 3% (w/v) bovine serum albumen (BSA, Sigma) or with 3% (w/v) BSA and 5% (w/v) blotting-grade non-fat dry milk (Bio-Rad)). Each dilution buffer was then tested using three different concentrations (1:75, 1:150, and 1:300) for serum samples.

Assay protocols that used the optimized serum dilution and buffer conditions described above were then applied to studying samples from the SCOT trial[8]. A total of 212 samples (n=192 SCOT samples who completed the treatment, n=63 subjects); and 20 HC were diluted 1:125 in

0.05% PBS-Tween supplemented with 3% (w/v) BSA and transferred into 3×96-well plates in a randomized layout. Across these 3×96-well plates, 14 well positions were randomly assigned as serum-free negative control wells, and 13 well positions were randomly assigned as serum pools. The bead array was distributed into a 384-well plate (Greiner BioOne) by transfer of 5 µl bead array per well. Using a liquid handler, we then transferred 45 µl of the 1:125 diluted sera into the 384-well plate containing the bead array. Samples were incubated for 90 min on a shaker at room temperature. Beads were washed with 3 × 60 µl PBS-Tween on a plate washer (EL406, Biotek) and 50 µl of 1:500 diluted R-phycoerythrin (R-PE) conjugated Fcγ-specific goat anti-human IgG F(ab')2 fragment (Jackson ImmunoResearch) was added to the 384-well plate for detection of bound human IgG. After incubation with the secondary antibody for 45 min, the plate was washed with 3 × 60 µl PBS-Tween and re-suspended in 60 µl PBS-Tween prior to analysis using a FlexMap3D instrument (Luminex Corp.). A minimum of 100 events per bead ID were counted. Binding events were displayed as median fluorescence intensity (MFI).

Initial array construction and validation

To optimize and establish the bead-based methodology to specifically study human SSc samples, we first generated an array with limited content composed of 43 unique antigens including 16 cytokines and chemokines and 27 traditional autoantigens including Ro52 and Scl-70. Six human and anti-human antibodies served as control analytes. This array was used to (i) optimize antigen-bead conjugation conditions; (ii) identify optimal buffer composition and dilution; (iii) determine serum sample concentrations; (iv) optimize conditions for secondary detection reagents; and (v) ensure that all reagents and instruments were functional prior to performing the assay on the SCOT samples.

A majority of the 43 antigens and all control human and anti-human antibodies were conjugated as a three-point dilution series, while two different antigen dilution buffers (1×PBS or 0.05M MES) were also compared. The array was first characterized and validated using 16 commercial antibodies specific for individual cytokines and chemokines (anti-Scl-70, anti-IL-17A, anti-Ro52/SSA, anti-La/SSB, anti-IL-6, anti-IL-2, anti-IL-5, anti-IL-4, anti-IL-10, anti-CCL2, anti-GM-CSF, anti-IL-13, anti-IL-1β, anti-IFNγ, anti-TNFα and anti-histone H2b). ***Supplementary Figure 3A*** shows a heat map depicting reactivities to these different commercial antibodies. Each antibody was specific for its target antigen and displayed low or negligible levels of cross-reactivity to unrelated targets, with the exception of cross-reactivity between anti-SSB and histone H2b (***Supplementary Figure 3A***, first column on the left) and anti-H2b and GM-CSF (***Supplementary Figure 3A***, sixth column from the left). Low level fluorescence was observed for IL-2 (eleventh row from top, ***Supplementary Figure 3A***) and to a lesser extent histone H2b and GM-CSF, even when using only assay buffer (first column on the left, ***Supplementary Figure 3A***). Taken together, these results confirm that all of the tested antigens were successfully conjugated to beads and retained conformational epitopes, and further demonstrate highly-specific binding of monoclonal and polyclonal antibodies to their cognate antigen targets.

We next analyzed well-characterized prototypical human sera from six individuals with SSc (three with diffuse cutaneous SSc who were Scl-70 positive, and three with limited cutaneous SSc who were centromere positive), obtained from the Stanford Systemic Sclerosis Biorepository. In addition, a total of nine commercially-available human autoantibody-negative control sera, and sera with known reactivities to SSA, SSB, Jo-1, U1-small nuclear ribonucleoprotein complex (U1-snRNP), whole histone, Sm (Smith), and single-stranded (ss)-DNA were utilized for validation. As shown in ***Supplementary Figure 1B***, binding profiles of the six SSc patient sera and nine

