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CXCL13 Is an Indicator of Germinal Center Activity and Alloantibody Formation Following Transplantation

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Background. Donor-specific antibodies (DSA) are a recognized cause of allograft injury, yet biomarkers that indicate their development posttransplant or guide management are not available. CXCL13 (chemokine [C-X-C motif] ligand 1) is a chemoattractant produced within secondary lymphoid organs necessary for germinal center (GC) and alloantibody formation. Perturbations in serum CXCL13 levels have been associated with humoral immune activity. Therefore, CXCL13 may correlate with the formation of HLA antibodies following transplantation. **Methods.** A murine skin graft model was utilized to define the production and kinetics of CXCL13 in response to alloantigen. Human Tfh:B-cell in vitro cocultures were performed to evaluate CXCL13 production by human lymphocytes, and serum from healthy controls and human transplant recipients with and without de novo DSA was tested for CXCL13. **Results.** CXCL13 was detectable in the blood of allografted mice and correlated with Tfh and GC B-cell responses. Greater CXCL13 expression was observed in the draining lymph nodes of allografted mice as compared with naïve or syngeneic graft recipients, and serum levels preceded the detection of DSA posttransplant. Similarly, productive human Tfh:B-cell interactions that led to plasmablast differentiation and IgG formation also exhibited CXCL13 expression. CXCL13 levels in human transplant recipients with de novo DSA were greater than in healthy controls and stable transplant patients and also correlated with the development of alloantibodies in a small cohort of serially monitored recipients. **Conclusions.** CXCL13 indicates GC alloreactivity and alloantibody formation and correlated with DSA formation in kidney transplant recipients, thereby introducing CXCL13 as a potential biomarker for HLA antibodies.

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INTRODUCTION

Donor-specific antibodies (DSA) are associated with decreased graft survival following renal transplantation.[1,2] Despite increased recognition of alloantibodies as mediators of early and late immunologic injury, reliable biomarkers to detect early or ongoing humoral alloreactivity have not been established. The ability to detect nascent alloantibody responses may be highly beneficial in predicting DSA formation and guiding clinical management to prevent or lower the risk of subsequent injury and premature graft failure. Although acute rejection rates in kidney transplantation are excellent for low- and short-term outcomes,[3] long-term outcomes remain suboptimal and are negatively affected by the development of DSA.[1,4] Solid-phase HLA antibody detection systems have greatly aided in detecting alloantibodies pretransplant and preventing hyperacute and early antibody-mediated rejection due to a preformed antibody, but their diagnostic utility posttransplant is limited. The need for biomarkers that identify DSA formation early and allow for therapeutic intervention and monitoring response to treatment persists.[2,5]

Durable antibody responses are a product of the germinal center (GC) reaction that occurs in secondary lymphoid

organs upon antigen exposure and depends on T follicular helper (Tfh) cell interactions with cognate B cells that promote immunoglobulin class switching, the formation of plasma cells, and the subsequent production of antigen-specific antibodies.[6-11] Given the dependence of antibody formation on GC reactivity and the inaccessibility of lymph node tissue, it has been postulated that circulating chemokines or cells involved in the GC reaction may function as a surrogate for antibody formation.[12,13] Thus, GC-related biomarkers could indicate active humoral alloreactivity and predict the development of detectable DSA. In fact, circulating Tfh (cTfh) cells have been observed to correlate with GC activity, predict DSA after transplantation in mice,[14] and parallel HLA sensitization in human renal transplant recipients.[11,15] Although cTfh cells are indeed a promising biomarker for GC activity and DSA formation, they are a highly heterogenous subset that are not yet entirely understood.[16] Additionally, their detection and processing, as with any potential cellular biomarker, requires isolating peripheral blood mononuclear cells with subsequent staining and flow cytometry analysis. The methods required for this analysis are cumbersome, difficult to reproduce, and high cost and require a large amount of time, which limits feasibility and can prove difficult to deploy in a clinical setting.[17] Therefore, serum-based GC-associated chemokines may act as a simpler, more feasible surrogate of GC reactivity.

