

# Variation in the Human Immune System Is Largely Driven by Non-Heritable Influences

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## SUMMARY

There is considerable heterogeneity in immunological parameters between individuals, but its sources are largely unknown. To assess the relative contribution of heritable versus non-heritable factors, we have performed a systems-level analysis of 210 healthy twins between 8 and 82 years of age. We measured 204 different parameters, including cell population frequencies, cytokine responses, and serum proteins, and found that 77% of these are dominated (>50% of variance) and 58% almost completely determined (>80% of variance) by non-heritable influences. In addition, some of these parameters become more variable with age, suggesting the cumulative influence of environmental exposure. Similarly, the serological responses to seasonal influenza vaccination are also determined largely by non-heritable factors, likely due to repeated exposure to different strains. Lastly, in MZ twins discordant for cytomegalovirus infection, more than half of all parameters are affected. These results highlight the largely reactive and adaptive nature of the immune system in healthy individuals.

## INTRODUCTION

The study of monozygotic (MZ) and dizygotic (DZ) twin pairs has provided a powerful means for separating heritable from non-heritable influences on measured traits for almost 100 years (Jablonski, 1922). Such studies have been used to study autoimmune diseases, vaccine responses (Jacobson et al., 2007), serum cytokines (de Craen et al., 2005), or the frequencies of major immune cell populations (Clementi et al., 1999; Evans et al., 2004). Most of these studies have found that both heritable

and non-heritable factors contribute to the resulting phenotype. Recent advances in technology now allow much more comprehensive surveys to be conducted across the many different components of the immune system, and thus we performed a very broad “systems-level” study in which we measured 51 serum cytokines, chemokines, and growth factors; the frequencies of 95 different immune cell subsets; and cellular responses to cytokine stimulation (Figure 1A). Our results show that these functional units of immunity vary across individuals primarily as a consequence of non-heritable factors, with a generally limited influence of heritable ones. This indicates that the immune system of healthy individuals is very much shaped by the environment and most likely by the many different microbes that an individual encounters in their lifetime.

## RESULTS

### A Systems-Level Analysis of the Immune System in Healthy Twins

Our study cohort was recruited from the Twin Research Registry at SRI International (Krasnow et al., 2013) in the years 2009–2011, with demographic data detailed in Table S1 (available online). The subjects were all apparently healthy, without any symptoms of disease (Experimental Procedures, “Twin Cohort”). To minimize biological variability, the time between blood sampling of each twin in a pair was kept to a minimum (Experimental Procedures, “Blood Sampling, PBMC Preparation, and Zygosity Testing”). Immunological assays were performed by the Human Immune Monitoring Center, where assays are continually benchmarked to minimize technical variability (<http://iti.stanford.edu/himc/>) (Maecker et al., 2005). However, some technical variability is inevitable, and thus we corrected for this in all of our models. We did this by analyzing aliquots of the same control sample many (>17) times to estimate the technical variance and subtracted this from our estimates of heritability (Experimental Procedures, “Correction of Model Estimates for Technical Variability”). We also analyzed longitudinal samples in an unrelated cohort over 2–5



**Figure 1. Systems-Level Analysis of Healthy Human Twins**

(A) Overview of data collected covering the functional units of the immune system, the cells, and proteins in circulation.

(B) Summary of all heritability estimates for 72 immune cell population frequencies as determined by flow (2009) and mass cytometry (2010–2011) (Experimental Procedures, section 3). See also Table S3.

(C) Heritability estimates of 43 serum proteins as determined by a fluorescent bead assay. See also Table S4. Error bars represent 95% confidence intervals for the heritability estimate. Gray area is heritability <0.2, our detection limit.

consecutive, yearly samplings and found that the variation was largely due to technical variability (Table S2). A total of 204 different immune measurements were included in our analyses.

**Estimating Heritable and Non-Heritable Influences**

Heritability for each parameter was estimated by comparing observed MZ and DZ covariance matrices to the expected

values based on a structural equation model that partitioned the observed variance into three components: heritable (A), shared (C), and unique (E) non-heritable factors. This model is based on the assumptions that: (1) heritable factors correlate perfectly between MZ twins ( $r_{MZ} = 1$ ) but only to 50% between DZ twins ( $r_{DZ} = 0.5$ ) and (2) that shared non-heritable influences are equally similar ( $r_{MZ} = r_{DZ}$ ) between MZ and DZ twin pairs

([Experimental Procedures](#), “Structural Equation Modeling to Estimate Heritable and Non-Heritable Influences”). For each measurement, we subtracted the technical variance estimate from the e-component prior to normalization to correct for noise ([Experimental Procedures](#), “Correction of Model Estimates for Technical Variability”). We also corrected all measurements for the effects of age ([Dorshkind et al., 2009](#)) and gender ([Furman et al., 2014](#)) by regressing out such effects and using only residual variance for estimating heritability. Finally, we performed jackknife bootstrapping tests to obtain 95% confidence intervals ([Experimental Procedures](#), “Structural Equation Modeling to Estimate Heritable and Non-Heritable Influences”). Importantly, as our model estimates heritability by comparing MZ and DZ twins, heritable influences include genomic and shared epigenetic traits ([Bell and Spector, 2011](#)), and non-heritable influences include environmental factors and stochastic epigenetic changes ([Fraga et al., 2005](#)).

We first ran a simulation experiment to verify that our cohort size of 210 twins (78 MZ and 27 DZ pairs) would be enough to test our hypothesis that most immunological traits are explained more by non-heritable than by heritable influences. We found this to be the case, and we estimate 20% heritability to be our detection limit, under which we cannot distinguish small heritable influences from zero ([Figure S1](#)).

### Most Cell Population Frequencies and Serum Proteins Are Dominated by Non-Heritable Influences

Although it is well known that the frequencies of different types of immune cells in blood often vary widely between individuals, in most cases it is not known how much of this can be attributed to heritable or non-heritable factors, respectively. To address this question, we used antibodies against cell surface markers to quantify 95 different cell subset frequencies but used the 72 most non-redundant ones and estimated the influence of heritable and non-heritable factors on their variation ([Experimental Procedures](#), “Immune Cell Phenotyping by Mass Cytometry and Flow Cytometry”). Among these, a few had very strong influences from heritable factors, especially naive, CD27<sup>+</sup>, and central memory CD4<sup>+</sup> T cells ([Figure 1B](#) and [Table S3](#)), but for most, non-heritable influences were clearly dominant. In fact, for 61% of all cell populations, the influence of heritable factors was undetectable (<20% of the total variation) ([Figure 1B](#) and [Table S3](#)). This was true of both adaptive (T and B cells) and innate cell types (granulocytes, monocytes, and NK-cells).

Serum cytokines and chemokines also have important functions as immune mediators and biomarkers of disease ([Villeda and Wyss-Coray, 2013](#)), and thus we measured 51 serum proteins but eliminated eight that were often at or below the limits of detection ([Experimental Procedures](#), “Serum Protein Quantification”). This left 24 cytokines, 10 chemokines, 6 growth factors, and 3 other serum proteins for which we estimated the influences of heritable and non-heritable factors ([Figure 1C](#) and [Table S4](#)). Some cytokines were particularly heritable, such as IL-12p40 ([Figure 1C](#) and [Table S4](#)). Interestingly, variants in the IL12B gene that contribute to the IL-12p40 protein have been associated with immune-mediated diseases such as psoriasis ([Nair et al., 2009](#)) and asthma ([Morahan et al., 2002](#)). In the latter condition, the susceptibility locus was also associated with a

reduced serum concentration of IL-12p40 ([Morahan et al., 2002](#)). For many other measurements, such as IL-10 and a group of chemokines, the heritable influence was low ([Figure 1C](#) and [Table S4](#)).