commercially-available human prototype sera revealed reactivity against the expected corresponding autoantigens, as well as additional antigens contained within the array. For example, anti-centromere positive serum samples derived from SSc patients with the limited cutaneous form of SSc recognized both centromere proteins (CENP-A and CENP-B), and several EBV-specific antigens (EBNA-1 and EBV p-18). Two samples (Patients #2 and #3) also recognized the Ro-52 autoantigen but not the structurally-related Ro-60 protein (top panel, ***Supplementary Figure 3B***). Anti-Scl-70 positive serum samples derived from patients with the diffuse cutaneous form of SSc exhibited broader reactivity, including all four EBV antigens (EBNA-1, EBV p-18, EBV EA and EBV EA-D), thyroperoxidase (TPO), Ro-52, and components of the U1-snRNP such as U1-A, U1-C, and U1-70 (middle panel, ***Supplementary Figure 3B***). Excellent concordance was observed when comparing reactivity against bovine Ro60/SSA and human recombinant Ro60/SSA from a different vendor (columns 6 and 7, ***Supplementary Figure 3B***). Similar results were obtained for the commercially-available prototype sera, with the broadest reactivity observed for the histone positive serum sample (bottom panel, ***Figure 1B***). Although not a goal of our initial studies, we also demonstrated that bead-based arrays are useful for epitope mapping, as Scl-70 positive patient #3 had high-titer antibodies that recognized full length topoisomerase I, but not a truncated version lacking the first 190 amino acids (middle panel, compare columns 3 and 4, ***Figure 1B***). We conclude from these pilot experiments that the bead-based approach provides results that are comparable to those obtained using planar arrays[3], but with far greater sample throughput, multiplexing capacity, and flexibility.

Construction of a high content SSc array for autoantigen discovery

Following technical validation of the "limited content" array and optimization of assay conditions described above, a "full-content" 280-plex array was designed and generated (***Supplementary Table 9***). Different classes of antigens were included in the array design: "Standard SSc antigens" such as Scl-70, CENP-B, Th/To, and RNA polymerases I/III[9] were included to determine whether the newly-reconstituted repertoire retained the original reactivities, or alternatively adopted entirely new autoantibody profiles following engraftment. Patients with SSc often evolve naturally over time to develop additional connective tissue diseases such as SLE, myositis, and Sjögren's Disease, and other autoimmune manifestations such as autoimmune thyroiditis. We thus constructed a subpanel of "Traditional Autoantigens" such as Ro, La, Smith proteins, RNP proteins, thyroperoxidase (TPO), and thyroglobulin (TG). In addition to the 109 unique proteins previously identified as autoantigens in connective tissue diseases, the array also included a "Discovery Antigen Panel" comprised of 107 unique antigens to determine whether autoantibodies against secreted proteins or cell surface receptors might exist in serum derived from diffuse SSc patients, mirroring our findings and those of other investigators in autoimmune diseases such as SLE[3 10-12] and a growing number of immunodeficiency disorders[4 5 7 13]. Among these 107 unique antigens, 80 were secreted proteins including cytokines, chemokines, growth factors, acute phase proteins, and extracellular matrix proteins. The remaining 27 antigens included enzymes, nuclear proteins, and cell surface receptors. To explore whether protective antibodies directed against viral antigens or other pathogens changed over time, we also included four antigens derived from EBV as well as HBSAg. Finally, dilution series of a number of control human and anti-human antibodies was included that served as internal assay controls.

Serum samples

Serum samples from SSc participants were collected at specific time points throughout the duration of the SCOT trial, with ethical approval for the trial described in our previous publication[14]. A total of 192 serum samples from the SCOT trial were provided to Stanford University by the clinical drug development & contract research service Rho (http://www.rhoworld.com/) as barcoded samples with no identifiers. In addition, 20 specimens from healthy controls with age and sex distributions similar to the SCOT participants, to which we were also blinded to identity, were analyzed. All samples were randomly distributed throughout the wells in the assay plates and analyzed in parallel. To establish the bead-based autoantibody assay, prototype serum samples derived from patients with diffuse and limited SSc were obtained through the Stanford Systemic Sclerosis Biorepository (Stanford IRB #12047). To eliminate batch effects, all samples were analyzed in the same assay run, using the same instrument, randomly distributed across assay plates.

Data analysis and statistics

All data analysis and statistics were performed using R and various R packages[15]. Data were log2 transformed. For normalization, mean MFI values for "bare bead" IDs were subtracted from MFI values for antigen conjugated bead IDs. To identify statistically-significant differences in array reactivity in various group comparisons ***(see Figure 1C)***, we applied the SAM algorithm to log2 transformed, normalized MFI values. We determined reactivity to be significantly correlated within each analysis (*e.g.* SSc vs Control, or SCT vs HSCT) when the SAM-reported log of fold

change (logFC) was greater than 0.6, using a False Discovery Rate of <20% (q < 0.2), unless otherwise specified. For completeness, all statistical analyses were also performed using non-normalized (raw) data in which MFI values for "bare bead" IDs were not subtracted yielding similar results (data not shown).

Linear mixed models analysis was used to identify autoantibodies in which serum levels exhibited significantly different trends over time between the two treatment arms, and for clinical response status (with response defined as survival event-free at month 54). In each model, we included the random effect due to individual subject differences. The analysis was performed using the R package *lmer.* Autoantibodies were considered to have significantly different trends if the appropriate coefficient for the model had an FDR-corrected p-value <0.2. Correction was performed with the Benjamini-Hochberg method for correction for multiple comparisons.

Subject-specific antigen trends were obtained by fitting a standard linear model to each patient-antigen intensity profile. We then built a distribution of the slopes of the regression lines by ordering them based on their magnitudes. From the assembled distribution, we selected patient-antigen pairs with slopes that had >2 standard deviations from the distribution mean.

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