Molecular signatures of antibody responses derived from a systems biology study of five human vaccines

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Many vaccines induce protective immunity via antibodies. Systems biology approaches have been used to determine signatures that can be used to predict vaccine-induced immunity in humans, but whether there is a ‘universal signature’ that can be used to predict antibody responses to any vaccine is unknown. Here we did systems analyses of immune responses to the polysaccharide and conjugate vaccines against meningococcus in healthy adults, in the broader context of published studies of vaccines against yellow fever virus and influenza virus. To achieve this, we did a large-scale network integration of publicly available human blood transcriptomes and systems-scale databases in specific biological contexts and deduced a set of transcription modules in blood. Those modules revealed distinct transcriptional signatures of antibody responses to different classes of vaccines, which provided key insights into primary viral, protein recall and anti-polysaccharide responses. Our results elucidate the early transcriptional programs that orchestrate vaccine immunity in humans and demonstrate the power of integrative network modeling.

Published studies have used systems biology approaches to identify molecular networks that orchestrate immunity in response to vaccination in humans1–3. Analyses of the immune response to the vaccine against yellow fever virus (YF-17D) have provided proof of the concept that molecular signatures in the blood of humans, induced within a few days after vaccination, can be used to predict the magnitude of the later immune responses to a vaccine4 and are beginning to yield insights about the nature of the innate and adaptive responses to vaccination.5,5. Subsequently, systems biology approaches have been extended to identify predictive signatures of vaccines against influenza virus6, and are being used to study immune responses to other vaccines7–11. The new field of ‘systems vaccinology’ has emerged from such data and is poised to address the mechanisms that control immune responses to vaccination and identify predictors of vaccine efficacy12,13.

A central question in systems vaccinology is whether there are universal predictors of vaccine-induced immunity. For example, given that many vaccines stimulate protective immunity through antibodies, are there molecular signatures that can be used to predict the antibody response to any vaccine? In studies of the vaccine against yellow fever virus4,5, a robust but transient type I interferon response has been seen in the blood transcriptomes of vaccinated subjects. Studies of the trivalent inactivated vaccine against influenza virus (TIV) have found that a distinct gene signature of antibody-secreting cells (ASCs) is detectable 7 d after vaccination6. Both studies have found that expression of genes such as TNFRSF17 (which encodes the receptor for the B cell growth factor BAFF) is highly predictive of the later antibody response4,6. How the early molecular and cellular events induced by vaccination affect the later antibody response remains a central question. Published work on live attenuated antiviral vaccines (YF-17D and the live attenuated vaccine against influenza virus (LAIV)) and an inactivated protein vaccine (TIV) suggests that different programs are induced by different vaccines6. The question of whether there are common programs that drive antibody responses to different vaccines remains unanswered. For example, YF-17D triggers the activity of Toll-like receptor 2 (TLR2), TLR7, TLR8 and TLR9, as well as that of the RNA helicases RIG-I and Mda5 (refs. 4,14), and LAIV triggers TLR7 activity15,16. However, bacterial polysaccharide does not trigger those receptors, which are involved in sensing viruses. Do carbohydrate-containing vaccines induce molecular signatures that reflect distinct molecular pathways that stimulate antibody production? To address this issue, we initiated a program aimed at comparing molecular signatures induced by different vaccines. As part of this effort, we made a detailed analysis of the innate and adaptive responses to vaccination

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with the polysaccharide-containing or conjugate vaccine against meningococcus. The key questions we addressed were as follows: can the molecular signatures induced early after vaccination be used to predict the later magnitude of the titers of antibody against those vaccines? Are these signatures similar to those elicited by other vaccines, such as the vaccines against influenza virus or yellow fever virus, and if so, to what extent can they be used to predict antibody responses to those vaccines?

*Neisseria meningitidis* is a leading cause of meningitis and septicaemia, with 1.2 million cases per year worldwide. Two major classes of vaccines against meningococcus available in the USA are the polysaccharide-containing vaccines, such as the quadrivalent polysaccharide vaccine (MPSV4), which contains polysaccharides from *N. meningitidis* serogroups A, C, Y and W-135, and the polysaccharide-protein conjugate vaccines, such as the quadrivalent conjugate vaccine (MCV4), which contains those same four polysaccharides conjugated to diphtheria toxoid (DT). Vaccination induces antibodies to the capsule that are able to fix complement and trigger lysis of the bacteria, as measured by an assay of serum bactericidal activity (SBA), which correlates with protection from clinical disease. Both classes of vaccines against meningococcus induce high titers of functional antibodies 1 month after vaccination; however, polysaccharide-containing vaccines are believed to induce T cell–independent antibody responses, which leads to waning humoral immunity and impaired memory, especially in infants. Moreover, repeated vaccination with polysaccharide vaccines can result in hyporesponsiveness to serogroups C and W-135 (refs. 20, 21). Despite the fact that these two vaccines contain the same polysaccharide antigens, the molecular mechanisms by which they elicit immunity may differ and are poorly understood.

In this study, we made a detailed characterization of the innate and adaptive immune responses to vaccination with MPSV4 or MCV4 in healthy young adults. We did a comparative analysis of five vaccines, combining the new data on MPSV4 and MCV4 with the published data on the vaccine against yellow fever virus and two vaccines against influenza virus. We developed a large-scale network integration of publicly available transcriptomes of human blood, with ‘interactome’, ‘bibliome’ (defined below) and pathway databases and specific biological contexts to deduce a set of transcription modules in blood, which we used to evaluate the correlation between the antibody response and the blood transcriptome. This approach revealed distinctive transcriptomic signatures that correlated with vaccine-specific antibody responses, which provided insights into primary viral, protein recall and anti-polysaccharide responses. Our results demonstrate the power of integrative network modeling and show that immunological mechanisms can be successfully inferred from blood transcriptomes shortly after vaccination.

