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Review

A systems biology approach to nutritional immunology – Focus on innate immunity

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A B S T R A C T

Innate immunity and nutrient metabolism are complex biological systems that must work in concert to sustain and preserve life. The effector cells of the innate immune system rely on essential nutrients to generate energy, produce metabolic precursors for macromolecule biosynthesis and tune their responses to infectious agents. Thus disruptions to nutritional status have a substantial impact on immune competence and can result in increased susceptibility to infection in the case of nutrient deficiency, or chronic inflammation in the case of over-nutrition. The traditional, reductionist methods used in the study of nutritional immunology are incapable of exploring the extremely complex interactions between nutrient metabolism and innate immunity. Here, we review a relatively new analytical approach, systems biology, and highlight how it can be applied to nutritional immunology to provide a comprehensive view of the mechanisms behind nutritional regulation of the innate immune system.

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1. Innate immunity and systems biology

The innate immune system is ancient and has evolved as the first line of defense against invading pathogens working in concert with the adaptive immune system to clear infections, initiate repair and promote a return to homeostasis (Zanker, 2008). Recently, non-hematopoietic cells such as epithelial cells and adipocytes have been recognized as important regula-

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tors of innate immunity. Innate immunity involves between 1500 and 5000 proteins and other biomolecules that work as a complex network of interwoven components involving numerous regulatory circuits, pathways and effector mechanisms. The complexity and interdependence of the immune response limits the usefulness of reductionist approaches that examine a single gene or cell type in isolation. As such, deeper insights into innate immunity require its investigation using a network biology approach that is often termed "systems" biology.

Systems biology is a comprehensive and quantitative approach to studying the interactions between all components of a biological system. It utilizes high-throughput techniques to collect extensive data sets for classes of biomolecules such as genes, mRNA transcripts, proteins or metabolites (Zak and Aderem, 2009). Mathematical modeling generates predictions on the behavior of systems, which are then experimentally tested to confirm or provide insight as to how the predictions can be further refined. These detailed *in silico* models of the system can then be used to predict how the system will respond under untested conditions (Gardy et al., 2009). As a predictive method, the outcomes are framed in terms of hypotheses that must then be experimentally tested. The application of the unbiased global analytical processes of systems biology to the innate immune response is likely to be critical to an enhanced understanding of this complex system. The major high-throughput techniques applied to systems biology are genomics, transcriptomics, proteomics and metabolomics.

2. Bioinformatics in systems biology

Bioinformatics is used to manage the vast datasets generated in systems-wide studies and for analysis, and identification of novel components, pathways and functions. To enable deeper understanding of the biological significance of these measurements, the data should be integrated with a biomolecular interaction network whereby relationships are established between dysregulated genes through analysis of their physical, biochemical or regulatory interactions within the cell (reviewed in Gardy et al. (2009)). A number of studies have identified overrepresented biological pathways in microarray datasets revealing novel functional information for numerous biological processes. However, pathway analyses utilize somewhat simplified linear cascades of established biological pathways; a more complete picture can be generated through network analysis, which examines the interactions between genes, proteins, RNA, metabolites and other biomolecules by utilizing interaction and pathway databases in conjunction with visualization tools. The resulting networks can be used to identify new biological pathways, key sub-networks, major regulatory elements and the central molecules (termed "hubs") associated with them. A vast array of tools are available and have been described in overview (Gardy et al., 2009). One user-friendly bioinformatics tool that has that has proved particularly valuable for pathway and network analysis with respect to innate immunity, and nutritional immunology, is InnateDB, a comprehensive open-access database of human and mouse molecular interactions as well as a complete analysis platform (Lynn et al., 2008). Continued development of bioinformatics tools has allowed for more complex workflows such as multiple experimental conditions, time-course studies and dynamic systems analysis of individual cells, providing researchers a more comprehensive and potentially dynamic picture of the innate immune response.

3. Nutritional immunology

Metabolism is a fundamental process that encompasses numerous metabolic pathways utilized to varying degrees for energy generation and the production of the metabolic precursors required for macromolecule synthesis. Innate immune cell survival and function has been intrinsically linked to nutrition and cellular metabolism. Malnutrition due to insufficient intake of macronutrients is a striking example of the critical role that nutrition plays in innate immune responses (Cunningham-Rundles et al., 2005). Dietary restriction results in reduced neutrophil trafficking to sites of inflammation, due to reduced integrin expression and chemokine production (Ikeda et al., 2001). Interestingly, even brief nutritional replenishment or provision of a single nutrient such as glutamine or arginine is able to restore lost cellular function (Ikeda et al., 2001, 2003; Ueno et al., 2010). Clinically, malnutrition is associated with more frequent and chronic infections (Cunningham-Rundles et al., 2005; Russell et al., 2004) leading to increased morbidity and hospitalization. Malnourished patents must often undergo nutritional rehabilitation in order to control infections (Bahwere et al., 2004).

