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Understanding the role of the immune system in the development of cancer: new opportunities for population-based research

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Abstract

Understanding the precise role of the immune system in cancer has been hindered by the complexity of the immune response and challenges in measuring immune cell types in health and disease in the context of large epidemiologic studies. In this review, we present the rationale to study immunity in cancer and highlight newly available tools to further elucidate the epidemiologic factors driving individual variation in the immune response in cancer. Here, we summarize key studies that have evaluated the role of immunological status on risk of cancer, discuss tools that have been used in epidemiological studies to measure immune status, as well as new evolving methodologies where application to epidemiology is becoming more feasible. We also encourage further development of novel emerging technologies that will continue to enable prospective assessment of the dynamic and complex role played by the immune system in cancer susceptibility. Finally, we summarize characteristics and environmental factors that impact the immune response, as these will need to be considered in epidemiological settings. Overall, we consider the application of a systems biologic approach and highlight new opportunities to understand the immune response in cancer risk.

Introduction

The evolving understanding of complexity of the immune system poses many significant research challenges for epidemiology as we seek to discover the factors that stimulate, repress and modulate the totality of the immune response, impacting cancer risk and cancer survival. Understanding the precise role for immunology in the genesis and modification of chronic diseases will depend upon our ability to assess the interaction between individual genetic composition, epigenetic profiles and environmental factors as they shape and modulate the immune response. The emergence of new tools has led us to the realization that it is the right time to expand the field of cancer immunology to include population-based research; these tools will move us considerably further along in the search to understand the role of the immune response in the early development of tumor. The technology to measure the immune status in large population studies (i.e., prior to cancer diagnosis) is available, but needs to be improved and fine-tuned; research to pursue this area of enquiry needs to be addressed and encouraged. This review will summarize studies that support the role of the immune response in cancer risk; discuss existing measures of immunological status for epidemiological studies; describe newly developed technology available to measure the immune response; summarize characteristics and environmental factors that impact the immune response; and, highlight progress that is needed to develop and improve our understanding of the immune response on cancer risk.

There is no question that there are a number of well-established infectious agents that are causally linked to cancer (e.g., HPV and cervical cancer; *H. pylori* and stomach cancer; HBV and liver cancer)(1), yet the long latency period between infection, changes in inflammation and immune status, and the onset of cancer has often hindered our ability to measure and evaluate the role of the immune response. In addition, the lack of immune response markers available to epidemiologists has limited progress in understanding the mechanisms and causal underpinnings of non-viral infections. Recent epidemiological studies suggest that the adaptive immune response (in addition to the innate immune response) may play a role in the development of cancer. The adaptive immune response is partially determined by genetic variants but a large component of the response is modulated by lifetime exposures to infection and allergens. Much more research that interrogates the specific nature of this response is needed to fill the knowledge gaps.

A. Immune system basics

While the immune system is extremely complex, it can be broken down into two main subsystems: the innate immune system and the adaptive (or acquired) immune system. The innate immune response, found in almost all forms of life, is the dominant system of defense and consists of the immediate (and fast) response to detection of pathogens. This immediate response is non-specific (any pathogen or foreign body is detected) and the response does not include immunological memory of the targeted pathogen or object. In contrast, the adaptive immune response, found in most vertebrates, is

antigen-specific, and detects specific antigens that are foreign to the host (i.e., not their own antigens). The adaptive immune response has the ability to form an immunological memory, maintained by memory cells, allowing the immune system to mount an efficient and quick response upon secondary exposure to the same antigens.

The key effectors of both of these responses are white blood cells (leukocytes).