CXCL13 (chemokine [C-X-C motif] ligand 13) is one such candidate biomarker. Alternatively referenced as B cell–attracting chemokine 1 (BCA-1), CXCL13 is the ligand for CXCR5 and is produced in lymphoid tissue by follicular cells as a homing chemokine for B cells and other CXCR5$^+$ cells, such as Tfh cells.[18,19] Together, these assist with the formation of the B-cell zone and GCs in secondary lymphoid tissues. Interestingly, CXCL13 is detectable in human blood, and its plasma levels have been shown to be associated with GC reactivity, HIV infection, and autoimmune disease activity.[20,21] Because GC reactions are necessary for alloantibody formation, plasma CXCL13 levels may have the potential to also function as a biomarker for DSA formation in patients posttransplantation. Although CXCL13 has been detected in the serum, urine, and tissue of transplant recipients with allograft dysfunction and rejection,[22-24] CXCL13 expression and kinetics have not been examined in response to alloantigen, nor has its potential to function as a biomarker for GC activity and subsequent DSA formation in transplantation. In this study, we sought to examine the production, kinetics, and detection of CXCL13 in a murine transplant model, as well as evaluate its potential as a biomarker in human transplant recipients with DSA.

MATERIALS AND METHODS

Mice and Skin Transplants

B6-Ly5.1/Cr and BALB/c mice were obtained from Charles River Laboratories. All mice were housed in pathogen-free facilities and maintained in accordance with Emory University Institutional Animal Care and Use Committee guidelines. Bilateral dorsal full thickness tail or ear skin were transplanted from B6-Ly5.1/Cr (syngeneic) or BALB/c (allogeneic) to B6-Ly5.1/Cr mice.

Human Samples

Human serum and lymph node samples were obtained from the Emory Transplant Center (ETC) biorepository via an IRB-approved immune monitoring protocol (IRB00006248) and the Emory HLA laboratory (IRB00113648). Lymph nodes were harvested from the pelvis of transplant recipients at the time of transplantation. Isolated serum samples were randomly selected from the ETC biorepository for healthy controls and stable transplant recipients. Isolated DSA+ samples were from kidney transplant recipients with de novo DSA detected within 2 wk of the stored sample date in the HLA laboratory. Serial serum samples used for longitudinal analysis in healthy controls, stable recipients, and 3 transplant recipients with de novo DSA formation were gathered from the ETC biorepository. These recipients were late in their transplant course with failing allografts and underwent immunosuppression discontinuation over the period of sample collection. Induction therapy and maintenance immunosuppression for each patient at the beginning of sample collection was as follows: Patient 1, basiliximab, tacrolimus, and prednisone; Patient 2, thymoglobulin, rapamycin, mycophenolate mofetil, and prednisone; Patient 3; basiliximab, tacrolimus, mycophenolate mofetil, and prednisone.

Flow Cytometry and Cell Sorting

Graft-draining axillary and brachial lymph nodes were processed into single-cell suspensions. Cells were surface stained with their appropriate markers followed by the fixable blue cell viability kit for UV excitation (LIVE/DEAD, Invitrogen) before fixation. Intracellular staining was performed with the use of the Foxp3 Fixation/Permeabilization Buffer Kit (eBioscience, Invitrogen). All antibodies were obtained from Biolegend and BD Biosciences. Samples were run on either LSR Fortessa or FACSymphony (BD Biosciences) and analyzed using FlowJo Software, version 10 (Flowjo, LLC). For sorting, cells were obtained from graft-draining axillary and brachial lymph nodes following skin transplantation. CD4+ T cells were enriched using magnetic bead negative selection (Miltenyi Biotec) and then sorted into CXCR5$^-$ (CD19$^-$CD4$^+$CD44^{hi}PD1^{lo}CXCR5$^-$GITR$^-$) and CXCR5$^+$ Tfh (CD19$^-$CD4$^+$CD44^{hi}PD1^{hi}CXCR5$^+$GITR$^-$) cell populations.

Real-time PCR

Graft-draining axillary and brachial lymph nodes were processed into single-cell suspensions, and total RNA was extracted using the RNeasy Plus Micro MiniKit (Qiagen) and then converted to cDNA using a high capacity cDNA reverse transcriptase kit (Thermo Fisher). The cDNA was used in a quantitative real-time PCR reaction with PCR TaqMan probes for mouse CXCL13 (Mm00444533_m1), IL-21 (Mm00517640_m1), and GAPDH (Mm99999915_g1). Quantitative PCR was performed using the QuantStudio Flex systems (Applied Biosystems). Data were calculated by the $2^{-\Delta\Delta Ct}$ method as described by the manufacturer's protocol and were expressed as a fold increase over the indicated control. Similar methods were carried out to determine the relative gene expression for human in vitro coculture as well as sorted mouse Tfh (CD19$^-$CD3$^+$CD4$^+$CD44^{hi}PD1^{hi}CXCR5$^+$GITR$^-$) cells. Human TaqMan probes for CXCL13 (Hs00757930_m1), IL-21 (Hs00222327_m1), and GADPH (Hs02786624_g1) were also from Applied Biosystems.

