CXCL13 is a plasma biomarker of germinal center activity
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Significantly higher levels of plasma CXCL13 [chemokine (C-X-C motif) ligand 13] were associated with the generation of broadly neutralizing antibodies (bnAbs) against HIV in a large longitudinal cohort of HIV-infected individuals. Germinal centers (GCs) perform the remarkable task of optimizing B-cell Ab responses. GCs are required for almost all B-cell receptor affinity maturation and will be a critical parameter to monitor if HIV bnAbs are to be induced by vaccination. However, lymphoid tissue is rarely available from immunized humans, making the monitoring of GC activity by direct assessment of GC B cells and germinal center CD4+ T follicular helper (GC Tfh) cells problematic. The CXCL13–CXCR5 (chemokine (C-X-C motif) receptor 5) chemokine axis plays a central role in organizing both B-cell follicles and GCs. Because GC Tfh cells can produce CXCL13, we explored the potential use of CXCL13 as a biomarker to indicate GC activity. In a series of studies, we found that plasma CXCL13 levels correlated with GC activity in draining lymph nodes of immunized mice, immunized macaques, and HIV-infected humans. Furthermore, plasma CXCL13 levels in immunized humans correlated with the magnitude of Ab responses and the frequency of ICOS+ (inducible T-cell costimulator) Tfh-like cells in blood. Together, these findings support the potential use of CXCL13 as a plasma biomarker of GC activity in human vaccine trials and other clinical settings.

Significance

A major challenge for vaccine science is that there is no way to measure germinal center activity in humans. This challenge is particularly acute for human clinical trials ofcandidate vaccines (and most nonhuman primate studies of candidate vaccines), because germinal centers are the engines of Ab affinity maturation, and generation of highly affinity-matured Ab responses is the goal of all Ab-elicting vaccines. Here, we report that we have identified the chemokine CXCL13 [chemokine (C-X-C motif) ligand 13] as a biomarker of germinal center activity. We show explicit relationships between plasma CXCL13 concentrations and germinal center frequencies in lymph node in a series of different conditions, including licensed and experimental vaccines, and in humans, nonhuman primates, and mice.


The authors declare no conflict of interest.

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1 A complete list of the International AIDS Vaccine Initiative (IAVI) Protocol C Principal Investigators is in SI Text.

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for fear of removing the primary site of the ongoing immune response. Therefore, identification of a plasma biomarker for GC activity would be of great value in immunization studies as well as have utility in a number of other biomedically relevant contexts.

The CXCL13 [chemokine (C-X-C motif) ligand 13]–CXCR5 [chemokine (C-X-C motif) receptor 5] chemokine axis plays a major role in organizing both B-cell follicles and GCs (12, 13). CXCL13 is expressed by both follicular dendritic cells (14) and GC Tfh cells (15, 16) in the B-cell follicles. B- and T-cell expressions of CXCR5, the receptor for CXCL13, are necessary for migration to the B-cell follicle. Although CXCL13 acts locally, it can also be detected in human plasma in the steady state, and perturbations in plasma CXCL13 have been associated with immune activity (17–21). Because GC Tfh cells regulate the size of the GC response and can be major producers of CXCL13, we explored whether plasma CXCL13 changes may reflect lymphoid tissue GC activity.