Macrophages and neutrophils rely on glucose and glutamine and withdrawal of these nutrients or inhibition of their metabolic pathways substantially reduces many cellular functions such as cytokine production and phagocytosis (Barghouthi et al., 1995; Jun et al., 2010; Murphy and Newsholme, 1999; Newsholme et al., 1996; Pithon-Curi et al., 2002). In addition to their structural role, certain fatty acids also modulate macrophage and neutrophil responses. Polyunsaturated fatty acids (PUFAs) can be metabolized into either pro or anti-inflammatory mediators. ω-6 PUFAs promote pro-inflammatory functions such as chemotaxis, ROS production and pro-inflammatory cytokine production (Calder, 2009), while ω-3 PUFAs are anti-inflammatory and promote wound healing and resolution of inflammation (Endres et al., 1989; Lee et al., 1985; Schmidt et al., 1992; Serhan et al., 2008). Although the same enzymes metabolize both ω-3 and ω-6 PUFAs, ω-3 PUFAs can prevent the production of ω-6 PUFAs through competitive inhibition (Rees et al., 2006).

Evidence suggests that macronutrient excess can promote inflammatory responses in adipocytes and macrophages, in part through the activation of stress responses. Acute induction of hyperglycemia in healthy individuals and stimulation of monocytes with high glucose levels *in vitro* induces IL-6, IL-18 and TNF-α production (Esposito et al., 2002; Morohoshi et al., 1996). Similarly, excess extracellular and intracellular free fatty acids (FFAs) and triglycerides trigger endoplasmic

reticulum (ER) stress responses or Toll like receptor (TLR) activation (e.g. TLR-2 and -4) in adipocytes and macrophages (Ozcan et al., 2004, 2009; Schaeffler et al., 2009; Shi et al., 2006). Stimulation of these pathways then activates NF-κB and leads to production of proinflammatory mediators including resistin and MCP-1 in adipocytes and IP-10 in macrophages (Laine et al., 2007; Schaeffler et al., 2009; Schwartz et al., 2010; Zhang et al., 2006).

Micronutrients, such as vitamins and minerals are metabolites, required by cells in low quantities, which regulate numerous physiological processes. Deficiencies in various micronutrients are strongly associated with the development of immune defects and increased susceptibility to infection (Shankar and Prasad, 1998). For example, zinc deficiency affects one third of the world's population and is a major risk factor for pneumonia, malaria, diarrhea (Caulfield et al., 2004) and poor wound healing (Grommes et al., 2011; Sharir et al., 2010). Zinc deficiency is also associated with increased systemic inflammation, organ damage and disease severity (Bao et al., 2010; Besecker et al., 2011; Knoell et al., 2009). Zinc is critical to innate immune cell development and activity, and zinc-deficient conditions impair macrophage phagocytosis and intracellular killing (Sheikh et al., 2010; Wirth et al., 1989). Zinc supplementation in malnourished children and children with *Escherichia coli*-induced diarrhea resulted in reduced phagocytic and fungicidal activity and oxidative burst (Schlesinger et al., 1993; Sheikh et al., 2010). Conversely, high zinc concentrations have been reported to suppress macrophage chemotaxis, activation, phagocytosis and oxidative burst *in vitro* (Allen et al., 1983; Chvapil et al., 1977; Karl et al., 1973). This highlights the fact that nutrients must be present at appropriate levels to ensure proper immune function.

The active form of vitamin D (1,25D3) is an important micronutrient involved in many cellular processes, including autophagy (Hewison, 2011). Vitamin D-induced autophagy acts as an important regulator of host defenses against *Mycobacterium tuberculosis*. Stimulation of *M. tuberculosis*-infected macrophages with 1,25D3 leads to the enhanced production of the host defense peptide LL-37, increasing the activation of autophagy, autophagosome–lysosome fusion and antimycobacterial responses (Liu et al., 2007; Yang et al., 2009; Yu et al., 2007). Vitamin D supplementation in combination with traditional therapy is now being investigated clinically as a potential new treatment for tuberculosis (Nursyam et al., 2006). Vitamin D-mediated LL-37 induction also occurs in other cells such as neutrophils and epithelial cells, although its role in anti-infective activity for these cells has yet to be elucidated (Gombart et al., 2005).

The studies summarized above establish a strong relationship between nutrition, metabolism and the immune response. It is clear that nutrition affects a large number of biological processes vital to the immune response, including gene expression, protein synthesis, modification and degradation, metabolism, signal transduction, and cellular proliferation and survival. However, research to date has failed to explore the obvious complexity behind nutrient-mediated regulation of the innate immune system, since it has followed a reductionist approach, focusing generally on a single cell or tissue type, nutrient, and/or enzyme/pathway.

To understand how nutrition regulates so many biological processes, and the innate immune response as a whole, requires analytical approaches that integrate all of the components of the cell (through analysis of the genome, transcriptome, proteome and metabolome), and their interactions with the environment. Systems biology provides the tools required for unbiased global analysis of this complex biological system, the pathogenesis associated with its dysregulation and the mechanisms by which potential treatments act. Critically while single component studies are important drivers of hypothesis-driven research, unbiased "omic" approaches have the potential to deliver fundamentally new perspectives and hypotheses.