### RESULTS

**Antibody responses induced by vaccines against meningococcus**

In a longitudinal study of over 2 years, we immunized 30 healthy young adults with a single dose of either MCV4 (*n* = 17) or MPSV4 (*n* = 13) and did a comprehensive analysis of their innate and adaptive responses (Table 1). We measured serum antibody responses to meningococcal serogroups A and C at days 0, 7, 30, 180 and 750 after vaccination (Fig. 1a). Serogroup A is the cause of large pandemics, especially in sub-Saharan Africa. Serogroup C is among the common types that cause meningococcal infections in the USA, along with serogroups Y and B (http://www.cdc.gov/abcs/reports-findings/survreports/mening11.html). There is no licensed vaccine against serogroup B in the USA. Both vaccines induced robust polysaccharide-specific immunoglobulin G (IgG) antibody responses to serogroups A and C, as measured by enzyme-linked immunosorbent assay (ELISA) (Fig. 1a). Those responses peaked at day 30 (MCV4) or day 180 (MPSV4) (Fig. 1a). The magnitude and duration of the polysaccharide-specific IgG response was greater with MPSV4 than with MCV4 (Fig. 1a). Both MPSV4 and MCV4 induced polysaccharide-specific IgG2 and IgG1 antibody responses (Fig. 1b), as well as specific IgM antibody titers, which continued to rise until at least day 30 (Fig. 1c). At 2 years after immunization, there was still a significant concentration of polysaccharide-specific IgG induced by MPSV4, whereas the response induced by MCV4 had decreased, although it stayed substantially above baseline (Fig. 1a).

Both vaccines induced a rapid increase in SBA, which was detectable as early as day 7, peaked at day 30, began to decrease by day 180, yet remained substantially above baseline at day 750 (Fig. 1d). Although there was a trend for MPSV4 to induce greater SBA titers than MCV4 did (Fig. 1d), the difference in the magnitude of the SBA titers was not as pronounced as that observed with the magnitude of the ELISA titers (Fig. 1a). An SBA titer of 1:8 or higher, a correlate of protective immunity, was maintained in the majority of subjects vaccinated with MPSV4 or MCV4 at 2 years after inoculation, with no difference observed between the two vaccine groups (Fig. 1d). MCV4, which contains DT protein as a conjugate, elicited a strong anti-DT IgG response at days 7 and 30, whereas MPSV4, which contains polysaccharide alone, did not (Fig. 1e).

We quantified ASCs in peripheral blood that were induced by vaccination. The ASCs showed a peak at day 7 in response to each vaccine (Fig. 1f). Unexpectedly, even though a greater polysaccharide-specific antibody response was induced by MPSV4 than by MCV4 (Fig. 1a,d), the magnitude of the ASC response induced by MPSV4 was lower than that induced by MCV4 (Fig. 1f). Polysaccharide- and DT-specific enzyme-linked immunospot analysis of a subset of subjects vaccinated with MCV4 revealed that the majority of IgG-secreting ASCs detected in the blood at day 7 were specific for DT (Supplementary Fig. 1a,b). Indeed, the percentage of total ASCs in the blood at day 7 after MCV4 vaccination strongly correlated with the abundance of anti-DT IgG in serum (Fig. 1g), not with the anti-polysaccharide IgG response (data not shown).

Together those data demonstrated that both the polysaccharide-containing and conjugate vaccines induced robust polysaccharide-specific antibody titers in healthy young adults, but the magnitude and isotypes of the antibody responses vary substantially for the two vaccines. Notably, MPSV4 induced a greater magnitude of titers of binding antibody than did MCV4 (Fig. 1a), although the differences in SBA titers were more modest (Fig. 1d). This suggested that despite the noticeable difference in the binding antibody response, MCV4 may be more efficient at inducing functional antibodies, perhaps as a result of increased

| Table 1 Study design |
|----------------------|
| Time (d)            |
|                      |
|                      |
| 0    | 3    | 7    | 14   | 30   | 180  | 750  |
| Gene-expression profile | X  | X  | X  | X  | X  | X  |
| SBA   | X  | X  | X  | X  | X  | X  |
| Anti-PS IgG (ELISA)  | X  | X  | X  | X  | X  | X  |
| Anti-PS IgM (ELISA)  | X  | X  | X  | X  | X  | X  |
| Anti-DT IgG (Luminex) | X  | X  | X  | X  | X  | X  |
| 27-plex cytokines (Luminex) | X  | X  | X  | X  | X  | X  |
| ASCs (flow cytometry) | X  | X  | X  | X  | X  | X  |

*Analyses done (left column) for blood obtained from healthy adult cohorts (18–45 years of age; *n* = 13 for MPSV4 and *n* = 17 for MCV4) at various days after vaccination (top row) with vaccines against meningococcus: whole blood cells were used for flow cytometry; PBMCs were isolated for DNA microarray analysis; plasma samples were used for the remaining assays. ‘X’ indicates the assay was done at that time point.*
antibody affinity. Notably, MCV4 induced a robust ASC response that was directed largely against the carrier protein, DT, whereas MPSV4 induced very little IgG-producing ASC response. These data prompted the question of what cells produce carbohydrate-specific antibodies to MCV4 and MPSV4. It is possible that such cells emerge more rapidly (or later) than do the plasmablasts that produce DT-specific antibodies or that such cells may not circulate in the blood. Further experimentation involving more detailed kinetic analysis of the response in humans will provide additional insights into these issues.

**Workflow to compare vaccine transcriptome signatures**
To gain insight into the molecular mechanisms underlying the responses to vaccines against meningococcus, we used DNA microarray to analyze the transcriptome profiles of peripheral blood mononuclear cells (PBMCs) of the vaccinated subjects days 0, 3 and 7 (Table 1). We identified 1,150 genes with a difference in expression at day 7 after vaccination relative to their expression before vaccination (‘differently expressed genes’ (DEGs)) in subjects vaccinated with MCV4 (Fig. 2a). Those DEGs bore the hallmark of ASCs, consistent with the enhanced frequency of such cells (Fig. 1f). A published study of TIV has shown that such an ASC response is characterized by a transcriptional network regulated by XBP1, which is a key transcription factor associated with endoplasmic reticulum stress and the differentiation of plasma cells. That XBP1 network also showed considerable enrichment for upregulated genes at day 7 after vaccination with MCV4 (Supplementary Fig. 2).

