Correction

MEDICAL SCIENCES

The authors note, “We inadvertently omitted a statement about institutional review board (IRB) approval of our study. The study was approved by Institutional Review Board of the National Cancer Institute (NCI). All patients signed a consent form approved by the IRB.”

www.pnas.org/cgi/doi/10.1073/pnas.1510832112
Humoral response to a viral glycan correlates with survival on PROSTVAC-VF

Christopher T. Campbell, James L. Gully, Oyindasola Oyelaran, James W. Hodge, Jeffrey Schlom, and Jeffrey C. Gildersleeve

*Chemical Biology Laboratory, National Cancer Institute, National Institutes of Health, Frederick, MD 21702; and Laboratory of Tumor Immunology and Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Edited by Chi-Huey Wong, Academia Sinica, Taipei, Taiwan, and approved March 27, 2014 (received for review August 5, 2013)

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Antiglycan immune responses to glycans also could provide new insights for immune responses. Although immune responses to carbohydrates could contribute to clinical efficacy in several ways (inducing antibody-dependent cell-mediated cytotoxicity, blocking metastasis, or directly killing tumors), technical challenges have limited studies into this class of responses. It is particularly difficult to predict relevant responses, and traditional methods for profiling antiglycan responses lack the necessary throughput for comprehensive evaluations. Although relatively few studies of humoral responses to glycans have been published, several associations between clinical outcome and antiglycan antibody responses have been reported (17–19). These studies analyzed only a few glycans in a small number of patients; more in-depth studies could uncover other important antiglycan responses. Studies on immune responses to glycans also could provide new insights for improving vaccine design.

Glycan microarrays (20, 21), which contain hundreds of carbohydrates immobilized on microscope slides, provide a multiplex assay for analyzing serum antiglycan antibodies that uses only minute amounts of valuable glycans and clinical samples (e.g., only 2–4 μL of serum). This approach, referred to as “glycomimmunomics,” generates a comprehensive profile of antiglycan immune responses. In this study, we applied glycan microarray technology to PROSTVAC-VF, a prostate cancer vaccine currently in a phase III clinical trial (22). PROSTVAC-VF induces immunity for prostate-specific antigen (PSA) using genetically modified vaccinia and fowlpox encoding PSA and three co-stimulatory molecules (LFA-3, B7.1, and ICAM-1, designated “TRICOM”) coadministered with GM-CSF. In two phase II clinical trials (23, 24), PROSTVAC-VF lengthened median survival by 8–9 mo, surpassing the survival benefit of the only FDA-approved cancer vaccine, PROVENGE (4.1 mo) (1). Although PROSTVAC-VF was not designed to simulate immunity to glycans, cancer vaccines can induce responses beyond the target antigen(s) through antigen spreading (25). This fact, coupled with our previous finding that preexisting IgM to blood group A correlates with survival (26), prompted us to evaluate whether PROSTVAC-VF induces immune responses to glycans. Here we demonstrate that this vaccine does induce antibody responses to glycans and that responses to the Forssman glycan correlate positively with survival. In addition to providing a potential biomarker which may be useful for evaluating the efficacy of PROSTVAC-VF, these studies highlight the importance of antiglycan immune responses for cancer vaccines, including vaccines that are not specifically designed to induce responses to glycans.

Results

Antibody Responses Are Induced by PROSTVAC-VF. PROSTVAC-VF is designed to stimulate T-cell responses to PSA, but several features of the vaccine could result in antiglycan responses. First, glycans associated with the vaccine could induce immune responses. In particular, PSA is a glycoprotein containing an N-linked glycan at Asn45 that accounts for ~8% of the protein’s weight, and PROSTVAC-VF’s enveloped viral vectors are glycosylated. Second, prostate tumors abnormally express tumor-associated carbohydrate antigens, and immune responses to these glycans could occur through antigen spreading (25). Because many glycans could trigger responses, and nothing was known about anticalbohydrate responses induced by PROSTVAC-VF, a high-throughput approach was needed to study antiglycan antibody responses.

For our initial assessment, we profiled free antiglycan antibody levels in sera of 28 patients collected before and after priming and at least one booster from a phase II study of PROSTVAC-VF (23). We used glycan microarrays containing 171 structurally distinct glycans and glycopeptides to compare serum antiglycan IgG, IgM, and total Ig profiles in patients before and 2–4 mo after initiating treatment with PROSTVAC-VF. All clinical and survival data were blinded during the profiling of serum samples during this initial discovery phase. In a previous study of healthy

![Fig. 1.](https://www.pnas.org/ cgi/doi/10.1073/pnas.1314722111) Postvaccination changes in levels of serum antiglycan antibodies. Heat maps show postvaccination fold changes in antiglycan antibody levels (IgM + IgG + IgA) for (A) discovery and (B) validation sets measured at a serum dilution of 1:200. Columns correspond to 204 array components (ordered alphabetically within glycan families), and rows are individual patients (arranged by expected survival calculated using the Halabi nomogram). Increases are colored red, and decreases are green. Black indicates no significant change (increase <160% or decrease <61%).
humans (27), spontaneous changes of ≥2.6-fold occurred only rarely over a 3-mo period; therefore, this threshold was applied to this study. Interestingly, numerous antibody responses to glycans and glycopeptides were observed 2–4 mo after the initial vaccination (Fig. 1A). Increases significantly outnumbered decreases by a ratio of 4:1, supporting a response to vaccination rather than normal physiological variation. The magnitude of the responses ranged from a 52-fold increase in antibodies to the terminal disaccharide of the Forssman antigen (Fsαd) in one patient to an 8.3-fold decrease in antibody levels for Galβ1–3GalNAcβ (Galα1) in another patient. Although many responses were observed in the group, each patient had responses to only a small percentage of glycans, so that their overall antiglycan antibody profiles were largely unchanged after receiving PROSTVAC-VF. These results are consistent with our previous studies showing that serum antiglycan antibody profiles were stable over weeks to months in normal controls (27). Neither immunomodulation with low-dose GM-CSF given at the vaccine site nor disease progression systemically altered antibody levels.