4. Systems biology approaches to nutritional immunology

Genomics is essentially the study of the genome of a given organism through mapping, sequencing and analysis of all genes, in order to identify genetic variations within a population and how these variations relate to specific phenotypes. In nutrition research, nutrigenetics and nutrigenomics are two approaches utilized to explore the interaction of nutrients and genes in an effort to determine the mechanism behind disease development.

Nutrigenetics focuses on the genetic makeup of an individual, identifying and characterizing genetic variations (polymorphisms) associated with differential responses to nutrients, which is then related back to specific disease states. Most genetic variations arise from single base pair changes referred to as single-nucleotide polymorphisms (SNPs), and can have substantial effects on an individual's health and susceptibility to disease. Using nutrigenetics, several genes have been identified with polymorphisms that alter the metabolism of nutrients such as folate and consequently susceptibility to nutrition-related chronic diseases including atherosclerosis and cardiovascular disease (Miyaki et al., 2005). Several vitamin D receptor (VDR) polymorphisms have been identified including VDR-*Fok1*, which is associated with reduced dietary calcium absorption, and VDR-*Bsm1* and VDR-*Apa1* associated with glucose intolerance and insulin resistance, respectively (Ames et al., 1999; Oh and Barrett-Connor, 2002; Smolders et al., 2009). VDR polymorphisms are also associated with the severity of autoimmune disorders such as multiple sclerosis (Smolders et al., 2009) and several infectious diseases such as tuberculosis (Motsinger-Reif et al., 2010). The direct effects of the four known VDR polymorphisms on immune cell function are ill-defined. One recent study examined their influence on vitamin D-mediated phagocytosis of *M. tuberculosis* by macrophages, since, as mentioned above, in healthy individuals vitamin D is able to enhance *M. tuberculosis* phagocytosis by macrophages. This effect of vitamin D on phagocytic potential was more pronounced in macrophages from individuals with the *BsmI*, *ApaI*, and *FokI* polymorphisms (Selvaraj et al., 2004). The major drawback of current approaches to nutrigenetics is that, much like traditional methods used in nutritional immunology, they only generate leads on the role of individual components in a system.

Nutrigenomics on the other hand measures nutrition-responsive genome activity through systems level studies and thus generates a far more comprehensive and mechanistic view on how nutrients regulate the immune response and disease development. Nutrigenomics relies heavily on the high-throughput techniques of transcriptomics, proteomics and metabolomics to assess the effects of nutrient stimuli on a biological system.

4.1. Transcriptomics

Macro- and micronutrients are major environmental regulators of gene expression. Nutrient-mediated regulation of gene expression occurs primarily through the altered activity of transcription factors, including several from the nuclear receptor superfamily, such as the peroxisome proliferator activated receptors (PPAR), which bind fatty acids (Kliewer et al., 1997; Straus and Glass, 2007). Nutrients can also affect the concentrations of other metabolic substrates or intermediates involved in gene regulation or can alter signal transduction pathways. Interestingly, many of these transcription factors influenced by metabolites have dual roles within cells of the innate immune system, regulating the expression of a variety of metabolic and inflammatory genes (Baker et al., 2011; Hong and Tontonoz, 2008).

Transcriptomics utilizes a variety of technologies, most commonly microarrays and next-generation sequencing (Metzker, 2010; Ricciardi-Castagnoli and Granucci, 2002), to assess gene expression changes at the mRNA level in response to different stimuli. Most transcriptomic studies related to nutritional immunology have been aimed at identifying metabolic genes that are not classically associated with innate immunity, and/or that are altered in response to infectious agents or inflammatory stimuli. For example, genome-wide expression profiling of whole blood from patients with dengue shock syndrome found the upregulation of genes associated with a pro-inflammatory innate immune response, altered cholesterol homeostasis in macrophages and the arachidonic-acid metabolism pathway at the time of cardiovascular failure (Devignot et al., 2010). This gene expression pattern resembles a pro-inflammatory lipid-enriched macrophage subtype, similar to foam cells, that has been implicated in inflammation, vascular damage and atherosclerosis (Moore and Tabas, 2011). Although these lipid-laden macrophages have not yet been identified in DSS patients, the presence of this gene expression profile implicates a novel mechanism for the systemic vascular dysfunction observed in dengue shock syndrome and warrants further study.