However, we identified only a small number of DEGs at day 3 after vaccination for both MPSV4 and MCV4 and at day 7 for MPSV4 (Fig. 2a). Consistent with that, analysis of cytokines in plasma revealed little change induced by either vaccine (Supplementary Fig. 3). That was in contrast to results obtained for three other vaccines, YF-17D, LAIV and TIV, for which we did the same analysis of differences in expression (Fig. 2a). We obtained similar results when we applied the additional gene-filtering and false-discovery rate in studies already published (Supplementary Table 1). Among the

**Figure 1** Antibody responses of subjects who received vaccines against meningococcus. (a–d) Concentration of total IgG (a), IgG1 and IgG2 (b) and IgM (c) and SBA titers (d) specific to polysaccharides from *N. meningitidis* serogroup A (MenA) or C (MenC), measured in subjects vaccinated with MPSV4 or MCV4. (e) DT-specific IgG response subjects vaccinated as in a. (f,g) Frequency of ASCs (selected as CD3 CD20 CD38 CD27 CD19+ cells) at various times after vaccination with MPSV4 or MCV4 (f) and quantification of ASCs at day 7 correlated with DT-specific serum IgG at day 7 after vaccination (g). Light lines (b–f) represent individual subjects. *P < 0.05, **P < 0.01 and ***P < 0.001 (unpaired t-test for comparisons between vaccine groups; paired t-test for comparison with baseline in a group; Pearson’s correlation in g). Data are from one experiment (mean and s.e.m. of 13 subjects (MPSV4) or 17 subjects (MCV4)).

**Figure 2** Analysis of data on the blood transcriptome for five human vaccines. (a) Difference in gene expression at day 3 or day 7 after vaccination compared with baseline at day 0 (horizontal axis) versus results from a paired t-test (vertical axis), for each vaccine and each time point. Red dots indicate DEGs (P < 0.001), numbers in plots indicate number of DEGs. Data are from one experiment. (b) Work flow for comparison of the transcriptome signatures of five human vaccines (interactive figures are in the online data portal).
Five vaccines, YF-17D and LAIV are composed of live attenuated viruses, TIV is an inactivated vaccine that contains a viral coat protein (hemagglutinin), MPSV4 contains meningococcal polysaccharides, and MCV4 contains the meningococcal polysaccharides conjugated to DT. Except for LAIV, all vaccines induce a robust antibody response in the blood, which is considered to be the principal correlate of protection for these vaccines. Whether such differences in transcriptomes reflect fundamental differences in the mechanisms of antibody response to these different vaccines is a key issue.

To address that issue, we devised a systematic framework for comparing the transcriptome signatures of five human vaccines (Fig. 2b). First, we applied to the lists of DEGs from the five studies a method that uses gene-interaction data (interactome and bibliome) to put them into a biological context. Second, we assessed molecular pathways for their association with vaccination and antibody responses through the use of a robust positional test (gene-set enrichment analysis (GSEA)) Finally, we devised a novel approach using blood transcription modules (BTMs) as an alternative to 'conventional' pathway analyses (Fig. 2b). Details of each step are provided below.

Given the complexity and depth of these data and analyses, we provide access to an online data portal (http://www.immuneprofiling.org/papers/梅西/). At this site, users can browse, filter and visualize the entire data set through a web interface. Online interactive versions of major figures and details of our BTM annotations are included as part of the website.

Comparative analysis of differences in gene expression

To find gene signatures shared by two or more vaccines, we cross-referenced the lists of DEGs (Fig. 2a) of all five vaccines. The DEGs common to any two vaccines are available online for interactive exploration, presented as blue curves linking two arcs on a circular plot (‘snapshot’ Fig. 3a). We present here some of the DEGs across these vaccine data sets (Fig. 3b). Several B cell genes were upregulated at day 7 in subjects vaccinated with MCV4 or TIV (Fig. 3b), including TNFRSF17, which encodes a B cell–differentiation molecule and whose expression is predictive of antibody responses to the YF-17D and TIV vaccines. Genes associated with innate immunity and interferon responses, such as OAS1, OAS2, STAT1, STAT2 and TRIM22, were upregulated at day 3 after vaccination with live attenuated virus vaccines (Fig. 3b).

To better understand the interactions between those DEGs and to put them into biological context, we adopted an approach that uses gene connections extracted from interactome and bibliome data (Supplementary Fig. 4a). The 'interactome' refers to pairs of genes associated with papers listed in PubMed. For each gene in the interactome or bibliome network, its association to DEGs in a vaccine response is assessed by statistical enrichment. If such association is significant, the 'linker' gene is added to the respective vaccine response network, even though it was not present in the original DEG list (Supplementary Fig. 4a). We note that those 'linker' genes are highly relevant to the study context. For example, among the 312 'linker' genes added to the MCV4 network from the interactome, 115 had P value below 0.05 in the gene-expression data (enrichment, P = 10^−19). Through this integrative approach, the overlap of DEGs plus 'linker' genes across the five vaccines were substantially increased (Supplementary Fig. 4b). The overlap network of MCV4 and TIV showed enrichment for genes expressed in ASCs (Supplementary Fig. 4d), whereas the overlap network of YF-17D and LAIV showed enrichment for genes encoding molecules associated with T cell antigen receptor signaling and interferon-related genes (Supplementary Fig. 4e). The network of 1,255 genes common to four or more vaccines (Supplementary Fig. 4c) showed substantial enrichment for various immunologically related processes, such as leukocyte differentiation and B cell activation (Fig. 3c and Supplementary Fig. 4f). Of those 1,255 genes, 1,231 were also present in the response network constructed from a published study of the RTS,S vaccine against malaria through the use of the same method (data not shown), which indicated that this common network may represent a general signature of the transcriptome response to vaccines.