Responses to a wide variety of glycans were observed, but there was considerable variability from one patient to another. In most cases, responses to individual glycans occurred in only one or in a small subset of patients. For example, antibody responses to tumor-associated carbohydrate antigens occurred only infrequently. For three glycans, however, responses were observed in many patients. The most common IgM, IgG, and total Ig (IgG + IgM + IgA) responses were directed at the terminal disaccharide of the Forssman xenantigen (Fsαd = GalNacβ1–3GalNAcβ) (Fig. 2A), the blood group A trisaccharide [BG–Aα3 = GalNAcβ1–3(Fucβ1–2)Gal] (Fig. 2A), and a nonfucosylated substructure of blood group A (BG–Aα3 = GalNAcβ1–3Galβ) (Fig. 2A). Beyond these three glycans, we detected humoral responses in at least 25% of patients for only one other case: Eight patients showed statistically significant changes for total Ig against the glycoprotein asialo-fetuin when serum was diluted 1:50.

Over the entire array, total Ig responses to low-density Fsαd (four copies per BSA molecule) typically were the largest in magnitude and occurred in the most patients (discovery set = 64%; maximum fold change = 52×) (Fig. 2B). A number of patients also showed changes for BG–Aα3 (discovery set = 46%; maximum fold change = 33.7×) and BG–Aα4 (discovery set = 39%; maximum fold change = 9.2×). However, spontaneous changes in antibody levels to these glycans were not found in healthy subjects or nonvaccinated prostate cancer patients treated with radiation in another study (28) (Fig. 2B). Thus we concluded that these responses are not general changes that occur physiologically or during disease progression.

To evaluate the consistency of these changes, we analyzed sera from several patients at multiple time points after initiating treatment with PROSTVAC-VF. Changes at 1, 2, and 4 mo after the first injection of PROSTVAC-VF displayed similar levels of change relative to prevaccination sera (Fig. S1). Moreover, these glycan-specific antibody changes are not the result of cross-reactivity for linkers used to conjugate glycans to carrier proteins because humoral responses did not occur for other glycans containing the same linker.

**Antibody Responses to Fsαd Correlate with Survival.** Once we had determined that antiglycan immune responses were induced by PROSTVAC-VF, we next evaluated whether any of the responses correlated with survival. Only responses that occurred in at least 25% of patients were considered, because disproportionately small strata (e.g., four vs. 24 subjects) show chance survival differences more frequently than predicted by log-rank *P* values. In addition, responses that occur infrequently would be more difficult to develop as biomarkers. The only glycans satisfying this criterion were BG–Aα3, BG–Aβ3, Fsαd, and asialo-fetuin, and antibody response to only one of these correlated with survival. In particular, overall survival correlated positively with change in anti-Fsαd total Ig measured at a dilution of 1:200 (*P* = 0.0155) (Fig. 3A).

**Antibody Responses and Survival Correlation Are Consistent in an Independent Patient Set.** Next, we focused on validating our initial findings in an independent group of patients treated with PROSTVAC-VF. We performed an analysis similar to that described above on sera from a second, separate phase II study of PROSTVAC-VF that included 76 vaccinated patients and 37 controls randomly assigned to receive wild-type viral vectors (24). As before, all clinical and survival data were blinded during the profiling of serum samples in this validation phase.

As seen in the discovery phase, numerous antiglycan IgM, IgG, and total Ig responses were observed 2–4 mo after the initial vaccination (Fig. 1B). Again, responses were directed most frequently at three structurally related glycans: Fsαd, BG–Aα3, and BG–Aα4. The frequency and magnitude of responses were similar in the discovery and validation sets (Fig. 2B). Once again total Ig responses to Fsαd were the largest in magnitude and showed statistically significant changes (≥2.6-fold) in the most patients (validation set = 64%; maximum change = 219×). Fewer patients showed changes for BG–Aα3 (validation set = 55%; maximum fold change = 74×) and BG–Aα4 (validation set = 32%; maximum fold change = 14×). Changes to these glycans tended to occur in many of the same individuals (Fig. 2C). Humoral responses to all other array components occurred in fewer than 25% of patients.

Humoral responses to Fsαd were the only responses that showed a statistically significant correlation with survival in our initial study; therefore, this was the only response that was examined in the validation set for a survival correlation. Importantly, we used the initial study to specify the threshold for...
stratifying patients according to anti-Fs$_{\text{dis}}$ responses in the validation phase. This conservative step of using the same threshold in the discovery and validation phases was necessary to prevent multiple hidden comparisons. Additionally, this step avoided the need to adjust for multiple comparisons because only one hypothesis (anti-Fs$_{\text{dis}}$ humoral responses at a specified threshold) was carried over to the validation set.

