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Rebooting Human Immunology

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Abstract

Recent progress in both conceptual and technological approaches to human immunology have rejuvenated a field that has long been in the shadow of the inbred mouse model. This is a healthy development both for the clinical relevance of immunology and for the fact that it is a way to gain access to the wealth of phenomenology in the many human diseases that involve the immune system. This is where we are likely to discover new immunological mechanisms and principals, especially those involving genetic heterogeneity or environmental influences that are difficult to model effectively in inbred mice. We also suggest that there are likely to be novel immunological mechanisms in long-lived, less fecund mammals such as human beings since they must remain healthy far longer than short-lived rodents in order for the species to survive.

Keywords

human immunology; systems immunology; CMV; human evolution

INTRODUCTION

For many decades, inbred mice have been the go-to system for any kind of advanced immunology, especially since the 1980s, when genetic methodologies allowed for sophisticated manipulations such as transgenics, knockouts, knockins, and CRISPR that were further enabled by whole-genome sequencing. Such developments have led to an explosion of knowledge about the immune system that continues to this day—and indeed it is hard to imagine how such major puzzles as antibody diversity and MHC-restricted recognition by T cells could have been solved without inbred mice and the cell lines derived from them. For those concerned with human diseases, the general paradigm was to create a mouse model of the disease, dissect how the model works, and develop a treatment that would then be translated to human patients. All seemed to be well, and exploration of immunological phenomena and mechanisms has arguably been one of the leading fields in

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the biological sciences. However, some assumptions—particularly the belief that mouse models of disease would lead inevitably to human treatments, which has rarely come to pass—have not been realized, although there have been notable exceptions (such as cancer checkpoint inhibitors, which have made it to the clinic only with a great deal of effort). Many authors over the years (1–4) have made this point, but it is just starting to be acknowledged.

Why are the mouse models not working? There could be many reasons, including the obvious one of rodents being short-lived relative to long-lived primates, the lack of environmental or genetic diversity, and the inevitable compromises involved in trying to create a mouse model of a disease (injecting tumor lines for cancer models, for example, or infecting mice with pathogens they do not normally encounter). Nonhuman primates (e.g., macaques) have been imperfect models as well. But one important reason why we do not know the precise answer to this question is that, until recently, we have not had very many data on human beings and their diseases. If we had, then we could have been more judicious in the creation and validation of these models. So why do we lack these data? Why did such data acquisition not march hand in hand with the explosive growth in murine immunology? In our view, a multitude of factors were involved. One factor is that the methodology for mouse work was powerful and advancing so rapidly that it attracted many basic scientists, while the technology needed for human work, which has many more constraints, was relatively stagnant. The general consensus that mouse models of disease were best for translational work also led many of the best physician scientists to this area, with many interesting results but very few cures. For example, type I diabetes in NOD mice and an induced demyelinating disease (EAE) have been prevented hundreds of times, but not in any humans to date. So, is it defensible to argue that preventing these diseases in mice in hundreds of additional ways is a good use of resources? Mostly not, and since the immune system is involved in many diseases, not developing a better paradigm for applying it to human health more efficiently would be a terrible loss. And the risk to the field of immunology of continuing to focus on mice over humans could be considerable because work in this area could be stereotyped as interesting biology but irrelevant to medicine, as the area of cancer immunology once was before its ninth-inning rally in recent times.

In addition, reviving human immunology also holds great promise for basic immunology as well, as recent results are starting to show and as we wish to emphasize in this review. The human model has many of the characteristics—genetic diversity and broad exposure to environmental microbes, both pathogenic and commensal—that inbred mice models lack; humans are also subject to many interventions (every vaccine and an ever-increasing list of medicines). Plus, there is no need to worry that a disease is an artifact of the experimental conditions. What follows is a discussion of strategies, ideas, and results that provide a way forward for reinvigorating human immunology—not as a replacement for inbred mice but as a vibrant and freestanding endeavor that both enriches our understanding of the immune system in general and is a reliable and important engine of translation. Below we outline some of the principal strategies for investigating the human immune system. We then discuss some interesting results that have emerged or could emerge.

STRATEGIES AND TACTICS

Systems Immunology

Human immunology has long been in need of a strategy that can enable work broadly across both health and disease [see a recent review for a more comprehensive discussion of this approach, especially as applied to vaccination (5)]. Since most approaches that are used in mouse immunology are not an option in human immunology, a strategy that fits best with available clinical material and is still relevant to immune function is critical. Most researchers in the field have settled on a systems analysis of blood since this approach involves sampling of white blood cells as well as many other cytokines that represent communications between immune cells and tissues. White blood cells are not a perfect substrate or substitute for studies directly in lymphoid organs, as several authors have pointed out, but these cells are an abundant resource in almost every disease cohort and are relatively easy to obtain in healthy individuals. For large quantities of cells for repertoire studies, for example, blood banks throw away a billion or so white blood cells with each donation; this represents a largely underutilized resource. In addition, in response to an immunological stimulus, antigen-specific and clonally expanded B and T cells surge through the blood, beginning from a local response to spread throughout the body. For example, Wilson and colleagues (6) showed that 7 days after a flu vaccination, plasmablasts in the blood were between 50–80% flu specific. Similarly, Sollid and colleagues (7) showed that gliadin-specific $CD4^+$ T cells were highly enriched in the blood 6 days postgluten challenge in celiac patients. Thus, the blood is a dynamic conduit through which immune responses traverse and, if the timing is right, a way to capture lymphocytes that are highly enriched for cells specific for a given response (8). The other aspect that makes blood worthy of attention is that we now have the technology to interrogate lymphocyte subsets and cytokines very broadly: Mass cytometry can distinguish between more than 100 different cell types and subsets (and fluorescence-based instruments are closing the gap quickly) (Figure 1a). In our immune monitoring facility at Stanford, we are routinely assaying 63 different cytokines by using Luminex Technology in blood samples, and there are technologies emerging that can monitor hundreds of molecules (9–11). Thus, it is feasible to survey many of the immune system components in an unbiased fashion in blood samples, and these technologies should improve significantly in the future. These novel high-dimensional technologies, including mass cytometry, offer the alternative of either very detailed analysis of well-defined and rare cell populations, such as antigen-specific T cells (Figure 1b), or broad coverage across all cell types in the blood and subsequent analyses of their interdependencies in a given condition (Figure 1c).

Also significant are technologies to measure pathogen exposure in the blood, principally through the measurement of serum antibodies. In theory, an individual’s infectious disease history should be represented in this way, and multiple groups have been developing this capability (12, 13). More difficult, but possible, are innovative ways to assess responses directly in human immunological organs. Fine-needle lymph node aspirates, for example, are being used to assess vaccine responses in healthy volunteers. Cox and colleagues (14) vaccinated more than 150 children with the live attenuated flu vaccine at various times (3–20

days) prior to scheduled tonsillectomy to study the immune responses in that type of specialized lymph node.

Another value of the systems approach is that it does not depend on a specific hypothesis. Since most of our knowledge about the immune system comes from decades of work on inbred mice, much of our hypothesizing must necessarily come from that knowledge, but it is more limited than if data had come from organisms that are exposed to a larger spectrum of microbes, are genetically heterogeneous, and have a much longer life span. But interestingly, for many of the uncovered phenomena, such as the inefficiency of negative selection on the existence of memory phenotype T cells (detailed below), what at first seemed unique in humans turned out not to be. Because we have to analyze human immune responses differently than what is the common practice in mice, some phenomena that at first appear to be differences may upon closer examination be found to occur in mice in much the same way. Nevertheless, there will certainly be significant differences between mouse and human immune responses; we simply have not gotten far enough in human studies to know what those differences are. For example, major differences in specific genes or families of genes in NK and other cells, the fact that there are three ICAM genes in humans versus one ICAM gene in mice, and the expression of class II MHCs in activated human T cells, which is not seen in mice, indicate that such differences exist, but whether there are profound differences in immune mechanisms remains to be seen (15). One can also argue that a short-lived rodent does not need as effective an immune system as does a long-lived primate, which not only is much less prolific but also needs to survive for more than 20 years to be able to have children and contribute to the next generation.