Pathways associated with vaccination and antibody responses

Beyond single-gene-level analysis, the expression of pathways can be also evaluated directly, which provides a more specific biological context and increasing statistical power. We adopted the positional test framework of GSEAs and the pathway-interaction database of the US National Cancer Institute and the Nature Publishing Group for pathway-level analyses. Several pathways related to cell proliferation (ERBB1 downstream signaling; signaling via the...
BMs provide a sensitive and robust statistical framework. (a) Construction of BTMs through large-scale data integration (full details, Supplementary Note). GO, gene ontology; TF, transcription factor; KEGG, Kyoto encyclopedia of genes and genomes; PID, pathway-interaction database. (b) Discriminative power of BTMs and canonical pathways (key), or simulated pathways consisting of randomly selected genes (Random), in assessing the transcriptome data for MCV4 at day 3 (relative to day 0), by t-score (additional examples, Supplementary Fig. 9). (c) Analysis of the statistical significance of the correlation of BTMs to antibody data, for the MCV4 transcriptome data comparing day 3 with day 0 (baseline): each module is collapsed to a single activity score (mean value of all member genes), and Pearson correlation to antibody data is calculated across all subjects (red bars); gray shaded curve, distribution of random data generated by permutations of module gene memberships and sample labels. (d) Classification of genes in BTM M156.1 by products encoded (key); each ‘edge’ (gray line) represents a coexpression relationship learned from public data. (e) Correlation of the activity of module M156.1 and the later DT-specific antibody response to MCV4.

GTP-binding Rho-family protein Cdc42; the transcription factor E2F network; and targets of the transcription factor c-Myc) were significantly induced by different vaccines, as were innate pathways, including the interferon-γ pathway (induced by YF-17D), the nutrient sensor mTOR pathway and signaling via the tumor-necrosis factor (TNF) receptor (induced by MPSV4) and signaling via the receptor PDGFR-β (induced by YF-17D, MCV4 and MPSV4) (Supplementary Fig. 5).

We also identified pathways whose expression was correlated to antibody responses (Supplementary Fig. 6), such as ‘B cell antigen receptor (BCR) signaling pathway’ for TIV and MCV4, and ‘ATF2 transcription factor network’ for YF-17D. ATF2 is a central transcription factor network, could be related to B cell responses.

Despite those results, we note that predefined pathways are inherently limited for analysis of such blood transcriptome data. The pathway databases now available are biased toward oncology and provide limited coverage of immunology. Also, many canonical pathways were defined in extreme or controlled experimental situations (for example, knockout experiments, pathological conditions, drug treatment, etc.), which makes them less suitable for describing how a healthy immune system responds under nonpathological conditions, such as vaccination. Additionally, genes in a pathway are not necessarily coexpressed. Furthermore, heterogeneous cell populations such as PBMCs, in which expression signals from different cell types are mixed, can limit the sensitivity of pathway analysis. Therefore, as described below, we undertook the novel approach of constructing gene-transcription modules for human blood through large-scale data integration.

**BTMs as a novel framework**

Transcriptional networks can be computationally inferred from compendia of gene-expression-profiling data. Large volumes of data on the transcriptome of human blood have already accumulated in public repositories, which provides the opportunity of reconstructing gene networks specific to the context of immune responses in blood tissue. A special form of gene networks, gene modules, has become a powerful tool of systems biology. This module approach was pioneered for human immunology through the use of in-house microarray data. However, one of the greatest challenges is finding modules that can be applied to the thousands of heterogeneous samples across the many different studies and platforms available (Supplementary Note). To fully leverage the power embedded in these public data, we developed a large-scale data-integration approach that reconstructed gene networks from publicly available microarray data and identified context-specific gene modules from the reconstructed networks.

We present here a detailed description of how we constructed the BTMs (Fig. 4a; full technical details, Supplementary Note). We compiled over 30,000 transcriptomes of human blood from more than 500 studies available in public repositories. From each of those studies, we assessed the statistical interdependence of gene expression through the use of mutual information (assessing if the change in expression of one gene was associated with the change in expression of another gene, which would suggest that their expression might depend on each other) and extracted significant gene-coexpression patterns. We connected genes whose coexpression relationships were confirmed in three or more studies to form a high-quality coexpression network, the ‘master network’ (Fig. 4a). Next we obtained subnetworks of specific biological contexts by intersecting that master network with a gene-ontology category, cell type–specific expression, interactome or bibliome. Finally, we implemented a massive parallel strategy to search through all networks for gene modules, defined as densely connected gene groups in a context-specific subnetwork. We also integrated pathway databases and transcription factor–binding data into the search algorithm. This approach identified 334 BTMs,
which we then annotated according to their biological functions and/or tissue-specific expression patterns. Only 37% of those BTMs had an overlap of over 25% with pathways available at present, which indicated that substantial new information had been brought in. Full details of BTMs, including member genes and associated contexts, are provided in our online portal (http://www.immuneprofiling.org/papers/ meni/) and in the Supplementary Tutorial, which presents a step-by-step demonstration of how BTMs can be used by multiple methods.

The BTM-construction process recovered many known protein complexes (Supplementary Note), which demonstrated that our reverse-engineering approach had great sensitivity. For example, 79 human genes from the ribosome complex are presented in the master network and are highly interconnected by 2,418 'edges' (Supplementary Fig. 7a). Additional examples of protein complexes, such as 'spliceosome' and nuclear pore complexes, are represented as BTMs (Supplementary Fig. 7b,c). A large majority of the BTMs represented specific biological processes (80% were annotated). For example, the literature fully supported the finding that the genes in BTM M18 encode molecules involved in T cell differentiation (Supplementary Fig. 8a), yet no database described a pathway that consisted of these genes. Many of the BTMs were self-evident; for example, M240 contained genes mostly specific to the Y chromosome (Supplementary Fig. 8b).

