Immunocompetence and the Hygiene Hypothesis

Katherine Wander

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Reading Committee:

Bettina Shell-Duncan, Chair

Kathleen O'Connor

Steven Goodreau

Jonathan Mayer

Program Authorized to Offer Degree:

Department of Anthropology

University of Washington

Abstract

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Katherine Wander

Chair of the Supervisory Committee:
Professor Bettina Shell-Duncan
Anthropology

Background: Evidence from the United States and Europe supports the *hygiene hypothesis*— exposure to infectious agents during immune system development protects against immune-mediated disorders (allergy and autoimmunity). An evolutionary perspective suggests that this protection may represent adaptive priming in immune system development, evolved to tailor immune responses to the local infectious disease (ID) ecology and/or to minimize risk of immune-mediated disease. This project evaluated 1) the utility of biomarkers of immunocompetence (cell-mediated reactivity to pathogen antigen); 2) the association between early exposure to infectious agents and allergy; and, 3) the association between early exposure to infectious agents and immunocompetence among children in Kilimanjaro, Tanzania.

Methods: Biomarkers of immunocompetence and allergy, diagnosed allergy, and indicators of early exposure to infectious agents (family size, housing materials, BCG vaccination, and hospitalization in infancy with infection) were evaluated among ~300 2-7 yo children.

Results: Delayed-type hypersensitivity (DTH) to *Candida albicans* was associated with known predictors of immunocompetence (age and adiposity). Allergy was inversely

associated with family size (OR: 0.24; 95% CI: 0.07, 0.85) and positively associated with earth housing materials (OR: 2.03; 95% CI: 1.21, 3.41) and hospitalization history (OR: 6.26; 95% CI: 1.84, 21.27). DTH was positively associated with family size (OR: 2.81; 95% CI: 1.04; 7.61), BCG vaccination (OR: 3.10; 95% CI: 1.10, 8.71), and hospitalization history (OR: 4.67; 95% CI: 1.00, 21.74).

Conclusions: Allergy was inversely associated with family size, and positively associated with hospitalization and earth house materials, suggesting that during immune system development, routine ID (acquired from siblings) protect against allergy, while severe ID (requiring hospitalization) and soil-derived helminth infections (earth housing materials) promote allergy. These findings reflect Kilimanjaro's complex ID ecology, and expand, rather than contradict, the hygiene hypothesis. Immunocompetence was positively associated with multiple measures of early exposure to infectious agents, supporting the hypothesis that immune development responds to early stimulation from infectious agents to enhance later protective immune responses to pathogen antigen. Together, these findings are consistent with the hypothesis that T-helper type 1 (Th1)/Th2 regulation adapts to exposure to infectious agents during immune system development, with lasting consequences for both pathological and protective immune reactivity.

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PREFACE

This dissertation has been organized around the production of four manuscripts intended for publication as stand-alone, peer-reviewed journal articles. One of these, *The Hygiene Hypothesis in Evolutionary Context*, is a review article and contains much of the background and rationale for the research described herein (and constitutes the bulk of the *Introduction*). The additional sections of the introduction provide supplemental information (e.g., an overview of human immune system development) which, for the sake of brevity, was not included in the review article manuscript. The results of the research undertaken for this dissertation are organized as three journal article manuscripts: *Comparison of Delayed-Type Hypersensitivity to* Candida albicans *and Epstein-Barr Virus Antibody as Biomarkers of Immunocompetence among Children in Kilimanjaro, Tanzania; Early Life Exposure to Infectious Agents Predicts Diagnosed Allergic Disease and Total Immunoglobulin E among Children in Kilimanjaro, Tanzania; and, Expanding the Hygiene Hypothesis: Early Exposure to Infectious Agents Predicts Delayed-Type Hypersensitivity to* Candida *among Children in Kilimanjaro*. These manuscripts are included here in as close to their submitted form as possible. As a result, there is some redundancy (in, for example, the *Materials and Methods* sections of each submitted manuscript). For the purposes of this dissertation, all cited references have been consolidated in the *Bibliography* and tables and figures have been numbered consecutively; as such, reference, table, and figure numbers in published journal articles may not correspond to those assigned here.