### Homeostatic Cytokine Responses Are Largely Heritable, whereas Most Other Cell Responses Are Highly Non-Heritable

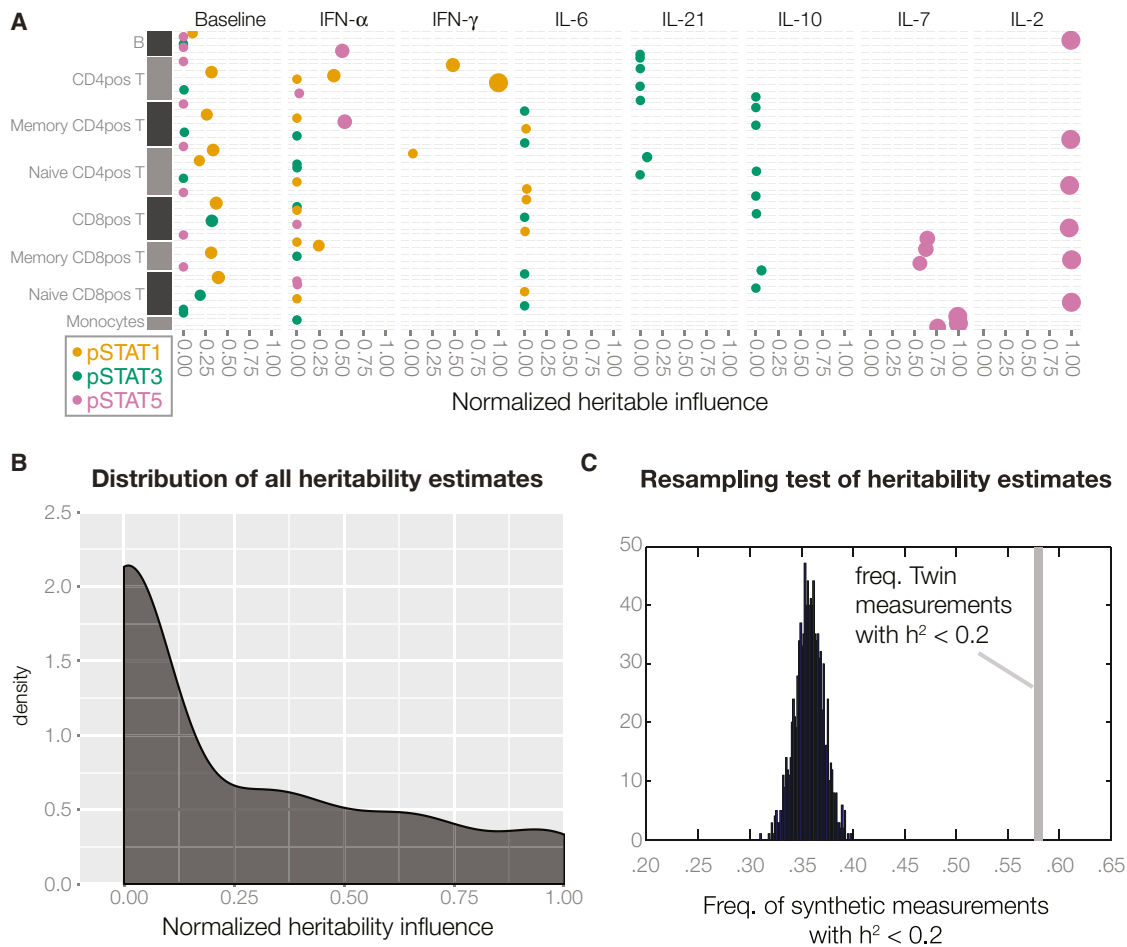
Because these serum proteins often regulate immune cells, we assessed the responses of eight different cell populations stimulated in vitro with seven different cytokines for the phosphorylation of three important transcription factors, STAT1, 3, and 5, using phospho-specific antibodies in flow cytometry ([Krutzik and Nolan, 2006](#)). We performed a total of 192 different measurements but focused on the 24 baseline measurements and the 65 strongest induced responses ([Experimental Procedures](#), “Immune Cell Signaling Experiments”). Baseline measurements were generally driven by non-heritable factors, with possible minor contributions from heritable factors ([Figure 2A](#)). The important homeostatic cytokines IL-2 and IL-7, known to stimulate the proliferation and differentiation of T cells, were found to induce STAT5 phosphorylation in both CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations, and these responses were highly heritable ([Figure 2A](#) and [Table S5](#)). In contrast, most signaling responses such as interferon-induced STAT1 phosphorylation and, in particular, the IL-6-, IL-21-, and regulatory IL-10-induced phosphorylation of STAT3, were dominated by non-heritable influences ([Figure 2A](#) and [Table S5](#)). In total, 69% of all signaling responses had no detectable heritable influence (e.g., <20%) ([Figure 2A](#) and [Table S5](#)). This lack of heritability was not related to the strength of responses or explained by a bias toward weak and variable responses ([Figure S2](#)).

### Non-Heritable Influences Are Major Factors Determining Immune Variation

Taken together, these results show that variation in blood cell frequencies and functions and soluble factors is largely driven by non-heritable factors, with 58% of all measurements having <20% of their total variance explained by heritable influences ([Figure 2B](#)). There was no relationship between the absolute degree of measurement variability in the cohort and estimated heritability ([Figure S3](#)), and we could also rule out any underestimation of heritability due to the skewed ratio of MZ/DZ twin pairs in our cohort by a resampling test. In brief, by creating 1,000 synthetic data sets with uniform heritability and the same MZ/DZ ratio as in our cohort, we estimated heritability and found that none of the 1,000 data sets ever had >40% of measurements with an estimated heritability < 0.2 ( $p < 0.001$ ) ([Figure 2C](#)), thus suggesting that the low heritability estimates obtained are not a result of study design or overall measurement variation in the cohort.

### Heritable and Non-Heritable Measurements Are Interrelated in the Immune Network

Our analysis also allowed us to analyze the interrelationships between the different components of the immune system. To construct such a network model, we calculated a precision matrix derived from a Spearman correlation matrix ([Liu et al., 2012](#)).



**Figure 2. Heritable Factors Explain Only a Fraction of the Variation for Most Immune Measurements**

(A) Heritability estimates for immune cell signaling states upon stimulation with the indicated cytokines. Only unstimulated controls and induced responses  $>1.5$ -fold are shown. See also Table S5.

(B) The overall distribution of heritability for all 204 measurements.

(C) The maximum number of measurements with heritability  $<0.2$  across 1,000 synthetic data sets with the same MZ/DZ ratio as in our twin cohort is  $<40\%$ , significantly less than our results of  $58\%$  of measurements with heritability  $<20\%$  (gray bar).  $p < 0.001$ .