B and T Cell Repertoire Analysis

B and T cell repertoire analysis could be considered part of a systems approach, and indeed it is, as it is an intrinsic way to understand adaptive responses. However, the T cell aspect in particular has advanced rapidly in recent years, and it receives special emphasis here.

For many years, immunologists who wanted to study specific B or T cell responses needed to grow cell lines or make hybridomas, a time-consuming and often problematic enterprise. Furthermore, one rarely knew how representative the cells that grew out, or formed successful hybrids, were. But recently, advances in single-cell antigen receptor sequencing have greatly simplified the process and expanded the scope of what can be done dramatically, as well as giving a less biased survey of the lymphocytes involved in a given response. We can now take B or T cells directly from the blood or tissue and sort them into wells where both chains of their antigen receptors can be reverse transcribed, amplified, barcoded, and then sequenced directly. In the case of T cells, the TCR pairs can be immortalized in repertoire T cells lacking endogenous TCRs, and in the case of B cells, one can directly make recombinant antibodies and test for specificity in yeast display libraries or in other assays. T cells are more complicated than B cells, but we can now outline a complete set of procedures that allow one to go from any T cell of interest to identify dominant specificities and ligands.

The procedure for clonal analysis of individual T cells is illustrated in Figure 2. It starts with a population of T cells that is enriched for some response: tumor-infiltrating cells, for

example, or T cells that respond to a given pathogen. In our procedure (16), we sort single T cells into 96-well plates; lyse them; and amplify both chains of the TCR with a large collection of (more than 60) nested primers to capture all the V regions, together with another set of primers for more than 20 genes associated with particular T cell types (FoxP3, IL-2, etc.). The amplicons in each well are then tagged with a unique oligonucleotide bar code, and all the wells (>500) are combined and sequenced. A pipeline program is then used to determine the exact sequence of each TCR chain (normalizing for PCR errors) and to assign TCR sequences and the phenotypic markers to a given well. At this stage, it is readily apparent which T cell clones have expanded, that is, which clones occur more than once. TCRs, especially in humans, are so diverse that one would almost never find the same sequence by chance, except in the special case of public TCRs, which are rare and likely serve some purpose other than diverse antigen recognition. Although one can derive TCR pairs using other schemes (17, 18), this single-cell method is more efficient and definitive, with 70–90% success in obtaining both α and β chain TCR sequences.

Once TCR sequences have been obtained, the next problem is how to compare them to each other and especially between individuals, in whom TCR diversity is such that the TCR sequences can be quite different, even if they are responding to the same peptide-MHC complexes. Here, recently developed programs to cluster TCRs according to their likely shared specificities are proving to be crucial. Our own lab (19) and the Thomas lab (20) recently provided algorithms that work from sequence data alone to group TCRs according to their likely specificity. They rely on different aspects, but key is the fact that the CDR3 regions of αβTCRs are the main contact points for peptide specificity (21, 22), and contact with the antigenic peptide in the binding groove of the MHC is typically mediated by highly conserved motifs of 3 to 4 amino acids in the CDR3. Other features, such as the spatial relationship between the end of V-beta and this CDR3 motif, are also often important. An example of this analysis is shown in Figure 3, which shows a schematic of the GLIPH (grouping of lymphocyte interactions by paratope hotspots) methodology and a sample product of TCR clusters derived from multiple individuals that are latently infected with *Mycobacterium tuberculosis*. Here peripheral blood mononuclear cells (PBMCs) were stimulated with *M. tuberculosis* peptides, and $CD4^+$ T cells that became activated were isolated and sequenced (19). GLIPH analysis clustered these sequences together in many groups; the five that were most widely shared are shown in Table 1. HLA typing data showed that each of these clusters were enriched for particular class II MHC alleles, immediately suggesting what the restricting element was (of the 69 different alleles expressed by this very diverse group of 22 South Africans!). For each of these five groups, we were able to confirm the validity of the major class II allele indicated and to identify the correct *M. tuberculosis* peptide. The peptide candidates came from the painstaking work of the Sette group, who screened thousands of peptides from *M. tuberculosis* to produce a megapool of 300 CD4+ T cell epitopes (23). Other sources of candidate antigens may come from mass spectrometry analysis of peptides eluting from MHC proteins, and this approach has been used to identify tumor antigens (24), but in many cases the possibilities are much more open ended. But a solution to this difficult problem has emerged in the work of Garcia and colleagues (25, 26), who have developed a new powerful and general method of screening for novel T cell ligands by using a yeast display system. Here a particular class I

or class II MHC molecule, either intact or in a truncated form, is expressed as a fusion protein on a yeast surface molecule. An N-terminal extension is randomized to provide a peptide mimic that can bind to the groove of the MHC and be presented on the yeast surface. In this way, Garcia and colleagues have been able to display 108–109 peptides in a given MHC in a library. These libraries can then be panned with soluble TCRs attached to beads or in a tetramer format to isolate peptide-MHC complexes that bind to the TCRs. Since 108–109 is still three to four logs short of what is needed to suggest what exact peptide might be the correct target, specific algorithms are used to assess the spectrum of peptides that can bind a given TCR, and candidate peptides from protein databases that fit that spectrum are found (25, 26). The peptide candidates are then screened against reporter T cells to confirm which ones can stimulate.

Together, this collection of methods means that we can finally go from T cells of interest to identifying the relevant antigens, even when no candidate antigens are known. The TCR clustering programs are also critical in identifying shared specificities if that is desired. Defining these specificity clusters is important for many aspects of human immunology. We almost always lack crucial information about T cell specificity in, e.g., autoimmunity, infection, and cancer, and yet such information is vital if we want to develop antigen-specific tolerance in autoimmunity or tumor vaccines or to understand infectious disease responses.

Accelerating Human Immunology

A common and all too accurate complaint about human work is that the lead times between conceptualizing an experiment and getting a result are long. There is much work involved in designing an appropriate study, obtaining funding and human subject approvals, and then accumulating sufficient subjects to get a clear result. But there are strategies that can dramatically shorten the timespan needed to arrive at a conclusion or at least focus the initial efforts to enhance the likelihood that there will be a clear answer. One is to use new bioinformatics tools that can mine existing transcriptional data sets to find gene signatures that characterize a given infection or vaccine response. This approach has been taken in a number of systems; see in particular the efforts by Khatri and colleagues using data deposited in GEO (27–30), and the review by Tsang and colleagues (31). These studies can give important clues as to what genes are particularly relevant to a given response or can characterize the immune response to one pathogen versus another. There are also databases, such as the NIAID-funded ImmPort (32) and ImmuneSpace (33), in which human immunological studies are curated in ways that are accessible to noncomputational biologists. These databases are being used quite a bit and will become increasingly useful for meta-analysis as more data are incorporated.

Another key resource that is important to develop and use is biobanking, particularly in immunology, where PBMCs (mostly lymphocytes and monocytes) and serum are relatively easy to collect and store and allow one to address questions that come up or to test new techniques in real time if one has the right samples. There should be national efforts to establish such banks, which could be accessible to investigators with good project ideas. Extending such biobanks to also include immune cells harvested from human organs, along

the lines of the innovative work of Farber and colleagues (34), would also be incredibly useful.

A third resource that would not only accelerate human work but also allow for detailed mechanistic studies would be in vitro organoid systems such as lymph nodes or spleens, where, if conditions could be established to mimic immune responses, many things could be rapidly tested and investigated in great depth. One such system being developed is tonsil organoids, which successfully promote antibody responses to flu vaccines (L. Wagar, C. Constance, C. Kuo, S. Boyd, & M.M. Davis, personal communication).

