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Adjuvanted influenza-H1N1 vaccination reveals lymphoid signatures of age-dependent early responses and of clinical adverse events

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Author Contributions

O.S. and E.B. co-ordinated experiments, designed and undertook phenotypic analyses of PBMC and sera, and contributed to data analysis; S. O'F. designed and undertook molecular analyses of PBMC gene expression and provided data analyses; A.L., J.P., Y.H., J.D., and R.S. developed and applied statistical and bio-informatic tools for data analysis and presentation; J.C. supervised and undertook PBMC sample preparation and bio-banking; M.Z. supervised and undertook HAI and MN assays of antibody titers; M.H.M. co-devised the study, oversaw bio-banking, and edited the manuscript; M.P. and A.C. oversaw and implemented T cell assays, clinical assessment measures and obtainment of ethical approval; I.C. and G.V.K. devised and implemented integrative data analysis strategies, prepared figures, and edited the manuscript. A.C.H. designed and supervised the study; co-wrote documents for ethical approval; reviewed all data sets; and wrote and edited the manuscript together with O.S. and S. O'F.

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Abstract

Adjuvanted vaccines afford invaluable protection against disease, while the molecular and cellular changes they induce offer direct insight into human immunobiology. Here we show that within 24 hours of receiving adjuvanted swine flu vaccine, healthy individuals made expansive, complex molecular and cellular responses that included overt lymphoid as well as myeloid contributions. Unexpectedly, this early response was subtly but significantly different in those aged over ~35 years. Wide-ranging adverse clinical events can seriously confound vaccine adoption, but whether there are immunological correlates of these is unknown. Here we identify a molecular signature of adverse events that was commonly and surprisingly associated with an existing B cell phenotype. Thus immunophenotypic variation among healthy humans may be manifest in complex pathophysiologic responses.

The profound impact of immunization on public health is well illustrated by the recently reported efficacy of an Ebola-specific vaccine[1,2]. Moreover, the potential applications of vaccines have broadened greatly as the biological implications of immunology have extended from infectious diseases, allergy and autoimmunity to metabolic disease[3], neuro-degeneration[4] and cancer[5]. Yet because most immunological experiments are performed in mechanistically tractable model systems, our understanding of human immune response dynamics has lagged behind.

This situation has been somewhat redressed by advances in high-throughput analytical technologies, and in informatics and biostatistics: so-called “systems vaccinology”[6]. Thus, several studies have shown that many immunological metrics show little day-to-day variation[7,8], thereby permitting reliable measurements of their responses to scheduled perturbations such as vaccination[6], immune checkpoint-blockade[9], or iatrogenic immune-suppression[10]. Nonetheless, more studies are required to help establish general principles, for example, whether there are consensus immune signatures predicting poor responsiveness to particular modalities, and whether discrete immunological phenotypes might be linked to adverse events (AEs). Transient AEs are often belittled as “man-flu”, but they can undermine herd immunity by provoking diminished vaccination compliance and even halting vaccine trials[11]. Additionally, there have been highly public concerns that vaccines may exacerbate pre-existing pathologies.

Influenza (flu) virus causes serious, potentially life-threatening disease. Because of seasonal strain variation, annual vaccination is recommended[12]. However, due to year-to-year similarity among seasonal flu strains, vaccine responses commonly reflect flu-specific memory cell re-activation[8,13]. Conversely, a Human Immune Response Dynamics (HIRD) study described here monitored the response to H1N1 “swine flu” which circulated in 2009 and was the first major H1N1 outbreak in several decades[14]. Whereas those aged over 40-years might harbor swine flu-specific memory cells, this was unlikely in younger individuals who comprised the bulk of the HIRD cohort. Thus, the study offered an opportunity to investigate responses to flu vaccination that were not obviously dominated by recall responses.

Importantly, and in contrast to flu vaccines used in American immune-monitoring studies[8,13,15,16,17], the UK H1N1 Pandemrix vaccine included an adjuvant, AS03, designed to enhance immunogenicity[18,19]. However, whether the early response differs quantitatively and/or qualitatively from non-adjuvanted flu vaccines has not hitherto been investigated by systems vaccinology. Interestingly, rather than being the sole province of myeloid cells, early phases of immune responses are increasingly acknowledged to include contributions from innate lymphoid cells and innate-like T cells, as in γδ T cell cross-priming of $CD8^+$ T cells[20,21,22]. Whether adjuvanted vaccines might promote such lymphoid stress-surveillance is unknown.

The HIRD trial longitudinally tracked 178 healthy volunteers, through two pre-vaccination and four post-vaccination time-points. The trial's outcomes confirmed the practicality of high-throughput immune monitoring, which is important as immunotherapies are more widely adopted. They established that within 24 hours of receiving adjuvanted flu vaccine, healthy humans mount large and complex immune responses including overt lymphoid contributions. Surprisingly, this early response was slightly but significantly different in individuals aged over ~35 years. By contrast to some reports[8,13,15,16,17], vaccine non-responsiveness seemed to reflect broad human phenotypic variation rather than a consensus immune signature. Conversely, post-vaccination AEs reported by ~20% of the healthy volunteers were associated with a discrete molecular signature, that was frequently and surprisingly associated with an atypical, pre-vaccination B cell phenotype. Thus, individual variation in immune composition can have complex pathophysiologic manifestations. This notwithstanding, vaccination did not obviously exacerbate existing immune dysregulation.

Results

Expansive transient responses to adjuvanted vaccination

From March 2010 to August 2011, the HIRD study consented 178 healthy adult volunteers (aged 18-65 years) with no known history of cancer, immunodeficiency, autoimmune or inflammatory diseases, and no recent infection or vaccine exposure (Table 1; Supplementary Table 1). Each volunteer underwent a standard health examination, including blood and urine analysis, whereafter they completed six morning visits on pre-vaccination days −7 and 0, and post-vaccination days +1, +7, +14 and +63 (Supplementary Figure 1). At each visit, fasting peripheral blood was sampled and banked. Completing the day 0 visit, volunteers received a single intramuscular injection of Pandemrix™ swine flu vaccine, comprising 3.75 mg antigen from the A/California/7/2009 H1N1 virus strain in adjuvant AS03 (GSK) containing DL-a-tocopherol (vitamin E), squalene and polysorbate 80. At day +63, serological vaccine responses were measured at the UK Health Protection Agency (HPA) by both hemagglutination inhibition (HAI) and micro-neutralization (MN). Responders were defined by convention as those making ≥4-fold increases in titer in one or both assays. Approximately 20% of volunteers were non-responders (Table 1) (note that occasional test failures meant that the denominator for any one test shown in Table 1 may be lower than 178). At all visits, volunteers completed Likert wellness assessments[23], revealing that ~20% of males and ~25% of females experienced above-average clinical AEs, primarily at day +1 (Table 1). Of the 178 vaccinees, 139 individuals had sera assessed by multi-parameter

Luminex, of whom 60 had white blood cells analyzed by flow cytometry, of whom 46 representative individuals, selected without bias, had peripheral blood gene expression assessed by microarray at day −7, day 0, day +1, and day +7 (Supplementary Fig. 1a). Thus, all subjects whose gene expression was assessed were also assessed by all other methods. All platforms were subject to rigorous quality controls (**Methods** and Supplementary Materials).

Essentially all volunteers analyzed showed expansive changes in peripheral blood gene expression by day +1 (significant changes in ~9,000 gene probes; Supplementary Fig. 2a), highly consistent with other studies that did not employ adjuvants[8,16,24,25]. Quantitative RT-PCR of 30 randomly selected genes across ~20 individuals provided independent validation of the microarray quantification at each time-point (Supplementary Fig. 2b,c). Collective gene expression patterns on day +1 differed significantly from pre-vaccination time-points (day −7; day 0), and from gene expression six days later (day +1 *versus* day +7) (red dots, Fig. 1a). Predictably, the genes most highly upregulated at day +1 were enriched in those encoding and/or associated with antigen-presentation and interferon (IFN) responses (Fig. 1b). Largely explaining this, most individuals at day +1 displayed transient but significant (average =1.6-fold) increases in the blood monocyte fraction, set against slight but significant decreases in recoverable peripheral blood cell numbers (Fig. 1c; Supplementary Fig. 2d). Of note however, for the 46 individuals assessed by microarray, the increases in monocyte numbers were significantly less than the fold-changes (up to 160x) in expression of over 135 genes including those encoding activation-sensitive regulators (e.g. *STAT1* [>16x] and *IRF1* [6x], *IRF7* [>3.5x], *IRF9* [>2.5x]), and markers of cell activation (e.g. multiple *HLA-DR* genes [from 2x to 2.5x], and *TLR8* [3.5x]) (Supplementary Fig. 2e). Thus, vaccination rapidly induced changes in PBMC gene expression over and above the changes induced in PBMC cell composition. Most day +1 changes returned to baseline by day +7, by which time blood plasma cell counts were greatly increased, as was the expression of >1000 genes, many associated with plasma cells (Fig. 1a,b,d; Supplementary Fig. 2a).