Results

Plasma CXCL13 Is Elevated in bnAb+ HIV-Infected Individuals. The high levels of SHM seen in bnAbs generated against HIV and the association of circulating memory Tfh cells with the generation of bnAbs (22) suggest that individuals able to generate HIV bnAbs may have superior GC responses (6). Because directly measuring these tissue-resident GC responses in humans is not generally feasible, we asked whether CXCL13 was higher in the plasma of individuals able to generate HIV bnAbs than in the plasma of individuals who were not. We tested plasma samples from ART (antiretroviral therapy)-naive HIV+ individuals enrolled in International AIDS Vaccine Initiative (IAVI) Protocol C, a large longitudinal cohort of HIV+ individuals monitored early after infection and followed every 3–6 mo. This cohort has been extensively characterized for the ability of each individual to produce neutralizing Abs against HIV (22, 23). A neutralization score was calculated based on both the breadth and the potency of the neutralizing Ab present in individual samples for each time point after infection; 15% of 228 individuals followed beyond 4 y postinfection were found to have a neutralization score of greater than one and were termed top neutralizers (Fig. 1A). Top neutralizers had Ab responses capable of neutralizing an average of 73% (27 of 37) of HIV pseudoviruses using a principally tier 2 virus panel. Most HIV-infected individuals had Ab responses that scored less than 0.5, neutralizing an average of 27% (10 of 37) of HIV strains; these individuals were termed low neutralizers. Plasma from each individual was tested for neutralizing Abs and CXCL13 concentration at both the earliest time point available after HIV infection (~4 mo postinfection) and the time of bnAb development (~40 mo postinfection). Broad neutralization at the later time point was associated with elevated concentrations of plasma CXCL13 at both the ~4-mo (top neutralizers median: 92.7 pg/mL vs. low neutralizers median: 31.3 pg/mL; P = 0.012) and the ~40-mo (top neutralizers median: 78.9 pg/mL vs. low neutralizers median: 32.2 pg/mL; P = 0.008) time points postinfection (Fig. 1B and C). A positive correlation between Ab breadth and CXCL13 was also observed in an independent cohort containing a small number (five) of individuals with neutralizing breadth (24). The generation of HIV neutralizing Abs is also positively correlated with HIV viral load (25, 25), and viral load could affect plasma CXCL13 levels (24, 26). We, therefore, asked whether viral load and CXCL13 were independent variables. The difference in plasma CXCL13 between top and low neutralizers remained significant at the ~40-mo time point and continued to show a strong trend at the ~4-mo time point. Multivariate analysis determined that plasma CXCL13 and viral load are largely independent factors [early time point ANCOVA (analysis of covariance), P = 0.021; bnAb development time point ANCOVA, P = 0.066]. Therefore, elevated plasma CXCL13 in top HIV neutralizers suggested that these individuals may have stronger GC responses.

Plasma CXCL13 Is Correlated with Lymphoid Tissue GCs in Humans. GC Tfh cells are a producer of CXCL13 in secondary lymphoid tissue, such as tonsil (15, 16, 27). We found that GC Tfh cells were uniquely able to produce CXCL13 when analyzed by intracellular FACS analysis (Fig. 2A). Other cell types have been reported to produce CXCL13 under various immunological conditions (26, 28). On examination, CXCL13 protein was not produced in the other cell types present in tonsil cell preparations (Fig. 2B) or monocytes in peripheral blood mononuclear cells (PBMCs) (Fig. S1 A and B). Similar results were obtained from human spleen and lymph node tissues, showing CXCL13 expression to be restricted to GC Tfh cells (Fig. 2C).

In an additional cohort of HIV+ and HIV− individuals at Massachusetts General Hospital, we obtained lymph node biopsy tissues, allowing us to directly compare plasma CXCL13 to GC activity in human lymphoid tissue. GC Tfh cells (CXCR5+ PD-1+) were identified (Fig. 3D) and quantified. In 14 matched plasma and lymph node samples, plasma CXCL13 concentration positively correlated with GC Tfh cells in the lymph node (r = 0.75; P = 0.003) (Fig. 2E). Furthermore, plasma CXCL13 also correlated with GC B cells (r = 0.62; P = 0.02) (Fig. S2A). The strong correlation observed between plasma CXCL13 and lymph node GC Tfh cells within a relatively small human donor set together with the ability of GC Tfh cells to produce CXCL13 suggest that CXCL13 can be a biomarker of GC activity.