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INTRODUCTION

The Hygiene Hypothesis in Evolutionary Context

Author:
Katherine Wander
Department of Anthropology, University of Washington

Corresponding author:
Katherine Wander
Department of Anthropology
Box 353100
University of Washington
Seattle, WA 98195
Phone: 425-443-4230
Fax: 206-543-3285
Email: kwander@u.washington.edu

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About the author: Katherine Wander is a PhD candidate in the Department of Anthropology at the University of Washington. She has an interdisciplinary focus, with training in both biological/biocultural anthropology and epidemiology. Her current research evaluates the roles of infectious disease morbidity and nutrition in immune system development among children in East Africa. Email: kwander@u.washington.edu

The *hygiene hypothesis* asserts that exposure to infectious agents plays a causative role in preventing immune-mediated disorders. Stimulation by microbial agents seems to shape immune system development and the future functioning of the immune system to prevent allergy and autoimmunity. A consideration of the hygiene hypothesis in evolutionary context suggests it be expanded to explain both immune-mediated diseases *and* protective immune responses to pathogens. Two regulatory mechanisms, the Th1/Th2 balance and suppressive regulation, seem to respond adaptively to microbial exposure in early life. Priming of the Th1/Th2 balance by infectious disease stress in early life may shape future immune responses to best combat locally prevalent pathogens. Priming of suppressive regulation by commensal microbes and pathogens may minimize the threat of immune-mediated damage. Consideration of both the substantial benefits and costs of immune function suggests that priming of these regulatory systems during immune system development may have evolved to minimize the overall risk posed by diverse infectious agents and the immune system itself.

Understanding of the intricate functioning of the human immune system has increased dramatically in recent decades. A detailed picture of how protective and pathological immune responses are mounted and regulated is emerging; however, there is still much in immunobiology to be learned. Immune system development is an area ripe for exploration. The human immune system undergoes rapid development during early life; exposures during this time can lead to lasting variation in immune phenotypes. Another area ripe for exploration is the evolutionary and ecological context of immune function. This review focuses on the intersection of these two areas, exploring the evolutionary context of human immune system development.

Because the human immune system is largely similar to that of all vertebrates, much of what is known about human immune function is based on the study of model organisms. However, much insight into immune system *development* comes from epidemiological study of allergy and autoimmune disease in humans. After a very brief overview of the mechanics of immune function, this paper summarizes 1) patterns revealed in the epidemiology of allergy and autoimmune disease; 2) the immunological mechanisms that have been hypothesized to explain these patterns; and, 3) corresponding evolutionary hypotheses. This paper then proposes, 4) to integrate some of these evolutionary hypotheses, to further understanding of human immune system developmental plasticity.

The Human Immune System

The immune system's primary function is to protect against invasion and colonization by pathogenic organisms (infection); other functions include protecting against cancer and aiding in the repair of injured or damaged tissues. Although an immune system is clearly critical to combating infectious disease stress, its protection is not without cost. The central challenge of an immune response is to maximize the damage done to an invading organism while minimizing damage done to the host organism's own tissues. This balance is, in humans, imperfectly realized: the immune system mediates both protective and pathological immune responses (summarized in Table 1, adapted from Janeway et al., 2001). This section provides a brief overview of the components of an immune response; more detailed description can be found in Janeway et al., 2001, the source for this overview (unless otherwise indicated).

Table 1. Immune Responses (adapted from Janeway et al, 2001)

Antigen	Effect of response to antigen	
	Response	No response
Infectious agent	Protective immunity	Recurrent infection
Innocuous substance	Allergy	No allergy
Self tissue	Autoimmunity	Self-tolerance
Tumor	Tumor immunity	Cancer

Healthy; Pathological

The innate immune system is ancient. Innate immunity relies on three primary components, reinforced by mutual positive feedback: phagocytes (e.g., macrophages and neutrophils), inflammation, and the complement system. Phagocytes recognize, engulf, and destroy pathogens. Macrophages also release chemical signals which induce inflammation, recruit additional phagocytes to the site of infection, and potentiate initiation of an adaptive immune response. Inflammation includes multiple processes, which in combination serve to recruit phagocytes and lymphocytes to the site of infection and physically isolate infectious agents. The acute phase response component of inflammation up-regulates liver production of molecules capable of recognizing and opsonizing (marking for phagocytosis) certain pathogens and triggering the complement system (e.g., C-reactive protein and mannose-binding lectin). Other important aspects of inflammation are increased blood flow and vascular permeability around the site of infection, and expression of adhesion molecules by endothelial cells. These vascular changes allow leukocytes to migrate into infected tissue in large numbers, and cause an influx of fluid and proteins into an infected area, causing edema and preventing the spread of pathogens. The complement system is made up of multiple plasma proteins, which, when stimulated by pathogen, interact in a cascade of reactions. The products of the complement cascade are molecules capable of recruiting and activating phagocytes, inducing inflammation, opsonizing pathogens, and directly damaging bacteria (through the action of the membrane-attack complex, which forms a pore in the bacterial membrane, inducing lysis of the bacterial cell).