Tfh:B-cell Coculture

Naïve human cells were obtained from benign inguinal lymph nodes of transplant recipients at the time of

transplantation. In accordance with previously published methods,[25,26] B cells were enriched using magnetic bead negative selection (Miltenyi Biotec), and T cells were FACS sorted into CXCR5$^-$ (CD4$^+$TCR$^+$CXCR5$^-$CD45RA$^+$) and CXCR5$^+$ Tfh (CD4$^+$TCR$^+$CXCR5$^+$CD45RA$^-$) cells. The prototypical Tfh cell markers ICOS and PD-1 were not used because of the absence of sufficient ICOS^{hi} or PD-1^{hi} effector Tfh cells normally present in pathologic or reactive lymph nodes. Cells were then cocultured for 5 d in media containing either CD3/CD28 Dynabeads (Life Technologies) or the superantigen staphylococcal enterotoxin in 96-well round bottom plates and then collected for flow cytometry and RT-PCR as described earlier. Results were equivalent between both T-cell stimulation techniques.

CXCL13 ELISA

Serum was collected from mice and humans at specified time periods. All serum samples were cryopreserved at –80°C. For murine serum samples, the mouse CXCL13/BLC/BCA-1 DuoSet kit (R&D Systems DY470) or an in-lab assay using anti-mouse CXCL13 (R&D MAB470) as a capture antibody and R&D anti-mouse CXCL13 biotinylated antibody (BAF470) as a detection antibody were used. The human CXCL13/BLC/BCA-1 Quantikine or DuoSet ELISA kit (R&D Systems DCX130 or DY801) was used to test human samples.

Antibody Assessments

For DSA measurements, flow cytometric crossmatch was performed. BALB/c splenocytes were processed into single-cell suspensions and incubated with recipient murine serum at 4°C, then stained with surface markers including anti-mouse IgG for measurement of anti-BALB/c IgG by flow. For human IgG antibody, the supernatant from wells was collected on D5 of human coculture experiments, and IgG was measured using ELISA. The assay was made using R&D capture (MAB11013) and detection (MAB11012) antibodies with recombinant human IgG (1-001-A) standard.

Statistics

The Mann-Whitney *U* nonparametric t test was performed for the analysis of unpaired groups. All analyses were performed by using GraphPad Prism (GraphPad Software, Inc). Statistical significance was attributed to $P<0.05$ (*<0.05, **<0.01, ***<0.001).

RESULTS

Serum CXCL13 Is Expressed in Graft Draining Lymph Nodes (DLNs), Correlates With GC Alloreactivity, and Indicates DSA Formation Following Transplantation

CXCL13 has been shown to be produced and detectable in murine models of infection, vaccination, and rheumatologic disease[21,27,28] but has not been evaluated in experimental transplant models. A full MHC mismatched BALB/c to B6 murine skin allograft model was used to test for CXCL13 in the serum and graft-DLNs of transplanted mice (Figure 1A). CXCL13 was measured in the serum of skin-grafted mice at the peak of the GC reaction 10 d posttransplant.[14] CXCL13 was indeed detectable following skin-grafting, and levels were significantly greater in allogeneic graft recipients as compared with naïve and syngeneic skin-grafted mice (Figure 1B). Given that GCs within B-cell follicles are known to be the primary source of CXCL13,[18,19] we next examined graft-DLNs for CXCL13 production. Unsorted lymphocytes from graft-DLNs 5 to 7 d after primary and secondary skin grafts were examined for mRNA expression. Similar to IL-21, CXCL13 expression was greatest (3-fold higher) in lymphocytes from allogeneic graft recipients relative to naïve and (>1.5-fold higher) relative to syngeneic-grafted mice following a primary graft (Figure 1C). Interestingly, the secondary memory response demonstrated an even larger (2.5-fold) increase in CXCL13 expression when compared with syngeneic controls. Closer examination of sorted Tfh (CD4$^+$CD44^{hi}CXCR5$^+$PD1^{hi}GITR$^-$) and non-Tfh (CD4$^+$CD44^{hi}CXCR5$^-$) lymphocytes demonstrated ~20-fold greater IL-21 and CXCL13 expression by Tfh cells (Figure 1D).