Leukocytes consist of cells originating from myeloid progenitor cells (neutrophils, eosinophils, basophils, monocytes), and from lymphoid progenitor cells (T lymphocytes, B lymphocytes and natural killer cells). Both the myeloid and lymphoid progenitors originate from multipotent hematopoietic stem cells (HSCs). HSCs, through a process of developmental signals, become epigenetically programmed to develop into the myeloid (myeloid-biased) or lymphoid lineages (lymphoid-biased), and these myeloid or lymphoid progenitors can further be programmed into their specific cellular fates. The innate immune response relies on neutrophils, eosinophils, basophils and monocytes (that can transform into macrophages in the tissue), as well as mast cells and dendritic cells (found in tissue), while the adaptive immune response is controlled by lymphocytes. Lymphocytes, primarily located in the lymphatic system, are made up of B cells, T cells and natural killer cells. While natural killer (NK) cells play an important role in innate immunity, it is now recognized that these cells have a “memory” and play a role in adaptive immunity (2-4). B cells make antibodies against pathogens, and T cells have a complex role in orchestrating the adaptive response that involves numerous subtypes of T cells, including helper T cells, regulator T cells, cytotoxic T cells, and

memory T cells. Finally, it is worth noting the presence of tissue resident gamma delta T cells, a specialized subset of T cells that bridge adaptive and innate immunity (5), and might be particularly relevant in the surveillance of precancerous cells in epithelial tissues.

B. Role of immune response in cancer risk

Despite the widespread and decade-long efforts to understand the role of the immune response on tumor growth, prognosis and treatment of cancer, surprisingly little research has been invested in investigating the direct role of immune response on de-novo development of cancer, i.e., its role in cancer etiology. Mechanisms related to the immune function are likely to vary by organ and tumor type; as with other known risk factors, each tumor type is uniquely susceptible to its environment.

We know that risk of developing certain cancers is extremely high among patients who experienced immune suppression as a result of organ transplants; these include skin cancers, non-Hodgkin's lymphoma, and kidney cancer (6). Yet, the risk of developing common cancers is not dramatically elevated among patients who received organ transplantations (relative risks 1.3-2.5 for lung, colorectal and breast cancers), and no increase in risk has been noted for prostate cancer (6-9). . While immunosuppression is an extreme example of immune dysfunction, there is abundant and convincing evidence that shifts in the distribution of blood leukocytes are important determinants of clinical outcomes in cancer patients. The common five-part WBC

differential (neutrophil, basophil, eosinophils, monocytes, lymphocytes) is used routinely in clinical practice to signal the presence of infection, overt immune disorders, leukemias, myelodysplastic and myeloproliferative disorders. In clinical studies, this basic test has been used to construct a metric known as the neutrophil-lymphocyte ratio (NLR); the NLR reflects the relative balance of the myeloid lineage in peripheral blood compared with the lymphocyte lineage (which includes subtypes of T cells (CD4, CD8), B cells and natural killer (NK) cells). A high NLR indicates chronic inflammation and immune stress and has been extensively examined as a prognostic factor for survival in cardiovascular and malignant disease (10-12). An NLR <3.0 is widely considered a favorable predictor for solid tumors as well as related disease mortalities, and NLR > 5 has often been used as the threshold that predicts poor outcome (10). A recent meta-analysis of solid tumor prognosis including 100 studies and 40,559 subjects showed that a higher NLR was significantly associated with overall survival, cancer specific survival, progression free and disease free survival (13). While there are no published studies on NLR and cancer risk, this measure has been examined in relation to risk of hypertension (14), cardiovascular disease (15) and diabetes (16). An elevated NLR at baseline was associated with subsequent risk of hypertension; subjects with elevated NLR had significant 23% higher risk of developing hypertension over a 6 year period (14). In an NHANES III cohort analysis, an elevated NLR increased risk for subsequent coronary heart disease mortality by 2.5 fold among subjects with no CHD at baseline, controlling for CRP, hypertension and smoking (15). A similar association for NLR and cardiovascular risk had been previously reported in a smaller prospective cohort study, although CRP

was not included in the multivariate analysis in this earlier study (17). It has been suggested that the NLR reflects a bone marrow 'stress response' and the activation of myeloid suppressor cells that cannot be phenotypically distinguished in blood smears or in automated differential counters (18, 19). These cells may inhibit the helpful immune response, leading to a more adverse outcome.