Some mediators of innate immunity are capable of recognizing highly-conserved pathogen antigens; however, this represents only a subset of pathogen antigens. In

contrast, adaptive immunity is capable of recognizing novel antigens. Adaptive immune responses employ two types of lymphocytes: B lymphocytes (which mature in the bone marrow) and T lymphocytes (which mature in the thymus). B lymphocytes produce antibodies, or immunoglobulins, which recognize and bind to pathogens. There are three types of T lymphocytes: helper T lymphocytes coordinate immune responses; cytotoxic T lymphocytes recognize infected host cells displaying pathogen antigen on their surface with major histocompatibility complex (MCH) class I proteins, and induce the death of these cells; and, suppressor, or regulatory, T lymphocytes down-regulate immune responses (e.g., when an infection is cleared and the threat has passed). Adaptive immune responses progress through three stages: the naïve lymphocyte phase (lymphocytes remain unstimulated by antigen); the primary immune response (stimulated lymphocytes mature into effector cells and memory lymphocytes); and, the secondary immune response (memory lymphocytes are stimulated by antigen and proliferate to mount an immediate response). Adaptive immune responses can only be mounted in the presence of an independent signal from the innate immune system; this mechanism likely serves to partially protect the host from the autoreactive capacity of adaptive immunity.

Although they are often discussed separately, as two distinct "systems", innate and adaptive immunity interact extensively when an immune response is mounted. Dendritic cells are the major interface between innate and adaptive immunity. Dendritic cells scavenge for pathogen antigen, and when activated, employ MHC class II proteins to present small fragments of pathogen proteins to helper T lymphocytes maturing in the lymph nodes. These lymphocytes then coordinate an immune response to the pathogen.

Immune responses can be categorized by the type of coordinating helper T lymphocyte into T-helper type 1 (Th1) or T-helper type 2 (Th2) mediated responses (see Table 2). Th1-mediated responses are coordinated by Th1 lymphocytes, and are associated with viral, bacterial, or protozoan infectious agents—small, usually intracellular, organisms. Th1-mediated responses rely on two means of destroying pathogens: the activity of cytotoxic T lymphocytes, which identify and destroy infected host cells, and the production of antibodies (in particular, immunoglobulin G, IgG), which bind to pathogens outside of host cells, marking them for phagocytosis by macrophages and other phagocytes. When mounted against healthy host tissue, Th1-mediated responses cause autoimmune disease (however, a minority of autoimmune diseases seem to result from Th2-mediated responses).

Th2-mediated responses are coordinated by Th2 lymphocytes, and are associated with infection by helminths—large, extracellular pathogens which are themselves multicellular eukaryotes and not susceptible to phagocytosis. Th2-mediated responses involve production of antibodies (in particular, IgE) and the activity of leukocytes, including basophils and eosinophils; these cells (through release of histamine and other mediators) promote intestinal permeability and mucous secretion, resulting in a "weep and sweep" (Anthony et al., 2007) response to eliminate parasites. Local production of noxious proteins and chemicals by leukocytes stimulated by IgE damages pathogens. When mounted against otherwise innocuous antigens (e.g., pollen or pet dander), Th2-mediated responses cause allergic disease.

Table 2. The Th1/Th2 Paradigm

Immune response:	T-helper type 1	T-helper type 2
Associated with:	Viruses, bacteria, protozoa (predominantly intracellular pathogens)	Helminths (extracellular pathogens)
Pathological responses:	Autoimmune diseases (most)	Allergic diseases
Mediated through:	Cellular and humoral immunity (immunoglobulin G)	Humoral immunity (immunoglobulin E)
Cytokines:	Interferon-γ, interleukin (IL)-12	IL-4, IL-5, IL-13