Humanized and More Humanlike Mice

Significant progress has also been made in developing mouse models that have the advantages of inbred mice but have more human characteristics or tissues. Pioneering work by Weissman, McCune, and colleagues first introduced SCID-hu mice (35) with human lymphoid organs and lymphocytes, and subsequent improvements have produced mice that have more and more human immune characteristics that work reasonably well, although there are still many differences and incompatibilities (36–38). Progress has also been made in finding ways to introduce genetic and microbial diversity into inbred mice, such as the Collaborative Cross mice (39) made by randomized crossing of eight founder strains into new recombinant inbred strains to be used for systems genetics analyses in which traits can be correlated quickly with genetic regions (40). Also promising is the production of so-called dirty mice by cohabitating pet shop mice with inbred strains to produce more pathogen exposure, which, if it does not kill the inbred mice, has a dramatic effect on resident memory T cells (41).

INTERESTING RECENT RESULTS

Human Immune System Variation

The composition of cells and proteins that constitute human immune systems varies substantially between individuals but is largely stable within a given individual over the course of weeks and months (42, 43) and even years (44). This relative stability allows for predictions of specific immune responses based on baseline immune states. Several successful attempts have been made, specifically in predicting vaccine-induced immune responses. There is now a growing understanding that such predictions cannot be made using only individual features but must take into account the many simultaneous measurements made at the systems level to capture the complexity of the immune system and to allow for robust predictions. Most studies to date have focused on gene expression analysis in combination with, for example, serum protein concentrations and cell frequency changes. Successful examples include the prediction of antibody responses to the yellow fever vaccine (45) and to seasonal flu vaccines (43, 46–49). These systems-level analyses have also provided leads that have been investigated further in mechanistic follow-up studies, revealing, for example, a previously unappreciated role for the gut microbiota in vaccine responses (50) and a role for the integrated stress response and autophagy in dendritic cells during the priming of adaptive immune responses to a vaccine (51).

A general problem in the most basic medical application of immunology is the lack of reliable metrics of immune system health. Such metrics would be an extremely useful guide for a clinical diagnosis of immune dysregulation and to predict responses to vaccine and immunomodulatory therapy (3). Ideally, such metrics of immune health should be based on blood immune parameters, given the accessibility of peripheral blood and its role as the conduit for immune cells circulating the body (8). Flow cytometry is a robust technology already used in clinical practice, and mass cytometry is a more highly parametrized cytometry method increasingly being established as a robust methodology for profiling all immune cell populations in blood. An attempt at establishing better metrics for describing immune variation and for future definition of immunological health was recently reported in nominally healthy human beings (52). One could hypothesize that interdependent immune parameters could be either clustered or dispersed. By analyzing the relative frequencies of immune cell populations in the blood in three different cohorts, the authors (including us) found that in every case, individuals were continuously distributed; that is, there were no discrete clusters of individuals. They did see, however, that older individuals as a group were more heterogeneous than younger individuals, forming a cone-like pattern of variation along an age axis. They also found that the composition of immune cells in the blood of these individuals was predictive of a diverse set of functional responses, suggesting that the balances between specific cell populations that stimulate and inhibit each other may be a key determinant of global immune competence in an individual (52). This represents just one approach to defining immunotypes, and others may be able to find discrete subsets. In any case, there is a clear need to discover general indices of immune function that can robustly predict who is or is not at risk for infectious diseases, autoimmunity, and/or cancer. At the very least the immunology community needs to counter the booming “immune booster” industry with actual science!

Nature Versus Nurture in the Immune System

The relative stability of immune phenotypes over time enables investigation of the underlying factors shaping an individual’s immune system. In the recently reported ExAC (Exome Aggregation Consortium) data set, on average 85 heterozygous and 35 homozygous protein-truncating variants were found in every given individual when exome-sequencing data were combined from 60,706 individuals (53). The number of nonsynonymous single-nucleotide variants is on the order of several thousands. Although heterozygosity rescues many such variants, even heterozygous deficiencies can give rise to aberrant immune phenotypes, such as the monogenic autoimmunity described in patients with heterozygous mutations in the *AIRE* gene (54, 55) and the immune dysregulation in patients with heterozygous mutations in the *CTLA4* gene (56). Apart from these findings on heterozygous gene variants, there is a vast literature on complex genetic associations with autoimmune diseases, most convincingly involving genes in the *HLA* locus, such as *HLADQ8* in celiac disease and type I diabetes and *HLA-B27* in ankylosing spondylitis. All these data clearly suggest a role for genetic influences in shaping human immune systems, but how do such influences compare to nonheritable influences, and to the influences of environmental factors?

In a recent attempt at quantifying the relative contributions of heritable and nonheritable influences, we and our colleagues performed a systems-level analysis in healthy human twins. By comparing monozygotic and dizygotic twins, the amount of variance explained by heritable and nonheritable factors was quantified and found to vary broadly between different immune components and functional responses. The overall heritability was low, and the majority of cell population frequencies, functional response, and serum protein concentrations were determined by nonheri-table factors (68% with >80% of variance explained) (57). Similar findings were reported by others with respect to cell frequencies (58, 59), although there is some variation in the interpretation of such results (60). Nevertheless, several additional lines of evidence suggest a broad influence of environmental exposures in shaping immunity, such as an increased divergence of monozygotic twin phenotypes with age (57) and the phenotypic convergence of unrelated individuals living together (42). We also found that influenza responses in adult twins were not strongly influenced by genetics (61), in contrast to results in infants vaccinated for hepatitis B (62, 63), presumably as a consequence of less environmental exposure.

Cytomegalovirus as a Symbiote

Although cytomegalovirus (CMV), a member of the herpes virus family, is typically classified as a pathogen because of its often lethal properties in individuals who are immunodeficient, such as patients undergoing bone marrow transplantation, most of the world's population has been or will be infected with CMV without obvious deleterious consequences. In fact, roughly 50% of adults in developed countries and 90% or more in less developed countries are carriers of this virus. And CMV has an enormous effect on the composition and function of the immune system. Up to 10% of all memory T cells can be specific to this virus in infected individuals (64), and 58% of all immune cells and proteins in blood are perturbed in CMV-discordant monozygotic twins (57) (Figure 4a). It has also been implicated in premature aging of the immune system (65), but the data have been controversial. However, our immunotype work reveals that, in every age bracket tested (8–89 years), CMV clearly induces a more aged phenotype, as shown in Figure 4b (52). Surprisingly, despite this effect, CMV seropositivity in young adults correlates with a more robust influenza vaccine response, and in mice it is clearly protective against flu infection (66), as shown in Figure 4c,d.

In addition, the work of Picker and colleagues has shown that vaccines using CMV as a vector are notably efficacious in stimulating a successful response to a simian immunodeficiency virus infection (67). Overall, the evidence strongly suggests that CMV is a symbiote (66), in most cases boosting the immune response as long as the individual's immune system is healthy.

In light of the evidence described above, heritable and nonheritable influences are important and collectively shape an organism's immune system, with nonheritable influences becoming increasingly dominant with age and environmental exposure. These influences are also interdependent, and environmental influences are dynamic during the course of life. Consequently, a lifestyle at odds with one's genetic makeup, shaped throughout evolution under pressures primarily from infectious disease, can be expected to lead to unexpected and

evolutionarily maladaptive consequences. Today examples of such evolutionary maladaptation are all around us in societies where living conditions during the last 100 years have drastically departed from the typical conditions of human history (and immune system evolution). In our modernized society, with its reduction in infectious disease burden, life expectancy has dramatically increased, but immune dysregulation such as allergies and autoimmune disease is increasing.

The hygiene hypothesis was formulated to explain the growing incidence of hay fever and eczema and their relationship with family size, with a possible mechanistic link with reduced exposure to infectious pathogens early in life (68). A growing body of evidence now suggests that specific environmental influences have different impacts at different time intervals during life. Most clearly, an early-life critical time window has been identified during which gut dysbiosis and immune perturbation have been associated with future allergy development in both mice (69, 70) and humans (71).