Plasma CXCL13 Is Correlated with Lymph Node GCs in Mice After Immunization. We next examined the relationship between plasma CXCL13 and GC activity after protein immunization in animal vaccination models (6, 29–31). We first immunized mice with 4-hydroxy-3-nitrophenyl acetyl haptenated ovalbumin in aluminum hydroxide adjuvant (alum + NP-OVA). One-half of the immunized mice received OT-II TCR (T-cell receptor) transgenic CD4 T cells to enhance the antigen-specific and GC Tfh CD4 T-cell response. Seven days after immunization, plasma CXCL13 was increased compared with that in unimmunized mice (Fig. 3A). Plasma CXCL13 was also increased 7 d after acute infection with LCMV (lymphocytic choriomeningitis virus) Armstrong or vaccinia virus (Fig. 3A). In alum + NP-OVA immunized mice, GC Tfh cells were quantified in the draining lymph node (CXCR5+PD-1+) (Fig. 3B), and plasma CXCL13 correlated with the frequency of vaccine-induced GC Tfh cells (Fig. 3C) (r = 0.82; P = 0.002) and GC B cells (r = 0.74; P = 0.008) (Fig. S2B).

In a larger study of immunized mice that did not receive transgenic CD4 T cells, keyhole limpet hemocyanin plus aluminum hydroxide (KLI1 + alum) immunized mice had higher plasma concentrations of CXCL13 after immunization compared with those at the preimmunization time point in the same mice, peaking at 2 wk postimmunization (Fig. 3D). The mice were given a booster immunization with KLI1 + alum 50 d after the primary immunization. Plasma CXCL13 concentrations were increased at both 10 and 18 d postboost (Fig. 3E) and again, correlated with the frequency of GC Tfh cells in the draining lymph node (r = 0.69; P = 0.023) (Fig. 3F). Thus, in small animal immunization models, plasma CXCL13 concentrations were a positive biomarker of GC activity and GC Tfh cells.
Plasma CXCL13 Is Correlated with GC Activity in Macaques After Immunization. NHPs are considered the best animal model for many preclinical vaccine studies. We considered that CXCL13 expression by GC Tfh cells in NHPs might be more similar to that of humans. We, therefore, examined the relationship between plasma CXCL13 and GC activity in rhesus macaques after protein immunization. Our previous identification of an anti-human CXCR5 Ab (clone MU5UBEE) reactive to rhesus macaque CXCR5 (used in the works of refs. 32-35) allowed detection of CXCR5hiPD1hi (programmed cell death 1) GC Tfh cells in macaque lymphoid tissue (Fig. 4A). In pilot experiments, we determined that GC Tfh cells in macaques express high levels of CXCR5, ICOS (inducible T-cell costimulator), and CD200 (Fig. 4C), directly analogous to human GC Tfh cells (8). Seven days after a protein and adjuvant immunization, GC Tfh- and GC B-cell responses were found in the draining lymph node but not in a nondondraining lymph node (Fig. 4B). We examined plasma CXCL13 in these animals at the same day 7 postimmunization time point. We observed a strong positive correlation between plasma CXCL13 concentration and GC Tfh-cell abundance in the draining lymph node \( (r = 0.71; P = 0.013) \) (Fig. 4C). A nonsignificant positive trend was observed with GC B-cell frequency (Fig. S2C). The correlation between plasma CXCL13 and GC Tfh cells in immunized rhesus macaques was confirmed in an additional study of 12 animals. In summary, plasma CXCL13 responses in both mice and rhesus macaques strongly correlated with GC Tfh-cell frequencies in draining lymph node after immunization.

Plasma CXCL13 Is Increased in Humans After Immunization. Because plasma CXCL13 was elevated after immunization in animal models, correlated with GC activity, and correlated with bnAb development in HIV+ individuals, we investigated plasma CXCL13 responses after vaccination in humans. We initially examined plasma CXCL13 in a small cohort of influenza vaccine recipients. We found mixed plasma CXCL13 responses after influenza immunization that did not exhibit a statistically significance change (Fig. S3). The lack of a clear increase in plasma CXCL13 could be because of the fact that there was low overall antinfluenza Ab response generated to the immunization caused by preexisting Ab titers (36). We, therefore, moved to study immunizations that generated more robust immune responses. Two separate cohorts were studied. The first cohort was a vaccine cohort immunized with the Food and Drug Administration-approved yellow fever virus vaccine (37). The second group comprised study participants in an HIV Vaccine Trials Network (HVTN) protocol testing a candidate HIV vaccine regimen (HVTN068) (38).