Th1- and Th2-mediated responses are antagonistically-regulated: the chemical mediators that promote Th1-mediated activity also inhibit Th2-mediated activity and *vice versa*, such that only one comes to dominate the response to a stimulus. Newborns start out life with a Th2 bias, associated with pregnancy; a Th1 bias may emerge in the course of immune system maturation. The precise mechanism by which an immune response breaks toward the Th1 or Th2 phenotype remains imperfectly understood; however, cytokine production by dendritic cells and helper T lymphocytes plays a critical role: Th1 responses are associated with production of interleukin (IL)-12 by dendritic cells and interferon-γ by helper T lymphocytes; Th2 responses are associated with IL-4 and IL-5 production by helper T lymphocytes (Romagnani, 2000; Szabo et al., 2003). Memory helper T lymphocytes persist long after a response has ended, enhancing the likelihood that future responses to the same antigen will be of the same type. For additional discussion of the Th1/Th2 paradigm, and its limitations, see Romagnani, 2000 and Kidd, 2003.

Allergy Epidemiology

In 1989, David Strachan noted that the most consistent aspect of the epidemiology of allergy in British children was an inverse association between allergic disease and family size or birth order (those from larger families and those born later in a sibship were at lower risk of allergy; Strachan, 1989). He suggested that infections, acquired from siblings, explained this observation, arguing that a dearth of childhood infections, due to small family size and other aspects of the modern world, promotes the development of allergic disease. This has been dubbed the *hygiene hypothesis*.

The hygiene hypothesis finds support from multiple lines of epidemiological evidence. Allergic diseases have been increasing in prevalence among the wealthy since the 19th century (Emanuel, 1988), with precipitous increases in the populations of Western Europe, North America, and Australia in more recent decades (Strachan et al., 1997; Isolauri et al., 2004). Allergic diseases have now reached epidemic proportions, affecting ~40% of young adults in some western populations (Strachan et al., 1997). By comparison, developing world nations, with higher prevalence of infectious diseases, have lower prevalence of allergy, and increases in the prevalence of allergy have been observed only much more recently (Strachan et al., 1997; van Ree and Yazdanbakhsh, 2007).

At the individual level, family size and birth order are very consistently associated with risk of allergic disease, including hay fever, eczema, and allergic asthma. Cross-sectional studies of English adults (Jarvis et al., 1997) and Italian Air Force candidates (Matricardi et al., 1998) demonstrate significantly lower odds of allergy associated with each additional sibling. Similarly, among children in Tucson, Arizona, each additional older sibling was associated with significantly lower odds of asthma (Ball et al., 2000; for complete review of the association between siblings and allergy, see von Mutius, 2001; Bach, 2002). Residence on a farm (Ernst and Cormier, 2000; Riedler et al., 2000) and day care attendance (especially in the first six months of life; Ball et al., 2000; Krämer et al., 1999; Haby et al., 2000; Infante-Rivard et al., 2001) are also consistently associated with lower risk of allergy and asthma, as is an Anthroposophic lifestyle, which includes restricted use of vaccination and antibiotics (Alm et al., 1999; Flöistrup et al., 2006), or residence in a group foster home (Stelmach et al., 2007). The single common thread in these disparate exposures—large family size, high birth order, day care attendance, farm residence, Anthroposophism, and group foster home residence—is enhanced exposure to pathogens, in most cases due to closer or more frequent contact with other children.

In addition to the consistent effects of these proxy indicators for exposure to infectious agents in early life, relationships between specific infectious agents and allergy have been investigated. Initial investigation (Shirakawa et al., 1997) suggested a protective effect of *Mycobacterium tuberculosis*: Japanese schoolchildren with positive responses to tuberculin skin testing exhibited significantly lower odds of allergy. However, it is possible that this result is attributable entirely to antagonism between Th1- and Th2-mediated responses: Th1-mediated delayed-type hypersensitivity reactions to tuberculin skin testing are likely weaker in children with (Th2-mediated) allergies. Subsequent investigations have shown inconsistent associations between tuberculin skin test results and allergy (Grüber et al., 2001; Ota et al., 2003; Obihara et al., 2005), and no

protective effect was observed when *M. tuberculosis* infection was evaluated by assay for active T-cells to *M. tuberculosis* antigen, rather than tuberculin skin testing (Sousal et al., 2008). Similarly, inoculation with bacille Calmette-Guérin (BCG vaccination against tuberculosis) has proved to be inconsistently associated with risk of allergy, but is inversely associated with asthma (Arnoldussen et al., 2011).