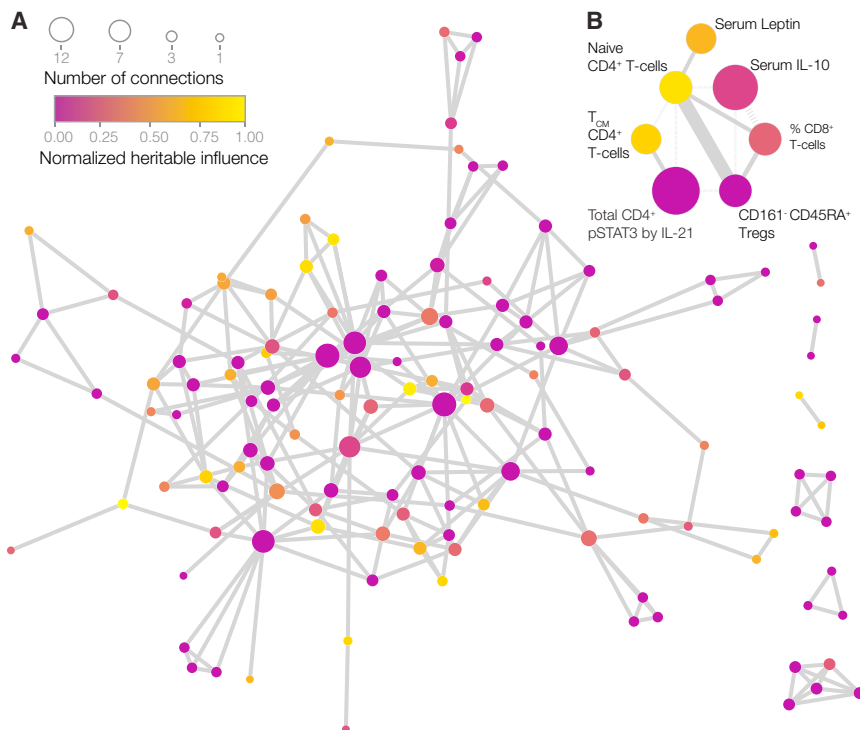
A matrix of this type captures partial correlations between variables and avoids spurious, indirect interactions that might occur in standard correlation analyses. By penalizing non-zero entries in this matrix (Friedman et al., 2008), we could pursue what is referred to as a sparse (rather than dense) network model, making it more interpretable. After removing unconnected nodes and validating the edges by a permutation test (Experimental Procedures, “Identification of Pairwise Dependencies between Measurements and the Creation of an Immune Network Model”), this model consists of 126 nodes and 142 edges (Figure 3A and Table S6). An interactive version is available online (<http://www.brodinlab.com/twins.html>). We found that heritable nodes (yellow) were generally connected to non-heritable nodes (purple) throughout the network (Figure 3A). One example shows how the weakly heritable cytokine IL-10 and CD161<sup>+</sup>CD45RA<sup>+</sup> regulatory T cells are connected to the strongly heritable frequency of naive CD4<sup>+</sup> T cells (Figure 3B). We found that all hubs in the network were dominated by non-heritable influences,

like the network as a whole, showing that heritable factors are not isolated by themselves but are buffered by connected non-heritable ones (Figure 3A and 3B). This may explain why the many gene polymorphisms found (for example, CTLA4 [Gergersen et al., 2009]) outside of the HLA locus that are associated with immune-mediated disease only explain a small fraction of the total disease risk (Todd, 2010).

#### With Age, Genetically Identical Twins Diverge as a Consequence of Non-Heritable Influences

As a major source of non-heritable influence is likely to be environmental, particularly microbial exposure, we reasoned that such influences would increase with time. To this end, we compared twin-twin correlations for all immune measurements between the oldest ( $>60$  years; median, 72 years) and the youngest ( $<20$  years; median, 13 years) MZ pairs in our cohort. Here, we also note that twins in the younger ( $<20$  years) cohort are in most cases living together, whereas the older ( $>60$  years) twins

## Network of Immune measurements



## Figure 3. Network of Dependencies between Immune Measurements

(A) Undirected network model of the healthy human immune system showing 126 nodes (measurements), connected by 142 undirected edges illustrating conditional measurement dependencies. Nodes are colored by their estimated heritability and sized by their number of edges. (B) Subnetwork exemplifying direct relationships between heritable and non-heritable nodes. Solid edges represent positive relationships, and dashed edges represent negative relationships. The edge weight represents the strength of relationships.

See also [Table S6](#).

have lived apart for decades, so concordance can also be a result of either shared environment and/or shared exposure, in addition to genetic similarity. For several cell population frequencies, we found much reduced correlations with age ([Figure 4A](#)). In the most striking example, the frequency of Tregs between the youngest MZ twins correlated very strongly at 0.78 but was highly uncorrelated at 0.24 between the oldest MZ twin pairs ([Figure 4A](#)). Similarly, several serum proteins showed remarkably reduced correlations between older as compared to younger MZ twins ([Figure 4B](#)). In particular, the chemokine CXCL10/IP10 showed a strong correlation (0.79) between the youngest MZ twins but was greatly reduced (0.18) in the older MZ twins ([Figure 4B](#)). Similar patterns were found for many cell signaling responses (data not shown), suggesting that this immune divergence between genetically identical twins with age is a common phenomenon, consistent with a major role for environmental exposure in driving variation, although some epigenetic changes could also contribute ([Fraga et al., 2005](#)).

### Cytomegalovirus Infection Has a Broad Influence on Immune Variation

As we postulate that microbial exposure is a likely driver of immune variation with age, a particularly interesting example is cytomegalovirus (CMV), a lifelong viral infection that has striking effects on the immune phenotypes of both humans and rhesus macaques ([Sylwester et al., 2005](#)). In our twin cohort, 16 MZ pairs were discordant for CMV seropositivity, and we compared their twin-twin correlations for all measurements to those of 26 CMV concordant (negative) MZ pairs. Here, we found that the CMV discordant MZ twins showed greatly reduced correlations

for many immune cell frequencies such as effector CD8<sup>+</sup> and gamma-delta T cells ([Figure 5A](#)), as compared to CMV-negative MZ twins. The same was true for cell signaling responses, especially in response to IL-10 and IL-6 stimulation ([Figure 5B](#)), as well as the concentrations of these same cytokines in serum ([Figure 5C](#)). In general, the influence of CMV was very broad, affecting 119 of all 204 measurements (58%) dispersed throughout the immune network ([Figure 5D](#)) and illustrating how at least one type of microbial exposure can dramatically modulate the overall immune profile of healthy individuals.

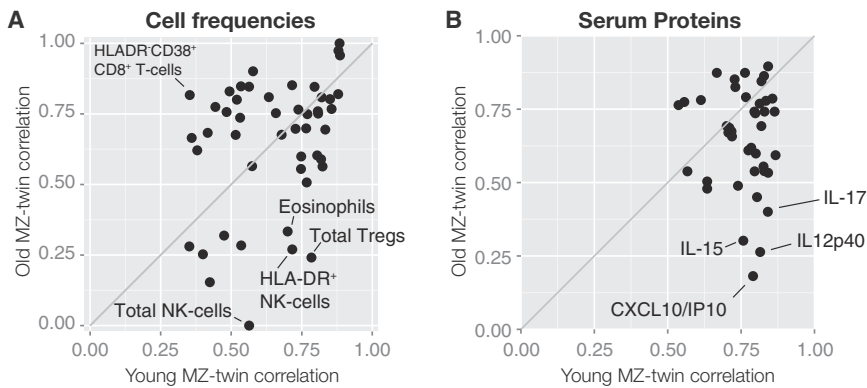
### Antibody Responses to Seasonal Flu Vaccines in Adults Have No Detectable Heritable Component

Finally, we immunized all of the subjects with seasonal flu vaccines in the year of participation (2009, 2010, or 2011) and assessed antibody responses using a standard hemagglutination inhibition (HAI) assay ([Experimental Procedures](#), “Hemagglutination Inhibition Assays”). We were surprised to find no detectable contribution from heritable factors on any of these vaccine responses ([Table 1](#)). As pre-existing antibodies are known to influence flu vaccine responses ([Sasaki et al., 2008](#)), we excluded subjects with a pre-vaccination titer above 40 but were still unable to find any heritable influences ([Table 1](#)). Though preliminary due to a small sample size, this result suggests that responses to seasonal flu vaccines in healthy adults (median age, 44 years) are dominantly influenced by non-heritable factors, likely due to multiple previous vaccinations and/or infections involving this pathogen ([Table 1](#)).

In summary, our findings strongly suggest that a healthy human immune system adapts to non-heritable influences such as pathogens, nutritional factors, and more and that this overshadows the influences of most, although not all, heritable factors.