A number of studies have examined associations between natural cytotoxic activity of peripheral-blood mononuclear cells and risk of cancer (20-22); these studies have found that individuals with lower cytotoxic activity have a higher risk of cancer. In a prospective study with 11-years of follow-up, strong associations were observed with cytotoxic activity, measured at baseline on 3625 residents in Japan, and subsequent cancer risk (at all sites); for both sexes, multivariate relative risk of cancer was 0.64 (0.44-0.94) and 0.60 (0.41-0.87), for high and medium cytotoxic activity, respectively, compared with low cytotoxic activity (21).

Although studies with direct measures of immune status and cancer risk are limited, there is substantial indirect and supportive evidence that the immune response plays an important role in cancer etiology. Epidemiological studies with measures on lifetime history to allergies, or other chronic inflammatory conditions, such as periodontal disease, provide data on immune dysregulation and their associations with cancer risk. A growing number of studies have observed inverse associations between allergies and risk of brain, pancreatic, colorectal, hematological and gynecological malignancies (23-25); in contrast, a history of allergies appears to be positively associated with lung and urological malignancies (25). The potential mechanisms have

been reviewed and include chronic inflammation, immunosurveillance, inappropriate Th2 immune skewing, and prophylaxis (25).

Other inflammatory conditions can impact immune status and consequently influence risk of cancer. Local inflammatory conditions, such as chronic pancreatitis, cirrhosis, and their association with pancreatic and liver cancer risk, respectively, are well known (26-28); these conditions directly impact the immune response. More recently, observational studies have reported consistent associations between periodontal disease, a chronic oral inflammatory disease, and subsequent risk of pancreatic and gastrointestinal malignancies (29, 30). Bacterial infections may also modulate immune response and influence cancer risk, as is now more clearly understood through research on *H. pylori* (31). Understanding the precise role of the microbiome on cancer will also require an in-depth understanding of the immune response. The microbiome assessment may be a phenotypic reflection of the relative individual state of immunotolerance; the interplay of microbial diversity and the immune response is complex and is just beginning to be addressed as the technology for microbiome research have become more available.

C. Measures of immune status: current status and future directions

A number of technologies for use in large population-based studies are currently available to characterize the immune response; both existing and novel technologies are summarized below.

1. Genomic/GWAS measures of immune response

Candidate-gene studies on cancer have focused on a large range of genes involved in pathways known, or suspected, to be involved in risk. In recent years, there has been a dramatic increase in interest in genes associated with the innate immune response (e.g., inflammatory response), and to a lesser extent in genes involved in the adaptive immune response. Unlike autoimmune diseases, where genetic factors are often strongly associated with risk, the effect of immune-related genetic variants on cancer has been, for the most part, quite small. It may be that much of the variability in immune function is not driven by genetic factors, or that the genetic variability leading to functional alteration occurs in regions that have not been measured using contemporary technology. Moreover, promising findings from studies with small sample sizes have not been reproduced in larger studies, calling into question the role of genetic variants in immune pathways on the pathogenesis of cancer.

GWAS analyses are often limited in their ability to infer variations in immune-related genes; some of the more complex and highly individually variable immune genes, such as human leukocyte antigen (HLA) genes and killer immunoglobulin-like receptor (KIR) genes, cannot be easily characterized using GWAS SNPs as they are highly polymorphic (up to 2000 alleles), or exhibit large copy number variations (CNV) (32, 33). Matching organ donors with recipients on polymorphisms in HLA genes has had great clinical impact in kidney and bone marrow transplantation (34), and HLA polymorphisms have also been linked to numerous autoimmune diseases, demonstrating some of the

strongest genetic factors for those diseases (35). HLA polymorphisms have been linked to risk of cancer, but more work is needed to better explore those associations using fine mapping (36).

2. Existing and novel serological measures of immune response

i. Markers of systemic inflammation in blood

Blood inflammatory markers commonly used by epidemiologists (i.e., WBCs, CRP, IL-6, TNF- α) provide a relatively crude measure of systemic inflammation. The widespread use of these biomarkers in large observational studies grew out of a literature demonstrating their stability (in different storing conditions, over time, and through freeze-thaw cycles) and reliability (acceptable intraclass correlations, i.e., demonstrating greater between-person variation compared to within-person variation). As new technologies are applied to epidemiological studies, opportunities to measure a wide-range of immune biomarkers simultaneously will increase, providing insight into the role of the immune system that will extend beyond the current existing markers of inflammation.