Postvaccination overall survival also correlated with humoral responses to Fs$_{\text{dis}}$ in the validation sample set. At the threshold identified by the discovery set, fourfold and larger increases in total serum Ig to Fs$_{\text{dis}}$ were associated with statistically significant improvements in survival (P = 0.008; Fig. 3 B). Patients with fourfold and larger responses had a median survival that was ~9 mo longer than that in patients with little or no response (Fig. 3 A and B). The odds ratio for living at least 3 y was 3.2 (95% confidence interval, 1.2–8.8; P = 0.02). Patients with fourfold and larger responses also lived significantly longer than control patients, with an improvement in median survival of about 11 mo (29.0 vs. 17.9 mo; P = 0.004). In contrast, patients in the vaccine arm who had little or no Fs$_{\text{dis}}$ response did not have a statistically significant difference in survival relative to the control patients (19.4 vs. 17.9 mo; P = 0.84). For comparison, the median survival for the 76 PROSTVAC-VF patients in this study was 25.5 mo. (Note that we did not receive serum samples from all the patients from this clinical trial; therefore, the median survival for our subset is somewhat different from the value reported for the entire group.) Taken together, these analyses demonstrate a significant improvement in survival for patients treated with PROSTVAC-VF who hadFs$_{\text{dis}}$ responses.

In addition to overall survival, we also evaluated survival relative to predicted survival based on the Halabi model. The Halabi model uses seven independent prognostic markers to predict survival of men with metastatic castration-resistant prostate cancer (mCRPC) treated with chemotherapy (23). It is based on multivariable analysis of more than 1,100 patients and provides a powerful tool for determining balanced prognostic criteria among groups of men. For each individual, the survival relative to expectations was calculated by subtracting the Halabi predicted survival (HPS) from the actual overall survival (OS). When a threshold of fourfold for Fs$_{\text{dis}}$ responses is used, the odds ratio for living longer than expected (i.e., OS - HPS > 0) was 3.67 (95% confidence interval, 1.38–9.75; P = 0.009). For patients above the threshold, the median improvement in actual survival relative to expected survival was 8.7 mo. For patients below the threshold, there was no increase in median survival relative to expectations (median OS - HPS = -1.0 mo). These data indicate that patients with an Fs$_{\text{dis}}$ response have both longer survival and longer survival relative to expected survival.

With the larger number of samples in the validation study, we could analyze survival correlations further over a broader range of anti-Fs$_{\text{dis}}$ humoral responses to assess consistency and robustness. Odds of long-term survival increased steadily with magnitude of response to Fs$_{\text{dis}}$ (Fig. S2). For example, patients with 10-fold or higher response had a median survival >3.5 y—more than twice as long as patients with little or no response and also twice as long as control patients vaccinated with wild-type poxviruses (Fig. S3). In addition, we also used the Kaplan–Meier survival estimator to evaluate all thresholds in which both strata had at least seven patients. Of the 69 thresholds studied for patients receiving PROSTVAC-VF, 20 had a statistically significant difference in survival relative to the control patients (19.4 vs. 17.9 mo; P = 0.84). For comparison, the median survival for the 76 PROSTVAC-VF patients in this study was 25.5 mo.

Fig. 3. Kaplan–Meier survival curves for patients stratified by postvaccination response to Fs$_{\text{dis}}$. Kaplan–Meier curves were generated using actual overall survival data (A–C) or HPS data (D–F) for patients stratified according to anti-Fs$_{\text{dis}}$ responses (IgG+IgM+IgA). (A and B) In both discovery (A) and validation (B) sets, humoral responses to Fs$_{\text{dis}}$ were associated with improved overall survival. (C) Increased anti-Fs$_{\text{dis}}$ antibody levels also were seen after inoculation with wild-type viral vectors; however, humoral responses did not correlate with improved survival for control patients. (D–F) To verify that there were no systematic differences between the strata at baseline, Kaplan–Meier survival curves were generated using the HPS based on prevaccination prognostic data.
Anti-Fsα Response Is Prognostic Specifically for Vaccinated Patients.

Once a consistent survival association for anti-Fsα responses was observed in patients from the two phase II trials, we next assessed the prognostic specificity of this potential biomarker. In particular, patients who are able to mount an immune response to Fsα may have less advanced disease or higher-functioning immune systems that could lead to longer overall survival regardless of treatment. To evaluate this possibility, we used a multipronged approach. First, we examined potential relationships between immune response to Fsα and various measures of general health and disease severity. We evaluated relationships between anti-Fsα responses and HPS. Before vaccination, patients with and without responses to Fsα had similar HPS (Fig. 3 D–F), indicating equally severe disease and comparable expected outcomes. In addition, anti-Fsα responses did not correlate with age, PSA level, or Gleason score (Fig. 4), indicating that patients with robust responses to the Fsα are not simply the healthiest patients or the patients with the least aggressive disease.

Second, we examined potential relationships between immune response to Fsα and measures of overall immune function. In particular, we calculated nonparametric Kendall correlations (τ) to evaluate associations between anti-Fsα responses and other aspects of immune function (Fig. 5). An absence of statistically significant associations would indicate that anti-Fsα responses provide unique information about immune function. First, we considered the possibility that immunosuppression blunts anti-Fsα responses. However, response to Fsα did not correlate with the number of circulating T-regulatory cells (Fig. 5A), suggesting that these immunosuppressive cells are not the cause of heterogeneity in anti-Fsα responses. As another measure of immune function, we assessed associations between responses to Fsα and the vaccine’s viral vectors, which provide general indicators of immune function. There was no significant association between humoral responses to Fsα and overall antivector titers (Fig. 5B and C). Additionally, there was a weak correlation between responses to Fsα and fold increase in PSA-specific T cells (Fig. 5D), which suggests that monitoring anti-Fsα responses provides additional information about immune responses not captured by assaying T-cell function in peripheral blood. These results indicate that patients with responses to the Fsα are not simply the ones with the highest immune capacity.