Based on previous studies, we know that Tfh differentiation and GC activity precede the elaboration of DSA and its detection in serum.[14] Therefore, we sought to examine the kinetics of CXCL13 in relation to GC reactivity and DSA formation. B6 mice were transplanted BALB/c skin, and graft-DLNs and serum were serially tested for Tfh cells, GC B cells, CXCL13, and DSA. Serum CXCL13 levels mirrored the expansion and contraction of the GC as measured by the frequency of Tfh and GC B cells (Figure 1E; gating strategy, Figure S1, SDC, http://links.lww.com/TXD/A379). When compared with alloantibody formation, the increase in CXCL13 levels preceded the generation of DSA and contracted back to baseline as DSA levels plateaued. Taken together, these data demonstrate that serum CXCL13 expressed in graft-DLNs by Tfh cells indicates GC reactivity and precedes DSA formation in response to transplanted alloantigen.

Human CXCL13 Correlates With GC-like Tfh:B-cell Coculture Interactions In Vitro

To evaluate whether CXCL13 production correlates with GC reactivity and antibody production in humans as was observed in mice (Figure 1), we utilized a Tfh:B-cell in vitro coculture system to simulate GC-like conditions. Lymphocytes from human pelvic lymph nodes were isolated and sorted into B cells (CD19$^+$) and CXCR5$^-$ and CXCR5$^+$ Tfh cells (Figure 2A) for coculture. After 5 d of culture, analysis demonstrated the superior differentiation of plasmablasts (CD27^{hi}CD38$^+$) over background and IgG antibody production in the Tfh group as compared with the B cell alone and CXCR5$^-$ T-cell groups (Figure 2B and C). Interestingly, IL-21 and CXCL13 production were only observed in the presence of Tfh cells (Figure 2D), and CXCR5$^+$ Tfh cells produced CXCL13 in contrast to CXCR5$^-$ T cells (Figure 2E). These results indicate human CXCR5$^+$ T-cell production of CXCL13 correlates with productive GC-like Tfh:B-cell interactions.

CXCL13 Levels Correlate With the Development of Alloantibodies in Kidney Transplant Recipients

Based on the observed relationship between CXCL13, GC reactivity, and antibody production in our murine transplant model and human in vitro coculture experiments, we hypothesized that serum CXCL13 levels may indicate alloantibody formation in human transplant recipients. To test this possibility, serum from healthy controls, stable postrenal transplant patients without DSA, and recipients with de novo DSA were evaluated for CXCL13. Among these groups, serum CXCL13 concentrations were greatest in transplant recipients with de novo DSA as compared with stable recipients and healthy controls (Figure 3A). We next examined the kinetics