Several serum biomarkers are available to measure systemic inflammation, such as C-reactive protein (CRP), interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α). These markers have been associated with numerous chronic diseases, including heart disease and diabetes. In contrast, inflammatory markers, such as C-reactive protein, IL-6 and TNF- α , have been less predictive of cancer risk, and associations have been weak, even

for those cancers with the strongest supporting evidence for a link to inflammation, such as colorectal cancer (37). Although the role of local inflammation at the site where cancer originates is well described, based on extensive experimental models, measuring local inflammation in the organs of healthy individuals is not possible, and observational studies are based on systemic markers of inflammation. Therefore, weak associations for inflammatory markers and cancer risk in observational studies (compared with cardiovascular disease) are likely due to the limitation of the biomarkers measured.

Mendelian randomization, which uses genetic determinants of known phenotypes of interest, has been used to examine causal relationships without bias (including reverse causation). Four studies have used this method to examine the role of CRP levels on cancer risk; two of these studies reported statistically significant positive associations for elevated CRP levels (predicted using genetic risk scores) and risk of colorectal cancer (38, 39), while the other two studies did not report statistically significant associations with colorectal cancer risk (40, 41). Additional measures of immune response could help us understand the role of the immune response in cancer risk.

Recent cardiovascular studies have measured other components of the immune profile, including biomarkers of monocytes and macrophages, which play a critical role in the development of atherosclerosis. Some soluble factors (of immune cell surface receptors) can be measured in the blood using ELISA, if levels are detectable. CD14, expressed on neutrophils and monocytes/macrophages, can be measured as a soluble

marker (sCD14), is a marker of abundance and activation of monocytes, and has been linked to cardiovascular disease risk and all-cause mortality in a healthy population (42). sCD14 has also been noted to be positively associated with subclinical atherosclerosis in HIV populations (43, 44). Other soluble markers have been measured in an attempt to clarify the role of different types of macrophages, and to better understand their role in cardiovascular diseases (43). In addition, as mentioned earlier, the NLR can be used as a measure of the balance between myeloid and lymphocyte lineage. To date, these markers of immune response have not been measured in relation to cancer risk.

ii. Flow cytometry measures of immune response

Clinically, the number of specific types of T cells within a patient sample has been used in understanding the severity of disease and the impact of treatment (e.g. CD4⁺ T cells in HIV), and the use of these measures in epidemiologic studies in the context of HIV, or organ-transplant related immunosuppression, have been informative. For example, although the role of different CD4⁺ T cell subtypes in disease is likely to vary by disease type, overall low CD4⁺ count, and poor CD4⁺ response to antiretroviral treatment among HIV patients, have been associated with an increased risk of heart disease, cancer, and non-AIDSs related mortality among HIV patients (45-48). In a prospective cohort study, HIV patients with low baseline CD4 count levels (≤ 200 cells/uL) had higher risk of subsequent cancers, including cancers with no known infectious etiology (lung, colorectal, and melanoma), suggesting that immune suppression may be impacting risk through pathways other than increased risk of infection. Similarly, cancer risk is elevated

among patients who are immune suppressed from organ transplants (6) and is greatest among those with low CD4 count (49).