Finally, we evaluated relationships between immune response to Fsα and survival in control patients, who also mounted anti-Fsα responses after inoculation with wild-type vectors lacking the key transgenes for PSA and the three costimulatory molecules. Unlike in the PROSTVAC-VF arm, however, humoral response to Fsα in control patients did not have a statistically significant correlation with improved survival (Fig. 3C), even though these patients showed IgG and IgM changes similar to those in vaccinated patients (Fig. S4). More specifically, we used the Kaplan–Meier survival estimator to evaluate the potential thresholds in the control arm in which both strata had at least seven patients. In the control arm, none of the thresholds yielded a statistically significant difference in survival or a trend toward a difference in survival between strata (i.e., none had a P < 0.10). The lack of a survival correlation in controls suggests that anti-Fsα responses are prognostic specifically for patients vaccinated with PROSTVAC-VF. Moreover, we used the Kaplan–Meier survival estimator to compare the survival of PROSTVAC-VF patients who mounted a fourfold or greater Fsα response with the survival of the subset of control patients who had a fourfold or greater Fsα response. Patients treated with PROSTVAC-VF were found to have significantly longer survival (P = 0.03), indicating that patients with robust responses to the Fsα are not simply the healthiest patients or the patients with the least aggressive disease.

Anti-Fsα Antibodies Correlate with Survival for Combined Therapy.

Having seen a consistent survival correlation in our discovery set and in an independent validation set, we next assessed the relationship between anti-Fsα antibody responses and survival for PROSTVAC-VF when used as part of a combination treatment, because combining PROSTVAC-VF with additional treatments may produce synergistic effects (29). Specifically, we analyzed anti-Fsα responses induced during a phase 2.5 trial of PROSTVAC-VF combined with the radiopharmaceutical Quadrant (Samarium-153 lexidronam pentasodium) in patients with two or more bone lesions related to their prostate cancer despite prior hormonal ablation and treatment with docetaxel (30). Interestingly, despite being associated with leukopenia (31), Quadrant did not blunt increases in total anti-Fsα Ig induced by PROSTVAC-VF. Of 13 patients who received Quadrant and PROSTVAC-VF, seven (54%) showed fourfold or larger anti-Fsα
responses, similar to the frequencies of response in the discovery (61%) and validation (51%) sets discussed above. Stratifying patients according to anti-Fs responses according to the threshold applied in Fig. 3 showed a statistically significant difference in overall survival ($P = 0.028$; difference in median survival, 1.9 y) (Fig. 6). This survival correlation further validates anti-Fs responses as a potential biomarker for PROSTVAC-VF and extends its clinical significance from mCRPC patients receiving PROSTVAC-VF as a single agent to include patients with radiographically evident metastases receiving combination therapy.

**Anti-Fs Antibodies Are Directed at Viral Vectors.** To understand better how humoral responses to Fs are linked with improved survival, we next investigated the source of the antigen that produced these humoral responses. Several factors led us to hypothesize that the anti-Fs, BG-A, and BG-A responses are triggered by glycans on the viral vectors. First, these antiglycan humoral responses were seen following inoculation with either PROSTVAC-VF or wild-type viral vectors but not in nonvaccinated patients, indicating that the response is caused by a vaccine component other than the transgenes or GM-CSF. Second, a nonhuman source of the Forssman antigen is more likely than a human source because humans cannot biosynthesize the Forssman antigen, except in very rare instances (32, 33). Third, the chicken embryo dermal cells used as host cells for production of the poxviruses express blood group A (34) and the Forssman antigen (35, 36). Carrying of host-cell glycans during production of viruses is well known (37–40) and could cause PROSTVAC-VF’s viral vectors and control viral vectors to display both blood group A and the Forssman antigen.

Two approaches were used to test the viral vectors for Forssman antigen. First, we used a competitive binding assay to detect binding of serum antibodies to the viral vectors (Fig. 7A). Sera from patients with the largest responses to Forssman antigen and blood group A were pooled and preincubated with the viral vectors. Binding of serum antibodies to the viral vectors was detected as a reduction in signal intensity on the array. Preincubation of pooled serum with vaccinia reduced binding of IgG antibodies to Fs by 80%, which was the most substantial reduction for any component on the array. Substantial reductions also were observed for BG-A-related glycans. ELISA experiments with monoclonal antibodies and lectins provided additional evidence that the viral vectors contain Fs and BG-A (26).

![Kaplan–Meier survival curves for combination therapy stratified by postvaccination response to Fs. Patients (n = 13) treated with PROSTVAC-VF and Quadramet were stratified according to their postvaccination humoral anti-Fs response. Probability of survival is plotted for patients with significant (solid blue) or little to no (dashed red) fold change in total Ig in response to Fs. The Kaplan–Meier curves were generated using the measurement technique and threshold used for stratifying Forssman responses in Fig. 3.](image)

**Discussion**
Therapeutic cancer vaccines are promising treatments, but clinical responses vary considerably from one patient to another. Strategies to distinguish responders from nonresponders could improve patient care substantially by allowing more personalized treatment decisions. For example, the ability to identify responders within the first 3 mo of treatment could enable clinicians to adjust treatmentsrationally many months to years before clinical assessments (e.g., tumor burden, PSA levels) become reliable indicators of an effective response. Although previous investigations of immune responses to vaccination have found potential associations with clinical responses (1, 5, 6, 10, 11), no clinically validated biomarker of efficacy is available for any cancer vaccine. Detailed investigations of T-cell responses, humoral responses to protein antigens, and immunosuppressive T-regulatory cells have not accounted consistently for variation in survival benefit among patients (2, 3, 5, 6).