We tested pre- and postvaccination plasma samples obtained from 17 yellow fever vaccine recipients. Seven days after immunization, statistically significant increases in plasma CXCL13 were observed \( (P = 0.04) \) (Fig. 5A). Plasma CXCL13 was 30% higher than prevaccinations levels in 9 of 17 individuals. We next assessed the kinetics of plasma CXCL13 in 11 vaccinated individuals in the HVTN068. After adenovirus-5 vector with HIV-1 gene inserts (Ad5/HIV) immunization, plasma CXCL13 concentrations were significantly increased over preboost levels at both days 7 \( (P = 0.001) \) and 14 \( (P = 0.014) \) (Fig. 5B). For each individual donor, peak plasma CXCL13 was detected at either the 7- or 14-d time point. Seven days after immunization, plasma CXCL13 was 50% higher than at the prevaccination time point in 9 of 11 individuals. In a larger set of samples available at only the 7-d time point, plasma CXCL13 positively correlated with the vaccine-specific gp140 Env IgG Ab response \( (r = 0.44; P = 0.037) \) (Fig. 5C) and showed a strong positive trend with the gp140 Env IgG Ab response \( (r = 0.54) \) measured 28 d after immunization. In a small number of individuals for whom cryopreserved PBMCs were available, we evaluated the frequency of activated ICOS+PD1hiCXCR5+ CD4+ T cells after vaccination. ICOS+PD1hiCXCR5+ CD4+ T cells are found in the blood of individuals with ongoing immune responses and may be a subpopulation of Tfh cells trafficking through the blood before becoming GC Tfh cells (8, 22, 39, 40). Even with only six individuals available for analysis, plasma CXCL13 concentration responses correlated with
the increase in activated ICOS⁺PD1⁺⁺CXCR5⁺ CD4 T cells found in the blood 7 d after immunization (r = 0.85; P = 0.03) (Fig. 5D). Thus, plasma CXCL13 correlates with GC-associated Ab and Tfh-cell responses in immunized humans.

**Discussion**

The GC response is a critical immune mechanism by which Ab affinity occurs, memory B cells develop, and long-lived plasma cells are produced. Here, we show a means to monitor GC activity in lymphoid tissues using a plasma biomarker. Plasma CXCL13 positively correlates with the lymph node GC response in mice, macaques, and humans. Increases in plasma CXCL13 were found in a number of different immune-activating conditions: aluminum hydroxide or TLR (Toll-like receptor) ligand adjuvants plus recombinant protein immunizations, acute viral infections, an adenovirus vector candidate HIV vaccine, the licensed yellow fever vaccine, and HIV infection. Based on the strong correlation of GC Tfh cells and plasma CXCL13 and the significant measurable change in plasma CXCL13 in two human vaccine cohorts, monitoring plasma CXCL13 could be useful in human and NHP vaccine trials, where direct analysis of lymphoid tissue is either

![Fig. 3. Plasma CXCL13 correlates with GC Tfh cells in mice after immunization.](image)

![Fig. 4. Plasma CXCL13 correlates with GC Tfh cells in rhesus macaques after immunization.](image)
not possible or undesirable for fear of disturbing the ongoing immune response.