## DISCUSSION

The vertebrate immune system consist of thousands of different components, and the application of systems biology ([Davis,](#)



**Figure 4. Increased Variability in the Immune System with Age**

(A) Twin-twin correlations (Spearman's rank) for all cell frequencies within the youngest MZ twin pairs ( $\leq 20$  years; median, 13.5;  $n = 25$  pairs), and the oldest MZ twin pairs ( $\geq 60$  years; median, 72 years;  $n = 16$ ).

(B) Twin-twin correlations (Spearman's rank) for all serum protein concentrations within the youngest MZ twin pairs ( $\leq 20$  years; median, 13 years;  $n = 26$ ) and the oldest MZ twin pairs ( $\geq 60$  years; median, 73 years;  $n = 13$ ).

2008; Duffy et al., 2014; Li et al., 2014; Querec et al., 2009; Sekaly, 2008; Tsang et al., 2014) holds great promise as a way to understand the interactions between these during immune health and disease. Here, we combine a classical twin study approach with the most recent advances in immune monitoring technologies to assess the balance between heritable and non-heritable influences on the functional units of the immune system, namely serum proteins and cell populations. In every category, we find that non-heritable influences dominantly influence 77% of all measurements ( $>50\%$  of variance) and almost exclusively drive 58% of the measurements ( $>80\%$  of the variance). Because most measurements made in this study focus on the adaptive immune system, partly due to the availability of reagents, one possibility is that these low levels of heritability are related to the stochastic nature of antigen receptor recombination. Indeed, previous work has shown that there are significant differences in the immunoglobulin-sequence repertoires of MZ twins (Glanville et al., 2011). But this is unlikely, as we find low heritability estimates also for many innate immune cell frequencies (NK cells, monocytes, and granulocytes [Figure 1B]) and differences within and between CD4<sup>+</sup> and CD8<sup>+</sup> subsets of T cells, which share the same antigen receptor apparatus.

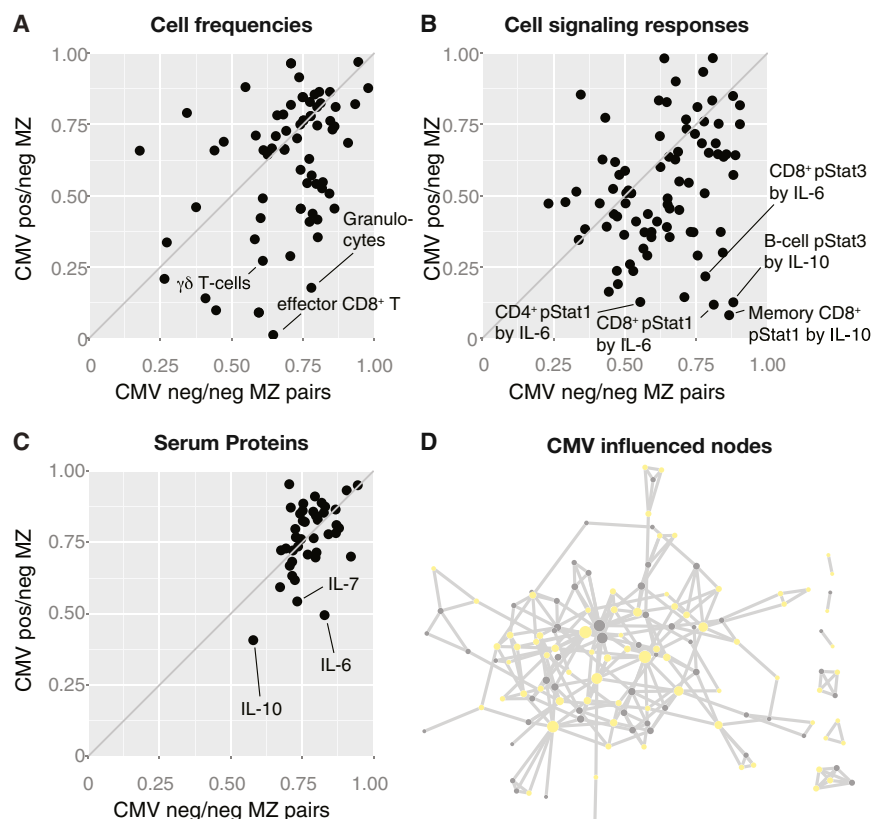
Although this study is not powered to completely rule out all heritable influences on any of the measurements made, the overall dominance of non-heritable factors is independent of this. The low estimates of heritable influences are also not explained by technical noise, as this is rigorously corrected for in our models (Figure S6A). We are also able to rule out that measurement variability over time is an important source of bias (Figure S6B). Therefore, the low heritability estimates for the majority of measurements cannot be explained by either technical noise or biological variability over time.

Given the concordance rates for common autoimmune diseases between 25% and 50% (Cooper et al., 1999) and the many studies finding associations between specific genetic variants and immunological traits and disorders, we were surprised to find such a dominance by non-heritable factors on these functional units of the immune system. Several large population studies have associated specific loci with white blood cell counts, showing some heritable influence though the amount of variation explained is typically low (Okada et al., 2011; Reiner et al., 2011). A recent study by Orrù et al. estimated the heritability of 272 immune cell traits from a non-twin cohort of healthy in-

dividuals on the island of Sardinia. They found that 220 of these ( $\sim 81\%$ ) had an estimated heritability lower than 50% (Orrù et al., 2013), which is comparable to our results. Although all the most heritable subpopulations identified by Orrù et al. expressed the marker CD39, which was not analyzed in our study, a number of the cell populations gave quite similar values, although others were different, such as NKT cells and central memory CD8<sup>+</sup> T cells (Table S7), possibly reflecting different environments (Bell and Spector, 2011) or a more diverse Palo Alto cohort versus a less diverse Sardinian one.

It is important to note that the twins in our cohort are healthy and without any known immunological deficiencies. Interestingly, two serious immunodeficiency syndromes are caused by defects in the genes IRAK-4 and MyD88 and are associated with invasive bacterial infections due to defects in TLR or IL-1R signaling (von Bernuth et al., 2008; Ku et al., 2007). Despite being associated with severe and often lethal infections in young children, both of these conditions improve significantly with age, starting in late childhood (von Bernuth et al., 2008; Ku et al., 2007). Although this could be explained by developmental immaturity, an alternative explanation for the improvement with age could be that these children's immune systems become more capable with greater environmental exposure.

A striking example of how broad the influence from one non-heritable factor can be is shown here for CMV, influencing 58% of all parameters measured in discordant MZ twins. These striking differences illustrate how non-heritable factors, alone or in combination, can affect the immune system broadly. We suggest that repeated environmental influences like herpes viruses and other pathogens, vaccinations, and nutritional factors cause shifts in immune cell frequencies and other parameters and, with time, outweigh most heritable factors. As an example, the life-long need to control CMV seems to cause a broad shift in the magnitude and complexity of many cell subsets (Chidrawar et al., 2009; Wills et al., 2011), and  $\sim 10\%$  of all T cells in CMV<sup>+</sup> individuals can be directed against this virus (Sylwester et al., 2005). The microbiome also clearly has a major influence on the immune system (Hooper et al., 2012; Mazmanian et al., 2005), and shifts in its composition might cause significant changes in the immune system. Also interesting in the context of how infectious disease exposure can shape subsequent immunity are the influenza vaccine results, in which we could not detect any heritable influence on the antibody responses to



**Figure 5. Broad Non-Heritable Influences in the Healthy Immune System**

(A) Twin-twin correlations (Spearman's rank) for all cell frequency measurements made in CMV concordant negative (neg/neg) MZ twin pairs ( $n = 26$  pairs) and CMV discordant (pos/neg) MZ twin pairs ( $n = 16$  pairs).