Major challenges in identifying promising biomarkers for cancer vaccines include difficulty in obtaining enough samples for independent discovery and validation stages as well as a lack of control groups for assessing a biomarker’s vaccine specificity. In this study, we were fortunate to have access to serum samples from multiple clinical trials of PROSTVAC-VF, a poxvirus-based cancer vaccine currently in phase III clinical trials for prostate cancer, comprising more than 100 vaccinated patients. In addition, we obtained sera from control subjects vaccinated with wild-type viral vectors as well as nonvaccinated control subjects treated with radiation. This unique collection of samples provided an opportunity to identify responses that correlated with survival and to assess the specificity and independence of these responses from other clinical factors.

In this study, we profiled antiglycan antibody levels during the first few months on treatment with PROSTVAC-VF. Although this vaccine is not specifically designed to generate antiglycan immunity, we demonstrate that antiglycan antibody responses are induced by this vaccine. In addition, we demonstrate that...
antibody responses to the Forssman antigen have a statistically significant correlation with survival for patients treated with PROSTVAC-VF as a single agent or in combination with Quadrant in mCRPC. The correlation was consistent in patients from three separate phase II clinical trials. Responses to FSG α induced 2–3 mo after the first vaccination were associated with an eight times better likelihood of surviving 3.5 y and an increase in median survival of ~9 mo. The correlation was specific to patients receiving PROSTVAC-VF, because antibody responses to the Forssman antigen did not show a significant correlation with general prognostic indicators, features of disease severity, or measures of general immune function. Moreover, no significant correlation was observed in controls vaccinated with wild-type poxviruses lacking the key tumor-associated antigen (i.e., PSA).

These results on antibody responses provide unique insights beyond our earlier studies on the relationships between preexisting antiglycan antibodies before vaccination and survival (26). In the previous study, we found that preexisting IgM to BG-A correlated positively with survival. Although this result was consistent with our central hypothesis that antiglycan antibodies are relevant to clinical outcomes for cancer vaccines, the correlations of preexisting antibodies with survival are not, in general, good indicators that an antibody response will be produced by a vaccine, nor are they good indicators that an antibody response will correlate with survival. This caveat is especially true for a vaccine designed to stimulate T-cell responses, such as PROSTVAC-VF. Given that (i) antibody responses to the overall viral vectors do not correlate with survival, (ii) preexisting IgM, IgG, and total Ig levels for FSG α do not correlate with survival (26), and (iii) the vaccine does not induce antibody responses to PSA (41), the induction of a response to a viral vector glycans and its correlation with survival was unexpected and represents an aspect of the immune response to PROSTVAC-VF, and to poxviruses in general, not previously appreciated.

Anti-Forssman humoral responses were traced to the viral vectors based on several lines of evidence. First, the Forssman antigen, a glycolipid composed of a pentasaccharide attached to a ceramide, is not expressed in humans, who lack a functional Forssman synthase to catalyze the addition of the penultimate GalNAc residue to a tetrasaccharide glycolipid precursor, Gb4 (also referred to as the “P antigen”). Although polymorphisms that render the Forssman synthase do occur in humans (33), they are extremely rare and are unlikely to account for the high frequency of anti-FSG α responses observed in this study. Therefore, the Forssman antigen likely is of nonhuman origin. Second, anti-Forssman responses were detected in subjects inoculated with either PROSTVAC-VF or wild-type vectors but not in nonvaccinated patients. This result indicates that vaccination with a poxvirus is necessary (i.e., changes in anti-Forssman antibody levels do not occur spontaneously in patients with prostate cancer), but the presence of a tumor-associated antigen and costimulatory proteins on the vaccine is not required for this antibody response. Third, the Forssman antigen was detected on viral vectors, and these vectors could competitively inhibit binding of serum antibodies to surface-bound Forssman glycans. Fourth, carriage of cell-surface glycans onto enveloped viruses is well known, and the chicken embryo dermal cells used to produce PROSTVAC-VF can biosynthesize the Forssman antigen. Interestingly, antibody responses to the Forssman antigen were not simply a surrogate for immune capacity or overall response to the viral vectors. Forssman responses did not correlate well with overall titers to the poxviruses. In addition, previous studies found no link between survival and overall antivector titers (23, 24). Therefore, responses to FSG α on the viral vectors provided unique information not captured by overall titers to the viral vectors, which may overlook heterogeneity in how individuals recognize specific epitopes and do not distinguish between neutralizing and nonneutralizing antibodies.

Responses to anti-Forssman antibody could be linked to clinical outcomes in a variety of ways. One possibility is that the antibodies act directly on prostate tumors; however, this seems unlikely, considering that human cells rarely produce the Forssman antigen and that no survival correlation was observed in control subjects who mounted an anti-Forssman response. Rather, the presence of the Forssman antigen on the virus suggests this xenoantigen may function as an adjuvant. Several reports have demonstrated that serum antibodies against xenogenic carbohydrate antigens on vaccines can enhance immune responses (42–44). For example, recognition of α-Gal on viruses by antibodies facilitates trafficking to dendritic cells, opsonization, and cross-presentation (45); these effects are notable, considering that immune complexes have been shown specifically to enhance the presentation of PSA on dendritic cells (46). Moreover, genetic modification of cancer cell vaccines to express α-Gal can enhance immune responses and break tolerance (42, 47). Because Forssman antigen and α-Gal are both T-cell–independent antigens (not presented on the MHC), and are both capable of inducing hyperacute rejection, anti-Forssman and anti–α-Gal antibodies may act through similar mechanisms. Alternatively, the activated B cells that produce the Forssman antibodies may play a key role in shaping the immune response. Additional studies will be necessary to evaluate the mechanistic link with survival more fully.