Changes in peripheral blood monocyte composition and gene expression at day +1 were associated with increased blood concentrations of cytokines and chemokines, notably CCL4, CCL2, interleukin 6 (IL-6) and G-CSF, mostly attributable to myeloid cells (Fig. 1e)[7,13,26]. Unexpectedly however, the biggest fold-change was in IFN-γ, a cytokine associated with T-helper 1 (T_H1), cytolytic T, γδ T, NKT, NK and Type-1 innate lymphoid cells (Fig. 1e). Thus, the expansive, multifaceted transient changes to blood cell composition and gene expression that occurred within 24h of receiving adjuvanted H1N1 vaccine included a distinct lymphoid contribution.

Early myeloid and lymphoid activation

The changes in day +1 peripheral blood monocyte composition and gene expression (Supplementary Fig. 3a) were reflected in monocyte activation, as evidenced by significant increases in the percentage of cells expressing HLA-DR, and in the amount per cell of surface-expressed HLA-DR (Fig. 2a,b). By contrast, there was a significant, transient decrease in T cell representation and in transcripts encoding T cell functions, particularly

signaling molecules (for example, *CD247*, *ZAP70*, *LCK*, *LAT*, and *FYN*) (Supplementary Fig. 3a; Fig. 2c). These findings do not simply counter-balance the increases in blood monocytes, because the losses among $CD8^+$ αβ T cells and γδ T cells were mostly confined to HLA-DR^-CD38^- cells. Thus, the blood became *de facto* enriched in activated lymphocytes, particularly HLA-DR^+CD38^+ cells (Fig. 2d).

A functional consequence of early myeloid and lymphoid activation was the overt upregulation of myriad IFN-stimulated gene transcripts (ISGs) (Fig. 2e). Although many ISGs probably reflected the actions of Type I/III IFN (IFNα/λ), they also included *CXCL9* (arrowed – Fig. 2e; red dots, Supplementary Fig. 2c) which is much more strongly upregulated by IFN-γ. Moreover, the day +1 peak in blood protein concentration of CXCL10, which can be upregulated by IFN-α or IFN-γ, paralleled IFN-γ expression (Fig. 2f) rather than IFN-α expression that peaked at day +7. Intriguingly, the striking increases in IFN-γ and IFN-α serum protein concentrations were not mirrored by *IFNG*, *IFNA* (or *IFNL*) transcript abundance on either day +1 or day +7 (Fig. 2e). This paradox might reflect enhanced IFN protein synthesis and/or secretion rather than increased *IFN* gene expression. Additionally, peripheral blood ISGs may have been upregulated by IFN proteins leaking into the blood from IFN-expressing cells within the vaccine injection site and/or the associated lymph nodes.

Among serum cytokines and chemokines, the most striking early marker of immune challenge was CXCL10, in part because baseline concentrations were consistently low: whereas >90% of 130 subjects displayed pre-vaccination CXCL10 concentrations of less than 1ng/ml, this become the median concentration by day +1 (Fig. 2f). Although significant increases also occurred in tumor necrosis factor (TNF) and IL-6, the pre-vaccination concentrations of these cytokines showed high individual variation that increased following vaccination (Supplementary Fig. 3b). Likewise, inter-individual variation largely obscured dynamic responses of other myeloid and lymphoid mediators, with post-vaccination expression in some cases (e.g. IL-10) best described by a bi-modal distribution (Supplementary Fig. 3c). This notwithstanding, significant day +1 increases were evident for CCL5, another lymphoid cytokine (Supplementary Fig. 3d). In sum, adjuvanted H1N1 vaccine rapidly upregulated multiple myeloid and lymphoid effector functions, including Type I and Type II IFNs.

Age affects early responses

This study was not designed to explore the well-established compromise in adaptive responses in subjects aged over 70[27,28]. However, we unexpectedly identified subtle but significant impacts of age upon day +1 gene expression. 40 subjects for whom gene expression data were available across two pre-vaccination and two post-vaccination time-points were grouped into successive younger and older subsets by a barrier method: that is, the youngest five were compared to the remaining 35 (“i5”); the youngest 6 to the remaining 34 (i6); through to the youngest 35 being compared to the remaining five (i35) (Supplementary Fig. 4 and Fig. 3a *x*-axis). This revealed a pattern not seen when subjects were randomly partitioned into 10^5 different, comparably sized groupings. Specifically, the aggregate day +1 gene expression signature was significantly different when ranks above i19

(right of vertical red line, Fig. 3b) were compared with younger subjects. At this point of divergence in gene expression, the median age of the comparison groupings was >40 and ~22, respectively (Fig. 3a, solid blue and yellow squares). Moreover, at i21 where divergence was very significant, the minimum age of the older group was ~30 (Fig. 3a; open blue circles). This indicated that significant changes in the early response profile may occur at ≥30-40 years of age, much younger than is usually considered in the context of age-related immune dysregulation. Interestingly, transcripts underpinning the age-dependent differences included several functionally implicated in the early response (e.g. *TBX21, CD38*, and *CD28*), and others implicated in immune regulation, e.g *HDAC4* (Supplementary Table 2).

Based on these unexpected findings, subjects were segregated into those aged above or below 35 years, respectively. Clearly, the day +1 increases in IFN-γ and CXCL10 serum concentrations, and the PBL depletion of $CXCR3^{+}CD8^{+}$ T cells were all significantly less in the older cohort (Supplementary Fig. 5a,b). Although the most profound impact of age on gene expression was at day +1, there were also some significant differences in pre-vaccination lymphocyte subset counts in those ≥35 years (Supplementary Fig. 5c). Perhaps consistent with this, six age groupings (i27-i32) showed significant divergence in pre-vaccination gene expression (Fig. 3b, black line).

It might be hypothesized that the difference in day +1 responses reflected younger subjects making primary responses *versus* older subjects making efficacious recall responses. However, the changes in titer (0 - $>10^{2}$-fold) of influenza-specific antibodies, measured by either hemagglutination inhibition (HAI) or micro-neutralization (MN), were spread comparably across subjects with low and high pre-vaccination titers, and showed no correlation with age (Supplementary Fig. 5d). Consistent with this, the day +7 plasmablast-dominated gene expression pattern was mostly unaffected by age (Fig. 3b, red line), with only one group (i30) showing a substantial divergence, possibly reflecting its inclusion (relative to i31) of a profound non-responder, aged 48 years. In sum, the available data did not obviously support the view that the early response in those aged over ~35 years is altered by a shift toward making recall responses.

When an analogous assessment was made of subjects grouped by gender, the peripheral blood gene expression patterns at all time-points displayed striking sexual dimorphism, as reported[29] (Supplementary Fig. 5e). However, this dimorphism was removed by normalizing gene expression at each time-point to that at day −7 (pre-vaccination). Thus, the capacity of vaccination to induce expansive changes in gene expression was essentially unaffected by significant pre-existing differences in the gene expression profiles of males and females.

Broad phenotypic variation underpins vaccine non-response

Compliant with international guidelines, subjects making ≥4-fold increases in influenza-specific antibodies measured by HAI and/or MN were defined as responders. Approximately 80% were responders (Table 1; Fig. 4a) compared to ~70% reported in Pandemrix efficacy trials[18], probably reflecting HIRD's stringent exclusion criteria. Non-responders collectively displayed a complete spectrum of preexisting HAI and MN titers (Fig. 4a), including "baseline" non-responders whose low starting titers did not increase ≥4-fold, and so-called

"glass ceiling" subjects whose high starting titers made enhancements by ≥4-fold harder, albeit not impossible, to achieve.

The frequency distribution of the day +63 / day +7 MN titer ratios adhered to an additive model of two quasi-Gaussian distributions that respectively partitioned the HIRD cohort into one group with a very broad range of ratios between 0 and >30 (red line, Fig. 4b), and one group with a narrow range of MN ratios, mostly ≤5 (blue line, Fig 4b). This model suggests that non-responders may have arisen from at least two groups of subjects, challenging the prospect of a consensus origin of vaccine failure. To further investigate this, gene expression patterns of 23 responders and 18 non-responders were compared. The most common non-response pattern was reduced expression of genes associated with plasma cell development and antibody production (Fig 4c), as previously reported[8,17,24,30,31], coupled to a poor (albeit not absent) day +7 expansion of $CD27^{+}CD38^{hi}$ plasmablasts (Fig. 4d). Conversely, B cell genes highly expressed by responders included three (*MZB1*, *TNFRSF17*, and *XBP1*; labeled red, Supplementary Fig. 6a) likewise linked to responsiveness in other studies[8,17,24,31].