(B and C) (B) Twin-twin correlations (Spearman's rank) for cell signaling responses to cytokine stimulation and (C) serum protein measurements between CMV neg/neg and CMV pos/neg MZ twin pairs.

(D) 58% of all 126 nodes in the immune network model with reduced correlations in CMV pos/neg as compared to CMV neg/neg MZ twin pairs.

influenza vaccination (Table 1). This recalls the “original antigenic sin” hypothesis (Francis, 1960), wherein it was postulated that previous encounters with influenza strains strongly impact the response to a novel strain. But our finding contrasts with other vaccine studies in twins, most often performed in very young children and involving vaccines against pathogens less frequently encountered in the population (Table 1) (Jacobson et al., 2007). Specifically, vaccines against mumps, measles, rubella (Tan et al., 2001), oral polio, tetanus, and diphtheria vaccines (Newport et al., 2004) have all been shown to be strongly heritable (Table 1) in studies with young children. The one study that we know of from adult twins is a study of hepatitis A/B vaccine responses and was conducted across a similar age range (18–65 years) as ours and reported a heritable influence of 36% for hepatitis A and 61% for hepatitis B antibody responses (Table 1). However, two other studies of hepatitis B vaccine responses performed in young children showed much higher estimates of heritability of 91% (Yan et al., 2013) and 77% (Newport et al., 2004), respectively. In addition, responses to vaccines given at birth (oral polio vaccine [OPV] and Bacillus Calmette–Guérin [BCG]) are more heritable than even those administered only 2 months later (diphtheria and tetanus) (Newport et al., 2004; O'Connor and Pollard, 2013). In addition, Evans et al. analyzed 12-year-old twins in Australia (Evans et al., 1999). Although there are only six broad categories of immune cell subsets that can be compared with our study and those of Orrù et al., it is interesting that, in all cases, the estimates of heritability in the 12-year-old twins were higher than either ours or Orrù et al., in

which the mean age is about 38 and 40 years, respectively (Table S7). These observations are consistent with our data (Figure 4), showing an increasing non-heritable influence on many variables with age, and suggest quite strongly that many if not most of the less heritable traits that we measure here in our mostly adult population may be much more heritable if measured in young children.

Before the advent of childhood vaccines, antibiotics, and improvements in human hygiene, almost half of all children younger than 5 years of age died of infectious diseases. Casanova and colleagues have proposed a genetic theory of infectious disease to account for the interindividual differences in susceptibility (Alcais et al., 2009). Our data and the vaccine studies cited here suggest that such genetic predisposition would be most pronounced in young children but that, later on, the adaptive nature of the immune system is able to overcome many defects. This is similar to the hypothesis proposed by Alcais and colleagues to explain the discrepancies in genetic lesions underlying the susceptibility to primary and secondary infections, respectively (Alcais et al., 2010). Adaptations of the immune system with time could be the result of well-known immune mechanisms, such as specific antibodies and T cells or cross-reactive immunity (Su et al., 2013), or some as yet to be defined maturational process. We would also speculate that the immune system may have feedback mechanisms that allow it to skew its mix of cell types and functional properties in order to compensate for a given individual's particular mix of gene polymorphisms and microbial exposures.

In summary, we find that, in an examination of many of the component parts of the immune system, as well as some response metrics, much of the considerable variation in human beings is driven by non-heritable influences. This variation increases with age and is likely due in large part to exposure to pathogens and other microbes, as we see for CMV discordant MZ twins and in the responses to influenza vaccination. Lastly, we expect that other complex systems in higher organisms, such as the nervous system, will also show this pronounced

**Table 1. Influenza Vaccine Responses in Adults Are Determined Mainly by Non-Heritable Factors**

Vaccine Response	Age at Vaccination	Heritability	Comment
Measles	1–6 years <sup>a</sup>	89%	(Tan et al., 2001)
Polio (oral vaccine)	<1 year	60%	(Newport et al., 2004)
H. influenzae b	<1 year	51%	(Lee et al., 2006)
Diphtheria	<1 year	49%	(Newport et al., 2004)
Rubella	1–6 years <sup>a</sup>	46%	(Tan et al., 2001)
Tetanus	<1 year	44%	(Newport et al., 2004)
Mumps	1–6 years <sup>a</sup>	39%	(Tan et al., 2001)
Combined hepatitis A/B	18–65 years	36% (HAV)/61% (HBsAg)	(Höhler et al., 2002)
Hepatitis B virus	<1 year	77%/91%	(Newport et al., 2004; Yan et al., 2013)
B/Brisbane/60/2008	12–82 years (median, 45)	0% (<20%)	
A/Cal/07/2009 (H1N1)	12–82 years (median, 44)	0% (<20%)	
A/Perth/16/2009 (H3N2)	12–82 years (median, 44)	0% (<20%)	
B/Brisbane/60/2008	13–77 years (median, 49)	0% (<20%)	Pre-vaccine titer < 40
A/Cal/07/2009 (H1N1)	12–76 years (median, 45)	0% (<20%)	Pre-vaccine titer < 40
A/Perth/16/2009 (H3N2)	12–82 years (median, 44)	0% (<20%)	Pre-vaccine titer < 40

Table of published heritability estimates for vaccine responses to various vaccines as well as the seasonal influenza vaccine responses analyzed in this study with or without the removal of subjects with a pre-vaccine antibody titer  $\geq 40$ .

<sup>a</sup>Vaccine responses analyzed between 2 and 18 years of age.

influence of non-heritable factors, as there is also a need (and ability) of such systems to adapt to environmental stimuli.

## EXPERIMENTAL PROCEDURES

### Twin Cohort

In collaboration with the Twin Research Registry at SRI International (Krasnow et al., 2013), 105 healthy twin pairs were recruited over the years 2009, 2010, and 2011. The study protocol was approved by the Stanford University Administrative Panels on Human Subjects in Medical Research, and written informed consent was obtained from all participants. We excluded anyone having received the seasonal influenza vaccine in the last 6 months, anyone with known or suspected impairment of immunologic function, with clinically significant liver disease, diabetes mellitus treated with insulin, moderate to severe renal disease, or any other chronic disorder, including autoimmune diseases or severe hypertension. We also excluded anyone who had received blood products in the last 6 months and pregnant or lactating women. The complete inclusion/exclusion criteria are available (Data S1).

### Blood Sampling, PBMC Preparation, and Zygosity Testing

Blood samples were collected in heparinized vacutainer tubes by venipuncture at day 0 (and day +28 for HAI responses after seasonal influenza vaccination) at the Clinical and Translational Research Unit, Stanford University Hospital. Whole blood collected in sodium heparin tubes was either analyzed immediately using our whole-blood flow cytometry protocol below or enriched for PBMCs using 15 ml of Ficoll-Paque PLUS (GE Health Care) and frozen at  $-80^{\circ}\text{C}$  overnight, transferred to liquid nitrogen, and stored until further analysis. Zygosity was determined by comparing 384 SNP loci using a discriminatory DNA polymerase and ligase assay (GoldenGate genotyping Assay, Illumina) and were performed by IGenix. Twins were considered fraternal if similarities in DNA markers were below 99.0%. Twins of the same pair were almost exclusively analyzed in the same experimental batch, irrespective of technology used in order to minimize technical variation.