A study conducted in Guinea-Bissau (Shaheen et al., 1996) found that children with a history of measles infection had significantly lower risk of allergy than children who had been vaccinated for measles and had no history of infection. Similarly, among European children (including those living an Anthroposophic lifestyle), measles infection was found to protect against allergic eczema (Flöistrup et al., 2006); measles infection was also found to protect against allergy among a large cross-section of European children (Rosenlund et al., 2009). Contrary to these results, two large studies conducted in northern Europe documented an *increased* risk of allergy associated with measles infection, rendering a protective effect of measles questionable (Paunio et al., 2000; Olesen et al., 2003). The effect of measles or measles-mumps-rubella (MMR) vaccination, which might be expected to increase risk of allergy by protecting against natural infection, is similarly inconsistent, with some studies documenting an increased risk of allergic disease following measles/MMR vaccination (Flöistrup et al., 2006; Olesen et al., 2003), a decreased risk (Hviid and Melbye, 2008), or no relationship (Destefano et al., 2002; Roost et al., 2004; McKeever et al., 2004).

Other infectious agents have been less extensively investigated, but seem to be consistently associated with lower risk of allergic disease. Among young Italian men, cases of allergy were less likely than controls to have serological evidence of chronic or past infection with hepatitis A virus (HAV), *Toxoplasma gondii*, or *Helicobacter pylori*. Further, the protective effects of these infectious agents were additive: those with serological evidence of all three had the lowest odds of allergy (Matricardi et al., 2000). Similar patterns have been documented among US residents: serological evidence of infection with HAV, *T. gondii*, and herpes simplex virus type I (HSV-1) were each independently associated with lower odds of allergy; and cumulatively, seropositivity to all three agents was particularly protective (Matricardi et al., 2002). In addition, serological evidence of past infection with *cagA+* strains of *H. pylori* was found to be associated with lower odds of allergy (Chen and Blaser, 2007). Among an international sample of European adults, serological evidence of infection with *H. pylori*, HSV-1, *Chlamydia pneumoniae*, or cytomegalovirus (CMV) was associated with lower odds of allergy; again, these protective effects appeared to be additive: those with serological evidence of the fewest infectious agents had the highest odds of allergy (Janson et al., 2007). However, researchers found no association between HAV or *H. pylori* and allergen sensitization among young British men (Jarvis et al., 2004). Taken together, these studies suggest a protective effect of common infections (many, but not all, of which are transmitted via the fecal-oral route) against allergy.

Many studies have documented associations between the number of infectious disease episodes in early life and lower risk of allergy or asthma, without examining particular infectious agents. Among German children, those who experienced two or more episodes of "runny nose" before one year of age were at significantly lower risk of allergy and asthma than children who experienced none or only one episode; lower

respiratory tract infections (LRTI), on the other hand, were associated with higher risk of asthma (Ili et al., 2001). Similarly, among Norwegian children of allergic parents (presumably with a genetic predisposition for allergy), those who experienced otitis media (OM, ear infection) and LRTI, or OM alone, during the first year of life were at lower risk of developing allergy; among children without predisposition for allergy, those who experienced LRTI were at higher risk of developing asthma (Njå et al., 2003). Among US children, febrile episodes during the first year of life were associated with lower odds of allergy, such that each febrile episode decreased odds of allergy by ~30%; this effect was particularly strong for febrile episodes associated with upper respiratory tract infections (URTI; Williams et al., 2004). Thus, it appears that most routine childhood infectious disease episodes, such as URTI and OM, have the potential to protect against allergy, although the more severe respiratory infections (LRTI, which include pneumonia, bronchitis, and croup) seem to be independently associated with *increased* risk of asthma (also see Nafstad et al., 2005). Severe respiratory infections may have this effect by damaging lung tissues, rather than affecting allergic reactivity in any way: among children with asthma, fevers in the first year of life were inversely associated with allergen sensitization in Germany (von Mutius et al., 1999) and Italy (Calvani et al., 2002).

The evidence is clear that infectious diseases in early life, particularly infancy, can protect against the development of allergy. The protective effect of infectious diseases does not appear to be limited to any particular pathogen or route of transmission. The more severe infectious diseases, tuberculosis and measles, do not consistently protect against allergic disease. LRTI can increase risk of asthma, potentially not through any effect on allergic disease phenotypes. The magnitude of exposure to pathogens in general seems to be the most consistent predictor of later allergy, with each additional increment of exposure (additional sibling, episode of infection, or seropositivity) lowering risk.