Many statistical methods commonly used for pathway analyses can be applied to our BTMs. For this study, we combined the expression values of member genes into a single module activity score (the mean value). Then, we used a standard t-test and Pearson correlation to assess those activity scores. Using the t-test, we 'benchmarked' the discriminative power of BTMs in the vaccine data sets and additional test data sets and demonstrated superior performance of BTMs compared with that of canonical pathways (Fig. 4b and Supplementary Fig. 9). Using a pathway-module activity score is an established practice44,45. These methods are statistically powerful because random elements in a module are expected to have no net contribution to the score. We thus had a framework with which to assess the statistical significance of correlations to antibody data, for which BTMs could be distinguished from random data (Fig. 4c). We have presented the plasma cell–immunoglobulin module here to illustrate this (Fig. 4d). This module consists of mostly immunoglobulin-encoding genes and genes encoding molecules that bear sequence similarity to immunoglobulins. It is noteworthy that the four genes in this category, TNFRSF17, POU2AF1, MZB1 and CD27, encode known B cell regulators. Because of the inherent nature of this module, we expected its correlation to antibody production. Indeed, the increased activity score of this module at day 7 after vaccination with MCV4 was well correlated to the later DT-specific IgG response (Fig. 4e), and this was consistent with the increase in ASCs at day 7 after vaccination with MCV4 (Fig. 1f). We obtained a similar result for TIV (Supplementary Fig. 10), which also induces population expansion of ASCs at day 7 after vaccination2. These results indicated that gene interactions can be learned from public data and that BTMs provide a powerful tool with which to decrypt immune responses.

Distinctive transcriptional programs in vaccine antibody responses

We applied our BTM framework to examine the transcriptome programs that correlate with antibody responses to different vaccines. For each of the vaccines—YF-17D (live attenuated virus), TIV (inactivated), MPSV4 (carbohydrate) and MCV4 (polysaccharide-conjugate)—we computed the correlation of the module activity score to the antibody response for all BTMs and assessed statistical significance by permuting gene memberships and sample identity. The conjugate vaccine MCV4 yielded two sets of correlation profiles from the same blood transcriptome data: one for the antibody response to DT and one for the antibody response to polysaccharide. Given the distinct nature of those vaccines and variations between cohorts, a unique profile might have been expected for each vaccine, as suggested by the earlier pathway analysis (Supplementary Fig. 6).

However, unexpectedly, the early BTM correlation profiles at day 3 displayed three distinct patterns (Fig. 5a and Supplementary Fig. 11) that represented the protein recall response (shared by TIV and the anti-DT response to MCV4 (MCV4-DT)), the polysaccharide response (shared by MPSV4 and the anti-polysaccharide response to MCV4 (MCV4-Ps)) and the primary viral response (YF-17D). The YF-17D profile at day 3 was very different from that of other vaccines but closely resembled that of YF-17D at day 7. In this context, an important consideration is that differences in the signatures observed with MPSV4 versus MCV4 may have been due to the fact that one vaccine is given intramuscularly and the other is given subcutaneously. However, we feel that that is unlikely, as the signatures that correlated with the anti-polysaccharide responses to MPSV4 and MCV4 were similar (Fig. 5a).

In the primary viral response (Fig. 5 and Supplementary Fig. 12), a greater antibody response was associated with greater activity in interferon-response and cell-adhesion modules. Although it has been shown that interferon activity is induced after vaccination with YF-17D, no correlation has been found between the interferon response and antibody production4,5,37. Now, using our module approach, we identified such robust correlation for YF-17D (Fig. 5b), which was consistent with the ability of YF-17D to induce robust production of type I interferons in plasmacytoid dendritic cell (DCs)44. The cell-adhesion molecules are a hallmark of leukocyte migration and activation and are thus a sensitive indicator of vaccine immunogenicity. Inversely correlated to the antibody response were many cell-division modules, including PLK1 signaling events. The PLK1 pathway is generally an antagonist of interferon signaling46,47 and thus indicated concerted immunological modulation.

The anti-polysaccharide response at day 3 (Fig. 5a, top; Supplementary Fig. 12) involved many modules associated with antigen-presenting cells and their activation, as well as complement and proinflammatory cytokines (Fig. 5b). Such a profile suggested the active involvement of myeloid DCs (Supplementary Figs. 12 and 13), possibly through TNF–transcription factor NF-kB signaling. These modules included genes encoding DC markers (CD83, HLA-DR and HLA-DQ; Supplementary Fig. 14), as well as those encoding inflammatory cytokines (CCL20 and IL1B; Supplementary Fig. 13). We present the correlation of the DC surface signature module to anti-polysaccharide IgG in both MCV4 and MPSV4 (Supplementary Fig. 14b).

We therefore further investigated the involvement of DCs in polysaccharide vaccination. The polysaccharide-containing vaccine MPSV4 was able to induce the maturation of blood myeloid DCs isolated from human PBMCs (Supplementary Fig. 15). We detected upregulation of the expression of costimulatory molecules (Supplementary Fig. 15) and secretion of IL-6, TNF and IL-12p40 in human DCs stimulated by MPSV4 (Fig. 6a and data not shown). We obtained similar results for mouse splenic CD11c+ DCs (data not shown). Using such cells from mice with the appropriate gene knocked out, we demonstrated that the maturation of DCs induced by MPSV4 was dependent on the adaptors MyD88 and TRIF and TLR4, as well as inflammasome activation (Fig. 6b and Supplementary Fig. 16). These data suggested that vaccination with MPSV4 involves pattern-recognition receptors, a finding in line with published reports48. To determine whether those genes noted
Figure 5 BTM analysis reveals distinct mechanisms of antibody response. (a) Vaccine data sets (six segments in color along perimeter) with ordered list of all BTMs for each (bar code–like bands in innermost ring) adjacent to ‘histograms’ of modules (ring exterior to innermost ring) significantly correlated to the antibody response (red, positive correlation; blue, negative correlation; plotted on circumferential gray coordinates), with module names inside outermost perimeter. Curved lines (in color) in the center link significant modules that are common between vaccines (as in Supplementary Fig. 12b–d; gray links for modules omitted in Supplementary Fig. 12). An interactive version of this figure is available (Fig. 2 of the online data portal). D3/0, day 3 versus day 0; D7/0, day 7 versus day 0. (b) Illustration of module activity: black boxes indicate membership of genes (top margin) in the corresponding module (left margin). Right, Pearson correlation between module activity and antibody response in each study. Bottom, correlation between module member genes and the antibody response.
above (Fig. 6b) encode molecules essential for the immunogenicity of MPSV4, we vaccinated mice deficient in those genes with MPSV4 or MCV4. Vaccination with MPSV4 did not reliably induce antibody titers (Supplementary Fig. 17), even after two successive immunizations (Supplementary Fig. 17d), which posed a challenge for further investigation with animal models. However, despite the correlation of the DC surface signature module to anti-polysaccharide IgG (Supplementary Fig. 14b), MCV4 did not induce robust activation of DCs in vitro (Fig. 6a and Supplementary Fig. 16). This suggested that MCV4 may activate DCs in vivo through an indirect mechanism and highlighted the challenges in extrapolating results obtained in vitro to studies in vivo.