Human Evolution

Insights into the most important genes in the immune system for survival can be gleaned from human genomic data from different epochs. Particularly relevant is the transition from the hunter-gatherer era to the current agricultural one, when humans went from living in small, nomadic groups to densely populated agricultural communities in the beginning of the Neolithic period. This transition from hunter-gatherer began only approximately 10,000 years ago, and it likely resulted in much greater pathogen exposure and thus selection for more robust immune genes. These populations were largely stationary and had only vague ideas about sanitation, famously pouring human waste outside their doors until fairly recently. Moreover, these populations stored grain and other foodstuffs, which would have attracted rodents and other disease vectors. And as these communities grew, they became more densely packed together and traded with other communities. Thus, the conditions were ideal for the introduction and spread of disease, which regularly occurred in the more populated areas. Under these conditions, child mortality became rampant, with an estimated 50% of children under 5 years of age dying of infectious diseases, and many young adults dying also. These circumstances continued generation after generation and so would likely have had a significant effect on immunological gene polymorphisms that were beneficial. In this context Barreiro & Quintana-Murci (72) found more than 100 innate immunity gene polymorphisms that show evidence of selection in European populations, and this is likely the result of a constant exposure to disease for thousands of years. Netea and colleagues (73) also made use of this approach in a novel way by comparing the genomes of the Roma people, who migrated to Europe in 800–900 AD from Northern India. Whereas the European migrants had been exposed to the Bubonic Plague (in the fourteenth century AD), the original population had not been exposed, and Netea’s group found a number of polymorphisms, again in innate immune genes, that seemed to be the result of selection. This seems like a very fertile area for further work, especially in other long-settled populations (e.g., in China and India) that also endured repeated and lethal epidemics for thousands of years. These historic events also suggest a reinterpretation of the standard narrative that Europeans introduced diseases that were devastating for aboriginal populations in the Americas and elsewhere because of a simple lack of exposure to those particular

diseases in those peoples. Perhaps a more important factor in such decimation was that those peoples had not been selected for survival over the many generations that Europeans had been.

Another interesting facet of human immune system evolution comes from the analysis of ancient human genomes, specifically Neanderthals and Denisovans. Here interbreeding with modern humans left a residue of DNA from these populations that has resulted in clear evidence that at least one HLA class I allele (74) and a cluster of TLR genes have been positively selected for, at least in modern Europeans and Asians (75). This finding suggests that these loci conferred an infectious disease advantage from protohumans that had settled these regions hundreds of thousands of years before modern humans migrated out of Africa.

Recent Evolution

Another interesting aspect of evolution and genetics is what to expect in the modern era once the once massive childhood mortality rate tapers off, as it has in developed countries for roughly 100 years and in other countries more recently. Individuals carrying immunologically deleterious genes that were once selected against by infectious diseases now make a greater contribution to the gene pool. This increased abundance of individuals carrying deleterious immunological mutations may be responsible for the surge in childhood autoimmune diseases like type I diabetes and juvenile arthritis, or is perhaps contributing to the recent epidemic of food allergies, perhaps synergizing with microbial exposure deficit (i.e., the hygiene hypothesis) (68). In any event, one could expect a Poisson distribution of immunological defects: If the average number of defective genes in the immune system were, say, 10 (10% of 100 random defective genes), then individuals on one side of the curve (the 10%) could have one defective gene, but individuals on the other side of the curve (the trailing 10%) could have 100 defective genes. Furthermore, even though most individuals would be heterozygous, some individuals would have a deleterious phenotype, as discussed above. Interestingly, in areas endemic to *M. tuberculosis*, 90% of infected adults are able to control the pathogen, whereas 10% of infected adults progress to disease and need antibiotic therapy. Could these individuals be the unlucky ones in the genetic lottery? Or is their inability to control this pathogen due to some other factors, such as prior disease exposure or genetic or epigenetic variants in the pathogens?

The Role of Clonal Deletion in the T Cell Repertoire

In 1959, Macfarlane Burnet, one of the most pivotal thinkers in modern immunology, proposed that the deletion of self-specific immune cells could be the principal mechanism by which harmful clones were eliminated and autoimmunity suppressed (76). This proposal was debated for many years subsequently, with Nossal and others questioning whether this would result in “holes in the repertoire” leaving an organism vulnerable to infectious diseases (77). But in the late 1980s, a series of spectacular experiments gave convincing results that Burnet was correct with respect to this aspect of T cell repertoire. First, Kappler and colleagues showed that certain Vβ-expressing thymocytes were missing in mice that harbored particular MLS alleles (78), later found to be endogenous retroviruses (79–82). This effect was found to be the result of superantigens that could bind to both specific Vβ types and class II MHC molecules independent of antigens cross-linking thymocytes and

antigens presenting cells and then trigger thymic deletion (83). A year after the initial report in 1987, multiple groups (one of which included M.M.D.) found that specific TCR transgenics caused profound thymic deletion at the $CD4^+CD8^+$ (double-positive) stage in the presence of a self-antigen (84). This finding convinced almost everyone that clonal deletion was the key to what became known as central tolerance (versus the peripheral form). While some artifacts of TCR transgenics, such as disruption in thymic architecture and premature expression of TCRs, were noted, the clonal deletion results were so striking that the issue seemed settled. Meanwhile, the development of and improvements in the ability to label specific T cells by using peptide-MHC tetramer constructs (85–87) allowed for the detection of very rare (one in 10^6) antigen-specific T cells. This led to the discovery that self-antigen-specific $CD8^+$ T cells are generally almost as abundant as foreign antigen–specific T cells (88). So what happened to clonal deletion? A more focused experiment used a tetramer specific for a Y chromosome–expressed antigen HY or SMCY in humans and found that, while males tended to have fewer T cells, i.e., approximately one-third of the number in females, males still had many SMCY-specific T cells (88). Male and female mice had an even smaller difference. These cells did not skew toward lower affinity but had the same avidity range in both sexes. Clones could be raised in male and female cells with equivalent killing ability. The only difference was that the self-specific T cells could not be induced to proliferate with antigen plus CD28 alone, whereas the foreign antigen T cells could be (both groups of T cells were purified using collections of tetramers). Since both types of T cells can be stimulated to divide with anti-CD3 and anti-CD28 stimulation, our interpretation is that the self-specific T cells are not anergic, but that they simply require additional signals to be activated (88). Soon after the publication of this paper (88), Moon and colleagues published an equivalent result in a novel murine system that they had developed for cre-specific $CD4^+$ T cells (89). Specifically, they found that the fraction of cre-specific T cells dropped by half when cre was expressed ubiquitously in mice but that there was no change in the number of cre-tetramer-positive $CD4^+$ T cells if cre was expressed in the intestines or lungs. But the fraction of tet^+ regulatory T cells (Tregs) did increase two- to threefold in that situation, suggesting that additional cognate Tregs were needed to control self-reactivity (89). Taken together, these results show that, contrary to previous conclusions, negative selection can be a factor in neutralizing self-reactive T cells but is not as efficient as previously thought. Furthermore, cognate Tregs can be a factor in the balance. Su et al. also showed that cognate Tregs are ubiquitous for both self-antigens and non-self-antigens, although the ratio of effector T cells to Tregs becomes much larger with two different flu epitopes (90), suggesting that modulating this balance between effector/regulatory cells with the same specificity may be a way to facilitate the T cell response to a repeated challenge. So, why were the earlier results misleading? Firstly, one must be cautious about possible transgenic artifacts; the early expression and the disruption of thymic architecture were warning signs. Also, the TCRs chosen for the transgenics were typically the most robust in one's collection. The superantigen results, which did not involve any genetic manipulations, could probably be explained by the massively polyvalent nature of the interactions in the thymus. But the larger issue is, what do these new findings mean for our understanding of the immune system and the balance between self and nonself recognition? Here we suggest a radical reinterpretation. While autoimmunity is an important clinical problem, it is mostly a disease of the later years of life. Evolutionary pressures occur mainly in childhood and

young adulthood. Even in modern times, the frequency of autoimmunity in the population is less than 1%. In contrast, in the prevaccine era, as mentioned above, approximately half of children under 5 years of age, and many older children and adults as well, died of infectious diseases (4). Thus, by far the greatest evolutionary pressure would be on resistance to infectious diseases, and in both mice and humans, tolerance seems to work fine without a very efficient negative selection mechanism. Thus, having a complete TCR ligand repertoire would seem to be the most important priority for the immune system. That infectious diseases are likely to be a key driver of immune evolution is reinforced by the work of Barreiro & Quintana-Murci (72) and Netea and colleagues (72), who found that many gene polymorphisms in the innate immune system showed evidence of selection in human evolution. Thus, these two independent lines of evidence—the lack of complete negative selection and the selection for certain innate immune system genes, despite their contribution to autoimmunity—point to infectious diseases and not tolerance as being the key driver of immune system evolution, at least in humans and other mammals.