Autoimmune Disease Epidemiology

The hygiene hypothesis has, more recently, been investigated as a means of explaining patterns of autoimmune disease, as well as allergy, on the reasoning that early life infections may also protect against the pathological immune responses to "self" tissue that underlie autoimmune disease. Autoimmune disease, like allergy, has been increasing in prevalence in western populations (Bach, 2002); taken collectively, autoimmune diseases now constitute a top-10 cause of death among young and middle-aged women in the United States (Walsh and Rau, 2000).

The results of studies into the hygiene hypothesis and autoimmune disease defy any single conclusion. This may be due in part to the variability of autoimmune diseases, which attack diverse tissues (at least 24 diseases are accepted to arise from a pathological autoimmune response, with many more suspected to be autoimmune in nature; Jacobson et al., 1997), and vary in severity and underlying immunobiology: although the pathological responses that cause most autoimmune diseases (e.g., type 1diabetes, T1D, and multiple sclerosis, MS), are thought to be Th1-mediated, others appear to be Th2-mediated (e.g., systemic lupus erythematosus, SLE). Conflicting results may also be attributable to variability in the effect of different infections—some infectious agents are known (e.g., *Streptococcus pyogenes*) or suspected (e.g., Epstein-Barr virus) to induce autoimmune responses and cause autoimmune disease, while other agents or overall exposure may be protective.

Acute rheumatic fever (ARF) is the quintessential autoimmune disease. The autoimmune response underlying ARF is initiated during infection with Group A *Streptococcus* (GAS; *S. pyogenes*): left untreated, ~3% of GAS pharyngitis (strep throat) cases develop some manifestation of ARF (Guilherme et al., 2005). ARF can affect heart and nervous tissue: chronic rheumatic heart disease develops in ~30-45% of cases (Guilherme et al., 2005); the neurological disorder Sydenham's chorea ("Saint Vitus' Dance") develops in ~10-30% of cases (Kirvan et al., 2006). GAS infections (including both pharyngitis and skin infection, or impetigo) can also precipitate acute poststreptococcal glomerulonephritis (APSGN), wherein autoimmune responses affect the kidney (Rodriguez-Iturbe and Batsford, 2004), and possibly pediatric autoimmune neuropsychiatric disorder associated with streptococcal infection (PANDAS; Kurlan and Kaplan, 2004) wherein autoimmune responses affect the brain. With the exception of PANDAS, the role of GAS in initiating the autoimmune responses that cause disease is undisputed.

While many other infectious disease-autoimmune disease links have been hypothesized and investigated, none so clear and strong as the connection between GAS and ARF have been documented. Epstein-Barr virus (EBV), a ubiquitous human pathogen affecting ~90% of the adult population (Rickinson and Moss, 1997), has been investigated as a potential cause of multiple autoimmune diseases. MS is an autoimmune disease involving damage to the nerves of the brain and spinal cord. Infectious mononucleosis, resulting from EBV infection in adolescence or young adulthood, is associated with increased risk of subsequent MS (Thacker et al., 2006). MS patients demonstrate significantly higher rates of EBV seropositivity than healthy controls; this difference is most dramatic among children (Alotaibi et al., 2004; Pohl et al., 2006). MS risk is elevated among those with higher levels of serum anti-EBV antibodies (Levin et

al., 2005; DeLorenze et al., 2006). Seroconversion (production of anti-EBV antibodies in response to EBV infection) seems to precede the onset of MS symptoms (Levin et al., 2005; Ascherio et al., 2001) and seropositivity to other common herpesviruses (e.g., cytomegalovirus; Ascherio et al., 2001) does not differ between MS cases and healthy controls, supporting a causative role for EBV in the development of MS. A causative role for EBV in the development of RA has also been proposed, based on the observation that RA patients exhibit higher levels of B lymphocyte EBV infection and poorer control of EBV replication than healthy controls (Saal et al., 1999; Balandraud et al., 2003). Other hypothesized infectious disease-autoimmune disease links include *Mycobacterium tuberculosis* and *Escherichia coli* as causes of RA (Edwards and Cooper, 2005), coxsackie B viruses as a cause of T1D (Banatvala et al., 1985), EBV as a cause of SLE (James et al., 1997), and *Campylobacter jejuni* as a cause of Guillain-Barre syndrome (Bach, 2005).