Although these studies have been useful in those with gross immunosuppression, only a few epidemiological studies have measured CD4 subtypes directly to examine their role in the development of subsequent disease when measured in healthy individuals. A number of these studies have focused on cardiovascular disease given the strong experimental evidence supporting a role for the adaptive immune response in atherosclerosis (50). In the largest study, CD4⁺ T cells (both naïve and memory) were measured in healthy individuals and associated with past infections, inflammatory markers and subclinical atherosclerosis (51). Higher memory CD4⁺ cells and lower naïve CD4⁺ cells were positively associated with interleukin-6 levels, infection (cytomegalovirus and *H. Pylori* titers), and common carotid artery intimal media thickness (IMT) in European-Americans (51). Other studies have found similar associations between memory CD4⁺ cells with IMT of the carotid artery, using similar cross-sectional study designs (52, 53). To date, no study has directly measured the associations with cardiovascular risk using a prospective cohort design in a healthy population. The study of such cellular fractions has been hampered by the inability to apply flow cytometry in epidemiologic studies. This technology requires use of freshly collected whole blood samples and highly trained personnel in experienced laboratories to achieve reliable and consistent results.

iii. Using differentially methylated regions (DMRs) to measure immune response

The dynamic process by which hematopoietic stem cells give rise to the lymphoid (T cells, B cells, and natural killer cells) and myeloid (neutrophils, eosinophils, basophils, monocytes, macrophages, megakaryocytes, platelets, and erythrocytes) lineages (hematopoiesis) involves a complex signaling cascade driven by lineage-specific transcription factors and coordinate epigenetic modifications including DNA methylation and histone modifications (54). Because normal tissue differentiation and cellular lineage is regulated by epigenetic mechanisms (55), DNA methylation shows substantial variation across tissue types (56) as well as individual cell types, particularly distinct types of leukocytes (57). This understanding has led to a search for *differentially methylated regions* (DMRs) that distinguish specific cell lineages with high sensitivity and specificity (58). The importance of DNA methylation in this process was initially demonstrated in the control of the β -globin locus, which is highly methylated and transcriptionally inactive in non-erythroid cells and pluripotent stem cells, but undergoes sequential hypo- and hypermethylation at specific regions throughout the locus corresponding to the transcriptional control regions of each of the embryonic (ξ), fetal ($\gamma\gamma$) and adult (α , β) globin genes corresponding to the point of lineage differentiation (54).

A growing body of literature is now defining differentially methylated regions (DMRs): CpG loci characterized by differential methylation based on cellular differentiation. Such

DMRs have been identified in the 5'UTR of *PU.1*, which is hypermethylated in CD4+ and CD8+ T cells but not in mature B cells, where this transcription factor is expressed (59). A DMR in *GATA3* is hypomethylated in naïve and memory CD4+ cells, compared to CD34+, CD8+, T and B cells, while those in *TCF7* and *Etv5* are hypermethylated in B and T memory cells compared to their naïve counterparts (59). DMRs in the *FOXP3* locus are methylated in naïve CD4+CD25- T cells, activated CD4+ T cells, and TGF- β -induced adaptive T-regulatory (Tregs) cells, whereas they are completely de-methylated in natural Tregs, which are critical cells in autoimmune regulation (60). Moreover, DNA methylation may provide insight into previously undefined human Treg signature genes (61). This growing body of data suggests that methylation of these DMRs is cell type-specific, and can be used to characterize or fingerprint specific cell types. An analytical methodology based on hematopoietic lineage-specific DMRs has been developed and validated to utilize DNA methylation profiles to define the proportion of each of the leukocyte lineages in peripheral blood samples (62).

To date, only a few studies have used DMRs to examine associations between specific immune profiles and disease risk in epidemiological studies. In one study, Wiencke et al. reported statistically significant decreases in T-lymphocytes (measured with DMR *CD3Z*) and Tregs (*FOXP3*) in peripheral blood of glioma cases compared to healthy controls (63). The DMR *CD3Z* was strongly correlated with the CD3+ T-lymphocyte level when measured with flow cytometry (FACS) in a subsample of cases and controls ($r=0.93$). In a separate case-control study, a low level of natural killer cells (NK), estimated with a

known DMR in NK cells (*NKp46*), was associated with a 5-fold increase in risk of head and neck cancer (64).