Memory Phenotype, or Virtual Memory, T Cells

Another striking finding in human immunology, is that adults have clear, albeit small, populations of memory phenotype T cells for diseases they have never had (90). Most striking in the human case is that every adult surveyed had HIV tetramer–specific T cells with all the indicators—cell surface and internal RNA transcripts—identical to those of authentic memory $CD4^+$ T cells, and these indicators were not evident in newborns, indicating that the development of this memory phenotype occurs during postnatal development. And this is not just about HIV, since a number of other viral epitopes (such as CMV, herpes simplex virus) were also evident in a memory form in seronegative individuals. Since T cells with these specificities have all the characteristics of memory cells, they likely confer some protection, although this possibility has not been shown in humans. The murine data of Jameson and colleagues (91), who refer to these as virtual memory T cells, revealed a similar phenomenon whereby even in germ-free mice there is a small population of these $CD8^+$ T cells, but this may be due to homeostatic proliferation leading to cells with a memory phenotype. As to how these memory phenotype T cells come about, Su et al. (90) showed that cross-reactivity is a very likely explanation since T cell clones made with a dominant HIV epitope and flu epitopes often cross-react with similar peptides from other organisms. This finding suggests that the developmental process that makes these T cells so ubiquitous in adults is at least partly due to our exposure to a myriad of microbes, even in highly developed and hygienic societies, from birth onward. This possibility was first proposed by Welsh and colleagues (92), who noted that mice immunized with one pathogen had profoundly different, and often more beneficial, immune responses to a subsequent pathogen. They referred to this phenomenon as heterologous immunity. In terms of a mechanism, we have known for many years that TCRs have a significant degree of cross-reactivity, both operationally and structurally, but the biological purpose of cross-reactivity has been variously attributed to either the need for positive selection on endogenous peptides in the thymus or the task of recognizing many more epitopes than T cells (93). But the affinities needed for positive selection are much lower than for an agonist peptide that can activate mature T cells, so this does not seem to be a good explanation for TCR cross-reactivity (94, 95). Perhaps an even more important benefit of TCR cross-reactivity and the

generation of memory phenotype T cells is the protection afforded against pathogens that have not yet been encountered. This possibility led Su et al. (90) to speculate that this potential protective effect may partially explain the vulnerability of very young children to infectious diseases, that is, before they have had enough microbial exposure to generate a broad repertoire of such memory phenotype T cells. These findings also call into question the idea that the primary purpose of vaccination in a naive population is to stimulate the maturation of naive T and B cells into memory cells. Perhaps an even more important purpose is to expand the numbers of these existing memory phenotype lymphocytes.

Everyone Should Be an Immunologist

There is clearly a growing understanding among clinicians and researchers across the spectrum of human conditions that the immune system is omnipresent and important for nearly all of these conditions. Pharmacologists are accustomed to the ever-growing use of antibodies targeting cytokines and surface receptors in patients with inflammatory conditions, tumors, and more. Cardiologists have been long aware of the inflammatory basis for cardiovascular disease, and novel immune mechanisms are being pursued as therapeutic targets. More and more conditions are being investigated as autoimmune or autoinflammatory. Neonatologists and obstetricians have understood that the causes and complications of preterm delivery are often inflammatory in nature, and novel immunomodulatory therapies are being investigated. The state of a patient's immune system impacts her recovery after surgery. Still, the most publicized example in recent years has been the booming field of cancer immunotherapy, in which a growing number of malignancies are now being targeted by immune system interventions. Another field that has been infiltrated by immunology in recent years is microbiology. Modern microbiology is largely focused on the interaction between the microbiota and the immune system, and many microbiologists are now studying their immunology textbooks, while immunologists are venturing in the opposite direction down the phylogenetic tree of life to study immunity-microbe interactions. This growing appreciation for the role played by the immune system across human conditions is a further testament to the importance of studying immunology in humans.

CONCLUSION

There are major inflection points in fields in which long-held beliefs, or "paradigms" in the influential work of Thomas Kuhn (96), collapse in the face of accumulating evidence. What we try to argue here is that the belief that inbred mice are the only way forward both with respect to our knowledge of basic immunology and as a necessary precursor to translational studies is no longer viable. Instead, we believe that with the increasingly powerful technologies and approaches such as systems biology, human immunology can be a freestanding and important branch of the field and eminently complementary to murine immunology, with the added benefit of being more readily translatable.

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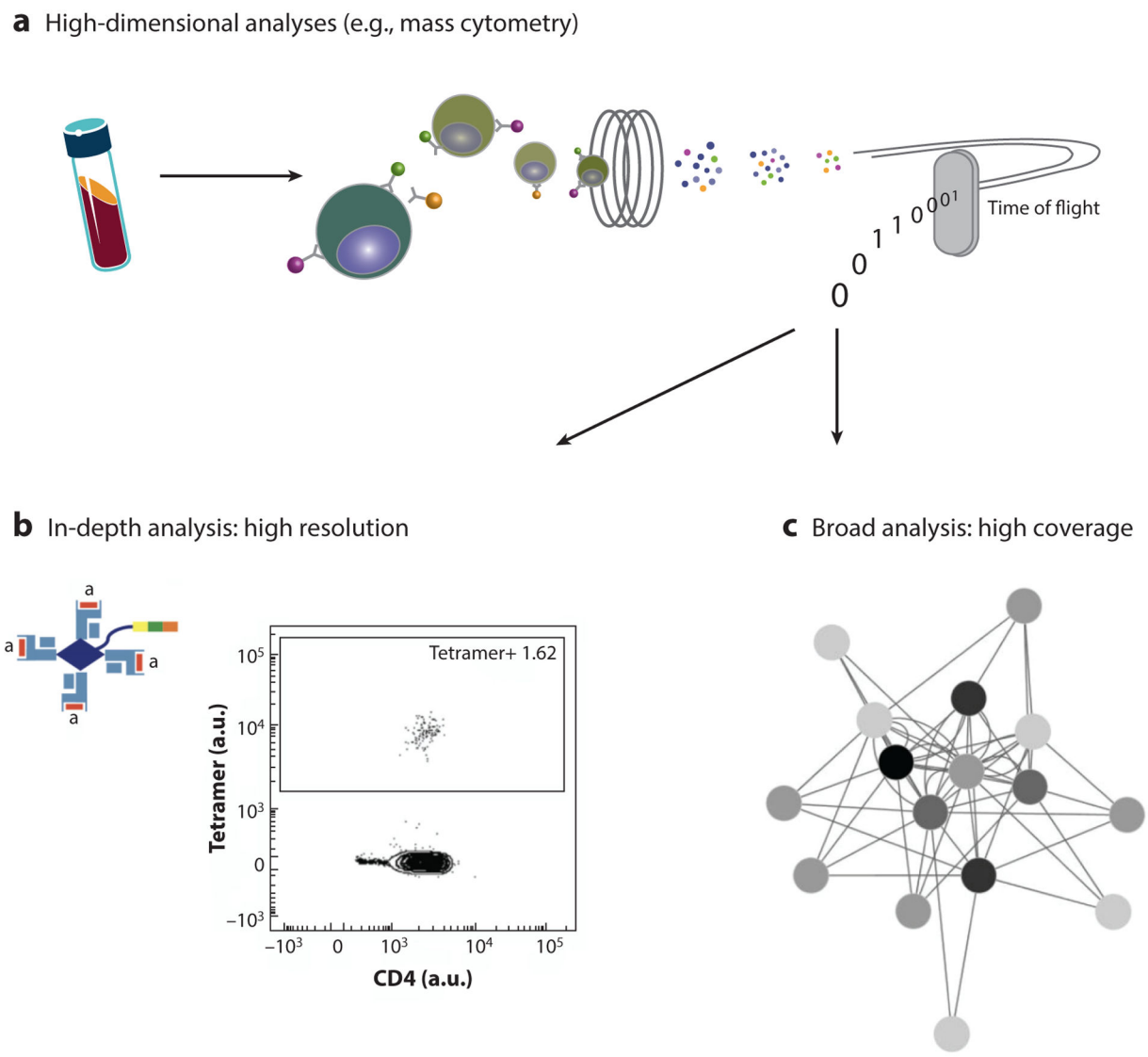