### Immune Cell Phenotyping by Mass Cytometry and Flow Cytometry

All experiments were performed by the Human Immune Monitoring Center at Stanford University. For years 2010 and 2011, 2 million thawed PBMCs were stained without prior resting or fixation using a panel of 26 different metal-

tagged probes to surface antigens and DNA (Data S2A). After repeated washes, cells were analyzed by mass cytometry (CyTOF, Fluidigm) with the following instrument settings: high-resolution mode analysis with cell length set to 10–75 pushes, a lower deconvolution threshold of 10, and instrument run in dual-count detection mode and noise reduction turned off. FCS3.0 files were manually analyzed using FlowJo v9.3 (TreeStar), as shown in Figure S4. Year 2009 PBMC samples were similarly processed but were instead analyzed using a set of custom-made Lyoplates (BD Biosciences) covering seven different antibody panels of fluorescently labeled antibodies (Data S2B and S2C). These samples were acquired using a LSRII flow cytometer (BD Biosciences) and were similarly analyzed manually using the same FlowJo v9.3 (TreeStar).

### Immune Cell Signaling Experiments

PBMCs were thawed in warm media, washed twice, and resuspended at  $0.5 \times 10^6$  viable cells/microliter. 200  $\mu\text{l}$  of cells were plated per well in 96-well deep-well plates. After resting for 1 hr at  $37^{\circ}\text{C}$ , cells were stimulated by addition of 50  $\mu\text{l}$  solutions of cytokines: IFN $\alpha$ , IFN $\gamma$ , IL-6, IL-7, IL-10, IL-2, or IL-21 (Data S2D), respectively, and incubated at  $37^{\circ}\text{C}$  for 15 min. Cells were then immediately fixed in 1.6% paraformaldehyde, permeabilized with 100% cold methanol, and kept at  $-80^{\circ}\text{C}$  overnight. Each well was barcoded by a unique combination of Pacific Orange and Alexa-750 dye concentrations (Invitrogen/Life Technologies), and the cells were washed with FACS buffer (PBS supplemented with 2% FBS and 0.1% sodium azide) and stained with our phospho-Flow antibody panel (Data S2E). Finally, cells were washed and resuspended in FACS buffer, and 100,000 cells per stimulation condition were collected using DIVA 6.0 software on an LSRII flow cytometer (BD Biosciences). Data analysis was performed using FlowJo v9.3 (TreeStar), as shown in Figure S5, and the mean fluorescence intensity (MFI) of the 90th percentile was used for downstream analysis. All stimulated samples were compared to unstimulated control (baseline) samples, and fold changes were calculated. Responses above 1.5 fold change as well as baseline MFI values were used for heritability estimates.

### Serum Protein Quantification

Blood samples were centrifuged and stored at  $-80^{\circ}\text{C}$  awaiting analysis. Human 51-plex were purchased from Affymetrix and were used according to the manufacturer's recommendations with modifications as described below. In brief, samples were mixed with antibody-linked polystyrene beads



on 96-well filter-bottom plates and incubated at room temperature for 2 hr followed by overnight incubation at 4°C. Room temperature incubation steps were performed on an orbital shaker at 500–600 rpm. Plates were vacuum filtered and washed twice with wash buffer and were then incubated with biotinylated detection antibody for 2 hr at room temperature. Samples were then filtered and washed twice as above and were resuspended in streptavidin-PE. After incubation for 40 min at room temperature, two additional vacuum washes were performed and the samples resuspended in reading buffer. Each sample was measured in duplicate. Plates were read using a Luminex 200 instrument with a lower bound of 100 beads per sample per protein. Each sample was measured in duplicate. Plates were read using a Luminex LabMap200 instrument with a lower bound of 100 beads per sample per protein per well. The Luminex LabMap200 outputs the fluorescence intensity of a given protein in a sample. For each well, we considered the median fluorescence intensity (MFI) for a serum protein as its abundance and averaged the MFI of these replicates. To ignore low-abundance proteins, only measurements with mean concentrations higher than a negative control serum were included in our analysis.

### Hemagglutination Inhibition Assays

The HAI assay was performed on sera from day 0 and day 28 post influenza vaccination. Fold changes day 28/day 0 were used for analyses. In one analysis, subjects with a pre-vaccination titer of 40 or more were excluded (Table 1). Serially diluted 25  $\mu$ l aliquots of serum samples in PBS were mixed with 25  $\mu$ l aliquots of virus, corresponding to four HA units, in V-bottom 96-well plates (Nunc). These were then incubated for 15 min at room temperature. At the end of the incubation, 50  $\mu$ l of 0.5% chicken red blood cells (cRBC) were added and the plate incubated for 1 hr at room temperature, and HAI activity was read as follows: (1) positive result: hemagglutination is present, the well is hazy with no cRBC button or (2) negative result: hemagglutination is absent, the well is relatively clear with cRBC button. The HAI titer is defined as the reciprocal of dilution of the last well that inhibits hemagglutination.

### Structural Equation Modeling to Estimate Heritable and Non-Heritable Influences

For all of the measurements made, a structural equation modeling approach was used to estimate heritability (Rijsdijk and Sham, 2002). This classical twin modeling approach is based on the assumption that MZ twins are genetically identical, whereas DZ twins share ~50% of their polymorphic genes and that MZ and DZ twin pairs are equally similar with respect to their shared environmental influence. The covariance matrix for each measurement made in MZ and DZ twin pairs can then be decomposed into three parameters: (1) additive genetic parameter, (2) common environmental parameter, and (3) environmental parameter unique to one twin. A linear ACE model then estimates the contribution of each of these parameters by maximum likelihood. After correcting the E-parameter for technical measurement errors, as described in detail below, all parameters were scaled as a proportion of the total variance (A+C+E). All data was corrected for age and gender prior to ACE modeling. We performed resampling tests on all heritability estimates, using a jackknife resampling approach leaving one twin pair out of the calculation in each iteration and using the mean values of all such iterations as our final estimate with 95% confidence intervals. All calculations were performed using both our own implementation of ACE fitting in MATLAB, version 8.3 and the openMX library version 1.4 running in R version 3.0.3. Both platforms were used in order to prevent any bias due to the software used.

### Correction of Model Estimates for Technical Variability

For all measurements, standard samples were analyzed repeatedly (>17 times). These were either PBMC aliquots frozen at the same time and thawed for every experiment or pooled serum used as standards for Luminex and HAI assays. By calculating pooled variance estimates for these technical replicates and subtracting this from the E-component in our ACE models prior to normalization, we prevented the underestimation of heritability due to such stochastic measurement errors. This procedure overestimates the technical noise level of the actual twin samples by being collected across multiple batches, whereas twin samples compared were always analyzed within the same batch, and after correction, no relationship between technical variability and heritability

estimates is seen (Figure S6A). We also assessed the biological variability over time in an unrelated cohort by calculating coefficients of variance (CVs) from longitudinal samples drawn once yearly for 2–5 years. No relationship between longitudinal CVs and estimated heritability was found (Figure S6B).

### Identification of Pairwise Dependencies between Measurements and the Creation of an Immune Network Model

To identify meaningful relationships between measurements, we used the recently developed non-paranormal SKEPTIC approach (Liu et al., 2012), with a transformed Spearman/rank correlation matrix as input. To make the network model interpretable, we pursued sparse precision matrices with a graphical lasso approach penalizing non-zero entries in the matrix (Friedman et al., 2008). A zero entry in the precision matrix encodes conditional independencies of pairs of measurements given the state of all other measurements and is less sensitive to spurious indirect connections as compared to simple correlation analyses. To validate the inferred structural relationships, two tests were performed. (1) A permutation of samples on per phenotype basis was done to obtain null distributions for entries of the precision matrix. By repeatedly producing permuted samples and running our procedure on those samples, we obtain a distribution of precision matrices that is mostly dominated by very sparse, diagonal matrices but that also has occurrences of precision matrices that have non-zero entries. The non-zero entries thus obtained were false positive, and hence we can estimate which entries obtained on the actual data exceed the size of these false positives. (2) Given relative infrequent occurrences of non-zero entries when fitting sparse precision matrices, we opted to estimate confidence intervals for each entry in the precision matrix. We deemed entries whose confidence interval contained 0 insignificant. To obtain these confidence intervals, we performed bootstrap analysis by resampling the real samples.