The modules whose activity on day 3 correlated with hemagglutination-inhibition titers (TIV) and the DT-specific antibody titers (MCV4-DT) were probably part of a transcriptome program associated with protein recall responses (Fig. 5a, bottom right, and Supplementary Fig. 12). That program has many features of monocytes, especially TLR signaling. Monocytes also have high expression of genes of the formyl peptide receptor–mediated response module. In those modules, TLRs had a coexpression relationship with Tnfrsf13b (which encodes BAFF module M25) and Tnfrsf13 (which encodes the proliferation-inducing ligand APRIL module M48) (Supplementary Fig. 12d and Supplementary Table 2). Together with signaling via the BCR, the correlation between those modules and antibody response indicated downstream activation of B cells. Plasma and memory B cell modules at day 7 were indeed positively correlated with the antibody response to both vaccines (Supplementary Fig. 18).

The distinctive early patterns of the protein recall response and the polysaccharide response become less apparent by day 7 (Supplementary Fig. 19). At that time point, several BTMs were in fact common to three of the five vaccines. For example, cell cycle–related modules were correlated with the antibody responses to MPSV4, MCV4 (anti-DT) and TIV (Supplementary Fig. 19). The antibody response to TIV and the DT-specific response to MCV4 still followed their pattern at day 3 (protein recall response), sharing among the top correlated BTMs plasma cell and immunoglobulin modules (Supplementary Figs. 19 and 20). Tnfrsf17, which encodes the receptor for BAFF and is reported to be a top ‘antibody-predictor’ gene,\(^4,6\) is a member of those modules. Notably, those plasma cell and immunoglobulin modules, as well as memory B cell modules, showed inverse correlation to antibody titers to YF-17D (Supplementary Fig. 12c and Supplementary Table 2), the opposite of the results obtained for TIV and MCV4, for which there was a positive correlation to the antibody responses (Supplementary Fig. 20). That difference may reflect some biology in the putative mechanisms that regulate the generation of short-lived plasmablasts during recall responses (TIV and MCV4) differently than the generation of long-lived plasma cells (YF-17D); this remains to be explored mechanistically.

In summary, our BTM analysis provided rich information about the underlying blood transcriptomes of vaccinated subjects. In addition to the most pronounced features discussed above, we obtained more detailed results (Supplementary Fig. 12 and Supplementary Table 2). For example, T cell activation seemed to be negatively correlated to antibody responses to MPSV4 (day 7) and TIV (days 3 and 7), consistent with the published report\(^6\). A male-specific module (M240) showed negative correlation to antibody responses to each vaccine with varying degrees of statistical significance, which suggested a sex bias in antibody production. That was in agreement with several epidemiological studies showing that vaccinated females have a greater immune response than that of vaccinated males\(^69\).

**DISCUSSION**

The major goals of systems vaccinology are to study the molecular mechanisms of the action of vaccines and to identify signatures that can be used to predict vaccine-induced immunity\(^2,50\). Those goals can be achieved more easily when applied to vaccines such as YF-17D and TIV, which induce robust cellular responses and changes in gene expression in the blood. However, vaccination with MPSV4 and MCV4 induced more subtle changes in the blood transcriptome of subjects vaccinated. Thus, delineating the molecular mechanisms underlying immunity induced by different types of vaccines poses a great challenge. An additional problem inherent to this field\(^27,50\) is the relatively limited size of many study cohorts, which poses challenges in achieving statistical robustness in data analysis. Another challenge stems from the use of canonical predefined pathways, which lack specificity in the context of the analysis of blood
transcriptomes. Using such predefined pathways introduces bias and limits the information that can be obtained from antibody-correlation analysis. To overcome those challenges, we developed a large-scale data-integration approach that leverages prior knowledge and data in the public domain to construct BTMs and to develop them into a robust yet interpretable statistical framework. We chose to use a straightforward module-activity score and to compute its Pearson correlation to antibody data with permutation tests. However, BTMs can be used as gene sets for GSEAs and for other pathway methods, which makes it easy for any researcher to study BTM activity in their own experiments.

We observed two sets of antibody-correlation profiles for the conjugate vaccine MCV4: one was specific to anti-polysaccharide and another was specific for anti-DT. Such resolution of ‘dual profiles’ was achieved only by our BTM approach, not by gene-level analysis, and therefore represented a considerable increase in sensitivity. That result also indicated that distinct molecular mechanisms are induced by different components of the same vaccine, and those may affect the study and development of conjugate vaccines in general.

An unexpected result of our study was that although both MPSV4 and MCV4 induced robust polysaccharide-specific antibody responses, the vast majority of plasmablasts induced by MCV4 secreted antibodies to the carrier protein rather than to the polysaccharides. This raises the possibility that carbohydrate-specific antibodies are produced by a particular B cell population that is not present in the blood.

Our BTM framework identified three distinct patterns in blood transcriptomes at day 3 after vaccination that were related to the nature of the vaccines studied. Among the BTMs whose activity correlated to antibody response at 1 month or later, we found type I interferon modules for the primary viral response (YF-17D), modules associated with DC and complement activation for polysaccharide responses (MCV4-PS and MPSV4) and modules such as BCR signaling and plasma cells–immunoglobulins for protein recall responses (TIV and MCV4-DT). Several additional BTMs were correlated to antibody responses, which indicated potential previously unknown networks of innate immunity that should be further characterized.