Figure 1.

Systems immunology. (*a*) Mass cytometry is an example of high-dimensional single-cell technology enabling systems immunology. (*b*,*c*) This method allows for two principally different types of analyses: (*b*) a very detailed characterization of well-defined and rare cell types, such as antigen-specific T cells identified by MHC-peptide multimer staining, or (*c*) broad analyses across all cell populations in the system and their interdependencies.

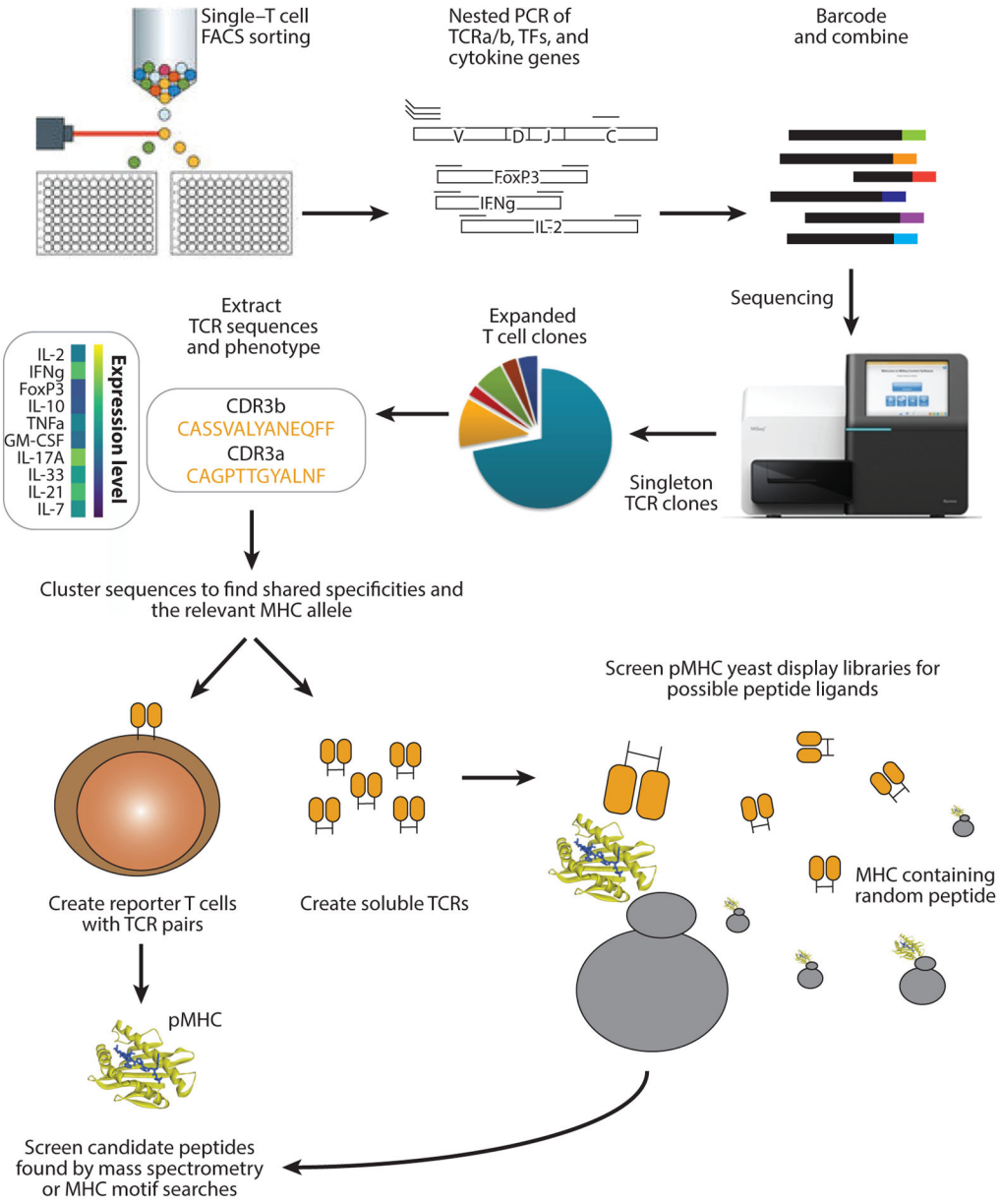

Figure 2.

(*Figure appears on preceding page*) From T cells to specificities. New methods allow an efficient path with which to go from a T cell of interest in a given disease or response to knowledge about shared specificities, clonal expansions, and specific peptide-MHC targets. Live T cells are extracted from blood or the tissue of interest and sorted into 96-well plates. This step can be done using index sorting to enable >15 cell surface markers to be associated with each well. The cells are lysed, poly A+ RNA is reverse transcribed, and both TCR chains and phenotypic markers are PCR amplified in three stages by using the protocol described in Reference 16. Finally, a bar code is attached to all the amplicons in a given well, and the wells can be combined and sequenced using a high-throughput device such as Illumina MiSEQ. Pipeline software is used to establish a consensus sequence for each well's

TCRs (to eliminate PCR errors) and to assign TCR sequences and phenotypic markers (on a positive or negative basis). Here clonal expansions typically indicate dominant clones in a given individual's response. At this point, GLIPH analysis of TCRs across individuals can be used to identify specificity groups and to indicate restricting MHC alleles (see Figure 3). Reporter T cells can be made by transfecting reconstructed TCR pairs in αβ T cells. Candidate antigenic peptides can thereafter be generated in a number of ways, such as by screening yeast display libraries or by sequencing peptides extracted from MHC molecules, and be analyzed by mass spectroscopy. Alternatively, relevant genomes can be screened for possible epitopes by using MHC binding motif algorithms such as those in the Immune Epitope Database. Abbreviations: FACS, fluorescence-activated cell sorting; GLIPH, grouping of lymphocyte interactions by paratope hotspots; TF, transcription factor.