Although non-responders shared a failure to activate plasmablasts, there were many different "routes to failure", as illustrated in Fig. 4e in which day −7/0 (pre-vaccination), day +1, and day +7 gene expression patterns, represented by principal components, were tracked for six baseline non-responders relative to the consensus responder pattern (blue line and areas). Thus, subject #402B (red) did not significantly alter any genes; #39 (yellow) altered many day +1 genes but returned to baseline; #3 (purple) started with an atypical pattern, regulated day +1 genes, but did not progress beyond that; #11 (orange) regulated some day +7 genes but without a preceding day +1 signature; #12 (black) regulated day +1 genes but did not reach the day +7 signature; and #164 (brown) started with a highly atypical pattern and reverted toward the consensus pre-vaccination signature. Thus, non-responders presented a broad spectrum of phenotypes.

Consistent with this, no common non-responder signature could be found among individual and combinatorial cell phenotyping and analyte expression patterns. There was a significant association with higher frequencies of activated γδ T and NK cells at all time-points (Supplementary Fig. 6b–d), but these frequencies were so variable among individuals that high frequencies could not predict non-responsiveness. Baseline non-responders often showed higher IL-17 concentrations than either responders or glass ceiling non-responders, but again IL-17 concentrations could not effectively segregate the groups (Supplementary Fig. 6e). In sum, neither molecular nor cellular data offered any consensus pre-vaccination predictor of non-responsiveness akin to those proposed in studies of non-adjuvanted vaccines[8,16,17,24,31].

Peripheral blood signature of pathophysiology

Using well-established methods for quantitating patient perception of symptoms such as pain[23], the short-term clinical outcome of all subjects was assessed across a spectrum of adverse events (AEs) (Fig. 5a). Most AEs related to day +1, with fewer prolonged events captured from day +1 to day +7. After pooling these two time-points, subjects were grouped

into those with high, medium and low AEs, respectively. There was no obvious correlation of AEs with gender or age, or with vaccine responsiveness (Table 1; Fig. 5b).

Given that the HIRD cohort was healthy with no known immunological dysregulation, we initially considered that high/medium AEs might reflect exaggerated, innate-like recall responses of memory lymphocytes. However, neither pre-vaccination memory T cell numbers (Supplementary Fig. 7a,b), nor baseline antibody titers correlated with AEs. Although subjects with high/medium AEs collectively showed higher day +1 CXCL10 upregulation, inter-individual variation confounded any prospect that CXCL10 concentrations might distinguish those with high/medium AEs (Fig 5c). Moreover, AEs did not correlate with circulating TNF, IL-6, or IFN-γ concentrations or with commonly employed markers of inflammation, including serum amyloid A (SSA) and C-reactive protein (CRP) (Fig. 5c,d). By contrast, those with high/medium AEs showed strikingly higher transient day +1 expression of a small set of genes, including *LNX2, BCL2L14, RARRES3, KRT9, CNDP2, RM12, METTL6,* and *TGM2* (Fig. 5e). While it remains to be seen whether any such genes directly relate to clinical symptoms, their capacity to segregate the HIRD cohort into those with high/medium AEs asserts a *bona fide* peripheral blood signature of self-reported vaccination-associated illness, commonly dismissed as “man flu”.

Transitional B cells, autoimmunity and adverse events

To investigate whether any pre-vaccination immunological profile might identify those prone to AEs, we further investigated differential gene expression. Most unexpectedly we found that relative to subjects with low AEs, the PBMC of those with high/medium AEs overexpressed many B cell genes, prior to and following vaccination (Fig. 6a; Supplementary Fig. 8a). This signature often correlated with an over-representation of $CD27^{-}CD38^{hi}CD24^{hi}$ transitional B cells prior to vaccination (Fig. 6b), a trend that gained statistical significance immediately post-vaccination (Fig. 6c). By contrast, AEs showed no association with total B cell counts (Fig. 6d).

Pre-vaccination transitional B cell numbers were previously linked with non-responsiveness following adjuvant-free flu vaccination[8]. However, the HIRD study found no correlation of vaccine efficacy with the percentages of transitional B cells, memory B cells, or naïve B cells (Supplementary Fig. 8b,c), whether responsiveness was classified as a categorical variable (≥4-fold increases in titer), or as a continuous variable. Indeed, baseline non-responders were spread across the full spectrum of transitional B cell subset representation (Supplementary Fig. 8b, red dots).

Interestingly, transitional B cell expansion has previously been associated with autoimmunity[32,33]. Therefore, to further test the unanticipated association of transitional B cells with pathophysiology, we investigated whether subjects with high/medium AEs might harbor autoantibodies, despite their being outwardly healthy. Remarkably, 8 of 31 subjects with high/medium AEs, compared to only 1 of 37 subjects with low AEs, displayed high-titer pre-vaccination reactivities to thyroglobulin and/or thyroid peroxidase, two specificities commonly tested for in the clinic (Fig. 6e). Of note, however, vaccination did not affect autoantibody titers, as assessed from day −7 to day +63 (Fig. 6f). In sum, there was within the healthy HIRD cohort a sub-population with increased frequencies of transitional B cells,

one quarter of whom unknowingly harbored autoantibodies of potential clinical significance, and this sub-population was more prone to developing AEs in response to adjuvanted H1N1 vaccine.

Discussion

Vaccines remain a cardinal plank of public health and several strategies are employed to promote their immunogenicity. For example, a recently developed Ebola vaccine used vaccinia virus to deliver DNA encoding an Ebola antigen[2]. An alternative strategy is to deliver antigens in the context of adjuvants, such as AS03, used in the Pandemrix™ H1N1 vaccine. AS03 dramatically enhanced B cell responses to bird flu when compared to another adjuvant, MF59 or to no adjuvant[34]. However, the human immune response to adjuvanted flu vaccines has not been assessed by systems vaccinology.

In that regard, this study shows that Pandemrix™ H1N1 vaccine provokes a rapid, expansive, yet transient activation of myeloid cells and effectors, comparable in pattern to changes induced by other vaccines, including flu vaccines lacking adjuvant[8,13,15,16,17]. However, the HIRD study also revealed a pronounced lymphoid contribution to the early phase of the immune response, most evident in the prominent transient upregulation of IFN-γ that was not apparent in most other virus vaccine studies. Indeed, IFN-γ increases apparent at day +2 following Yellow Fever vaccine[35] were not statistically significant. Conversely, IFN-γ RNA was upregulated in muscles of mice 4h after injection of AS03 plus viral antigens, with IFN-γ detected in the draining lymph nodes[19]. The functional consequences of IFN-γ upregulation remain to be clarified, but may at minimum have included CXCL9 and/or CXCL10 upregulation since their expression closely paralleled that of IFN-γ in the HIRD study and in AS03-treated mice.

The issues remaining to be clarified include: the degree to which rapidly induced IFN-γ and/or other lymphoid effectors may or may not explain the efficacy of AS03 and/or other adjuvants; the cell types most responsible for the early lymphoid response; and the molecular cues that promote the rapid activation of those cell types. For now however, the data offer strong support to the notion that in particular scenarios, lymphoid stress-surveillance may be a key component to the early phase of the immune response. In that regard, the early enrichment for HLA-DR$^+$CD38$^+$ activated effector T cells in the HIRD trial contrasted somewhat with delayed increases in those cells following smallpox or Yellow Fever vaccination[35,36].

Within the HIRD cohort, a prominent day +7 plasmablast signature correlated strongly with vaccine responsiveness, just as was true for several flu and other viral vaccines. Nonetheless, the size of the HIRD study exposed the large scale of human immunophenotypic variation, and therein, the many routes by which individuals could fail to appropriately activate plasmablasts. Thus, the HIRD study did not identify a consensus pre-vaccination predictor of non-responsiveness. This was true even when the convention of using ≥4-fold increases to define responders was substituted with a continuous range, and when markers and predictors of non-responsiveness reported by others[8,13,15,16,17] were specifically investigated, albeit

that no detailed examination was made of T follicular helper cells[8,37]. There was also no association of HLA haplotype with any criteria measured in the HIRD study.

The difficulty in identifying a consensus non-responder signature seems consistent with a recent twin study arguing that phenotypic, experiential variation more profoundly affected vaccine responses than did genetics[38]. Wide-ranging immunophenotypic variation may likewise underlie current difficulties in identifying predictive signatures of non-responsiveness in cancer immunotherapy. Conversely, the emergence in HIRD and other trials, of a few good markers, particularly CXCL10[24], that robustly reflect recent immunological challenge may form the basis of a consensus “Immune Responsiveness Signature” by which patient responses to a spectrum of interventions can be easily gauged in the clinic.

Age is a fundamental component of experiential variation, and marked declines in adaptive responsiveness in the elderly are well known and increasingly well understood[7,27,39,40]. By contrast, age-related differences in the early (day +1) response, such as those described here, have not been widely considered. Moreover, those differences appeared to emerge at younger ages (~35 years) than are usually considered in the context of age-related immune dysregulation. Neither the basis nor consequences of those differences are yet known, but the findings emphasize how responses to vaccines, allergens and infections might be different for younger adults, with the potential to influence the design of efficacious vaccines and immune-therapeutics.