If bnAbs against HIV are to be generated by vaccination, the GC response will play a central role. Measuring CXCL13 in vaccine studies can provide data on post-vaccination GC activity, a major driver of Ab quality by SHM. Furthermore, in some cases, antigen-specific Ab results are not measured until after a final boost 6 mo after the primary immunization. CXCL13 can be measured after each immunization, providing much earlier data on the progress of the immune response to the immunization scheme, which could be important for in-trial decision-making. Our studies detecting increases in plasma CXCL13 in the majority but not all of the immunized individuals suggest that GCs were not generated in certain individuals, a potentially critical observation. We do not suggest that CXCL13 analysis should replace antigen-specific Ab titer data, but rather that CXCL13 monitoring be added as a valuable parameter to gain an understanding of the magnitude of the GC activity that is necessary for the development of improved Ab quality. Given that GC B cells do not exist in peripheral blood, CXCL13 may be the best available proxy for those inaccessible cells.

Plasma CXCL13 has been proposed to serve as a biomarker of autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, Sjogren’s syndrome, and Myasthenia Gravis (41). Elevated plasma CXCL13 was detected in patients with systemic lupus erythematosus and further increased in individuals with severe disease presenting with nephritis or anti-DNA Ab responses (19). In rheumatoid arthritis, CXCL13 was not only followed as a plasma biomarker of disease, but also, CXCL13 blockade has been proposed as a treatment (42). It is important to note that analysis of plasma CXCL13 is not an antigen- or disease-specific readout. Plasma CXCL13 reports total GC activity, and the basal levels detected in unimmunized humans, macaques, and mice likely reflect ongoing GC activity in tonsillar and gut-associated lymphoid tissue. For these reasons, we consider a multiparameter approach to be the best approach. Analysis of plasma CXCL13 together with analysis of other potential biomarkers specific to the immunological and pathological setting under study are advisable.

We have shown a strong correlation between CXCL13 and lymphoid tissue resident GC Th cells. With the additional observation that GC Th cells are robust producers of CXCL13, it is suggestive of a direct relationship between GC Th cells and plasma CXCL13. We did not identify other cell types in lymphoid tissue, monocytes in PBMCs, or CXCR5+ Th cells in PBMCs as producers of CXCL13 by intracellular staining. Although follicular dendritic cells (FDC) and some dendritic cell subsets are likely lost during tonsil tissue processing, a histological study suggests that much of the CXCL13 observed in the tonsil GC coexists with PD-1, and PD-1 is an excellent marker of GC Th cells (27). In the same study, CXCL13 was only weakly associated with CD21+ FDC. Monocytes (26) and dendritic cells (28) have been reported to express CXCL13 and could contribute to plasma CXCL13 concentrations in different inflammatory settings. The observation that monocytes can produce CXCL13 in response to IFN-γ may be relevant in HIV infection and immunizations with viral vectors or adjuvants containing TLR ligands. However, we did not identify CXCL13-producing monocytes in PBMCs from HIV+ individuals ex vivo or after a short in vitro stimulation, and IFN-γ responses are normally short in duration, peaking at 24–48 h after immunization (43, 44). Therefore, monocyte-generated CXCL13 may not significantly impact plasma CXCL13 measured in our immunization studies. These later time points examined are contemporaneous with GC activity.

Here, we show that CXCL13 acts as a plasma biomarker for GC activity in generally inaccessible lymphoid tissue. Plasma CXCL13 correlates with GC activity after immunization in animal models and in HIV+ humans. Furthermore, plasma CXCL13 is associated with generation of HIV bnAbs, and CXCL13 was elevated in humans after immunization. Together, these findings support the use of CXCL13 as a plasma biomarker of GC activity useful for studying differences in humoral immunity among patients and vaccinees.

Materials and Methods

IAVI Protocol C and Human Vaccine Cohorts. The IAVI Protocol C cohort has been described elsewhere (22, 23). An HIV neutralization score for each plasma sample was determined as in the work by Simek et al. (45) to account for both breadth and potency. All donors were ART-free and had CD4 counts >200 cells per 1 mL at each time point tested.