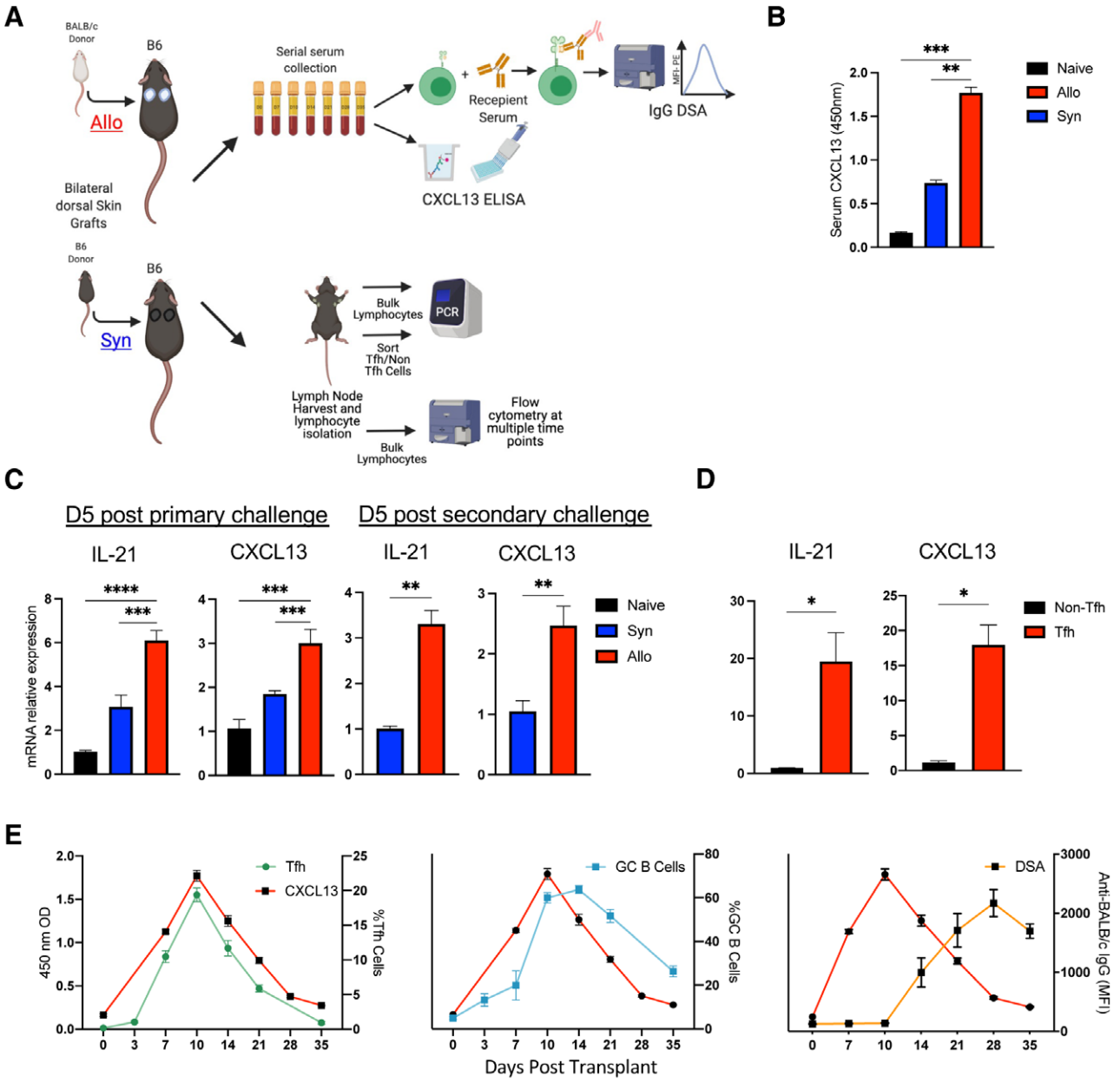

FIGURE 1. CXCL13 expressed in graft-DLNs correlates with GC alloreactivity and indicates DSA formation following transplantation. (A) B6 mice were transplanted with BALB/c (allogeneic) or b6 (syngeneic) skin grafts and draining lymph nodes collected for flow cytometric and PCR analysis. Mice were also serially bled, and serum collected for CXCL13 and antibody analysis. (B) Serum CXCL13 levels in naïve and syngeneic or allogeneic skin-grafted mice 10 d posttransplant. (C) IL-21 and CXCL13 mRNA expression in graft-DLNs 5 d after primary and secondary skin grafts. (D) IL-21 and CXCL13 mRNA expression in sorted murine Tfh ($PD1^{hi}CXCR5^{+}$) cells compared with $CD4^{+}$ non-Tfh ($CD44^{hi}CXCR5^{-}$) cells. (E) Serum CXCL13 levels over time relative to graft-DLN Tfh cell (green), GC B cell (blue), and DSA (orange) kinetics. Summary data represent mean (SE) and are representative of at least 2 independent experiments with a total of 5–10 mice per group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. CXCL13, chemokine (C-X-C motif) ligand 1; DLN, draining lymph node; DSA, donor-specific antibody; GC, germinal center; Tfh, T follicular helper.

of CXCL13 in a unique cohort of kidney transplant recipients serially sampled over the course of developing de novo DSA and third-party HLA antibodies. In these subjects, CXCL13 levels increased during the period of new alloantibody formation and paralleled the rise in HLA antibodies (Figure 3B) with a subsequent return to baseline once alloantibody levels plateaued. These CXCL13 kinetics in transplant recipients forming HLA antibodies were in stark contrast to the longitudinally stable CXCL13 concentrations observed in healthy controls (Figure 3C) and recipients without DSA (Figure 3D). Thus, CXCL13 in human kidney transplant recipients correlated with alloantibody formation.

DISCUSSION

Donor-specific alloantibodies are associated with decreased allograft survival and a barrier to improved outcomes following kidney transplantation.[1,2] The HLA antibody hurdle is confounded by the lack of biomarkers to indicate early DSA formation or guide clinical management posttransplant. In this study, we demonstrate that serum CXCL13 is produced by Tfh cells that drive GC formation and antibody production in transplantation.[29,30] CXCL13 was also observed to correlate experimentally with GC reactivity and early DSA formation, and most importantly, it longitudinally paralleled the development of alloantibodies in a small cohort of renal transplant recipients.