Whereas there may be many ways to fail to make an effective vaccine response, there may be only few routes to developing AEs of the broad spectrum assessed in this study. Vaccine-related AEs can pose serious obstacles to vaccine approval and to public compliance with vaccination regimens. Thus, concrete cellular and molecular patterns associated with symptoms often derided as “man flu”, may offer ways to limit AEs and/or stratify patients. In this regard, the HIRD study provided evidence that high/medium AEs reported equally by men and women were *bona fide*, being associated with a discrete post-vaccination gene signature. Most unexpectedly, however, AEs were also associated with a pre-vaccination gene expression pattern that was in turn commonly manifest in an atypical B cell phenotype. Moreover, this atypical phenotype successfully predicted that several subjects reporting AEs unknowingly harbored autoantibodies. Indeed, over one quarter of the medium/high AE cohort scored positive from testing only two, clinically relevant specificities. Thus, whereas it is well established that human immonophenotypic variation can affect immune responses to pathogens and/or provoke clinical autoimmunity, the HIRD trial has established how such variation in healthy adults may also be associated with complex, sub-clinical pathophysiology whose consequences only become manifest upon challenge and whose read-outs are not overtly immunological. Therefore, as algorithms and data-analysis tools grow in power, together with clinical phenotyping and molecular pathology, so practical monitoring of peripheral blood may identify discrete forms of human immunophenotypic variation that collectively predict a broad spectrum of pathophysiologic conditions and/or responses to treatments.

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Methods

Study design

From March 2010 to August 2011, 178 healthy adult volunteers (18–65 years of age) with no known history of cancer, autoimmune or inflammatory diseases were recruited for an experimental, before-and-after observational cohort study of Human Immune Response Dynamics (HIRD) following vaccination (Table 1). Each volunteer completed 6 visits: two pre-vaccination, at days −7 and 0, and four post-vaccination, at days +1, +7, +14 and +63.

At day −7, the subjects filled out a personal and family history health questionnaire, and underwent a standard health examination, including full blood and urine analysis. Study exclusion criteria (described in Supplementary Table 1) were largely applied at this point. At the end of the day 0 visit, participants received a single intramuscular injection, administered according to the manufacturer's instructions, of the GlaxoSmithKline (GSK) Pandemrix™ H1N1 swine flu vaccine (comprises 3.75mg antigen derived from A/California/7/2009 H1N1 virus strain, and GSK proprietary adjuvant system AS03 containing DL-a-tocopherol, squalene and polysorbate 80). Study participants donated 2 baseline blood samples on days −7 and 0 before H1N1 vaccination (total blood volume 150 ml), followed by further donations on days +1, +7, +14 (each 60 ml) and day +63 (150 ml), totaling 540 ml (similar to a standard blood donation volume). Blood was drawn between 7.30 am and 10.30 am following starvation from midnight. At each visit, subjects completed a self-reported wellness assessment on the Likert scale, to monitor for adverse events (AEs).

The study was reviewed by the Executive Board of the Biomedical Research Centre (BRC) of Guy's and St Thomas' Hospitals and King's College London, and approved by the Research Ethics Committee (REC study number 09/H0717/88). All volunteers gave informed consent to take part. The conditions of ethical approval required the study be completed for all volunteers after the day +63 visit, and that we inform relevant individuals of any deleterious metrics uncovered.

Sample processing and storage

Bloods were drawn in the Clinical Research Facility of St Thomas' Hospital, transported to the main investigators' laboratories at King's College London, and immune cells, serum and plasma purified by standard procedures. Cells, serum, DNA and plasma were preserved according to standard protocols dictated by downstream studies (e.g. cryopreservation of "live cells" for functional analyses; snap-freezing of cells in RNA preservative for analysis of gene expression). Samples were stored in the King's College London BRC BioBank facility. Cryopreserved cells were held in liquid nitrogen until required; all other samples were stored at −80°C.

Defining serological vaccine response

Immune response to vaccine was assessed by H1N1-specific hemagglutination (HAI) and microneutralization (MN) assays carried out by the Health Protection Agency, UK, according to standard protocols. Assays were conducted on day −7 and day +63 samples,

and day +63/−7 HAI and MN ratios determined. A vaccine responder was defined as an individual whose HAI and MN ratios were both >4.

Defining adverse events

The 16-parameter "wellness assessment" self-reported for every study participant the intensity of each parameter on the Likert scale of 0–3. Parameters assessed were: site reaction to vaccine, fever, headache, tiredness, muscle ache, joint ache, swollen glands, numbness, sleepiness, dizziness, diarrhea, nausea, itching, sleeplessness, general un-wellness, and any required use of painkiller. The AE score was calculated for each participant as a sum of all individual scores on every parameter on days +1 and from day +1 to day +7. The AE range for the HIRD study was 0–24, with a mean of 5.9 ± 4.8 and a median of 5 (Q1-Q3 2-8). For the purposes of our study, scores of 0–8 were defined as low AE, scores above the third quartile, 8–16, were defined as medium AE, and scores above 16 (over 2 standard deviations above the mean) were defined as high AE. Of note, even the high AE scores reflect an acute overt event, but of insufficient magnitude to merit medical consultation.

Experimental procedures and QC

Immune phenotypes were assessed by Flow Cytometry in the Department of Immunobiology and in the BRC core facility, according to standard operating procedures (SOPs). In brief:

1. Multi-parameter polychromatic flow cytometry was performed on PBMC samples of 60 study participants, to define white blood cell subset composition, activation state, and function. To achieve this, cryopreserved cells were thawed, washed and rested for 1 h, before Fc blocking with KIOVIG (human normal immunoglobulin, Baxter) followed by staining with relevant monoclonal antibodies (full staining panels described in Supplementary Table 3) and acquisition on the BD LSRFortessa analyzer, which was precisely calibrated to the same run parameters for each day of sample acquisition. Data were analyzed using the FlowJo software. To control for day-to-day variation, PBMC samples from 2 individuals were prepared in a way identical to the study samples, and stored as large batches. These 2 individual samples were included in each experiment, and used as an internal longitudinal standard in all flow cytometry assays.

2. Multiplex serum cytokine analysis was performed on cryopreserved serum aliquots using the Luminex Flexmap 3D analyser and the 39-plex and 16-plex human cytokine kits (Merck Millipore), according to manufacturer's instructions. Each analysis included its own standard curve for every analyte; in addition, to control for any day-to-day experimental variation, 2 longitudinal control serum samples were included in each run.

3. Serum Amyloid A (SAA); C-reactive protein (CRP), and autoantobody assays (anti-nuclear antibodies, ANA, using the Hep2 reporter cell assay; anti-rheumatoid factor, RF, using latex-enhanced turbidimetry; and anti-

thyroglobulin, TH, and anti-thyroid peroxidase, TPO, ELISA assays) were carried out according to standard clinical testing protocols at the Immunology Diagnostic Laboratory, King's College Hospital, London.

4. HLA haplotype analysis for HLA-A*01, *02, *03, and *11, HLA-B*07 and *27, HLA-DRB1, and DRB3/4/5* was carried out by the Clinical Transplantation Laboratory at Guy's Hospital, London, according to standard protocols, on DNA obtained from frozen granulocyte samples.

Microarray analysis

Day −7, 0, +1 and +7 samples for 46 of the study participants were selected without bias for microarray analysis. The samples were chosen from patients representative of our overall cohort, and include both vaccine responders (R, 34/46) and non-responders (NR, 12/46; 6/46 "baseline" non-responders, with no detectable pre-existing H1N1 antibody response and no increase in post-vaccine antibody titers by either HAI or MN), and low (32/46) and medium/high (14/46) AE subjects.

RNAs extracted from PBMCs were processed in two batches according to the Agilent protocol and with Agilent Gene Expression Hybridization Kit, then hybridized to Agilent 4x44K v2 Human Whole Genome microarrays, and scanned using Agilent's Microarray Scanner System (Agilent Technologies).

After QC, 173 arrays were retained in the analysis and the batch effect was removed with the method ComBat from the R package sva[41]. Further pre-processing (quantile normalization and subtraction of background estimated with method normexp) was completed with R package Agi4x44PreProcess[42]. Only probes passing default good quality features filter from Agi4x44 were retained, resulting in 27,879 probes.

Initial analysis of the microarray gene expression data was performed at the level of entire samples in order to assess data quality and detect potential differences between groups of samples (e.g. males versus females). To perform such analysis, each sample i is represented by the vector x_i of log2 intensity values over all probes. Overall dissimilarity between two samples i and j is quantified with $d_{ij} = (1 - c(\mathbf{x}_i, \mathbf{x}_j))/2$, where c stands for the Pearson correlation coefficient. Perfect similarity between two samples corresponds to d_{ij} =0 and perfect dissimilarity to d_{ij} =1. Dissimilarities can be used for instance to perform hierarchical clustering of samples.