At the same time, evidence suggests an overall *inverse* association between infectious and autoimmune diseases: at the population level, as with allergy, autoimmune diseases are rare when and where infectious diseases are common. The prevalence of many autoimmune diseases, including T1D, IBD, and MS, has been increasing in Western populations in recent decades; and, autoimmune disease prevalence tends to be highest in populations with the lowest infectious disease morbidity (Okada et al., 2010).

At the individual level, as with allergic diseases, factors promoting early life exposure to infectious agents are often found to protect against autoimmune disease. For example, day care attendance, older siblings, and large family size are inversely associated with T1D (Kaila and Taback, 2001; Stene et al., 2001; Cardwell et al., 2005). Studies directly examining the effect of infections on T1D risk demonstrate mixed results: the causative role of coxsackie B virus is not consistently supported across studies, and in fact, some support exists for a protective effect (Green et al., 2004); neonatal infections have been shown to increase T1D risk (McKinney et Al., 1999), as have some of the more severe childhood diseases (measles, mumps, rubella, chickenpox; Tenconi et al., 2007). Similar to allergy epidemiology, routine exposure to infectious agents during childhood seems to protect against T1D; episodes of severe infection may increase risk of T1D. A protective effect of routine early infections is supported for other autoimmune diseases, as well: large family or household size is inversely associated with MS (Montgomery et al., 2004; Ponsonby et al., 2005) and inflammatory bowel disease (IBD; Amre et al., 2006; Koloski et al., 2008); bedroom-sharing is inversely associated with RA (Edwards and Cooper, 2005); *H. pylori* infection is inversely associated with IBD (Koloski et al., 2008).

Thus, exposure to infectious agents seems to simultaneously increase and decrease risk of autoimmune disease, sometimes for the same condition (MS and RA). This may be in part attributable to spurious associations (e.g., treatment of autoimmune disease with immunosuppressive chemotherapies may enhance susceptibility to infection), or may reflect heterogeneity in pathogenesis across autoimmune diseases. However, these seemingly inconsistent results may also reflect real complexity: infections may act through multiple mechanisms to affect the immune system during development and afterward, with multiple effects on autoimmune disease risk. This review turns now to discussion of the immunological mechanisms hypothesized to

underlie these patterns in the epidemiology of allergy and autoimmune disease; Table 3 begins a summary of the pertinent epidemiological evidence.

Table 3. Summary of Epidemiological Evidence

Exposure	Outcome	Association	Strength of evidence
Early life intracellular infection	Allergy	Inverse	Strong
Severe early life intracellular infection	Asthma	Positive	Mixed
Early life intracellular infection	Autoimmune disease	Inverse	Mixed
Early life intracellular infection	Immunocompetence	Positive	Preliminary
Intracellular infection [a]	Autoimmune disease		
Group A Streptococcus	Acute rheumatic fever	Positive	Strong
Epstein-Barr virus	Multiple sclerosis	Positive	Mixed
Coxsackie B virus	Type I diabetes	Positive	Refuted
Severe early life intracellular infection	Autoimmune disease	Positive	Mixed
Early life helminth infection	Allergy	Positive	Preliminary
Chronic helminth infection	Allergy	Inverse	Mixed
Chronic helminth infection	Autoimmune disease	Inverse	Strong
Allergy	Autoimmune disease	Inverse	Weak

Strong: Many studies; little or no contrary evidence exists; preponderance of evidence clearly supports the association
Mixed: Many studies; contrary evidence exists; preponderance of evidence supports the association, but is not definitive
Weak: Many studies; effect small or limited to largest studies; preponderance of evidence supports the association, but suggests it may not be important
Preliminary: Few studies (<10)
Refuted: Meta-analysis or preponderance of the evidence does not support the association

[a] Only some of the many hypothesized infection-autoimmune associations are described; autoimmune sequelae of Group A *Streptococcus* infection are well-demonstrated, but have been less consistently documented following infection with other agents.

Immunological Mechanisms

Multiple mechanisms of interaction between infectious agents and the immune system have been proposed to explain the lasting effect of early infections on risk of allergy and autoimmune disease. Each hypothesized mechanism can explain one or more, but not all, of the observed epidemiological relationships (see Table 3). While often treated as such, these mechanisms are not mutually exclusive; in fact, all may be at work in interactions between infectious agents and the developing immune system.