Our results have a number of important implications for PROSTVAC-VF and other vaccines produced in nonhuman cells. First, tracking changes in a patient’s anti-Forssman antibody levels during the initial 2–3 mo of treatment with PROSTVAC-VF could enable clinicians to adjust treatment rationally many months to years before clinical assessments (e.g., tumor burden, PSA levels) become reliable indicators of an effective response (48). With treatment options for prostate cancer expanding rapidly (with five recent drug approvals and one more expected shortly) (1, 49–53), there is a clear need to determine which particular therapy is best suited for each of the >240,000 men diagnosed with prostate cancer annually in the United States (54). Because therapies with the fewest side effects, such as therapeutic vaccines, often are considered first for patients with minimal or no symptoms, a test that could determine the likelihood of eventual clinical benefit within a few months of taking the vaccine would be highly valued in this rapidly changing clinical landscape. This type of test eventually may function as a potential marker of disease progression and could determine the likelihood of eventual clinical benefit within a few months of taking the vaccine. In addition, there are a few months of taking the vaccine would be highly valued in this rapidly changing clinical landscape.

Second, the link between postvaccination survival and a xenogenic glycans on the vaccine’s viral vector suggests that immunogenic glycans on viral vectors may be an underappreciated feature of vaccine design and characterization, especially for vaccines that are not designed to induce immune responses to glycans. Analysis of a viral vector’s glycans may be an important aspect of quality control. The type of host cell and growth conditions, as well as other factors that influence glycans displayed on viral vectors, may influence clinical outcomes for poxvirus-based vaccines significantly. Moreover, engineering vaccines to display Forssman xenoantigen could be a generally useful strategy to enhance vaccine immunogenicity.

Third, Forssman antibodies may be relevant to a variety of other vaccines and biological agents. For example, the wild-type vaccinia vector used to immunize control subjects has been used as a smallpox vaccine for many years. There is considerable interest in defining the key antigens targeted by the immune system upon immunization with the smallpox vaccine and in understanding the factors that influence the development of protective immunity. In this study we specifically demonstrate
Human antibody responses to glycans on vaccinia. Therefore, our results reveal a previously unrecognized aspect of the immune response to this vaccine/virus. Because only 50–60% of patients had a response to the Forssman antigen, our results also demonstrate that the variability in responses to poxvirus glycans can be a previously unrecognized contributor to the heterogeneity of immune responses. A number of other enveloped viruses also are produced in chicken-derived cells and are likely to display the Forssman xenoantigen. Some examples that currently are in clinical trials or clinical use include other poxvirus-based cancer vaccines [e.g., PANVAC (56) and rT-NN-ESO-1 (57)], poxvirus-based HIV vaccines [e.g., ALVAC-HIV (58)], oncoviral poxviruses (59, 60), and the influenza vaccine. Moreover, enveloped viruses produced in other Forssman-positive host cells (e.g., CHO, MDCK, NIH 3T3, BHK21-F) also may display the Forssman antigen. Therefore, anti-Forssman immunity could influence the clinical efficacy of a broad range of vaccines and biological agents, even when they are not designed to induce antiglycan immunity.

Although these results are promising, certain limitations should be noted. In particular, our study was retrospective and observational, like other biomarker studies done in conjunction with randomized clinical trials. To guard against bias inherent in observational studies, we analyzed two independent sample sets and verified that strata defined by an anti-FsR response were balanced on several control factors that were not accounted for by our control analyses. Although our study comprised more than 100 vaccinated patients, as well as controls to demonstrate the prognostic specificity of anti-FsR responses, additional prospective studies with larger numbers of patients and control subjects will be an important focus of future research. An ongoing phase III clinical trial evaluating overall survival is anticipated to enroll 1,200 patients and could provide additional studies on Forssman responses as early indicators of a favorable immune response. A second limitation of our study is that only a small subset of the human and chicken glycomes is represented on our glycan array. Therefore, the array may have missed antibody responses to other glycans, such as additional xenogenic glycans, on PROSTVAC-VF’s viral vectors. Third, our analyses focused on antibody responses that occurred frequently in patients; therefore, infrequent responses that correlate with survival may not have been detected. Moreover, combinations of infrequent responses were not evaluated. Fourth, the glycan array detects only free antibodies in serum; antibody responses that result in antibody-antigen complexes would not have been detected.

Our results underscore the importance of studying antiglycan immune responses for biomarker discovery and vaccine development. Immune responses to carbohydrates traditionally have been very difficult to evaluate, when a complex assortment of glycans is present on an immunogen. With advances in glycan microarray technology, high-throughput analyses of antiglycan antibody responses to hundreds of potential glycan antigens in hundreds of patient samples can be accomplished rapidly and efficiently. Our study demonstrates how more comprehensive profiling of antiglycan responses can uncover previously unrecognized aspects of the immune response that are relevant to our basic understanding of vaccine immunology and may lead to the development of new biomarkers and the design of improved vaccines. In addition, these findings highlight the potential of antiglycan immune responses to advance personalized medicine.