More quantitative statements regarding separation between two groups G and H of samples are obtained as follows. First, the following multivariate statistic t (m.v.t) is estimated: $t(G,H) = d(G,H)/(s(G) + s(H))$ with $d\left(G,H\right) = \frac{1}{\mid G \mid \mid H \mid}\sum_{i \in G, j \in H} d_{ij}$, $s\left(G\right) = \frac{2}{\mid G \mid (\mid G \mid -1)}\sum_{i<j \in G} d_{ij}$ and where $|G|$ stands for the size of group G. Just like the univariate t-statistic, m.v.t takes into account both difference between groups and spread within each group. Next, observed values of $t(G,H)$ were compared to values $T(G,H)$ expected when randomly reassigning samples between the two groups. Namely, 10^5

realizations of the Fisher-Yates shuffle are utilized to estimate $p = p_I(T \geq t)$. An important advantage of such a permutation test is that it takes into account correlation between probes.

Analysis of potential effect on gene expression of subject age presented additional challenges, and was conducted as follows. Since factor day has a strong effect on gene expression patterns, effect of age is investigated at each day separately. To enable comparison of age effect at different days, the data set is reduced to the 40 subjects having gene expression data for the three days 0, +1 and +7. Utilizing m.v.t to examine age effect requires defining age groups for permutation testing. An exhaustive approach to define age groups consists in enumerating all possible partitions of subjects into "young" (Y) and "old" (O) groups of size at least 5 and reporting m.v.t *P*-value for each partition. Such enumeration is performed via a "barrier" approach: subjects are sorted by ascending age and the first r (Y) are compared to the remaining 40-r (O). The profile of m.v.t *P*-values as a function of age rank r ($5 \leq r \leq 35$) provides an estimate of effect of subject age on gene expression. When subjects have the same age on both immediate sides of the barrier, all possible partitions are enumerated and the reported m.v.t *P*-value is the average over these partitions.

To find specific genes changing expression levels upon vaccination, we used all QC-approved samples and fitted (with R package limma) a linear model with time point as categorical variable, treating both time points before vaccination as repeated measures and subject as a blocking variable. Genes with expression levels different between any two time points (before vaccination, one day after, 7 days after) were identified by moderated *t*-test; cut-offs for q.value and fold change were 0.05 and 1.5 or 2. For genes with several probes per gene, per-probe information was collapsed to per-gene information by arbitrary picking a probe with lower *P*-value in first day post-vaccination to pre-vaccination comparison in all individuals.

To find genes expressed differentially in vaccine responders and non-responders, only individuals with samples available from both post-vaccination and a pre-vaccination time points were analyzed (39 individuals). Three different approaches were used. To detect genes expressed differently between Rs and NRs at separate time points, a linear model was fitted with limma, with responder status and time point as categorical variables and individual treated as a blocking variable. To detect genes with change in expression between time points different between Rs and NRs, pre-vaccination expression level was treated as a baseline and subtracted from later measurements; then genes changing differently were identified with limma-fitted linear model (time and responder status as categorical variables) and moderated t-test, or by fitting time course quadratic profiles with maSigPro[43]. Unless otherwise mentioned, cut-offs for q.value and fold change were 0.05 and 0.5.

Differences between individuals with and without AEs were identified in the same way as described for differences between responders and non-responders, with low and medium/high AE defined as above.

Statistical analysis

Statistical analysis, significance cut-offs, and other analyses for the microarray data were performed following standard array analysis techniques as described in Methods. Flow

cytometry, serum biochemistry, and serum analyte data were analyzed using Prism software. Data are reported as mean (SD) or median (IQR). Multiple samples were compared using one-way ANOVA where all data points for each sample were available, and using the Wilcoxon rank sum test, Student's *t* test, or the Mann-Whitney non-parametric test otherwise, as appropriate to the analysis. Categorical data were compared by calculating the standard Normal deviate and two-tailed *P*-value, and are reported as mean (95% confidence interval).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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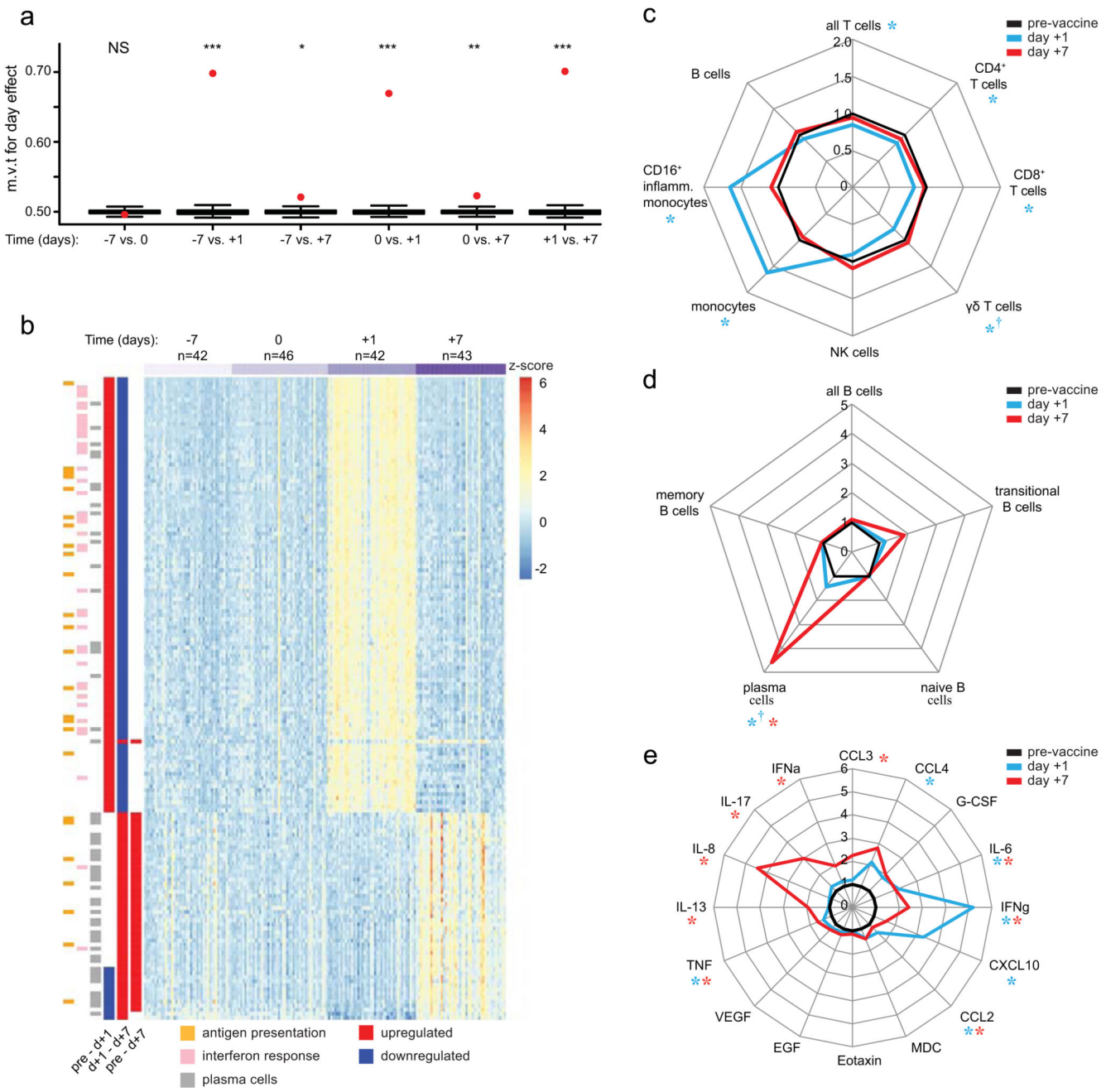

Figure 1.

H1N1 vaccination leads to rapid and reversible early changes in the immune compartment, followed by a B cell-rich signature at day +7. (**a**) Observed values of multivariate statistic t (m.v.t, quantifying global PBMC gene expression dissimilarity) when comparing two study days (red dots) to values expected when randomly assigning days between the two groups (boxes: median and quartiles; *=p<0.0001, **=p<0.00005, ***=p<0.00001, NS=not significant, p>0.05). (**b**) Heat map of gene expression levels of the 133 highest changed genes (log2FC>1; centered and scaled by gene across all subjects and time points), on pre-vaccine days −7 and 0, and post-vaccine days +1 and +7, n=42-46 study subjects. (**c**) PBMC

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immune cell subset composition (%) determined by flow cytometry, plotted as average of fold changes per subject at days +1 (blue) and +7 (red) compared to pre-vaccination time points (n=46-65 subjects; *=p-value of <0.05 in a two-tailed paired t test of % cell type at day +1, in blue, or +7, in red, compared to both pre-immune time points; †=only significant in comparison to pre-vaccine day −7). (**d**) B cell subset composition (%) determined by flow cytometry, plotted as average of fold changes per subject at days +1 (blue) and +7 (red) compared to pre-vaccination time points (n=23-25 subjects; p-values as in panel **c**). (**e**) Serum cytokine and chemokine concentration determined by Luminex analysis, plotted as average of fold changes per subject at days +1 (blue) and +7 (red) compared to pre-vaccination time points (n=123-134 subjects for most analytes, 96 for IL-13, and 24 for IL-8, GM-CSF and CCL4; *=p-value of < 0.05 in a two-tailed paired t test of serum analyte concentration at day +1, in blue, or +7, in red, compared to both pre-immune time points, black).