These data suggest that gene-expression predictors of antibody response are probably not ‘universal’ but are dependent on the type of vaccine, which is consistent with the proposal that different classes of vaccines would induce similar signatures of immunogenicity.

These analyses also enabled the formulation of hypotheses about some novel mechanisms of vaccine-induced immunity. For example, the ‘signaling in T cells (1)’ module showed strong correlation to the carbohydrate-specific antibody response to MPSV4 (a carbohydrate-containing vaccine). Contrary to the prevailing belief that immune responses to MPSV4 are T cell independent, our results raised the possibility that CD4+ T cells may be involved. That hypothesis is consistent with a published report describing polysaccharide–specific T cells.

Furthermore, unexpectedly, modules for RIG-I-like signaling and the transcription factor IRF2 showed strong correlation to carbohydrate-specific antibody responses to MPSV4 and MCV4. That raised the possibility that those vaccines may use the RIG-I pathway to induce antibody responses; this remains to be tested. Finally, the observation that the AT2F network correlated with antibody responses suggested a potential role for the integrated stress response in mediating antibody responses. That was consistent with the roles of EIF2AK4 as a predictor and a regulator of the later CD8+ T cell response induced by vaccination with YF-17D.

Finally, while our BTMs offer a tool with which to analyze data on the blood transcriptome, further research is needed to better model the system and understand it. Thus, the fine kinetics and potential regulatory mechanisms of genes in the BTMs could be elucidated by studies with more frequent sampling of blood. Given the relatively modest strength of the early transcriptional signatures induced by MPSV4 and MCV4, a key question is the extent to which such changes represent variations in environmental confounding factors, such as external temperature, time of collection of blood and atmospheric pressure. However, the biological relevance of many of these signatures to vaccine-induced immunity indicates that it is likely that these results were indeed due to vaccination. Nevertheless, future studies could benefit from the inclusion of groups of subjects given placebo. Furthermore, this analysis presents several hypotheses about the functional relevance of the networks identified. In summary, our findings highlight the utility of systems biology approaches in discovering novel correlates of immunogenicity to vaccination. The identification of a compendium of signatures that correlate with antibody responses to different classes of vaccines represents a first step in addressing the question of whether there are universal correlates of antibody responses to vaccination. Extending this analysis to a broader range of vaccines will pave the way toward the development of a ‘vaccine chip’ that could be used to predict vaccine-induced immunity for a broad range of vaccines.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. GEO: microarray data for MPSV4 and MCV4, GSE52245; for YF-17D, GSE13485; for TIV, GSE29617; and for LAIV: GSE29615. Access to Online data portal (Firefox and Safari recommended for best results): http://www.immuneProfiling.org/papers/meni/.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.L. and S.D. did experiments in nature immunology; S.R.-S., D.S.S., S.E.J., A.M., G.R. and G.M.C. did experiments in Immunity; S.P., C.O., D.C. and A.K.P. helped with study design and presentation; N.R. organized the clinical study; R.A. supervised the study in Atlanta (laboratory), the Georgia Research Alliance GRA and Emory University Research Committee and the Clinical and Translational Science Award Program (National Center for Research Resources of the US National Institutes of Health; to N.R.). The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the Centers for Disease Control and Prevention.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Clinical study organization. Healthy adult volunteers (18–45 years of age) were randomly assigned to groups to be vaccinated subcutaneously with MPSV4 (Menomune (Sanofi Pasteur); n = 13 subjects) or intramuscularly with MCV4 (Menactra (Sanofi Pasteur); n = 17 subjects). Whole blood samples were collected into cell-preparation tubes at days 0, 3, 7, 14, 30, 180 and 750 after vaccination. Random assignments were made by the pharmacist using a Research Randomizer Form. The trial was double-blinded for participants and laboratory staff during data collection. Informed consent was obtained from all participants before any study procedure. This research was approved by the Emory University Institutional Review Board and by the CDC Human Subject Office. The trial was designed as an exploratory study, without pre-specified effect size. All samples from the subjects that completed the study were included for analysis.

Cell and plasma isolation. PBMCs and plasma were isolated from fresh blood in cell-preparation tubes (Vacutainer with Sodium Citrate; BD) as described. PBMCs were frozen in DMSO with 10% FBS and stored at −80 °C, then were transferred the next day to liquid nitrogen freezers. Plasma samples from cell-preparation tubes were stored at −80 °C. Trizol (Invitrogen) was used for lysis of fresh PBMCs (1 ml of Trizol for ~1.5 × 106 cells) and to protect RNA from degradation. Samples prepared with Trizol were stored at −80 °C.

Blood transcriptome analysis. Total RNA was extracted from PBMCs stored in Trizol according to the manufacturer’s recommendation (Invitrogen). The quality of RNA was evaluated by a NanoDrop spectrophotometer and BioAnalyzer (Agilent Technologies). DNA microarray experiments were performed with one dose of poly-l-lysine conjugated vaccine (Menomune; Sanofi Pasteur) was added to the DT carrier protein was assessed at days 0, 7 and 30 by an anti-DT antibody (109-005-064; Jackson Immunoresearch) at a concentration of 1:4. Serum was also tested with N. meningitidis serogroup A strain 3125, L10 immunotype as described.

Measurement of anti-meningococcal antibodies by ELISA. Immulon 2 HB plates (Thermo) were coated with N. meningitidis serogroup A purified polysaccharide at a final concentration of 5 µg/ml or N. meningitidis serogroup C purified polysaccharide at a final concentration of 2.5 µg/ml (NIBSC). Mixed together for a final concentration of 2.5 µg/ml methylated serum albumin of serogroup A and of 5 µg/ml methylated serum albumin for serogroup C. A blocking step with 0.01 M PBS buffer (pH 7.2) containing 5% newborn BioAnalyzer (Agilent Technologies). DNA microarray experiments were performed with one dose of poly-l-lysine conjugated vaccine (Menomune; Sanofi Pasteur) was added to the DT carrier protein was assessed at days 0, 7 and 30 by an anti-DT antibody (109-005-064; Jackson Immunoresearch) at a concentration of 1:4. Serum was also tested with N. meningitidis serogroup A strain 3125, L10 immunotype as described.