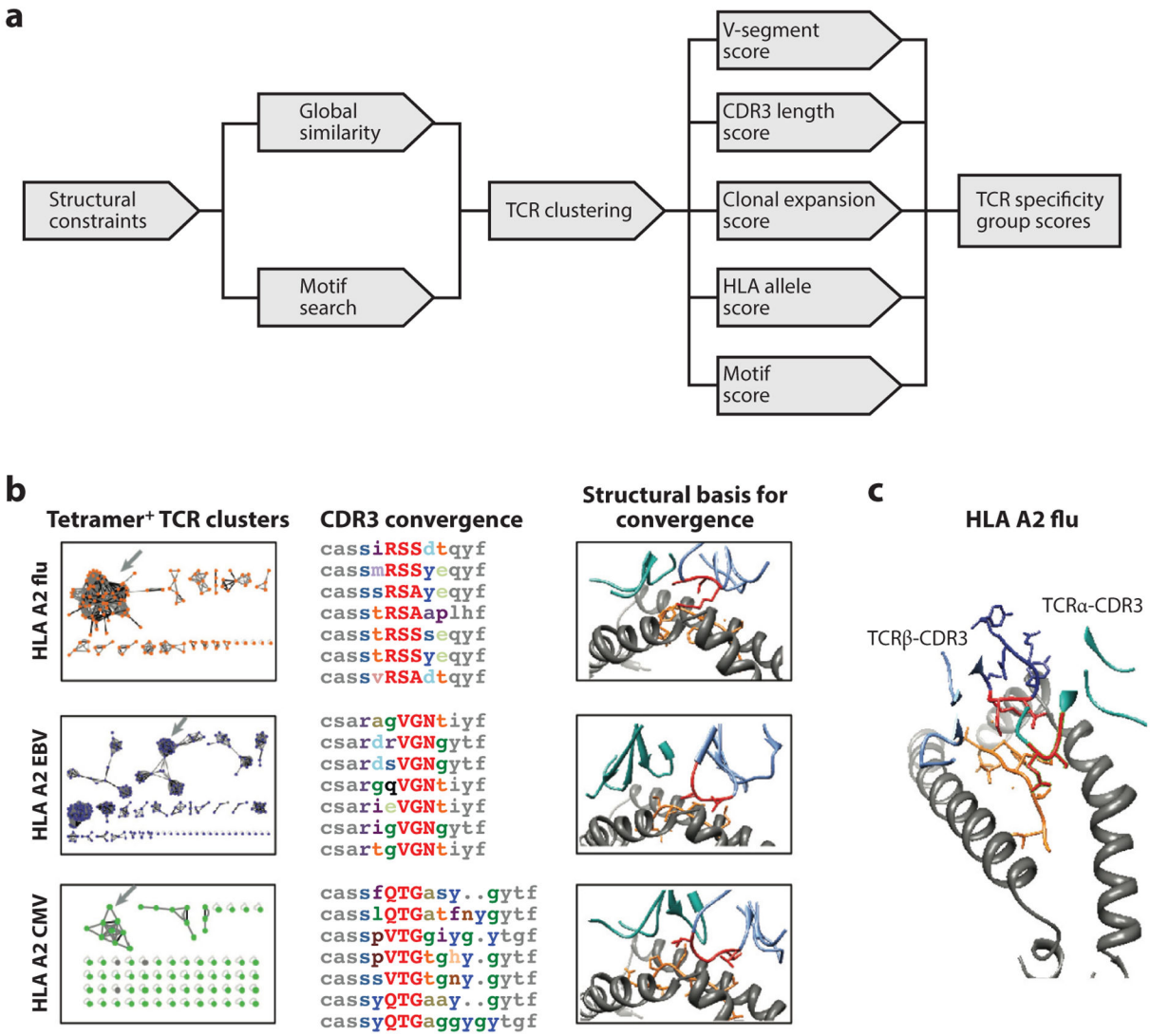

Figure 3.

Grouping TCR sequences according to peptide-MHC specificity. New algorithms such as GLIPH use the observed common properties of αβ TCRs that share specificities to take raw TCR CDR3 sequences and to group those likely to share specificities, especially across individuals. This is a multistep process, as indicated in panel *a*. The typical results are shown in panel *b*. (*c*) Of 5,700 TCR β sequences from 22 South African subjects with latent *Mycobacterium tuberculosis* infection, we were able to identify more than 100 different specificity groups. Of the first five groups, by fitting the most stringent criteria, we correctly assigned HLA alleles (Table 1)and found the correct peptide antigen from a large collection of $CD4^+$ epitopes curated by Sette and colleagues (23).

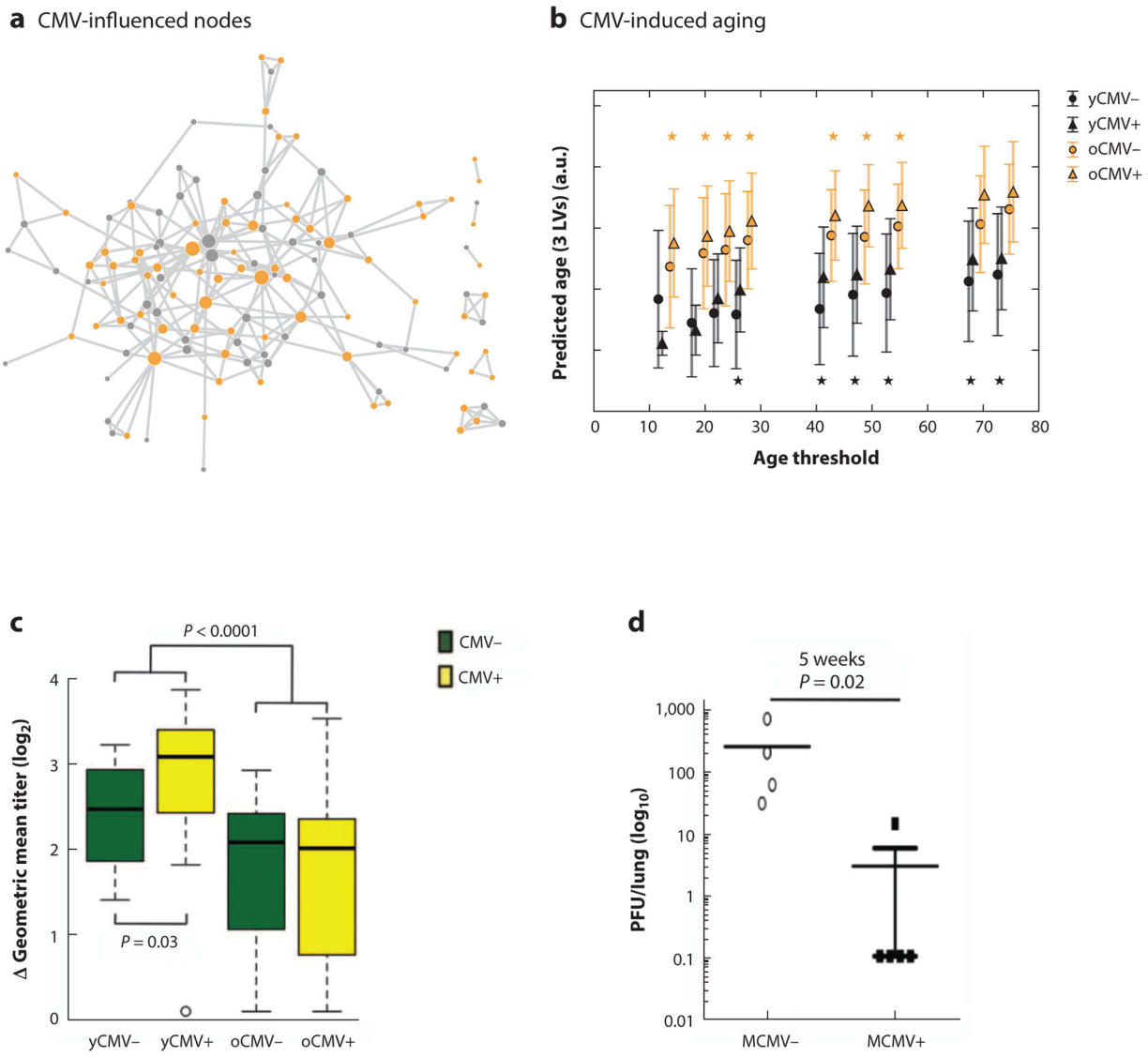

Figure 4.