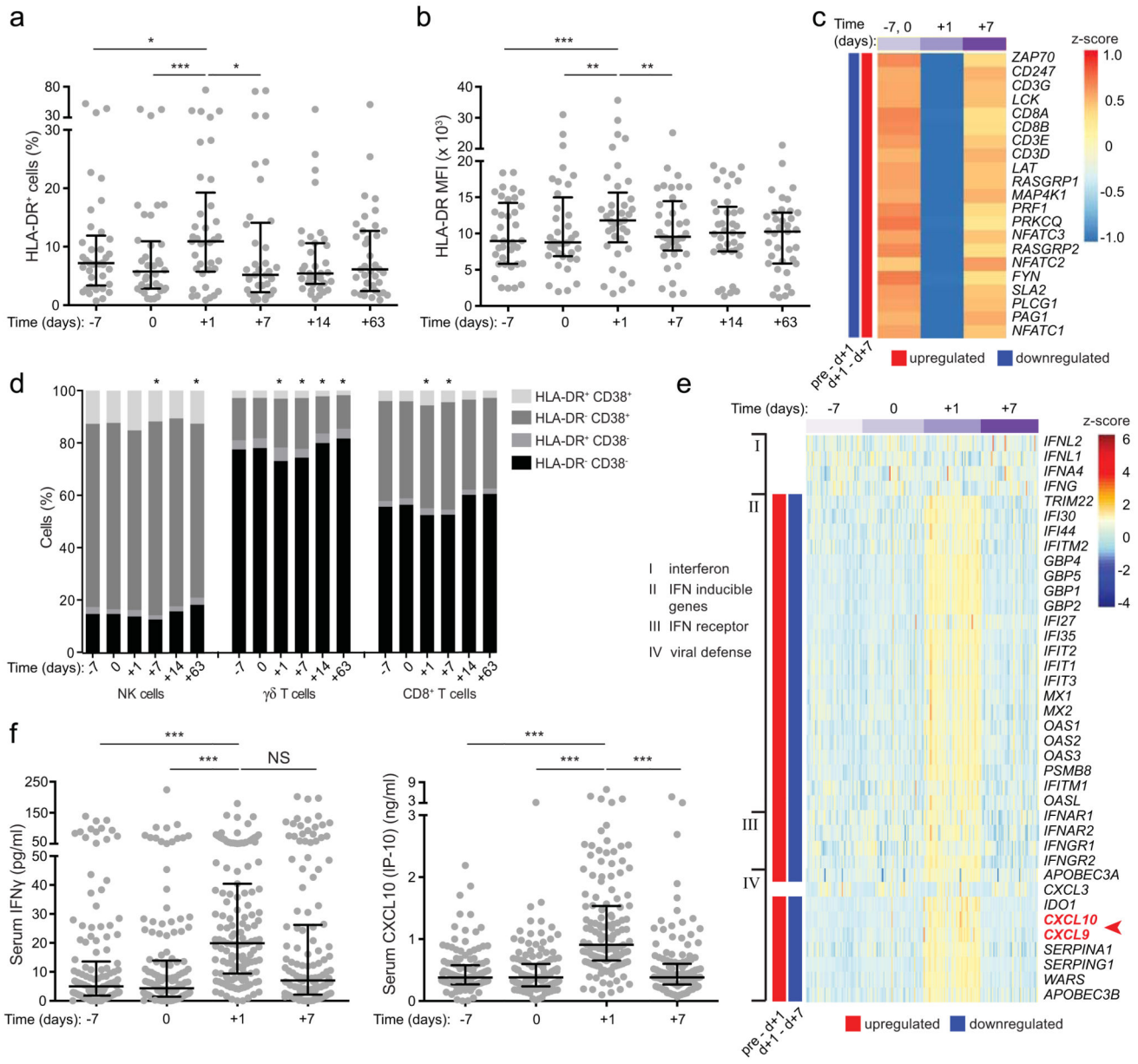

Figure 2.

Early post-vaccine activation of myeloid and lymphoid cells is detectable across multiple assays. (**a, b**) Monocyte activation, assayed by flow cytometry of HLA-DR expression and mean fluorescence intensity (MFI) of CD14+ live cells (n=36-37 subjects; plot of individual subject values, black lines indicating median and interquartile range; p-values two-tailed paired t test; *=p<0.05, **=p<0.01, ***=p<0.00001, NS=not significant, p>0.05). (**c**) Gene expression of T cell-specific genes (averaged across 46 subjects, centered and scaled by gene across time points). (**d**) Average % activation, assayed by flow cytometry HLA-DR and CD38 expression, of CD8+ T cells, γδ T cells, and NK cells, at all time points of the study (n=65 subjects; shaded bars represent average % cells expressing the indicated markers; *=p-value of <0.05 in a two-tailed paired t-test of % HLA-DR- CD38- cells compared to

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both pre-immune time points). (**e**) Expression of interferons and interferon-inducible genes following vaccination, compared to pre-immune time points (centered and scaled by gene across all subjects and time points; red font and arrowhead highlight CXCL9 and CXCL10 expression). (**f**) Luminex analysis of serum IFNγ and CXCL10 (IP-10) concentrations (n=130-133 subjects; plot of individual subject values, black lines indicating median and interquartile range; p-values two-tailed paired t-test, as in panels **a, b**).

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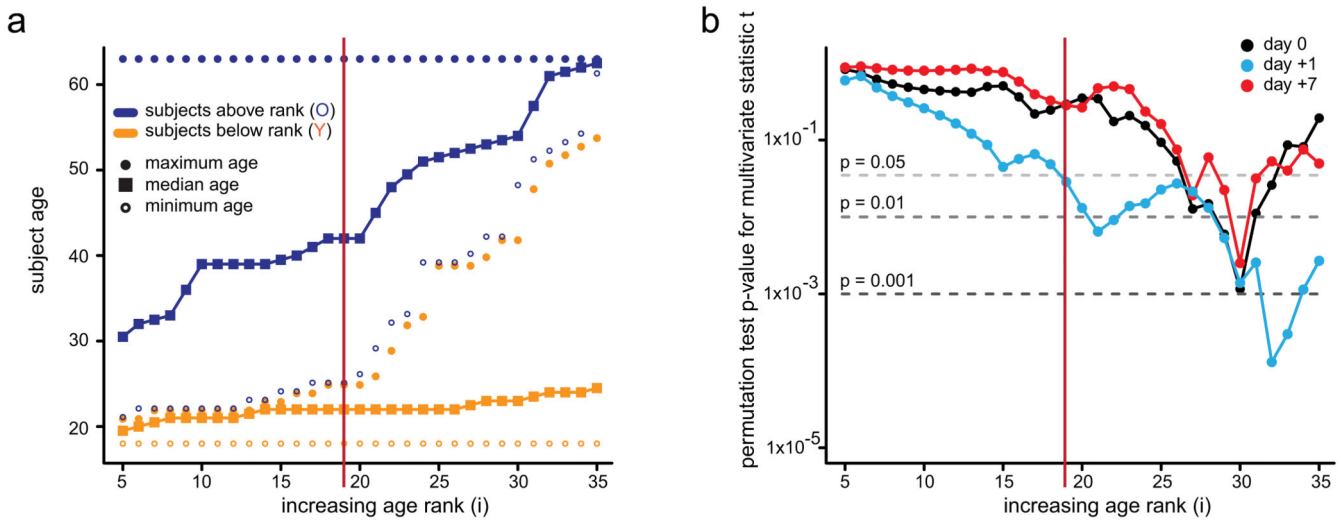

Figure 3.

Age factor in multivariate analysis of gene expression based on all genes. (**a**) Age statistics for groups considered in Supplemetary Figure 4, defining the maximum, median, and minimum age of subjects assigned as “young” and “old” for each value of i in our subject cohort. (**b**) Increasing subject age rank plotted against permutation test p-values for a multivariate statistic t, which quantifies Pearson dissimilarity between age groups, representing all possible partitions of subjects into two age groups of size of at least five. P-values are estimated with respect to 10^5 random reassignments of age to subject. The vertical red line in panels **a** and **b** indicates the inflection point at which age factor becomes a significant influence (multivariate statistic t permutation p-value <0.05) on gene expression in the day +1 data set.