Genome-wide DNA methylation (EWAS) array data taken from a mixed cell population, such as peripheral blood, can infer the underlying distribution of cells within the population and can provide a more comprehensive immune profile than measuring a subset of DMRs. In a recent study (65), a high correlation was observed between predicted and actual cell proportions of monocytes and lymphocytes (0.65 and 0.63, respectively) using DNA methylation profiles, with very low median absolute error between predicted and actual cell proportions (3% for both monocytes and lymphocytes). Additionally, a moderate degree of consistency was observed between the average predicted and actual proportions of lymphocytes and monocytes across the study samples (actual average proportion of lymphocytes and monocytes = 0.82 and 0.18 compared to predicted average proportion of lymphocytes and monocytes = 0.82 and 0.15). Importantly, these results have been experimentally validated using peripheral blood samples (66). The errors in estimates of leukocyte proportions using the DNA methylation methodology are comparable with other methods (including flow cytometry).

Using epigenetics to measure immune cell profiles offers unique advantages to existing methodologies for application to large epidemiological studies with archival samples. The methods are robust under varying conditions. Studies have shown that results are

not affected by type of anticoagulant used to obtain bloods, freeze/thaw and storage conditions, and when using whole blood or buffy coat (66).

D. Characteristics and environmental factors impacting immunological profiles

The environment plays an extremely important role in the development and shaping of the immune system. Certain environmental factors, such as cigarette smoke, have the ability to modify the adaptive immune response, and can interact with genetic variants to increase risk (67, 68). In a recent study of 210 healthy twins, 58% of the 204 immunological parameters measured were completely determined by non-heritable parameters (<20% of their total variance was explained by heritable factors), and 77% of these parameters were dominated by non-heritable influences (>50% of variance) (69). The study also observed more variation in some of the immunological parameters with age, suggesting the cumulative influence of environment exposures.

Here, we briefly review some of the environmental factors known to impact the immune response:

1. Race/ethnicity and socioeconomic (SES) status

Immunological differences, both in innate and adaptive immune responses, are seen in males and females, and across different ethnicity/race, raising the possibility that some of the disparities observed in cancer might be partially explained by these immunological responses. Ethnic-related differences in the prevalence of autoimmune diseases, such as systemic lupus erythematosus (70) and multiple sclerosis

(71) are well recognized, and it is also well known that there are striking differences in race/ethnicity in response to immunotherapies such as interferon (72) and belimumab (73), as well as stem cell transplantation(74).

Examples of well-described racial/ethnic differences in immune profile include “benign ethnic neutropenia” which has been found at an almost 100% prevalence in some African populations (75). This condition is now known to arise as a result of a -46 T to C substitution in the Duffy Antigen Receptor for Chemokines (*DARC*) gene. This variant has been associated with altered recruitment of leukocytes to sites of inflammation (76), and the gene on RBCs is capable of binding chemokines and may diminish WBC numbers in part by modulating chemokine signaling in the bone marrow (as it can sequester molecules through membrane binding). Numerous subtle immune alterations have been associated with this variant, including modulation of chemokine concentrations in vascular and tissue microenvironments (77), and alterations in endotoxin reactivity (78).

Among the other studies that have shown phenotypic differences in the immune response associated with race or ethnicity, Ford and Stowe (79) reported that there were very significant difference in Epstein-Barr virus antibody titers in black-African Americans compared with whites using data from the 2003-2020 National Health and Nutrition Examination Study (NHANES). Similarly, a number of Major Histocompatibility Complex (MHC) genes, known to contain large haplotypic variation and distinct patterns of lineage disequilibrium, have been linked to race; for example, one of the African

ancestry alleles, HLA-DRB1*15, has been consistently associated with risk of MS (80, 81) and with disease severity (82).

Finally, recent single cell network profiling of peripheral blood mononuclear cells revealed striking differences in normal signaling responses by race/ethnicity (83). This study reported that B cell signaling through the PI3kinase pathway was significantly altered when a discovery and test set were employed to rigorously avoid false positive results. These authors further speculate that this may indicate that there are race/ethnicity specific differences in NF-kappa-B responses that signal through the MAPK pathways.