More recently, transcriptomics has been utilized to examine direct nutrient-mediated modulation of immune cell activity as well as chronic inflammation observed in metabolic diseases such as obesity, type 2 diabetes mellitus and cardiovascular disease (Grayson et al., 2011). Genome-wide profiling of peripheral blood mononuclear cells from subjects consuming fish oil supplements was carried out to determine the effects of ω-3 PUFAs on human health status (Bouwens et al., 2009). High doses of ω-3 PUFAs resulted in a significant downregulation of proinflammatory genes as well as genes associated with atherosclerosis. Overall, the gene expression profiles of the PBMCs suggest that PUFA supplementation promotes an anti-inflammatory- and anti-atherogenic cellular state. This particular nutrigenomic study also showed that genomic profiling can be used to determine the effects that long-term nutritional adaptations have on other biological processes.

One major issue associated with transcriptomics performed using microarrays (largely avoided by RNA-Seq) is the large proportions of false positives and negatives present within the data sets generated (up to 30% or more with three experimental repeats using cut-offs of 2-fold changes in gene expression and p values of <0.05). Thus no single gene expression result can be considered reliable and for hypothesis generation it requires examination of patterns of altered expression. Data sets from multiple experiments examining related phenomena can be integrated and meta-analysis performed to identify genes that behave similarly or differently across the data sets. If the data sets are from very similar experiments, it allows for the reduction of false positive or negative results within a single study. Also, applying this method to more diverse studies allows for the identification of collections (termed clusters) of genes representing common pathways, ontologies (functional classifications), transcriptional factor targets, etc., that are commonly or differentially important under varying conditions (e.g. in the presence of an infection or nutritional deprivation) or in multiple pathophysiologies (e.g. asthma or cystic fibrosis as inflammatory lung conditions). From these clusters, functional and mechanistic information can be inferred.

A meta-analysis of whole peripheral blood gene expression profiles of type 2 diabetes mellitus, coronary artery disease and their precursor state, metabolic syndrome, was recently completed by comparing data sets from each disease state to control data sets and to each other (Grayson et al., 2011). Metabolic syndrome refers to a constellation of indications, including central obesity, hypertension and hyperglycemia that increase the risk for development of type 2 diabetes mellitus, coronary artery disease and stroke. Thus, differences in gene expression between the two disease states and the precursor metabolic syndrome may provide insights into the unique mechanistic features associated with disease progression. The progression from metabolic syndrome to cardiovascular disease was characterized by an increased number of genes associated with macrophage signaling and activation. Conversely, metabolic syndrome and type 2 diabetes mellitus were closely related, but in the type 2 diabetes mellitus profile there was an increase in genes associated with T-lymphocyte signaling and proliferation.

While the above study demonstrated the utility of meta-analysis, it required the completion of large numbers of costly microarrays for samples that can be difficult and time consuming to obtain, making studies like these prohibitive. There are several open access repositories, including Array Express and Gene Expression Omnibus (GEO), that house transcript data from numerous species, conditions and platforms. Meta-analysis tools that can utilize these datasets serve to minimize the requirement of resources for clinically relevant genomic studies, while maximizing the utility of generated data. Gene expression profiles of obesity and type 2 diabetes mellitus obtained from multiple experiments (Kaizer et al., 2007; MacLa-

Table 1
Selection of immunologically relevant genes differentially expressed in both obesity and diabetes identified using MetaGEX.

Gene ID[a]	Gene name	Obese vs. control (*P*-value)		Diabetes vs. control (*P*-value)		
		Omental obese vs. lean (GSE15524)	Subcutaneous obese vs. lean (GSE15524)	Type 1 diabetes vs. control (GSE9006)	Type 2 diabetes vs. control (GSE9006)	Diabetes mellitus vs. control (GSE16415)
	Cytokine/chemokine and receptors					
3552	Interleukin (IL)1α	–[b]	3.83E-02	2.00E-06	1.45E-03	–
3554	IL1R1 [IL1 receptor, type I]	4.60E-02	–	3.56E-02	1.52E-02	–
7850	IL1R2 [IL1 receptor, type II]	6.29E-03	–	1.37E-03	–	–
3598	IL13RA2 [IL13 receptor, alpha 2]	2.06E-02	–	–	4.57E-02	–
6363	Chemokine CCL19	3.98E-03	–	–	–	1.09E-02
3579	Chemokine receptor CXCR2	4.62E-02	–	3.61E-03	–	–
7852	Chemokine receptor CXCR4	1.21E-02	–	–	1.41E-03	–
	Complement components and receptors					
708	Complement component binding protein C1QBP	2.95E-05	–	–	5.82E-06	–
713	Complement component C1QB	2.71E-02	–	–	–	1.40E-02
730	Complement component C7	7.20E-04	–	–	1.40E-02	–
1380	Complement receptor CR2	3.42E-03	–	3.06E-04	–	–
	Interferon-associated molecules					
3454	IFNAR1	9.98E-04	–	–	1.69E-02	–
3460	Interferon-γ receptor IFNGR2	3.27E-02	–	3.06E-03	1.24E-03	–
3662	Interferon regulatory factor IRF4	1.13E-02	–	7.88E-03	–	–
10581	Interferon induced IFITM2	6.62E-03	–	9.74E-03	1.67E-02	1.21E-03
10561	Interferon induced IFI44	3.02E-02	–	2.09E-02	1.66E-02	–
10964	Interferon induced IFI44L	2.46E-02	–	2.41E-04	–	–
	Cell surface molecules/receptors					
958	CD40, TNF receptor superfamily	2.76E-02	–	–	2.25E-02	–
4481	Macrophage scavenger receptor MSR1	3.21E-02	–	2.40E-02	–	–
3111	HLA-DOA	1.40E-02	–	2.20E-03	–	–
3123	HLA-DRB1	1.38E-02	–	–	–	1.53E-02
3133	HLA-E	1.80E-02	–	3.20E-02	–	–
3135	HLA-G	3.04E-02	–	–	3.09E-03	–
	Transcription factors and signaling components					
5465	PPARA [peroxisome proliferator-activated receptor-α]	2.56E-02	–	–	–	4.31E-02
5871	MAP4K2 [MAP kinase kinase kinase kinase]	5.95E-03	–	–	3.11E-02	–
7786	MAP3K12 [MAP kinase kinase kinase]	8.28E-04	–	–	–	3.03E-02
8649	MAPKSP1 [MAPK scaffold protein 1]	1.41E-02	–	–	3.62E-02	–
6772	Transcription factor STAT1	3.21E-02	–	–	3.95E-03	–
7022	Transcription factor TFAP2C	4.56E-04	–	–	–	3.97E-02
10010	TANK [TRAF family member-associated NFKB activator]	4.56E-02	–	–	2.35E-03	–