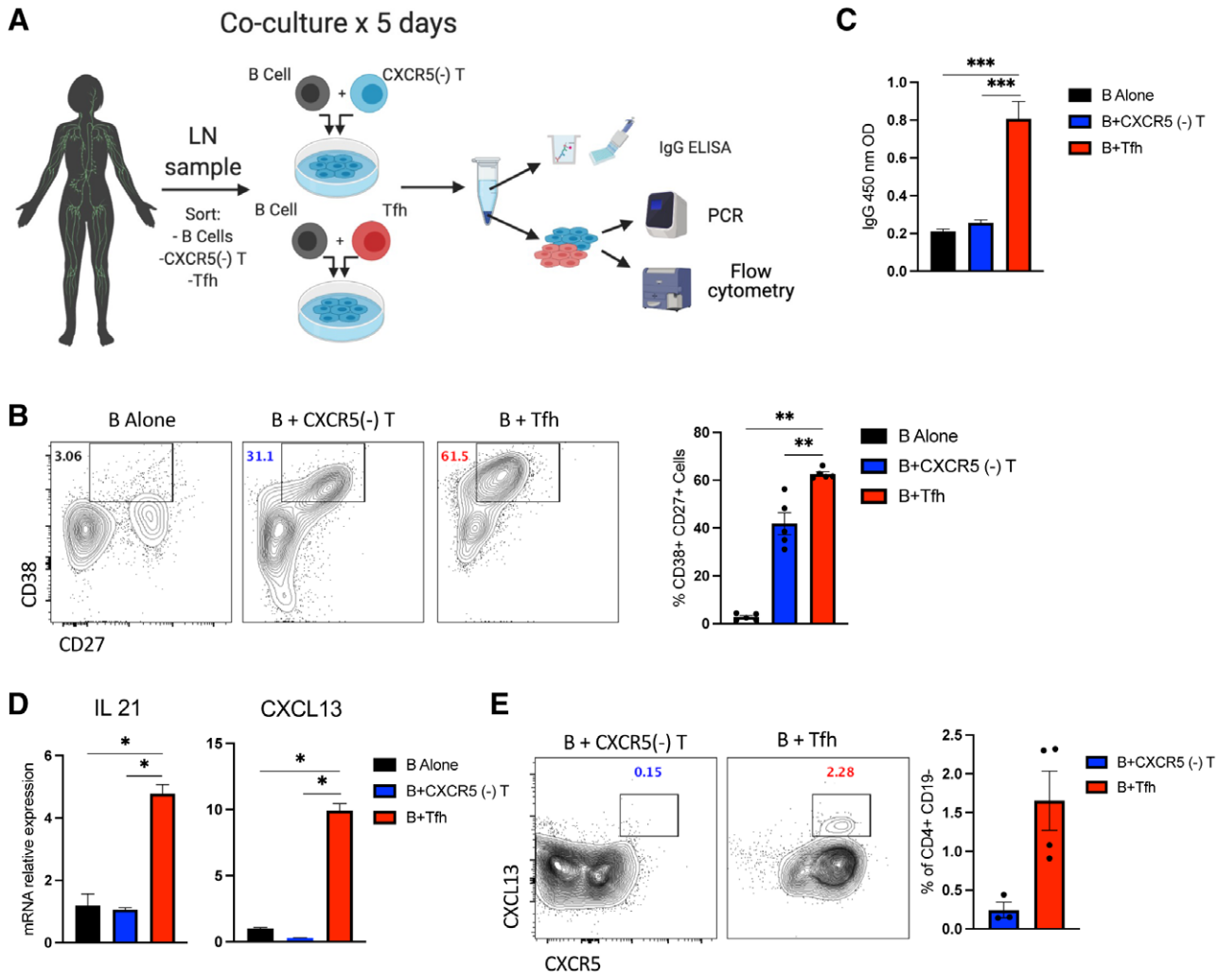

FIGURE 2. Human CXCL13 correlates with GC-like Tfh:B-cell reactivity in vitro. (A) Lymph node–derived human lymphocytes were sorted into CXCR5⁻ T and CXCR5⁺ Tfh cells and cultured in vitro with enriched B cells for 5 d. Cocultured cells were collected for flow cytometric and PCR analysis, and culture supernatants were collected for antibody assessment. (B) Representative flow plots and summary data of plasmablast ($MHCII^+CD27^{hi}CD38^+$ B cells) formation. (C) Summary data of IgG antibody production as measured by ELISA (450 nm OD). (D) Summary data of coculture IL-21 and CXCL13 mRNA expression. (E) Representative flow plots and summary data of CXCL13 production by $CD4^+$ T cells. Summary data represent mean (SE) and are representative of 2 independent experiments. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. CXCL13, chemokine (C-X-C motif) ligand 1; Tfh, T follicular helper.