CXCL13 was analyzed in the serum before and 1 wk after a single immunization of TIV Fluzone (Sanofi Pasteur) in vaccine recipients from the Stanford-Lucile Packard Children’s Hospital Vaccine Program (36). Yellow fever vaccine recipients received the Food and Drug Administration-approved 17D yellow fever vaccine (37). Plasma samples from HIV-uninfected candidate vaccine recipients from the HVTN068 trial (38) were analyzed for CXCL13 in plasma after a booster immunization (Ad5HIV) given at the 6-mo time point. Primary immunizations were either Ad5HIV at month 0 or DNAHIV at months 0 and 1. Some participants received placebo.

Human Lymph Node, Spleen, and Tonsil. Inguinal lymph nodes from HIV-infected persons and HIV+ healthy volunteers were obtained by excisional surgical biopsy under local anesthesia at Massachusetts General Hospital and processed as in the work by Lindqvist et al. (46) Additional nonidentifiable, discarded, excess spleen, lymph node, and tonsil tissues were obtained at Massachusetts General Hospital or Rady Children’s Hospital-San Diego. Spleen and lymph node tissues (46) and tonsils (15) were processed as previously described.

Macaque and Mouse Immunization. Macaques were immunized s.c. with gp140 (SIVmac239) Env and p55 Gag protein mixed with either aluminum hydroxide (Alhydrogel 2% adjuvant; Invogen) or MPL (monophosphoryl lipid A) + R848-encapsulated PLGA [poly(lactic-co-glycolic acid)] nanoparticles (47). A full description of the vaccine trial methods and results will be published elsewhere.

C57BL/6 (B6) mice (Jackson Laboratory) were immunized with alum + NP-OVA (Sigma-Aldrich); 10 μg NP-OVA was mixed 1:1 with 10% (wt/vol) aluminum hydroxide (aluminum potassium sulfate dodecylate; Sigma-Aldrich) in PBS. In some mice, 2 × 10⁹ OVA-specific OT-II TCR transgenic CD4 T cells were transferred.
5.3 before immunization. For KLH + alum immunized mice, 10 µg KLH was mixed 1:1 with 10% aluminum hydroxide in PBS. For LCMV immunization experiments, 2 x 10^5 PFU CBA/7 C57BL/6 transgenic cells (GC) were transfected before immunization with 2 x 10^6 pfu LCMV Armstrong by i.p. injection. For vaccinia virus immunization experiments, B6 mice were i.p. injected with 6 x 10^6 pfu.

**CXCL13 ELISA and Flow Cytometry.** The Human CXCL13 Quantikine ELISA Kit (R&D Systems) was used for both human and macaque (49) plasma samples as described in the instructions. The mouse CXCL13 DuoSet (R&D Systems) was used for standardization of the ELISA in mouse serum. All samples were tested in duplicate. GC Tfh cells were characterized by flow cytometry as previously described in mouse lymph node (50), human tonsil (15, 22), or human lymph node (46). Previously cryopreserved human PBMCs from HVTN068 subjects were stained. Intracellular CXCL13 was detected by intracellular cytokine staining as described (15). Cells were acquired on a BD Fortessa Analyzer.

**Statistics.** The Mann-Whitney test was used for evaluating differences among groups. The Wilcoxon test was used to evaluate differences between time points for the same individuals. The Spearman correlation test was used for all correlative analysis. Covariance of plasma CXCL13 and viral load was evaluated with the ANCOVA multivariate statistical test (VassarStats). Prism 5.0 (GraphPad) was used for all other statistical analyses.

**Study Approvals.** Informed, written consent was obtained from all human study participants before enrollment in the human studies listed above and approved by the local institutional review boards (La Jolla Institute Internal Review Board, The Scripps Research Institute Internal Review Board, Massachusetts General Hospital Partners Human Research Committee, the Institutional Review Board of the Research Compliance Office at Stanford University, Institutional Review Boards at Emory University, Centers for Disease Control, and the Institutional Review Board at the HVTN). Rhesus macaque study procedures were performed in accordance with USDA guidelines of Medicine Institutional Animal Care and Use Committee-approved protocols. Mouse study procedures were performed in accordance with approved animal protocols at La Jolla Institute for Allergy and Immunology.

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