[a] Gene ID indexed NCBI gene: http://www.ncbi.nlm.nih.gov/gene; data sets indexed in Gene Expression omnibus (GEO): http://www.ncbi.nlm.nih.gov/geo/.
[b] Signifies not significant.

ren et al., 2010) were compared using MetaGEX (http://www.cmdr.ubc.ca/metagex/; C. Fjell, M. Mayer and R.E.W. Hancock, unpublished), a meta-analysis tool under development in our lab. Preliminary analysis of genes differentially expressed in both disease states revealed a large number associated with immunity and inflammation, including cytokines/chemokines and their receptors, complement components, interferon-associated molecules and cell surface receptors (Table 1). In addition, analysis of over-represented biological processes identified pathways associated with leukocyte/lymphocyte differentiation and activation, initiation of cell signaling, the NF-kB signaling cascade and wound healing. This analysis supports the link between nutrition, metabolic disorders and immunity. While further work is needed to confirm the significance of these findings, this preliminary analysis shows the potential utility of such meta-analysis tools in nutritional immunology.

There are several more issues facing transcriptomics including the induction of gene expression changes by sample manipulation. Gene expression can be rapidly altered in response to the physical stresses induced by sample extraction. The time required for the extraction procedure can also affect transcript levels with longer extraction and storage times resulting in increased transcript degradation. Data analysis also poses a potential challenge as there is no universally applied method for analysis of transcriptome data, nor is there a consensus on which algorithms and statistical tools should be used (Shi et al., 2008). Finally, especially with microarrays, validation studies such as quantitative RT-qPCR are highly recommended and although gene expression patterns are often confirmed, the magnitude of change can be substantially different; this relates to the "background problem" whereby the signal level chosen as the background for subtraction (which in most platforms varies across the microarray) strongly influences the apparent magnitude of dysregulation. Similarly, expression patterns for genes of interest should also be confirmed at the protein level, since changes in mRNA level are not necessarily predictive of changes in protein level or function. In addition, metabolism is generally regulated by modulating protein activity, for example through activation (by phosphorylation or other chemical modifications) or inhibition (by marking for proteosome degradation), not gene expression, thus while transcriptomics provides a certain perspective, it is not a comprehensive systems biology tool; although it is the easiest and least expensive to perform. The emergence of RNA-Seq (Costa et al., 2010; Ozsolak and Milos, 2011), a high throughput sequencing alternative that tremendously reduces false error rates and provides a quantum leap in quality and quantifiability, by eliminating the background problem, has made this the go-to method of systems studies.

4.2. Proteomics

Many nutrients regulate cellular activity at the protein level both by activating specific signaling pathways as well as regulating global cellular protein metabolism. Nutritional status can affect the availability of amino acids needed for protein synthesis and can regulate post-translational modification of proteins and protein degradation (Kussmann et al., 2010). The abundance and activity of metabolic enzymes are critical to the flux of nutrients or metabolites through different biochemical pathways. Given the intimate connection between nutrition and the proteome, it is surprising that only a small number of nutritional proteomic studies have been completed and of those only a handful focus on nutrient mediated modulation of the immune response (Fuchs et al., 2005).