Thus, CXCL13 has the potential to function as a biomarker for DSA formation in the posttransplant setting and facilitate the prevention of antibody-related allograft dysfunction.

Because of its unique role in organizing GCs necessary for antibody formation, circulating CXCL13 has been previously identified as an indicator of GC reactivity and autoimmune disease.[20,21] Although the relationship between CXCL13 and the development of DSA in transplantation has not been previously reported, some preliminary studies have described an association between CXCL13 and posttransplant status in heart recipients, as well as chronic allograft dysfunction in renal transplant patients.[22,31] More relevant to humoral alloresponses, elevated tissue and urinary CXCL13 levels have been observed to correlate with B-cell infiltrates and renal dysfunction in cases of T-cell and antibody-mediated rejection, respectively.[23,24] In humans, CXCL13 has also been associated with active chronic graft-versus-host disease,[32] a disease process that has been demonstrated to depend on GC formation and alloantibodies.[33] Together, these studies corroborate our results linking CXCL13 to alloantibody production and support the potential diagnostic value of serum CXCL13 concentrations as a biomarker for DSA.

The experimental murine data presented here demonstrate that CXCL13 indicates GC alloreactivity and precedes DSA formation in comparison with naïve and syngeneic skin-grafted controls and correlates with active alloantibody formation in human transplant recipients; however, although CXCL13 is seemingly process-specific,[18,19] it is not alloantigen- or disease-specific. Therefore, concomitant viral illnesses or acute cellular rejection, autoimmune disease activity, or other inflammatory disorders that commonly afflict transplant recipients may also result in elevated CXCL13 levels and confound its interpretation in the clinical setting. Preliminary data on CXCL13 postkidney transplant have linked it with the occurrence of infections in 1 study, and increased levels have also been associated with ischemia reperfusion injury and autoimmune diseases like rheumatoid arthritis and systemic lupus erythematosus.[20,31,34] Although random banked serum samples from stable recipients and those with de novo DSA were used for the cross-sectional analysis of CXCL13 between