Measurement of ASCs by flow cytometry and enzyme-linked immunosorbent analysis. ASCs were identified by flow cytometry as CD3–CD20–CD38hiCD27hiCD19+ cells as described. Cells secreting antigen-specific total IgG, IgM and IgA were quantified by enzyme-linked immunosorbent assay (ELISPOT) analysis. Goat antibody to human IgG, IgM and IgA (capture antibody 109-005-064; Jackson Immunoresearch) at a concentration of 100 ng per well was used for capture of total immunoglobulin. For capture of antibodies specific for meningitis polysaccharides, the tetravalent meningococcal polysaccharide vaccine (Menomune; Sanofi Pasteur) was conjugated to the DT carrier protein was assessed at days 0, 7 and 30 by an anti-DT antibody (109-005-064; Jackson Immunoresearch) at a concentration of 1:4. Serum was also tested with N. meningitidis serogroup A strain 3125, L10 immunotype as described.

Integration of interactome and bibliome to vaccine response networks. The interactome data used in this study was retrieved from Pathway Commons (26 October 2011), including data from the following databases: the Human Protein Reference Database (HPRD), BioGRID (BioTek), IntAct (IntAct), the Molecular Interaction database (MIBiome), Reactome, the pathway-interaction database of the US National Cancer Institute and the Nature Publishing Group (Nature Publishing Group and HumanCyc). The Bibliome network was built with gene keywords in PubMed entries (retrieved 26 August 2010), for which the concurrence of two genes in the same paper constituted a connection between two genes. Papers with ten or more keyword genes were excluded, as they were probably based on high-throughput assays. From a data set of a blood transcriptome after vaccination, DEGs were identified with a P value of <0.001 in Supplementary Table 1, no gene filtering. A DEG network was first built by connection of the upregulated DEGs with the ‘edges’ from reference network (interactome or bibliome). Next, a ‘linker’ gene was added to the DEG network if its connecting neighbors showed significant enrichment in the DEG network relative to its neighbors in the interactome-bibliome network (Fisher’s exact test; P < 0.05 after Benjamini-Hochberg correction). The validity of those results could be evaluated by examination of the ‘linker’ genes. For example, among the 312 ‘linker’ genes added to the MCCV network from interactome, 115 had a P value of <0.05 in the gene-expression data (enrichment P value, 1.60 × 10−19). Data obtained with the RTS,S vaccine doi:10.1038/ni.2789
against malaria\textsuperscript{5} were used for further validation, for which the 1,000 genes with the lowest \( P \) values in the paired \( t \)-test between the third vaccination and 24 h after vaccination were used as input DEGs. The same integration method yielded an RT6,8 response network of 2,951 genes, including 1,231 genes found in our common network with four or more vaccines.

**Pathway and gene-ontology analyses.** We retrieved the XML version of the pathway-interaction database of the US National Cancer Institute and the Nature Publishing Group (14 September 2011)\textsuperscript{28} and ‘parsed’ them into plain-text gene sets. Those pathways were used as external file in the GSEA program\textsuperscript{23}. Genes were ranked by \( t \)-score (comparison of the transcriptome after and before vaccination) or Pearson correlation (correlation of gene expression with antibody response) and were input into GSEA as an externally supplied preranked list. This approach offers good sensitivity and robustness without relying on gene selection, as do other over-representation methods. The significance of pathway enrichment in either scenario was assessed by permutation in the GSEA program. The DAVID (Database for Annotation, Visualization and Integrated Discovery) bioinformatics server of the National Institute of Allergy and Infectious Diseases\textsuperscript{66} was used for the gene-ontology enrichment test (Fig. 3c).

**BTM construction and correlation to antibody response.** The construction and evaluation of BTMs is described fully in the Supplementary Note. The expression values of member genes in a module were combined into a single module activity score (the mean value). The module activity scores were used for subsequent analysis, such as Student’s \( t \)-test or Pearson correlation. All data obtained in vaccination studies were excluded from the BTM-construction process. In antibody-correlation analyses, the statistical significance of BTMs was estimated by comparison to random-permutation data (i.e., module memberships) and sample labels were permuted for estimation of the null distribution. Such significance was further enhanced when the same module was found in two independent data sets of vaccine studies. The analysis result with GSEA using BTMs was similar to that presented in this paper (Supplementary Table 3).

Data for polysaccharide-specific IgG (sum of serogroups A and C) at baseline (day 0) was subtracted from the results obtained at day 30 after vaccination, and the resultant values were used for the antibody responses to MCV4 and MPSV4, as they were robust indicators throughout the study.\textsuperscript{56} The results obtained at day 30 after vaccination and the resultant values were used for the antibody responses to MCV4 and MPSV4 vaccines (final concentration 0.1×, 0.01× and 0.001×) or lipopolysaccharide (130-090-506 and 130-050-201; Miltenyi Biotec). DCs were stimulated overnight (at a density of 1 × 10\textsuperscript{6} cells per ml) in 96-well plates with dilutions of the MPSV4 or MCV4 vaccines (final concentration 0.1×, 0.01× and 0.001×) or the following TLR-ligand controls (Invivogen): Pam\(_3\)Cy\(_4\) (10 \( \mu \)g/ml), poly(I:C) (100 \( \mu \)g/ml), LPS (10 \( \mu \)g/ml), FSL-1 (100 \( \mu \)g/ml), R-848 (10 \( \mu \)g/ml) or CpG (10 \( \mu \)g/ml). Cell supernatants were collected and IL-6, TNF and IL-12p40 were measured with ELISA kits according to manufacturer’s instructions (555220, 555212 and 555171; BD Biosciences). All mice were 12–16 weeks old and were matched by sex and age in all experiments. Mice were housed and handled according to local, state and federal regulations. All experimental procedures were carried out according to protocols approved by the Institutional Animal Care and Use Committee at Emory University.

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