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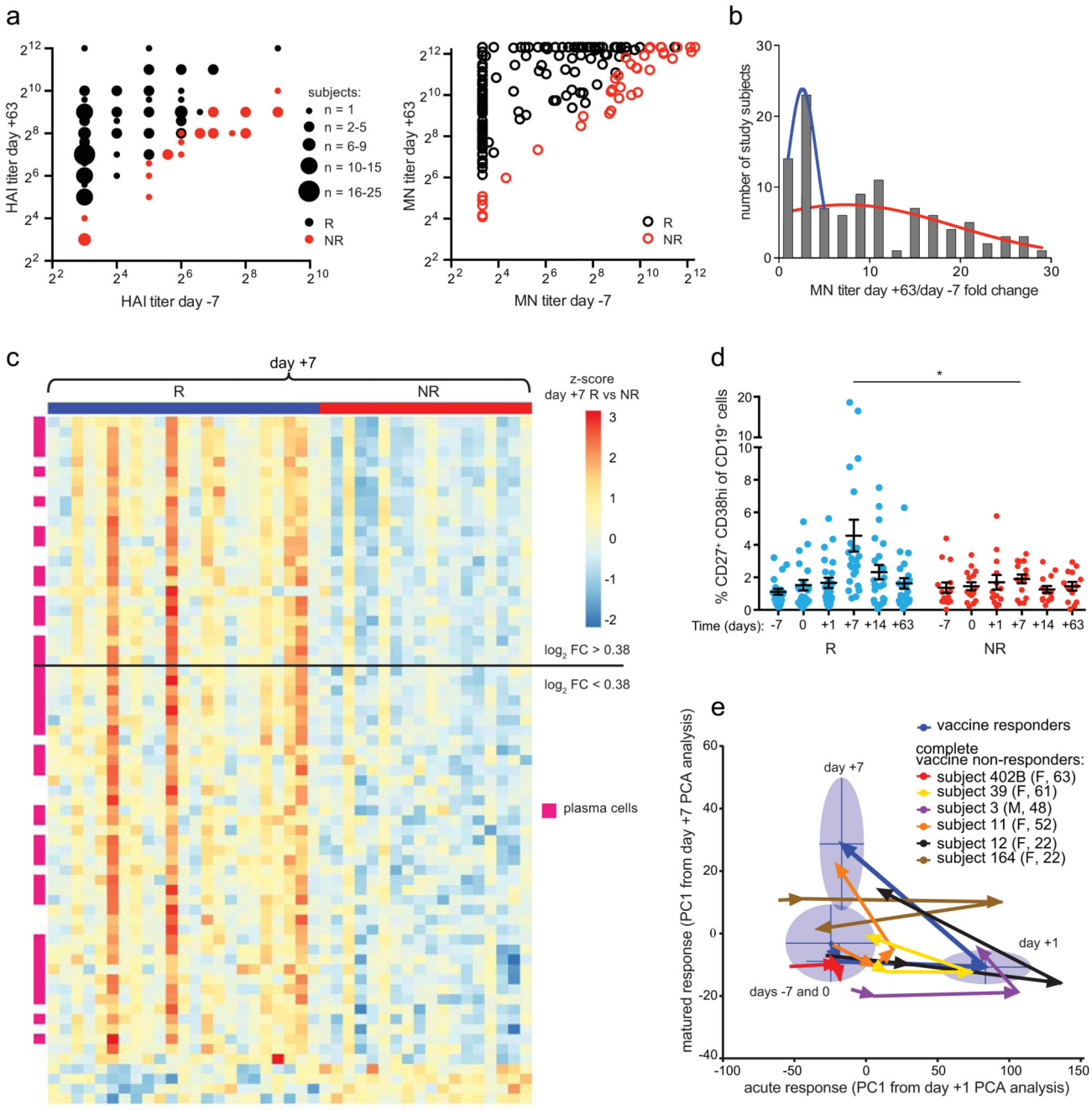

Figure 4.

A vaccine non-response signature is detectable by post-vaccination day +7. (**a**) HAI and MN titers among HIRD study subjects at days −7 and +63. Subjects failing to make a 4-fold increase in both titers (non-responders; NR) are highlighted in red, responders (R) in black. For the HAI assay, where the read-outs are discrete, the number of subjects with the same read-out is indicated by marker size. (**b**) Fold change in MN titer (focus on the low range, FC 0-30) is best described as a sum of two distributions, also identifying NR as a separate population (trendline fit R=0.8766). Distribution 1 (blue) mean: 2.128, standard deviation

(SD): 0.581, distribution 2 (red) mean: 7.183, SD: 12.30. (**c**) Genes expressed differentially between 23 R and 18 NR subjects at day +7 (q-value <0.05, centered and scaled by gene across all subjects). (**d**) FACS analysis of plasma cell abundance in R vs NR subjects at all time points (n=20-23 R, 13-15 NR; each point represents plasma cell % for an individual study subject, bars represent mean and SEM; two-tailed Mann-Whitney test comparison of R vs NR, *=p<0.05). (**e**) Principal component analysis (PCA) of genes significantly altered (p<0.0001) at day +1 and at day +7 compared to pre-immune time points day 0 and day −7. PC1 from probes significantly altered at day +1 (Acute Response) is plotted against PC1 probes significantly altered at day +7 (Matured Response). The average response for all vaccine responders at each time point is shown with blue arrows; blue bars and ovals indicate area within 1 SD of vaccine responder average. Colored lines show the trajectory of individual “baseline” NR, chosen as subjects whose initial HAI and MN titers were at baseline pre vaccination and failed to increase. Subject gender and age at vaccination indicated in parentheses.

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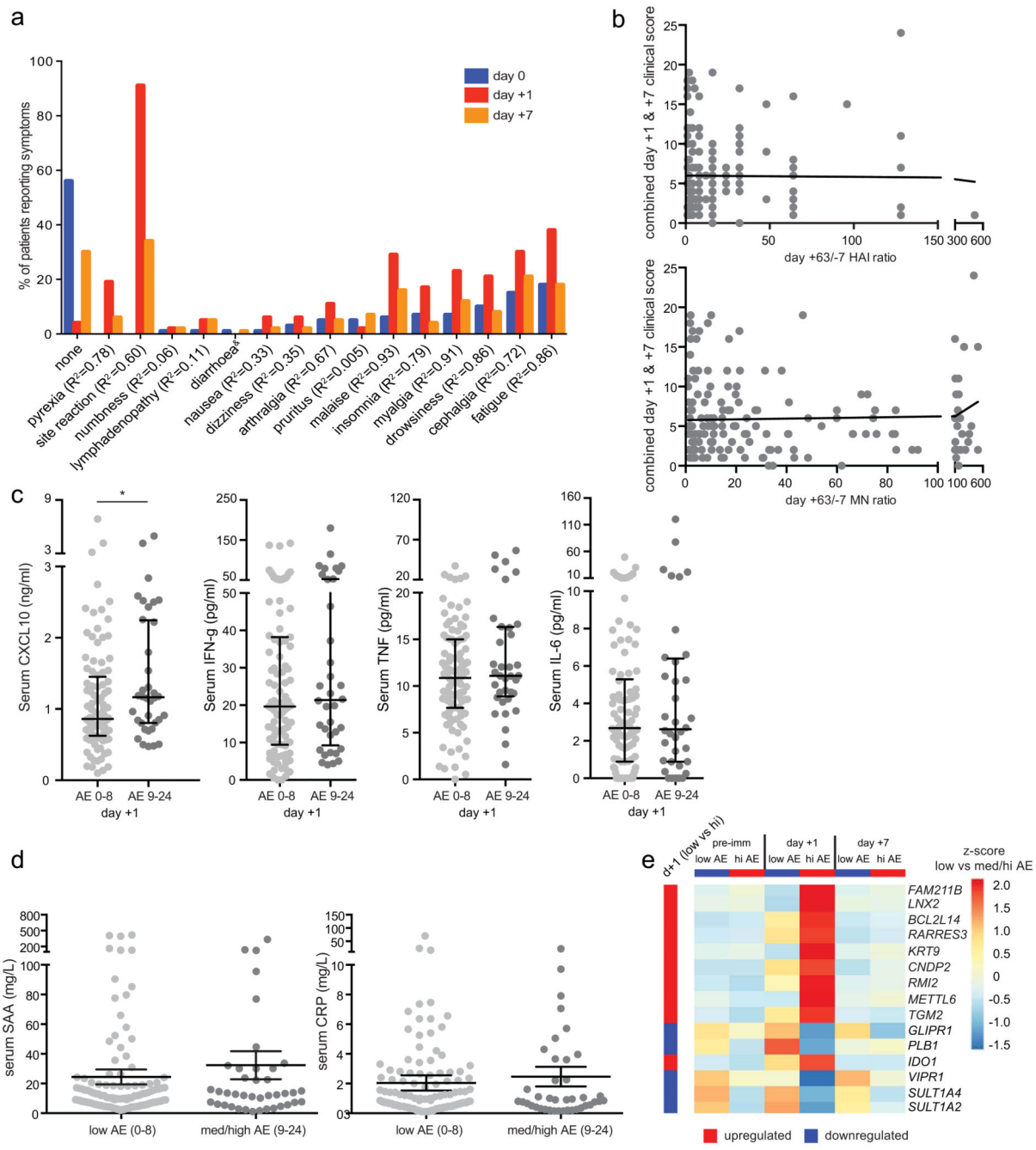

Figure 5.