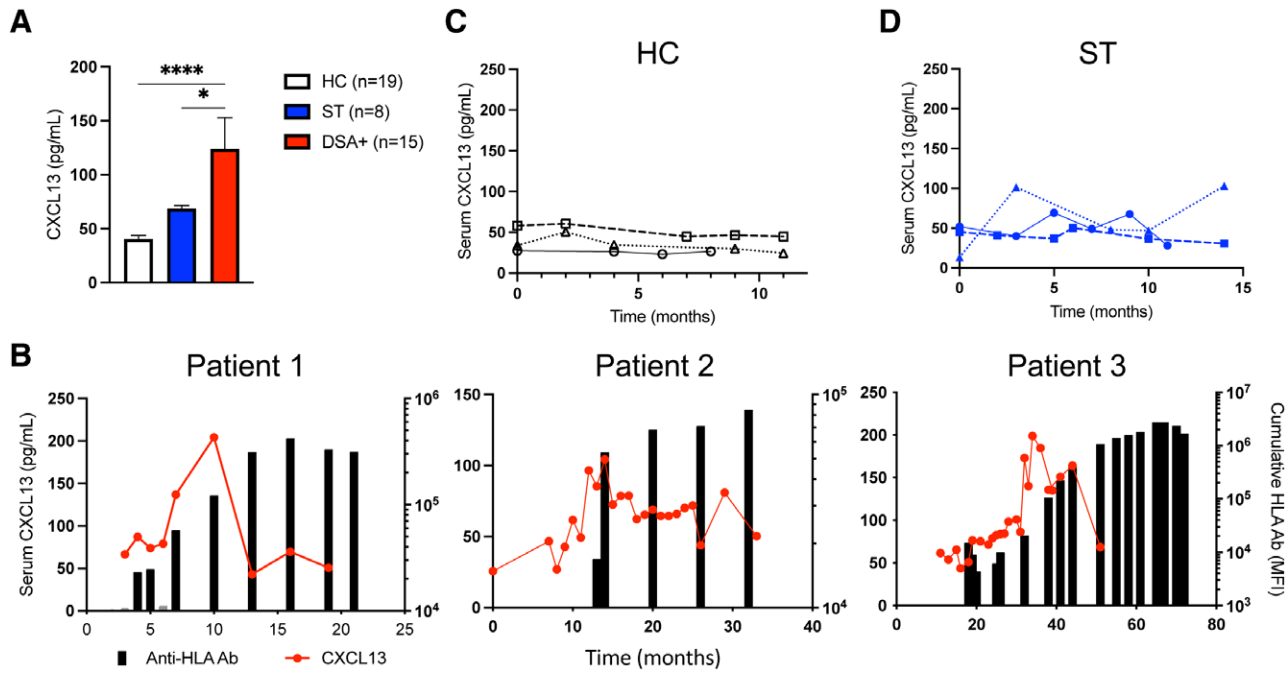

FIGURE 3. CXCL13 levels correlate with the development of alloantibodies in kidney transplant recipients. (A) Serum CXCL13 levels in HC (n = 19) and kidney transplant recipients with (DSA+, n = 15) and without (ST, n=8) de novo DSA. DSA+ samples were collected from patients within 2 wk of developing de novo DSA. (B) Serum CXCL13 levels (red line) and cumulative HLA antibodies (black bars) over time in 3 kidney transplant recipients with de novo DSA formation. Serum CXCL13 levels in 3 separate healthy controls (C) and 3 separate ST recipients without DSA (D) over time. Summary data represent mean (SE). *$P<0.05$, ***$P<0.001$. CXCL13, chemokine (C-X-C motif) ligand 1; DSA, donor-specific antibody; HC, healthy controls; ST, stable transplant.

the groups (Figure 3A), prospective validation studies will be needed to build on these preliminary data and better evaluate the utility and specificity of CXCL13 as a potential biomarker of DSA in larger, more heterogenous transplant populations.

Alternative biomarkers for DSA like cTfh cells have been proposed as indicators of GC reactivity and humoral sensitization,[12,13] but like other cellular biomarkers, the vast phenotypic heterogeneity and the inability to identify allo-specific cells limit their translatability to the clinic.[16] Although cTfh cells may signal humoral alloreactivity and possibly predict DSA formation,[14] in hematopoietic stem cell transplantation patients with active chronic graft-versus-host disease, Forcade et al observed high plasma CXCL13 levels but a lower frequency of cTfh cells in the circulation as they presumably migrate to secondary lymphoid organs to mediate their effector functions.[32] Thus, during active or prolonged periods of GC reactivity, CXCL13 and not-cellular surrogates like cTfh cells may be more clinically reliable indicators of ongoing alloantibody responses.

Similar to the murine transplant model, longitudinal analysis of kidney recipients with de novo DSA formation demonstrated an increase in CXCL13 at the same time as DSA and third-party HLA antibody development with a return to baseline as alloantibody levels plateaued (Figure 3B). Unlike HLA antibodies or cTfh cell memory that are likely to persist after a primary humoral alloresponse, CXCL13 may better indicate intermittent periods of humoral alloreactivity, late antibody-mediated rejection, or response to therapy over the life of a transplanted organ as it mirrors GC activity, and it was even more distinguishable from background reactivity upon the secondary challenge in our experimental mouse model (Figure 1C). In clinical practice, if cross-sectional or 1-time CXCL13 values are inconclusive, the observed CXCL13 kinetics and stability in clinically stable controls suggest that perturbations in serially collected values over time reflecting changes from the baseline may be of greater potential diagnostic value. Such an approach may be particularly beneficial early following transplant as DSA developed within 1 y post-transplant has been associated with lower graft survival when compared with later development.[35]

Our findings demonstrate that CXCL13 is generated by Tfh cells in secondary lymphoid organs in response to alloantigen and correlates with the development of DSA in kidney transplant recipients. These data support the continued investigation of CXCL13 as a potential biomarker for alloantibodies to either aid in the early diagnosis of de novo humoral alloreactivity or guide clinical management once antibodies have formed. Larger, well-controlled prospective studies will provide additional insights into the viability of this chemokine as a clinical indicator of humoral alloreactivity that promises to expand our ability to diagnose and treat deleterious alloantibodies in organ transplantation.

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