Proteomics is the analysis of all the proteins in a biological system, their interactions and their functional states although effectively, usually only the most abundant subset of 300 or so proteins is relatively easily analyzed. Quantitative mass spectrometry (MS) is a powerful unbiased approach used in proteomics to identify proteins and measure their abundance, cellular localization, post-translational modifications and complex formation (Wittwer et al., 2011). Within nutrition research, proteomics is primarily used to examine the physiological responses to changes in nutrient levels or to identify molecular signatures of specific pathological/physiological conditions induced or alleviated by nutrient supplementation (de Roos et al., 2008; Fuchs et al., 2007). Cardiovascular disease is considered a chronic inflammatory disorder, largely mediated by endothelial cells and circulating monocytes/macrophages. Nutrient supplementation has been proposed as a means to reduce the inflammation observed and several reports have indicated that soy-based diets are cardio-protective, especially in postmenopausal women who are at a high risk of developing cardiovascular disease (Scheiber et al., 2001). Fuchs et al. (2007) used proteomics to determine how dietary supplementation with the soy extract isoflavone affects protein expression patterns in the PBMCs of postmenopausal women. The levels of 29 proteins were altered in response to isoflavone intervention including heat shock protein 70, filamin A, 26S protease subunit 8 and prohibitin, that were elevated, and galectin-1 that was reduced. Overall these changes suggest that the cardioprotective activity of isoflavones found in soy may be mediated by inducing an anti-inflammatory phenotype in circulating immune cells (Fuchs et al., 2007).

Proteomic studies focused on post-translational modifications have the potential to identify alternative mechanisms behind nutrient and metabolite mediated regulation of innate immune responses. For example, certain fatty acids modulate neutrophil, macrophage and dendritic cell responses. They can be metabolized into lipid mediators such as prostaglandins and leukotrienes or used for protein modification (Yaqoob, 2003). S-palmitoylation ensures proper targeting and functions of many membrane-associated proteins and thus can potentially regulate numerous cellular pathways (Linder and Deschenes, 2007). Proteomic analysis of S-palmitoylation in dendritic cells, using metabolic labeling with an alk-16 palmitate reporter and CuAAC, and protein identification by MS, identified interferon-induced transmembrane protein (IFIT) 3, a potent inhibitor of influenza, dengue and West Nile virus replication (Brass et al., 2009). S-palmitoylation of IFIT3 was essential to its antiviral activity in influenza infected cells, controlling IFIT3 clustering in membranes. It was hypothesized that the antiviral activity of IFIT3 is associated with its ability to aggregate viral envelope proteins, preventing their activity.

As mentioned above, the major limitation to proteomics is sensitivity and even with the development of new technologies, the detection of low abundance proteins remains quite difficult. This is in part due to the huge dynamic range in the concentrations of different proteins in a given sample. In many biological samples there are proteins found at extremely high levels; for example albumins and immunoglobulins make up 90% of the plasma proteome. These samples must be pretreated to remove high abundance proteins in order to improve sensitivity (Wittwer et al., 2011). The sample processing and analysis associated with proteomic studies also make it quite costly and time consuming, limiting the study size. In many cases only a subset of samples from a study are submitted for analysis or samples are pooled to reduce the number of sample sets that need to be analyzed. This can result in significant variability between samples and can potentially mask identification of important changes to the proteome under the conditions being studied. Finally, as with microarray transcriptomics, proteomics suffers from high rates of false positive and negative hits. New bioinformatics tools for data analysis have been proposed to deal with this issue (Biron et al., 2006), but validation studies are still recommended.

Proteomics can be very useful in identifying functional consequences of altering metabolism or nutrition. However, when analyzing alterations in metabolism, protein levels may not accurately reflect the key metabolic pathways utilized by the cell, since multiple pathways and nutrients can be used in anabolism and catabolism. Direct analysis of metabolite levels via metabolomics would provide excellent insight in such situations.

4.3. Metabolomics

Food and diet influence mammalian physiology, metabolism and defense against diseases, but how they do so is poorly understood. There are more metabolites present in food than nutrients known to be utilized by humans and it is unclear how these metabolites influence different physiological responses and therefore how diets affect human health. A holistic study of the metabolome has the potential to provide insights into the relationship between diet and health. The metabolome consists of all of the non-proteinaceous, small molecules present in a biological system. Changes in the metabolome content reflect the biological responses to external stimuli (nutrients, hormones or infectious agents), which involves altered gene expression and protein production/activity associated with metabolic pathways (Wittwer et al., 2011). Thus the metabolome provides an indirect measure of a biological system's metabolic status under a given condition, as well as insights into genotype–phenotype and genotype–environment relationships.

Metabolomics utilizes nuclear magnetic resonance (NMR) or chromatography based separation techniques coupled to mass spectrometry (MS) to identify and quantify the majority of components making up the metabolome and how these metabolites interact (reviewed in Dunn et al. (2010)). These metabolic profiles can be compared to study how metabolism changes in response to stresses like disease, toxins or nutrient availability. Although it is a relatively new discipline, metabolomics has been used for drug discovery and toxicology (Harrigan et al., 2005) and to assess the pathophysiology of diseases in relation to normal cellular physiology. Aerobic glycolysis, *de novo* lipid synthesis and glutamine-dependent replenishment of TCA cycle intermediates (anaplerosis) have been identified as key pathways enhanced during physiologic cell proliferation, tumorigenesis, and viral infection (DeBerardinis et al., 2008; Yu et al., 2011). Vizan et al. examined glucose metabolism in growth factor activated endothelial cells to understand the metabolic changes associated with angiogenesis. Glycogen metabolism and the pentose phosphate pathway were found to be essential to endothelial cell proliferation and migration. The enzymes of glycogen metabolism could thus constitute a novel therapeutic target, limiting tumor-induced angiogenesis (Vizan et al., 2009).