High AE individuals show day +1 and pre-vaccine differences to asymptomatic study subjects. (**a**) Distribution of symptoms before and after vaccination in the HIRD study cohort, expressed as % subjects reporting a given symptom on study days 0, +1, +7 and +14. R values in linear correlation of average day +1 and +7 score/subject for each symptom with the overall combined AE score are indicated; & = not reported on day +1. (**b**) Combined day +1 and +7 clinical score, as compared to the HAI or NM day +63/day −7 titer ratio for the same individuals, shows no correlation between these variables (n=176 subjects), Pearson r

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= 0.00022 and 0.00723 for HAI and MN, respectively; p>0.05 in both cases. (**c**) Luminex analysis of serum analyte concentrations at day +1 in low AE vs medium/high AE subjects (plot of individual subject values, black lines indicating median and interquartile range; n=98-100 low AE and 36-38 med/high AE subjects, p-values in two-tailed Mann-Whitney test, *=p<0.05). (**d**) Pre-vaccine serum amyloid A (SAA) and C-reactive protein (CRP) levels (average of days −7 and 0) are not significantly different between low AE (n=132) and med/high AE (n=40) study subjects. (**e**) Genes expressed differentially in low AE (combined clinical score of 0-8) vs medium/high AE (combined clinical score of 9-24) study subjects on post-vaccine day +1 (q-value <0.05, centered and scaled by gene across all subjects).

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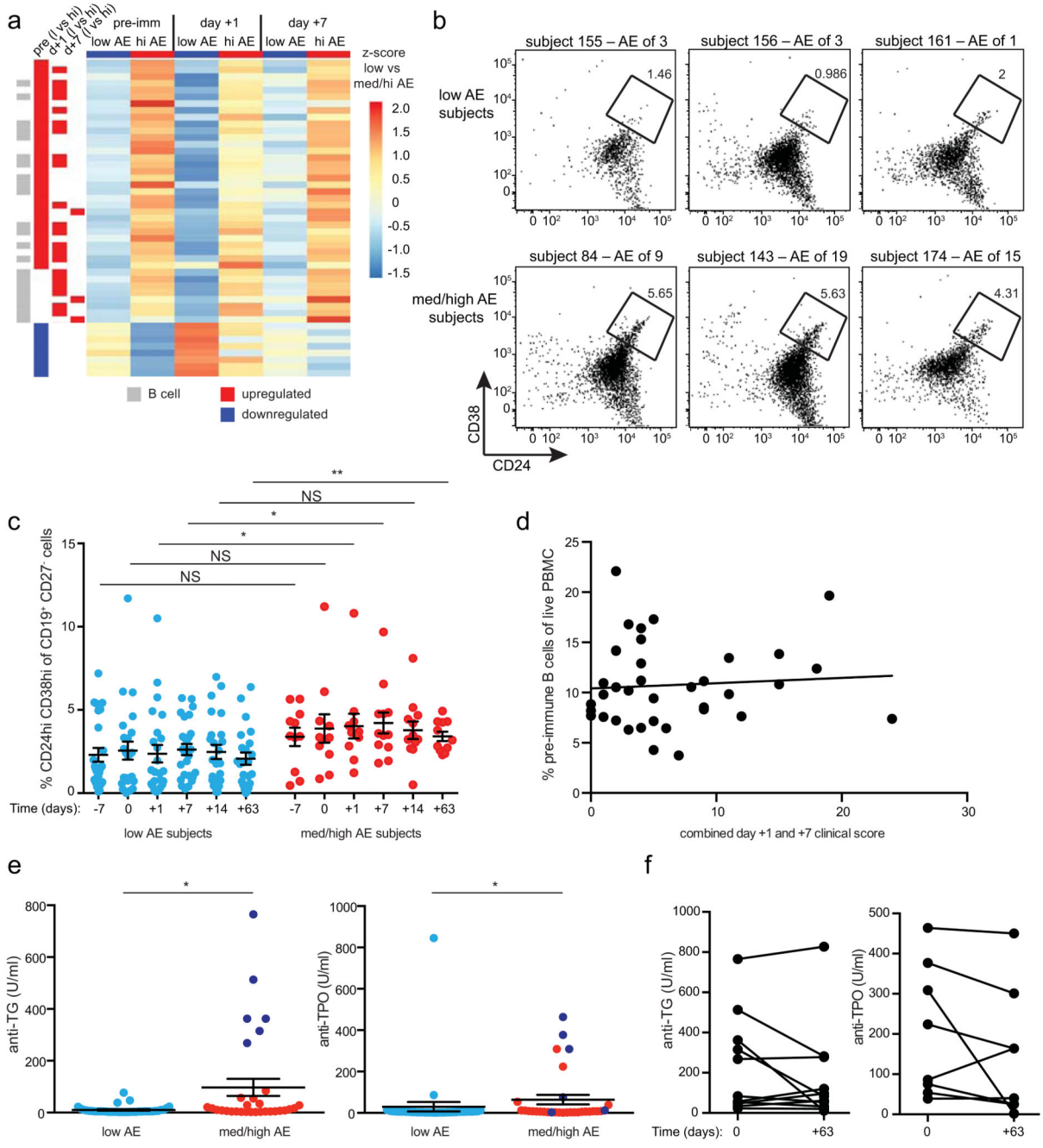

Figure 6.

Pre-vaccine transitional B cell levels and increased auto-antibodies correlate with post-vaccination AE. (**a**) Genes expressed differentially in low AE (clinical score 0-8) vs medium/high AE (clinical score of 9-24) study subjects, both pre-vaccination and on post-vaccine days +1 and +7 (q-value <0.05, centered and scaled by gene across all subjects). (**b**) Representative FACS plots of transitional B cell gating, showing % of live CD19+ CD27− naive B cells at day −7 in three low AE and three medium/high AE subjects. (**c**) FACS analysis of transitional B cells at all time points in low AE vs medium/high AE study

subjects (n=23-26 and n=11-12, respectively; each point represents transitional B cell percentage in an individual subject, bars represent mean and SEM for each time point; p-values in two-tailed Mann-Whitney test comparisons of low AE vs med/high AE at each time point, *=p<0.05, **=p<0.01, NS=not significant, p>0.05). (**d**) Pre-vaccination total B cell % (plotted as average of days −7 and 0 for each individual study subject, n=37), compared to the combined day +1 and +7 AE score for the same individuals, shows no correlation between these variables (linear regression trendline), R2=0.0055, p>0.05. (**e**) Anti-thyroglobulin (anti-TG) and anti-thyroperoxidase (anti-TPO) serum antibody (Ab) titres at day 0 in low AE vs medium/high AE; 6 subjects with highest anti-thyroglobulin Ab levels are tracked in purple across both assays (n=37 low AE, 31 med/high AE; each point is an individual study subject measurement, while bars represent mean and SEM for subject groups; p-values in two-tailed Mann-Whitney test, *=p<0.05. (**f**) Anti-TG and anti-TPO Ab titres at days 0 and +63; connected points represent repeated measurements from an individual subject with detectable pre-vaccine auto-antibodies; n=11 and 9, respectively.

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Table 1

HIRD study overall cohort and sub-groups of interest

	HIRD study cohort	serological vaccine non-responders (NR)	medium/high Adverse Events (AE)
Characteristic	**n**	**n**	**n**
Total Subjects (%)	178 (100)	35/166 (21.1)	40/177 (22.6)
Age			
Mean (SD)	28.7 (11.03)	29.1 (12.76)	27.6 (8.8)
Range	18.2 - 63.3	18.3 - 63.3	18.2 - 61.9
Gender			
Male (%)	70 (39.3)	14/66 (21.2) *	14/69 (20.3) *
Female (%)	108 (61.7)	21/100 (21.0) *	26/108 (24.1) *
Ethnicity			
Caucasian (%)	127 (71.4)	26 (74.3)	29 (72.5)
Asian (%)	24 (13.5)	6 (17.1)	5 (12.5)
Black/African (%)	10 (5.6)	2 (5.7)	2 (5.0)
Other (%)	17 (9.15)	1 (2.9)	4 (10.0)
Serological vaccine response			
HAI day −7, Mean (SD)	56.05 (98.1)	155.2 (157.0)	72.0 (119.7)
HAI day +63, Mean (SD)	451.5 (670.6)	265.1 (228.1)	461.5 (484.2)
HAI day +63/−7 ratio, Mean (SD)	19.6 (43.8)	1.9 (0.9)	21.1 (32.0)
MN day −7, Mean (SD)	361.4 (784.7)	1194.0 (1353.6)	524.3 (1123.6)
MN day +63, Mean (SD)	2419.3 (2008.4)	2264.9 (1941.6)	2650.2 (2072.1)
MN day +63/−7 ratio, Mean (SD)	45.04 (85.23)	2.3 (0.9)	59.0 (112.8)
Adverse Events			
combined day +1 & +7 score, Mean (SD)	5.93 (4.76)	6.03 (5.30)	13.30 (3.74)

* Percentage of males and females with the phenotype (NR or AE) of total subjects of that gender in relevant cohort