### CMV Serology

CMV serology was determined using a commercially available ELISA kit (CMV IgG, Gold Standard Diagnostics) as per manufacturer's instructions. In brief, sera stored at  $-80^{\circ}\text{C}$  were thawed to room temperature ( $20\text{--}25^{\circ}\text{C}$ ) and diluted 1:51 in kit diluent. Diluted samples were added to wells coated with CMV antigen from strain AD169 and were incubated at room temperature for 30 min. Wells were washed and drained, followed by the addition of goat anti-human IgG antibodies labeled with calf alkaline phosphatase, and incubated at room temperature for 30 min. Wells were again washed and drained, followed by addition of p-nitrophenyl phosphate substrate, and incubated at room temperature for 30 min. After the addition of 0.5 M trisodium phosphate stop solution, the absorbance of each well at 405 nm was read and results analyzed using the manufacturer's instructions.

### MZ Twin-Twin Correlations

All measurements performed in the youngest set of MZ twin pairs (<20 years) and oldest MZ twin pairs (>60 years) was extracted, and Spearman's rank correlation coefficients were calculated and compared between these independent groups for every measurement made. Similarly, CMV serologically negative/negative MZ-twin pairs were compared to CMV positive/negative (discordant twins).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, seven tables, and two data files and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2014.12.020>.

### AUTHOR CONTRIBUTIONS

P.B. and V.J. performed all analyses. T.G. helped with the network analysis. S.B. and S.S.-O. provided input on heritability estimates. All data was generated by the Human Immune Monitoring Center under the direction of H.T.M. except for the 2009 HAI data generated by D.F. and CMV serology by C.J.L.A. G.E.S. directs the twin registry and provided input on the twin analyses, and C.L.D. was responsible for regulatory approvals, protocol design,

study conduct, and clinical data management. P.B. and M.M.D. wrote the paper with input from co-authors.

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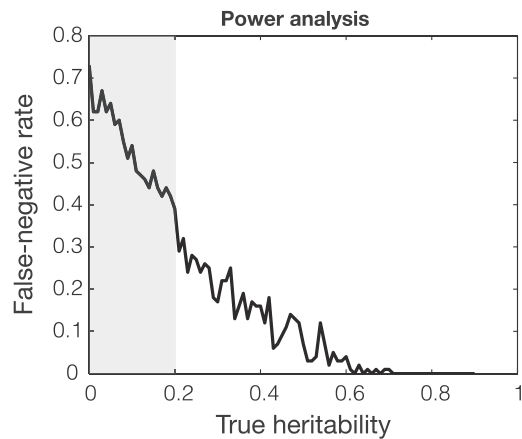
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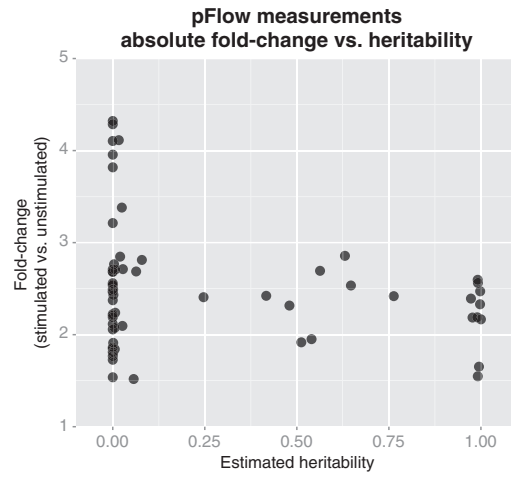
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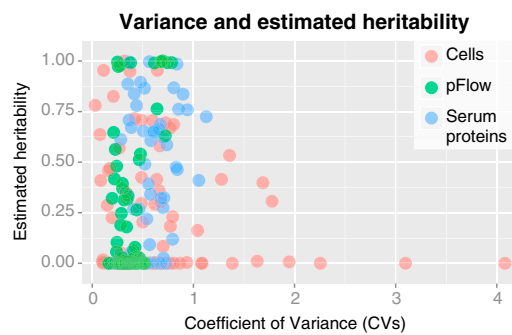


**Figure S1. Power Analysis, Related to Experimental Procedures**

One thousand randomly generated data sets with 78 MZ twin pairs and 27 DZ twin pairs in each and for every level of known heritability between 0 and 1 at increments of 0.01. False-negative rate is the number of simulations yielding  $h = 0$ , for each true value of  $h$ . Grey area marks  $h \leq 0.2$ , the estimated detection limit.

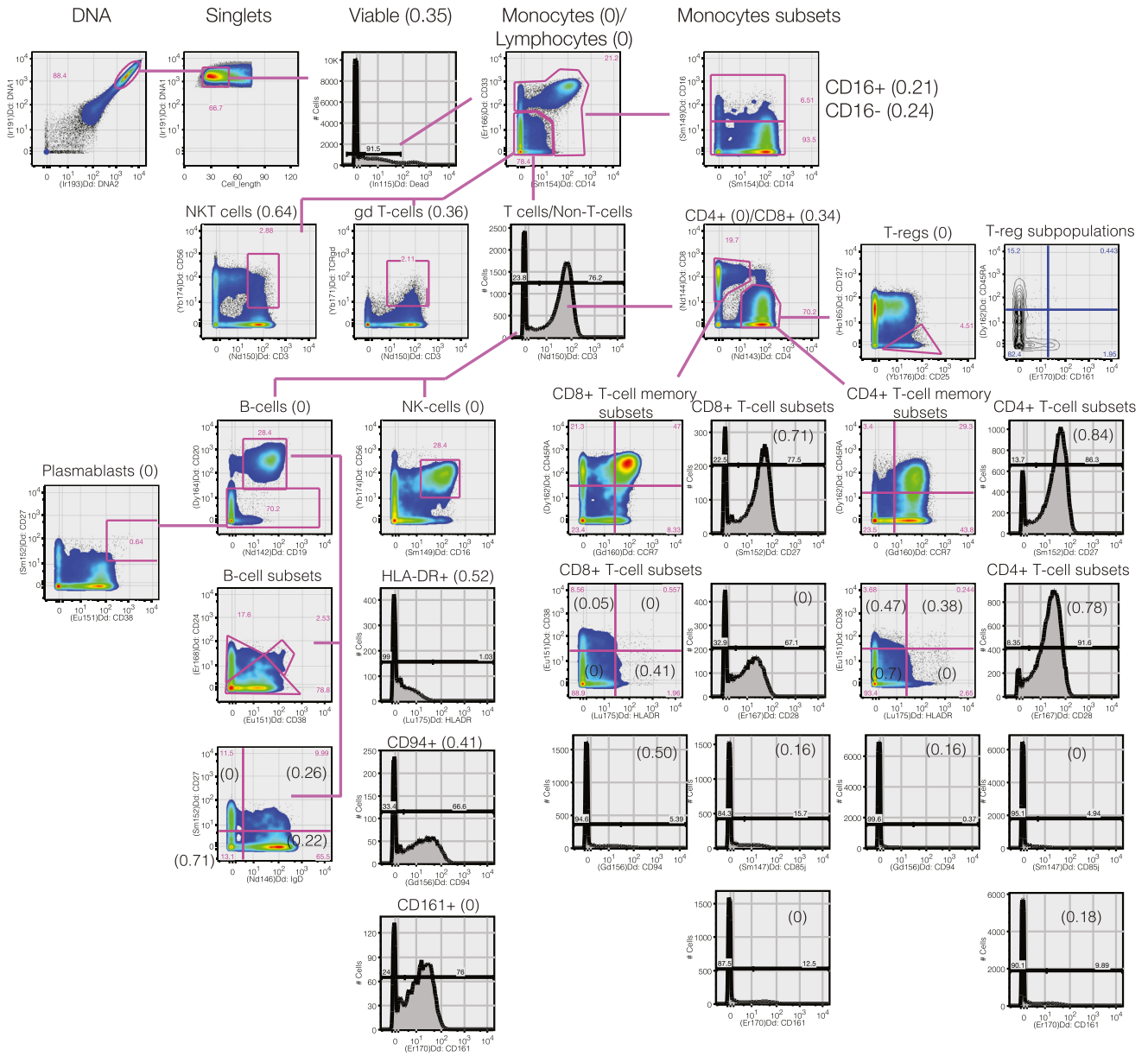


**Figure S2. Absolute Levels of pFlow Signaling Responses Does Not Impact Heritability Estimates, Related to Experimental Procedures**  
For each measurement above the (1.5 fold) background threshold, absolute fold-change (stimulated/unstimulated) of signaling responses upon cytokine stimulation is shown against the level of estimated heritability.