Many of the metabolomics studies in nutritional immunology have focused on how different infectious agents and signaling molecules alter the metabolic profile of immune cells (Brugger et al., 2006; Diamond et al., 2010; Wikoff et al., 2009). Metabolomics was used in a lymphocytic choriomeningitis virus (LCMV) infection model to elucidate systemic metabolic changes induced during an acute viral infection (Wikoff et al., 2009). The metabolites identified had similar kinetic profiles, whereby the metabolite concentration declined until day 7, when the peak viral load was observed, and either returned to baseline when the infection was resolved (day 14) or in some cases, remained low. LCMV infection caused a substantial decline in some of the tricarboxylic acid (TCA) cycle metabolites by day 7, but returned to baseline by day 14. This indicates that, at the peak of infection, the host response involves systemic alterations to central metabolism, potentially to allocate more nutrients to the activated immune cells, which exhibit a substantial increase in metabolic rate (Straub et al., 2010). Xanthine and hypoxanthine were depleted by day 7 of the infection and never recovered, indicating elevated levels or activity of xanthine oxidoreductase, an enzyme that catalyzes the conversion of hypoxanthine to xanthine, which is then converted to urate. Interestingly, xanthine oxidoreductase is also a major regulator of innate immune responses, promoting NF-κB activity, ROS and RNS production and phagocytic killing (Vorbach et al., 2003), implying that the innate immune response to LCMV infection might involve activation of xanthine oxidoreductase. This study provides a starting point for systems biology analyses, and the eventual development of comprehensive, predictive metabolic models of cellular responses to external stimuli (Peng et al., 2009).

To improve the utility of metabolomics, comprehensive metabolome databases for different organisms and tissues must be generated. In 2005, the human metabolome project was started, with the goal of identifying, quantifying and cataloguing all of the metabolites found in human tissues and fluids. Upon completion, the metabolome and metabolomics will provide tools to improve patient diagnosis and monitoring, analyze drug metabolism and toxicology, and provide insights into the human genome–metabolome relationship (Wishart et al., 2007, 2009). A research group associated with the LIPID Metabolites And Pathways Strategy consortium, recently completed the first "functioning" macrophage lipidome, characterizing

temporal changes in numerous lipid species in response to LPS and integrating the data with that on mRNA transcript levels (Dennis et al., 2010). The dynamics of metabolic and gene expression profiles indicated that the initial response to LPS involved arachidonic acid conversion into prostaglandins, followed by biosynthesis of sterols and sphingolipids, which have been implicated in cell-signaling, phagocytosis and induction of autophagy (Sims et al., 2010). The late stages (8–24 h) of stimulation were characterized by increases in the metabolic intermediates of glycerophospholipids/glycerolipids, including diacylglycerols and phosphatidic acid, suggesting that LPS-induced intracellular signaling continued for at least 24hrs. Based on the results of this study, it was evident that system-level analysis of metabolism can provide significant insights into the connection between biochemical pathways and the innate immune response.

In a recent study coordinated dynamic transcriptomic, proteomic and lipidomic profiling was performed during an *in vitro* hepatitis C virus (HCV) infection model to determine how viruses influence intracellular metabolism to promote their propagation (Diamond et al., 2010; Walters et al., 2009). Early in the infection, HCV-mediated modifications to the cellular proteome were associated with increased aerobic glycolysis and host biosynthetic pathways, such as the pentose phosphate pathway and anapleurotic pathways. Interestingly, transcriptomic analysis of the same infection model did not predict this shift in cellular metabolism, suggesting that it was a result of post-translational regulation by HCV. Another shift was observed in the late stages of infection characterized by increased fatty acid oxidation and amino acid catabolism for energy and decreased macromolecular biosynthesis. This was associated with an increased expression of cell cycle check point/arrest genes indicating a decline in cell cycle progression, which was later confirmed by flow cytometry (Walters et al., 2009). A similar metabolic profile was observed for growth arrested HepG2 cells (Miccheli et al., 2006) suggesting that inhibition of cell cycle progression elicits a specific shift in metabolism that promotes energy generation over biosynthesis.