Materials and Methods

Serum Samples. Serum samples were collected during two previously reported phase II clinical trials of PROSTVAC-VF (23, 24) and a completed phase 2.5 trial of Quadramet alone or combined with PROSTVAC-VF (30). For the discovery set, serum samples came from 28 patients with mCRPC who enrolled in a single-center phase II study of PROSTVAC-VF (23). The independent validation set (76 vaccinated patients and 37 controls randomly assigned to receive wild-type vaccinia and fowlpox) came from a placebo-controlled, multicenter study of PROSTVAC-VF (24) in which all patients received immunizations according to a single regimen. All patients received the same dose of vaccinia and fowlpox vectors, and the vaccination schedule was the same except that the validation subjects received an additional boost on day 14. Across all study centers, sera were obtained in serum-separator tubes, processed within 4 h, and stored at −80 °C until assayed. We included all study sites in our analysis after clustering showed no systematic differences in antiglycan antibody profiles that might have resulted from variations across different sites in sample collection or processing (Figs. S5 and S6). Sera were collected before vaccination and at least one postvaccination time point (Dataset S1). For the discovery set, we analyzed data from the time point closest to 3 mo after initial vaccination in cases when multiple post-vaccination samples were available. For the validation set, all reported post-vaccination changes were measured by comparing prevaccination antibody levels with postvaccination levels measured 2–4 mo after initial vaccination. Consistency of postvaccination measurements was verified by comparing pre-vaccination serum samples from a few patients to post-vaccination sera (Dataset S2). PROSTVAC-VF combination trial sera were analyzed from the available time point closest to 3 mo postvaccination (Dataset S1). Additionally, serum samples were collected over 2–3 mo from healthy subjects (n = 7) and from prostate cancer patients (n = 9) before and after treatment with local definitive radiotherapy instead of PROSTVAC-VF (28).

High-Throughput Profiling of Serum Anti-Glycan Antibodies. Serum antiglycan antibodies were profiled on a glycan microarray (array components are listed in Dataset S1). Before array printing, glycans were conjugated to albumin via short, flexible linkers with the goal of mimicking the density and spacing of native glycans (61, 62). For example, the Forssman disaccharide was conjugated to BSA via mercaptoethylamine glutarate to measure the anti-FsR antibodies reported here. The array format and assay have been described previously (63), along with analysis of reproducibility (27) and validation with numerous antibodies and lectins (61–64). Arrays were printed as previously described (63) using SMP2 pins (TeleChem). Print buffer included DyLight 649 (0.7 μg/mL) (Thermo Scientific) to assess print quality before being washed away before serum assays. Sera were diluted at 1:50 or 1:200 to obtain the necessary dynamic range to measure changes in both high- and low-abundance antibodies. Unless otherwise noted, all changes reported were measured at a dilution of 1:200. Bound antiglycan antibodies were detected with fluorescent secondary antibodies specific for IgM, IgG, or total Ig (Jackson ImmunoResearch) (Table S1 and Dataset S1). Clinical data were blinded during data collection and processing.

Because >2,000 arrays were required, precautions were taken to minimize technical variations and to monitor print quality (65). Slides came from the fewest possible print batches, and consistency of print batches was checked using reference serum. Samples were analyzed in a random order to ensure intermixing of controls and vaccinated patients with varied responses to PROSTVAC-VF. Additionally, the same experimenter collected all array data, and samples were analyzed on replicate slides to identify technical faults.

Glycan Profiling of Viral Vectors. Glycans on viral vectors were assayed by adapting the glycan microarray for a competitive binding assay. Post-vaccination serum (diluted 1:500 in 1% BSA and 0.3% HSA) was preblocked for 1.5 h at 37 °C with vaccinia (9.1 × 10^9 pfu) and fowlpox (6.9 × 10^10 pfu) that had been propagated in chicken embryo dermal cells, purified with a sucrose cushion, and UV-inactivated. The presence of particular glycans on viral vectors was detected as reduced binding of preblocked serum relative to binding of control sera.

Second, the expression of Forssman antigen on viral vectors was assayed via ELISA. Viral vectors (10 μg/mL in carbonate buffer, pH 9.6) were coated onto Maxisorp plates (Nunc) and incubated with monoclonal anti-Forssman antibody M187 (2 μg/mL) (Santa Cruz Biotechnology) or biotinylated Dolichos biflorus agglutinin (DBA) (10 μg/mL) (Vector Labs), which is highly reactive for the Forssman antigen. Controls included monoclonal antibody BRIC111 (10 μg/mL), which is specific for the Tr antigen (GalNAc linked to serine or threonine) and biotinylated Ulex europaeus agglutinin (10 μg/mL) (Vector Labs), which reacts with blood group H. All antibodies and lectins were diluted in PBS containing 3% (vol/vol) BSA. Next, bound monoclonal
antibody or lectin was detected with either an alkaline phosphatase (AP)-conjugated secondary antibody (2.4 μg/mL in PBS + 3% (w/v) BSA) (Jackson Immunoresearch) or AP-conjugated streptavidin (2 μg/mL in PBS + 3% BSA) (Vector Labs) that catalyzed the conversion of methylumbelliferyl phosphate (26 μg/mL in Tris, pH 9.0) into a fluorescent indicator.

Measurements of Peripheral T Cells and Overall Titers to Viral Vectors. Assays of peripheral T cells along with antivaccinia and antifowlipox Ig titers (Dataset 51) were previously reported (23, 24).

Statistical Analyses. To reduce unintentional overfitting common for high-throughput assays, leads were identified in a discovery set and were further tested with independent samples in a blinded manner. In the discovery set, antilycan antibody profiles were scored for statistically significant survival differences in patients with high or low fold-changes in antibody levels after inoculation (nonparametric log-rank P value < 0.05 for Kaplan–Meier survival estimator). Based on a previous study of typical variations in serum antilycan antibody occurring over 3 mo in healthy controls (27), we considered only increases > 2.6-fold (i.e., increase > 160%) or decreases < 2.6 fold (i.e., decreases < 62%). Additionally, analysis considered only strata containing seven or more patients because disproportionate strata (e.g., five vs.

23 patients) show chance survival differences more frequently predicted by log-rank P values. Lead validation required consistent, statistically significant survival differences in the independent, blinded validation set. The survival correlation for anti-Fsresponses was validated using samples from a multicenter phase II trial of PROSTVAC-VF and separately in a phase II phase trial of PROSTVAC-VF combined with Quadratum. Because only one change (anti-Fs total Ig) correlated with survival in the discovery set, adjusting for multiple comparisons was unnecessary.