Population based thresholds for the NLR have been established in primarily non-Hispanic white populations and there is a significant gap in our understanding of leukocyte profiles in AA populations. Importantly, the limited work that has been done on AA-specific NLR levels shows that this biomarker, although different in its distribution among AA subjects, is an important health indicator, as it is known to be among whites. For example, an extensive study of AA subjects examined the NLR and mortality following percutaneous cardiac intervention (angioplasty) (84). Previous studies had established that NLR was a significant predictor of mortality following angioplasty in whites (85). Among 1,283 AA patients undergoing angioplasty, NLR values, although shifted to lower levels in AA subjects compared to whites, were shown to be powerful and independent predictors of long-term mortality in AAs undergoing this common procedure (84).

There are abundant data showing that low SES plays an important role in immune response and contributes to racial disparities in immune function, greater risk of disease, more rapid disease progression and reduced survival (86). Many such studies have proven that low SES and related high life “stress” conditions lead to elevated antibodies titers to herpes virus, reflecting poorer immune control of chronic viral infection and cell-mediated immune function (87) (88). Low SES is related to several immunologically mediated diseases including asthma (89), kidney failure and kidney transplant outcomes (90). Stress has been linked to abnormal numbers of NK and B cells (91), and limited financial resources increases the percent of ineffective NK subtypes (NKCD57+) as seen in aging (92). Finally, measures of education and low SES have been associated with short telomere length in blood leukocytes (93, 94). African Americans have shorter leukocyte telomeres compared to whites after adjustments for age and gender (95). Telomere length decreases in blood leukocytes with age and this telomere attrition is accelerated by chronic inflammatory responses that drive immune cell mitosis and apoptosis.

2. Smoking

Cigarette smoking results in a strong immune response that has been well-characterized using both population and experimental studies. Recent reviews on this topic describe in detail the impact tobacco smoking has on the inflammatory response (96), and the concurrent immunosuppressive effect it has on the adaptive immune response (97, 98). Smokers have higher circulating serum levels of pro-inflammatory cytokines than

nonsmokers (99, 100). Smokers also have higher risk of infection (101). By pushing the balance of the immune response towards a pro-inflammatory response and decreasing the adaptive immunity response, the effect of smoking is akin to the overall effect of ageing on the immune response.

Moreover, nicotine has been shown to have important immune effects that are separate from the toxic chemical inflammation related to tobacco smoke exposure (as well as distinct from the mutagenic activity well known to be associated with this complex mixture). It has been convincingly demonstrated that lymphocytes express most of components of the cholinergic system, and respond to stimulations independent of cholinergic nerves, directly impacting the regulation of immune function and local circulation (102). This fact is now widely thought to account for the action of nicotine in preventing and treating ulcerative colitis (103). Similarly, smoking has been associated with worsening Crohn's disease via this same pathway (104). The thymus based maturation of T cells is dependent upon cholinergic signaling and nicotine exposure in mature T cells influences their responsiveness to T cell receptor mediated activation and effector functions (105). Hence, the direct action of smoking and nicotine on the immune system has been well described; however the consequences of this immune dysregulation clearly can be complex and difficult to predict in a differing tissue context.

3. Physical activity

The impact of physical activity on the immune response is well-established; moderate to high levels of exercise have consistently been linked to lower levels of inflammatory markers, such as CRP and IL-6, in observational studies (106). Intervention studies examining impact of exercise on inflammatory markers have been less consistent, but this is likely due to small numbers, short follow-up periods, and different levels of inflammatory markers at baseline. One of the largest randomized controlled trials to date observed a significant reduction in IL-6 levels after a 12-month period of moderate exercise (150 mins/week of walking) in elderly men and women, compared with a successful ageing intervention without exercise, although other markers, including CRP, did not change (107). In contrast, acute and intensive exercise, more common among athletes such as marathon runners, can lead to transient immunodepression (108). Exercise also has an impact on the adaptive immune response; CD4+ and CD8+ activation and proliferation decrease, while NKs increase, with exercise (109).