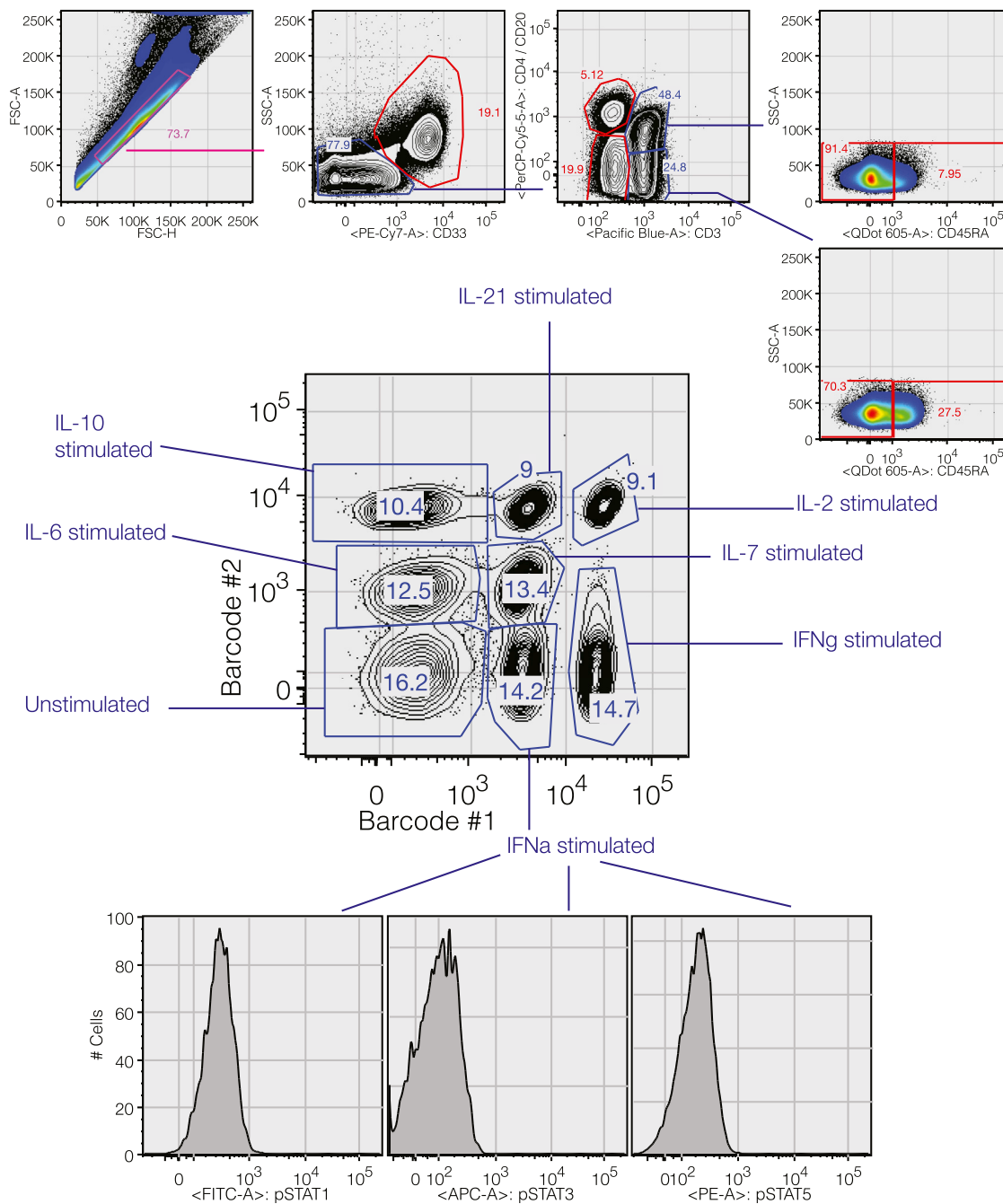


**Figure S3. Absolute Measurement Variance Does Not Impact Heritability Estimates, Related to Experimental Procedures**

For each measurement coefficients of variance (CV) in the complete twin cohort is shown against the corresponding corrected estimates of heritability for each measurement.



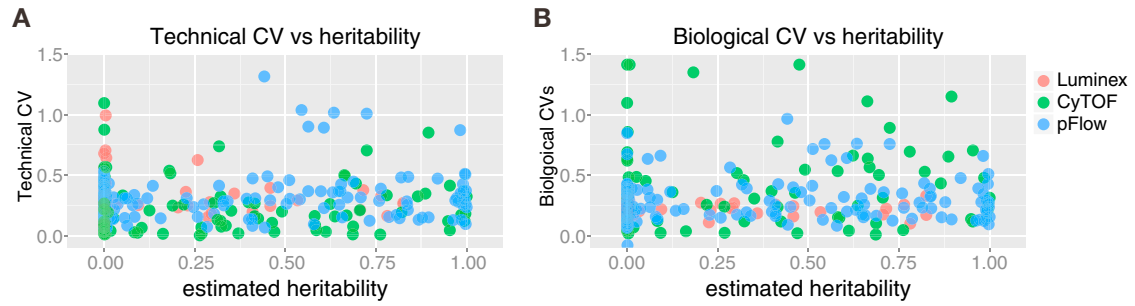
**Figure S4. The Identification of 91 Different Immune Cell Subsets from PBMCs by Mass Cytometry (CyTOF, Fluidigm), Related to Experimental Procedures**  
 Manual gating using the FlowJo v9.3 software (TreeStar, Inc). One representative FCS3.0-file generated by Mass Cytometry (CyTOF) is shown and in parentheses the estimated heritability of the gated population is shown for reference.



**Figure S5. Phospho-Protein Analysis of Signaling Molecules in Response to Cytokine Stimuli Using Flow Cytometry, Related to Experimental Procedures**

PBMC surface stained and fluorescently barcoded after being stimulated by either of seven different cytokines. After barcoding, samples are pooled and stained with antibodies to phosphorylated signaling molecules STAT1, 3 and 5 as well as surface markers. For the identified populations in red, the eight, barcoded stimulation conditions are analyzed for the level of phosphorylated transcription factors STAT1, 3 and 5.





**Figure S6. Heritability in Relation to Technical Variability and Longitudinal Variability of Measurements, Related to Experimental Procedures**  
(A) Coefficients of Variance (CV) for each measurement calculated from at least 17 technical replicate samples as an estimate of measurement “noise,” plotted against estimated level of heritability for the corresponding measurements in the twin cohort. (B) Coefficients of Variance (CV) for each measurement over longitudinal samples made in an unrelated cohort with yearly samples taken.