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New technologies and applications in infant B cell immunology

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Abstract

The human immune system changes dramatically with age, and early life exposures to pathogens and environmental antigens begin the formation of immune memory which influences subsequent responses later in life. To study infant immunity, sample-sparing experimental methods that extract maximal data from small samples of blood or other tissues are needed; fortunately, recent developments in high-throughput sequencing and multiplexed labeling and measurement of markers on cells are well-suited to these tasks. Here, we review some recent studies of infant immune responses to infectious disease, highlighting similarities and differences between infants and adults, and identifying important questions for future research. Recent clinical trials in food allergy have revealed the critical role of immunological events in the first year of life that determine an individual's risk of developing peanut allergy; these also warrant thorough evaluation using the new immune monitoring tools.

Introduction

The immune systems of infants and children, and the critical events stimulated by pathogens, vaccines and other exposures at the start of life are poorly understood, yet likely influence lifelong patterns of immune responses. Efforts to improve vaccination strategies for influenza are only now beginning to grapple with the potential impact of the humoral memory formed during initial viral antigen exposures in childhood on responses to new influenza strains encountered in adulthood, for example [1-4]. More broadly, there is evidence that infants and young children may respond differently from adults to particular infections or vaccines, and that improved knowledge of these differences could help to optimize medical care in early life. Infancy or early childhood are also crucial times when some immune pathologies develop, and when they can be prevented, as shown by the LEAP study of peanut allergy that demonstrated that feeding peanut-containing foods to infants in the first year of life could significantly decrease the incidence of peanut allergy later in childhood [5].

Our current limited knowledge of infant immunity is due in part to factors such as the small sample volumes of blood that can be safely collected from infants, the few opportunities for sampling of other tissues, and the lower levels of funding for pediatric studies compared to

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adult research. Fortunately, recent technological developments, particularly advances in DNA sequencing and highly multiplexed measurements of phenotypic markers on cells and soluble molecules in fluids such as the serum, have greatly expanded the scope of immunological measurements in humans. Because these approaches can be sample-sparing, they are of particular benefit for studies of the infant immune system, enabling researchers to maximize the yield of data from small blood sample volumes. Here, we outline recent progress in infant and early childhood immunology, with an emphasis on B cell studies and humoral immunity, and highlight key knowledge gaps for future research.

New Technologies and Systems Biology Perspectives on Immunity

The invention and commercialization of high-throughput DNA sequencing (HTS) technologies based on sequencing-by-synthesis or hybridization has transformed many areas of biomedical research, including genomics, microbiome studies, and analysis of the complex genomic rearrangements that form the genes encoding antibodies and T cell receptors [6-8]. Recent improvements in single-cell transcriptomics have also depended on HTS, and highlight the power of this methodology for revealing previously unrecognized subpopulations hidden among more abundant cell types, such as the pulmonary ionocyte in airway epithelia, and new types of monocytes and dendritic cells [9,10].

A second major area of innovation in the past decade has been the development of more highly-multiplexed methods for measuring phenotypic markers on cells in suspension or in histological sections. The CyTOF mass cytometry method uses isotopically pure elemental metal reporters instead of fluorophores to label monoclonal antibody reagents specific for particular cell markers, and uses a mass spectrometer to read out the markers expressed by individual cells [11,12]. A related methodology for histology, multiplexed ion beam imaging (MIBI), has recently been reported using mass-labeled antibody reagents to detect markers expressed by cells in tissue sections, and shows the same advantages of enabling dozens up to 100 markers to be measured simultaneously from each cell in a specimen [13]. In parallel, improved methods using DNA oligonucleotide-labeled monoclonal antibody reagents and cycles of fluorophore-labeled nucleotide extension or probe hybridization, have provided additional routes for highly multiplexed histological immunostaining [14-16].

These two core technological areas, HTS and improved multiplexing for cell labeling, enable much more extensive datasets to be harvested from very small samples, and are therefore well-suited to analysis of the small samples of blood or occasionally tissues that can be collected from infants. We describe some initial applications of these experimental approaches to infant immune system questions below.