The role of CMV in shaping immune systems. (*a*) Network model showing dependencies between serum proteins and cell populations in the immune systems of healthy twins. Yellow nodes represent the 58% of all 126 measurements with reduced correlations in CMV +/− compared with CMV−/− monozygotic twin pairs. (*b*) Individuals are projected onto the immunological age axis in ImmuneSpace. The positions on this line are compared between CMV+ and CMV− individuals across age groups (mean ± SD). (*c*) Young (22–32-year-old), but not old (62–89-year-old), CMV+ individuals respond with higher titers of anti-influenza antibodies (28/0 days postvaccination). (*d*) C57BL/6 mice mock infected or infected with MCMV (Smith strain) and challenged with flu (IAV) intranasally 5 weeks later and viral titers assessed. Abbreviations: CMV, cytomegalovirus; LV, latent variable; MCMV, murine cytomegalovirus; oCMV, old (62–89-year-old) CMV+ individuals; PFU, plaque-forming unit; yCMV, young (22–32-year-old) CMV+ individuals.

Table 1

TCR specificity groups and predicted HLA restriction among *Mycobacterium tuberculosis*–infected subjects[a]

TCRID	Donor ID	CDR3b	Frequency	CDR3a	Frequency	DQA1		DQB1		DRB1		DRB3/4/5	
Group 1													
TCR001	01/0873	CASSFEETQYF	2/168	CIVKTHSGGSNYKLTF	2/158	*05:02	*01:02	*03:19	*06:02	*11:01	*15:03	DRB3*02:02	DRB3*02:02
TCR008	09/0018	CASSLEETQYF	2/400			*05:01	*01:02	*02:01	*06:03	*03:01	*15:03	DRB3*02:02	DRB5*01:01
TCR010	03/0492	CASSPEETQYF	1/112			*01:02	*01:02	*06:09	*06:02	*13:02	*15:03	DRB5*01:01	DRB3*03:01
TCR012	09/0217	CASSPEETQYF	49/166	CIVHTNSGGSNYKLTF	47/135	*01:03	*01:02	*06:04	*06:02	*13:01	*13:02	DRB3*03:01	DRB3*02:02
TCR003	01/0430	CASSLEETQYF	1/82	CGMSGNTGKLIF	1/70	*03:03	*01:05	*02:02	*05:01	*10:01	*09:01	DRB4*01:01	DRB4*01:01
TCR004	01/0873	CASSLEETQYF	21/168	CIEHTNSGGSUYKLTF	21/158	*05:02	*01:02	*03:19	*06:02	*11:01	*15:03	DRB3*02:02	DRB3*02:02
TCR009	01/0873	CASSPEETQYF	2/304			*05:02	*01:02	*03:19	*06:02	‘11:01	*15:03	DRB3*02:02	DRB3*02:02
TCR011	09/0018	CASSPEETQYF	31/400	CAVPSGGANSKLTF	1/267	*05:01	*01:02	*02:01	*06:03	*03:01	*15:03	DRB3*02:02	DRB5*01:01
Group 2													
TCR022	01/0873	CASSVALAGAEYF	1/69	CAVGGLSGANSKLTF	1/67	*05:02	*01:02	*03:19	*06:02	*11:01	*15:03	DRB3*02:02	DRB3*02:02
TCR023	02/0152	CASSVALASGANVLTF	2/41	CAGAGGGGFKTIF	2/28	*05:01	*01:02	*02:01	*06:01	*03:01	*15:01	DRB5*01:01	DRB3*01:01
TCR024	03/0492	CASSVALQGVHTWQYF	2/112	CAGTNTGNQFYF	2/90	*01:02	*01:02	*06:09	*06:02	*13:02	*15:03	DRB5*01:01	DRB3*03:01
TCR026	09/0018	CASSVALYANEQFF	1/151	CAGPTTGYALNF	1/125	*05:01	*01:02	*02:01	*06:03	*03:01	*15:03	DRB3*02:02	DRB5*01:01
TCR036	09/0772	CASSVALLGETQYF	1/107	CAGAPTGNQFYF	1/98	*05:05	*01:02	*03:01	*06:02	*03:01	*15:03	DRB3*02:02	DRB5*01:01
TCR029	09/0328	CASSVALLGGEQYF	1/107	CAGLVGTSYGKLTF	1/73	*06:01	*04:01	*03:01	*04:02	*12:02	*03:02	DRB3*03:01	DRB3*01:01
TCR02S	03/0492	CASSVALATGEQYF	1/112	CAGPTGGSYIPTF	1/90	*01:02	*01:02	*06:09	*06:02	*13:02	*15:03	DRB5*01:01	DRB3*03:01
Group 3													
TCR051	02/0152	CASSLIEGGTEAFF	1/41	CVVSAITNDYKLSF	1/28	*05:01	*01:02	*02:01	*06:01	*03:01	*15:01	DRB5*01:01	DRB3*01:01
TCR052	09/0772	CASSLIEGLEQYF	1/107	CAVQPGAGGFKTIF	1/98	*05:05	*01:02	*03:01	*06:02	*03:01	*15:03	DRB5*01:01	DRB3*02:02
TCR0S3	09/0018	CASSLIENTEAFF	1/151	CAVTIGATQGGSEKLVF	1/125	*05:01	*01:02	*02:01	*06:03	*03:01	*15:03	DRB5*01:01	DRB3*02:02
TCR054	02/0152	CASSLIEQQPQHF	1/41	CASQSNTGNQFYF	1/28	*05:01	*01:02	*02:01	*06:01	*03:01	*15:01	DRB5*01:01	DRB3*01:01
Group 4													
	03/0492	CASSSGQGHYMEQFF	1/162			*01:02	*01:02	*06:02	*06:09	*15:03	*13:02	DRB3*03:01	DBBS* 01:01
	09/0328	CASSVGQGHYNEQFF	1/107	CAVISGGSNYKLTF	1/73	*06:01	*04:01	*03:01	*04:02	*12:02	*03:02	DRB3*03:01	DRB3*01:01
TCR098	03/0492	CASSLGQGHYNEQFF	3/162	CAVHGGGSNYKLTF	3/134	*01:02	*01:02	*06:02	*06:09	*15:03	*13:02	DRB3*03:01	DRB5*01:01

TCRID	Donor ID	CDR3b	Frequency	CDR3a	Frequency	DQA1		DQB1		DRB1		DRB3/4/5	
TCR099	09/0125	CASSPGQGHYMEQFF	4/56	CAVNSGGSNYKLTF	4/39	*06:01	*01:02	*03:01	*05:02	*12:02	*16:02	DRB3*03:01	DRB5*01:01
Group 5													
	01/0906	CSARSSGGEAKNIQYF	2/118			*02:01	*01:02	*02:02	*06:02	*07:01	*15:01	DRB4*01:03	DRB5*01:01
	09/0018	CSARKGGGEAKBIQYF	1/182			*05:01	*01:02	*02:01	*06:03	*03:01	*15:03	DRB3*02:02	DRB5*01:01
TCR087	03/0492	CSARAGGGEAKNIQYF	3/112	CAVSEAGAGSYQLTF	3/90	*01:02	*01:02	*06:09	*06:02	*13:02	*15:03	DRB3*03:01	DRB5*01:01
TCR088	01/0906	CSAEASGGEAKNIQYF	1/106	CAVRDPGNTDKLIF	1/72	*02:01	*01:02	*02:02	*06:02	*07:01	*15:01	DRB4*01:03	DRB5*01:01

[a]Of 5,700 TCR β sequences from 22 South African subjects with latent *M. tuberculosis* infection, we were able to identify more than 100 different specificity groups. Of the first 5 groups, by fitting the most stringent criteria, we correctly assigned HLA alleles (*yellow highlight*) and found the correct peptide antigen from a large collection of CD4+ epitopes. Curated by Sette et al. (23).