Lipidome profiling of the infected cells indicated differential regulation of a variety of lipid species including increased abundance of certain phosphatidyl choline and phosphatidyl ethanolamine species at the later stages of infection. This may reflect their importance in maintaining viral replication structures such as the lipid droplet and membranous replicase compartments. There was also a decrease in several sphingomyelin species with a concomitant increase in ceramides. The elevated levels of cytopathic, pro-apoptotic ceramides indicated that altered lipid metabolism may be one of the underlying causes of the cytotoxicity observed in HCV infections (Lang et al., 2007; Walters et al., 2009). Alternatively the elevated ceramides could represent a host defense mechanism, as they appear to inhibit HCV entry into cells (Voisset et al., 2008). This study highlights how integration of transcriptomic, proteomic and metabolomic profiling can yield substantial insights into a variety of areas in nutritional immunology.

Flux balance analysis involves genome-scale stoichiometric reconstruction of metabolic networks *in silico* in order to predict changes in metabolism induced by different stimuli or conditions (reviewed in Gianchandani et al. (2010)). This method was applied in the dynamic simulation of the arachidonic acid metabolic network, which is utilized by human neutrophils to generate leukotrienes and prostaglandins. The computational model developed was able to accurately simulate the effects of elevated extracellular arachidonic acid and inhibitors of cyclooxygenase-2 and 5-lipoxygenase on the flux through the network (Yang et al., 2007). Systems biology approaches such as flux balance analysis have the potential to accurately simulate changes in pathways relevant to nutritional immunology, and can be applied to drug development.

Metabolomics is a relatively new and extremely complex area of research and there are a number of challenges associated with it (reviewed in Dunn et al. (2011); Garcia-Canas et al. (2010)). One such issue can be the samples themselves. Metabolism is a rapidly changing process that is easily affected by numerous environmental cues, which can make it difficult to analyze and understand the data generated. Metabolomics can be costly and time-consuming and as a result, its feasibility is dependent on the study type. Small-scale studies consisting of a few treatments that cause substantial changes to metabolism are feasible because fewer biological replicates are required to reach statistical significance. Large-scale epidemiological studies are less feasible due to the large sample numbers required to account for the substantial physiological diversity.

In addition, metabolomics involves the measurement of multiple classes of compounds with different chemical and physical properties. This is in direct contrast to transcriptomics and proteomics, which focus on a single class of compounds, RNA and proteins. There is also substantial variation in the concentrations of different metabolites in a given tissue or biofluid. Therefore no single analytical platform is capable of identifying all the metabolites in a sample, and multiple methods must be used (Garcia-Canas et al., 2010). As mentioned above, the two most common platforms are NMR and MS coupled with a chromatographic (gas or liquid) separation technique. NMR is a relatively rapid, quantitative, and highly reproducible technique that, unlike MS maintains metabolite integrity because it does not require any extraction, derivatization or fragmentation steps. The major drawback to NMR is its low sensitivity (micro- to nanomolar levels), although there have been some recent advances in this area (Pan and Raftery, 2007). In cases were higher sensitivity is required, MS is used as it can detect and measure metabolites at picomolar to femtomolar levels (Lei et al., 2011). Unfortunately, the instrumentation required for MS is quite costly and in general MS requires more extensive sample preparation, making it time consuming. The choice in sample extraction, derivatization and separation method used poses another concern as it introduces an inherent bias towards certain compound classes. To limit this, a combination of multiple metabolite profiling tools must be employed (Dunn et al., 2011; Pan and Raftery, 2007).

The chemical identification of metabolites by NMR or MS can pose a problem, as it requires comprehensive spectral libraries. Mass spectral libraries are generated using chemical standards analyzed using specific MS platforms and methodologies; thus libraries must be generated for each method being used. There are several commercially available libraries for GC–MS and they are easily transferrable between methods. Unlike GC–MS, for LC–MS there is a limited availability of mass

spectral libraries and due to technical issues, those that are available are not transferable between methods (Dunn et al., 2011).

Because of technological advances in NMR and MS, several hundred metabolites can now be measured in sample volumes as low as a few microliters. However, because metabolomics is such a new field, most of the metabolomes recorded include a substantial number of unidentified compounds. Therefore only a subset of all cellular metabolites is analyzed, limiting our ability to identify novel interactions and pathways important to nutritional immunology (Wittwer et al., 2011).

5. Conclusion

The immune response and nutrient metabolism are two basic but complex biological systems that are essential to sustaining and preserving life. Each system is capable of modulating the activity of the other to ensure coordinated and appropriate responses under any condition. Immune competence is easily dysregulated as a result of changes to nutritional status; nutrient deficiency increases susceptibility to infection and over-nutrition is associated with chronic inflammatory diseases. Systems biology is an unbiased analytical approach that integrates all of the components of a system and their interaction with the environment. Many current methods are insufficient to analyze the global response to inflammatory stimuli and the interdependence between nutrition and innate immunity. However, recent advances in both the technical platforms and data analysis pipelines have significantly enhanced the use and utility of systems biology in nutritional immunology. Systems biology can be used to provide significant insights into nutritional regulation of the innate immune system, the pathogenesis associated with its dysregulation, and the mechanisms by which potential treatments act.

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