Humoral immune system development in early human life

B cell populations begin to develop *in utero*, and then undergo major changes in the newborn child upon exposure to the extra-uterine environment. The newborn infant B cell compartment primarily consists of naive and transitional B cells lacking evidence of antigen experience, with few memory cells [17-19]. Neonates and infants must respond appropriately to the range of pathogens as well as environmental antigens that they

encounter, despite their immunologically and anatomically immature adaptive immune system. There are many important non-B cell factors that influence the generation of an antibody response, such as the slow development of follicular dendritic cells as well as limited expansion of follicular T helper cells, which causes delayed and reduced germinal center B cell responses [20,21]. Nevertheless, rare B cells with somatic hypermutation (SHM) in their B cell receptors (BCRs) have been observed during fetal development [22,23], suggesting that fetuses have some capacity to respond to antigenic stimulation, although it is possible that some of these cells may represent maternal B cells that have entered the fetus [24]. Peripheral blood B cells in infancy show age-dependent maturation as measured by increasing frequencies of SHM and evidence of selection, but these changes have been studied for relatively few infections and vaccine types [18,23,25,26]. The studies of Ridings et al. before the advent of high-throughput DNA sequencing observed SHM in the first year of life using genomic DNA template from the B cell populations studied, and were therefore not able to distinguish between B cells expressing different isotypes, while Wendel et al. analyzed B cells under the specific circumstance of malaria infection.

Anatomical studies have made it clear that the infant immune system is immature in several important ways. Infant human splenic maturation occurs in stages, with essentially no germinal centers present at birth, and with the marginal zone B cell compartment remaining largely immature until the age of two years [27]. The slow marginal zone B cell development is thought to be a main reason for the weaker and shorter-lived antibody responses that are mounted after T-cell independent vaccine antigens [28]. However, T-dependent humoral responses to vaccines and natural infections have also been reported to be decreased, delayed and short-lived in neonates and infants compared to adult responses [reviewed in [29,30]].

Premature infants are exposed to the extrauterine environment before their immune cell development has progressed to that of term infants. A recent CyTOF multiparametric analysis of infant blood cells showed that although the frequencies of many immune cell types differ between premature and term infants, similar changes in the cell populations occur in the first three months after birth in both groups. In particular, CD27⁺ class-switched memory, CD27⁻ class-switched memory, non-class-switched memory, plasmablast and naive B cells show similar coefficients of variation of their frequencies over time [31••]. The premature and term infants converged on similar immune cell population phenotypes by three months of age, suggesting that the effects of environmental exposures on the premature infants accelerated their immune development, even for those premature infants that had prolonged stays in the hospital. We emphasize these results from B cell populations here, but the study examined all major leukocyte lineages in the small-volume blood samples, demonstrating the extended scope of investigation made possible by the CyTOF technology.

Infant antibody responses to infectious disease

Serological analysis of infant primary and memory humoral responses elicited by vaccination has been main laboratory tool used in vaccine development, and has been reviewed elsewhere [29,32,33]. More recent studies have focused on understanding the B cell and plasma cell populations that are responsible for secreted serum antibodies, and

defining the molecular features of the antibodies expressed by antigen-specific B cell clones. Infant antibody responses to several important human pathogens for which no vaccine currently exists, have been investigated using these new approaches.

Extensive studies in adults with HIV infection have highlighted the importance of rare, difficult-to-generate antibody lineages that are able to neutralize a broad range of HIV variant strains. These antibodies in adults typically have extremely high frequencies of SHM and other unusual features such as long CDR3 regions in the heavy chain [34]. One might have predicted that infants, given their generally decreased SHM frequencies compared to adults, would be unlikely to develop serum neutralizing breadth against HIV when infected. However, infants who contract HIV early in life (in utero, during birth, or from breast feeding) are able to mount a *de novo* plasma antibody response capable of neutralizing a range of cross-clade HIV-1 isolates within 1-2 years after infection [35]. A follow-up study from the Overbaugh group [36••] found that the neutralizing anti-HIV antibodies (nAbs) from one of the infants reported by Goo et al. [35] had low frequencies of SHM compared to adult HIV-neutralizing Abs. One antibody lineage with low SHM accounted for most of the HIV neutralizing breadth in the panel isolated from the infant, and bound an epitope on HIV similar to adult antibodies that target the N332 glycan and V3 loop of the HIV envelope [36••]. The capability of infants to generate these nAbs is speculated to result, in some part, from the high antigenic viral loads measured in infants [35], a finding which has been observed in adults with broadly-neutralizing HIV antibodies [37].

Infant antibodies for a more common but clinically important early- and late-life viral pathogen, respiratory syncytial virus (RSV) [38] have shown some similarities, but also important differences to the HIV antibody responses. Goodwin et al. studied neutralizing antibodies to RSV in infants and found that most lacked SHM in the youngest infants (< 3 months) but that SHM frequency increased with age; these antibodies were heavily biased in their heavy chain V gene usage toward IGHV3-21 or IGHV3-11, paired with the light chain V gene IGLV1-40 [39••]. In addition, antibodies in young infants primarily recognized the viral prefusion glycoprotein conformation (preF), while older infants had more antibodies recognizing the postfusion conformation (postF). Interestingly, none of these sites are major antigenic sites in adult RSV responses [40]. RSV and HIV responses therefore illustrate how infant humoral responses to infection may differ from those of adults.