Correlations between antilycan antibodies and clinical or other immuno- nological data were assessed by nonparametric Kendall correlations (r) and their associated P values (66). Analyses were performed in Microsoft Excel and Partek Genomics Suite (version 6.4).

ACKNOWLEDGMENTS. We thank Kevin Camphausen for providing sera from nonvaccinated patients with prostate cancer and Professor Tom Tolbert (University of Kansas), Professor Lai-Xi Wang (University of Maryland), and Dr. Joseph Barchi (National Cancer Institute) for contributing glycans for the array. This research was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute. C.T.C. received fellowship funding from the Pharmacology Research Associate Program of the National Institute of General Medical Sciences.


Supporting Information

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Fig. S1. Stability of postvaccination levels of anti-Forssman xenoantigen (anti-Fs\(_{\text{di}}\)) antibodies (Fs\(_{\text{di}}\) = GalNAc\(_{\alpha}1\)–3GalNAc\(_{\beta}\)). To assess the stability of postvaccination antibody levels within the first months of treatment, samples from three postvaccination time points (ranging from 2 to 4 mo after initial vaccination) were available from 10 patients within the discovery set. Postvaccination levels of total Ig (IgG + IgM + IgA) have been normalized with prevaccination levels.

Fig. S2. Probability of long-term survival as a function of anti-Fs\(_{\text{di}}\) response. Over a broad range of anti-Fs\(_{\text{di}}\) responses, the likelihood of long-term survival (≥3.5 y) was compared in individuals with and without postvaccination humoral responses to Fs\(_{\text{di}}\). The odds ratio (solid line), which shows the odds of long-term survival in individuals who responded to Fs\(_{\text{di}}\) relative to the odds for those who did not, improved steadily with increasing response to Fs\(_{\text{di}}\). Dashed lines indicate the 95% confidence interval of the odds ratio.
Graded survival correlation for anti-Fsdi humoral responses. Vaccinated patients were stratified according to their humoral responses to anti-Fsdi. (A) Patients with the largest fold changes (≥10x) in total Ig for Fsdi had longer overall survival than patients with intermediate (4x to 10x) or low (<4x) responses. (B) Interestingly, the patients with ≥10x changes in anti-Fsdi total Ig had the best actual overall survival despite a trend to have shorter Halabi predicted survival.

Fig. S4. IgM and IgG responses to Fsdi in vaccinated and control patients. Both IgM and IgG increased after vaccination with PROSTVAC-VF (green) or inoculation with its wild-type viral vectors (control; orange). According to the Mann–Whitney test, there was no statistically significant difference in the humoral responses between vaccinated and control patients. Whiskers of box plots reach to the 10% and 90% percentiles.

Fig. S5. Variation in antiglycan antibody profiles across clinical study sites. The validation study analyzed sera collected at 41 clinical sites participating in the multicenter phase II study of PROSTVAC-VF. Before including samples in our analysis, we checked for discrepancies in serum antiglycan antibody profiles of samples obtained at different sites. This heat maps shows normalized levels of antiglycan antibodies (total Ig measured at a dilution of 1:50). Rows correspond to the 259 individual serum samples grouped by the clinical study site. Columns correspond to glycans, which have been sorted alphabetically. There were no systematic differences in antiglycan antibody profiles for sera collected at different sites.
Fig. S6. Serum samples were frozen at −70 °C and were thawed immediately before analysis. To assess the effect of sample handling on data quality, freshly thawed reference serum was compared with identical aliquots that had been thawed for an extended period and/or freeze thawed repeatedly. (A and B) There was no significant change in antiglycan IgM (A) or IgG (B) levels after serum had been left thawed at 4 °C for 12 h. (C and D) Storing serum at 4 °C for 36 h also did not affect serum antibody levels. (E and F) Similarly, antibody levels were not changed significantly after thawing overnight at 4 °C and then freeze thawing an additional two times. Each circle indicates the average of 12 data points.

Table S1. Secondary antibodies for detection of serum antiglycan antibodies

<table>
<thead>
<tr>
<th>Description</th>
<th>Source</th>
<th>Stock concentration</th>
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<tbody>
<tr>
<td>Cy-3 conjugated goat antihuman IgM + IgG + IgA</td>
<td>Jackson ImmunoResearch (109-165-064)</td>
<td>1.5 mg/mL</td>
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<tr>
<td>DyLight 549 conjugated goat antihuman IgG</td>
<td>Jackson ImmunoResearch (109-505-008)</td>
<td>1.5 mg/mL</td>
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<tr>
<td>DyLight 649 conjugated goat antihuman IgM</td>
<td>Jackson ImmunoResearch (109-495-043)</td>
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Dataset S1. Clinical data, antiglycan antibody profiles, and other immunologic data for patients

Dataset S1

The Excel data file contains patients' full antiglycan antibody profiles along with the corresponding previously published clinical data collected during two phase II trials of PROSTVAC-VF. Separate worksheets contain data for the patients used for this study's discovery (n = 28) and validation (n = 113) sets. Data are organized by the sample set (discovery vs. validation), secondary antibody (IgM, IgG, or total Ig), and the serum dilution (1:50 or 1:200). Clinical data include postvaccination survival, Halabi predicted survival, age, prostate-specific antigen (PSA) levels, and Gleason score. The worksheets also contain T-cell reactivity for PSA, T-regulatory cell data, and IgG titers for vaccinia and fowlpox. Array data are reported on a log base 2 scale with a floor signal of 7.22. This data file also contains a description of the array components.