4. Obesity

It has been noted for some time that overnutrition and increased adiposity are linked to immune dysregulation. Hypertrophied adipocytes lead to increase production of adipokines, cytokines, and fatty acid which lead to stimulation of macrophages (110). The impact of obesity on immunity is partially mediated through the pro-inflammatory activity of adipokines, such as leptin. Leptin has been shown to impact both the innate and the adaptive immune response in humans, through a myriad of effects on various immune cell types (111). Other mechanisms through which adiposity impacts the

immune response include hypoxia and cellular stress in the adipocytes, which exacerbate the local inflammatory response (110). Obese individuals, like smokers, are more likely to develop infections, both primary and secondary infections, and also have a decrease antibody response to immunization (112).

5. Diet

There is extensive evidence that vitamin D levels have a direct impact on the immune response and Niels Finsen won the Nobel Prize in 1903 for the discovery that dermal tuberculosis (*lupus vulgaris*) could be cured with concentrated light rays (113).

Substantial research has been conducted on effects of vitamin D, with renewed interest in the past decade, especially with regards to the potential role of vitamin D on preventing chronic diseases and secondary infections; various clinical trials are underway (114, 115). Other vitamins have also been under scrutiny with regards to their impact on the immune function. While experimental studies have demonstrated the anti-oxidative properties of a number of vitamins and nutrients in inflammatory processes, the actual impact of taking vitamins, either as supplements or in the diet, on the immune response and to prevent disease in humans has not been consistent. For example, while vitamin C was initially heralded for its impact on the immune response to infections, based on experimental data, randomized clinical trials on the common colds have shown no benefit of vitamin C to prevent the common cold (116). Similarly, studies on antioxidants vitamins A and E, have not yielded expected results on diseases with strong inflammatory components, such as cardiovascular disease (117).

Other components of diet, including meat and high fat diets, may also impact the immune response by increasing systemic inflammation. Observational studies have noted higher circulating inflammatory serum markers (especially C-reactive protein) among individuals with high saturated fat intake (118) or Western diets (119), although not all studies were consistent. Data on the impact of diet on immunity are otherwise sparse.

6. Infection

While it is clear that exposure to pathogens shapes the adaptive immune response throughout a lifetime, the extent of the impact that infections have on immune variation are far-reaching. In a recent twin study examining heritable and non-heritable influences on the variation of the human immune system, cytomegalovirus (CMV) infection was found to have a wide-ranging influence on the overall immune profile of healthy individuals; 119 of the 204 immunological measures, including cell population frequencies, cytokine responses and serum proteins, were affected by CMV infections in MZ twins (69). Other viruses are likely to have a broad influence on the immune system (69). Research evaluating the role of the microbiome on the immune system is just at its infancy with the development of next-generation sequencing technologies (120). There are exciting opportunities for epidemiologists to understand how these exposures shape immunity and future disease risk.

E. Summary and directions

There are potentially 30 or more distinct types of leukocytes that may be relevant for differentiating health outcomes. In an ideal epidemiology study, reasonably precise cell counts (or proportions) would be available for the relevant types (e.g. Th vs. Treg cells or activated vs. non-activated NK cells). However, limitations in cell sorting technology (including collecting and processing fresh blood on a large number of participants) make this difficult or infeasible. An alternative to flow-sorting cell types is to use a DNA-based method of profiling, where the DNA methylation profiles obtained from whole blood are deconvolved into proportions of relevant types. Assuming adequate sensitivity and availability of reference profiles for the target cell types, this represents an extremely efficient approach to immunoprofiling. However, some target cell types may be quite rare in whole blood, making use of arrays and a deconvolution-based approach problematic. A long term solution is to develop reference data sets for a large panel of cell types that is able to quantify DNA methylation, allowing for lineage specific markers to emerge. Newer approaches to detect these sentinel demethylated regions using bisulfite sequencing or digital droplet PCR may offer sensitive solutions to this problem.

Advances in clinical epidemiology will also be made by applying these new technologies to examining prognosis of cancer. Many studies have demonstrated the critical importance of immune cell profiles and proportions in predicting survival and prognosis of cancer, using traditional methodologies. Refining the ability to measure the immune function in patients should afford new advances in the field of prognosis. There are exciting opportunities for epidemiologists to understand how these exposures shape immunity and future disease risk.

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