Under some circumstances, it appears that infants can also generate antibodies with surprisingly high levels of SHM. Wendel et al. [18] compared infant (<12 months) and toddler (12-47 months) responses during acute malaria infection, finding that the infants had high frequencies of SHM in their BCRs, albeit somewhat lower frequencies than toddlers. Infants and toddlers showed similar increases in Ig transcript counts that could reflect B cell clonal expansions, and these were similar to changes seen in young adults with acute malaria infection. Infant responses featured more IgM expressing B cells compared to toddlers. The authors also report greater evidence of positive selection in CDRs and negative selection in framework regions in infants compared to toddlers.

One additional study of BCR gene rearrangements compared infants and adults with acute rotavirus (RV) infection [41], another common and clinically significant early-life pathogen

[42]. Although few immunoglobulin transcripts were sequenced from antigen-specific B cells in this paper (11 heavy chains and 11 light chains for antibodies specific for the VP7 protein, and 3 heavy chains and 2 light chains from antibodies specific for VP6), the infant sequences showed increased VH1 and VH4 usage (particularly VH1-46) that was similar to findings in adult patients. In addition, the infants showed no difference in D or J gene segment usage, junctional diversity, or CDR3 length compared to adult antibodies, in this small sample of antibodies evaluated.

These examples of HIV and RSV infection show that infant humoral responses differ from those of adults, while responses to malaria and RV infection show more prominent similarities between infant and adult responses in the features that were evaluated. It is clear that assessment of infant antibody responses needs to be carried out on a case-by-case basis for different pathogens, and no simple summary of immune system immaturity is sufficient.

Immunological diseases: Allergy

The LEAP clinical trial study of peanut allergy in children overturned conventional medical thinking and has ignited intense interest into the effects of infant dietary exposures on allergy development [5]. In the trial, infants were randomized into either a peanut consuming group or peanut avoiding group and were assessed for peanut allergy at 5 years of age, with the result that 17% of the avoidant children had become allergic in contrast to 3% of the peanut-consuming children. Initial mechanistic studies found that peanut consumption was associated with gradually decreasing peanut-specific serum IgE levels, and higher levels of peanut-specific IgG4 [5]. IgG4 is an antibody isotype thought to compete with IgE for binding to allergen, and potentially to downregulate immune responses through other mechanisms.

HTS analysis of immunoglobulin gene rearrangements has been applied to study IgE, IgG4, and other isotype repertoires in patients with peanut allergy, finding that most IgE shows somatic hypermutation indicating prior antigen exposure, and evidence of potential class-switching to IgE from other switched isotypes such as IgG1 [43,44]. However, the youngest participant in these studies was 7 years old, and there is currently very little known about the IgE repertoires in infants, or how early peanut exposure beginning in the first year of life may alter the maturation of B cell repertoires to decrease the risk of peanut allergy later in childhood. Similarly, application of the CyTOF methodology as in Olin et al. [31] with the addition of labeled allergen proteins or peptide-MHC complexes to quantify and phenotype allergen-specific lymphocytes should provide a more comprehensive way of studying the development of allergic diseases in future research.

Conclusions

Many aspects of infant immune responses represent gaps in our knowledge (Table 1). Improved understanding of these responses could advance clinical care for infants, and help to predict subsequent immunity later in life. This is particularly for B cell and T cell responses that are affected by prior immune memory. Approaches using HTS and highly-multiplexed labeling in cytometry and histology are a perfect fit for the challenges of infant

immunology research, as they can capture extensive fine-grained immune phenotypic data from even very small sample volumes. The studies we have highlighted here represent initial forays into an intensely interesting and medically relevant research area that could be described as being in its own infancy, with a bright future ahead.

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Highlights

- New immune monitoring technologies enable sample-sparing analysis of infant specimens
- Differences between infant and adult immune responses remain underexplored
- Infant neutralizing antibodies can be potent without high somatic mutation levels

Table 1:**Knowledge Gaps in Infant Immunology**

- How do infections and vaccinations in infancy and early childhood shape the clonal B cell and T cell populations forming the immunological memory to particular pathogens, such as influenza virus, and how does the early memory pool affect subsequent responses later in childhood?
- What are the effects of non-pathogen exposures in infancy and early life (including the microbiota, and food and aeroallergens) on adaptive immune development and development of immunological diseases such as allergy?
- What environmental exposures are responsible for the increasing rates of allergy in children?
- How do routes of antigen access to the infant immune system shape responses?
- What are the specific mechanistic effects on adaptive immune system development of vaginal versus caesarian section birth, and breast-feeding versus formula feeding?
- How can vaccines be designed to optimize infant immune responses and durable immunity?

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