Immune deviation: Antagonism between Th1- and Th2-mediated immune responses may explain the inverse association between early life infectious disease exposure and allergic disease risk. Infants are born with a bias toward Th2-mediated responses, perhaps related to the Th2 bias that normally accompanies pregnancy (which may protect the fetus from attack by the maternal immune system; Holt and Jones, 2000). Exposure to infectious agents, especially viruses, bacteria, and protists (primarily intracellular agents), during immune system development has been hypothesized to protect against allergy by inducing Th1-mediated responses and shifting the immune system toward a Th1 bias, inhibiting later Th2-mediated responses, including those to allergens (Martinez and Holt, 1999). Figure 1 summarizes the predicted effects of the early pathogen burden on Th1/Th2 deviation.

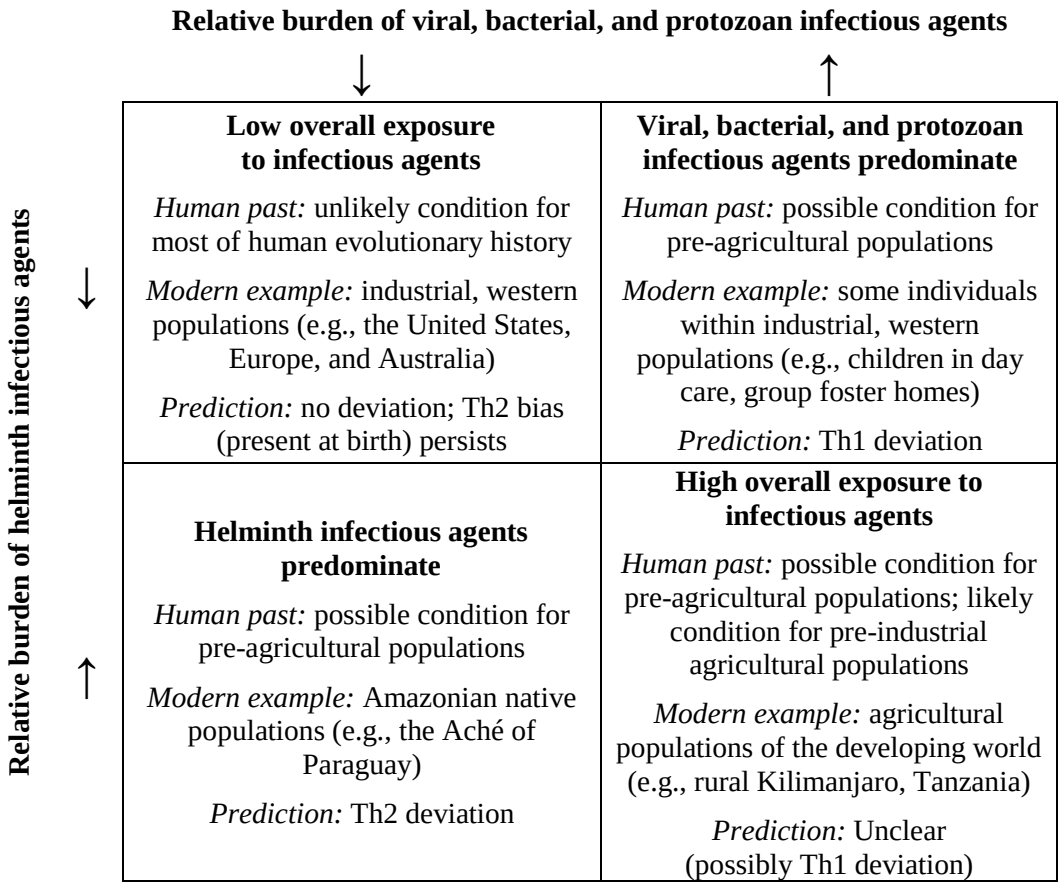

Figure 1. Predictions of the Immune Deviation Hypothesis. The condition of a low overall burden of infectious disease (upper left quadrant), characteristic of modern, industrial populations, and the condition in which helminth infectious agents predominate (lower left quadrant), characteristic of some modern Amazonian native populations and potentially many populations throughout human evolution, are predicted to result in T-helper type 2 (Th2)-biased immune reactivity (and thus, elevated risk of allergy). The condition in which viral, bacterial, and protozoan infectious agents predominate (upper right quadrant), characteristic of some conditions within modern, industrial populations (e.g., children attending day care) and potentially many populations throughout human evolution, is predicted to result in Th1-biased immune reactivity (and thus, lower risk of allergy). It is unclear from the immune deviation hypothesis what phenotype is predicted to result from the condition of a high overall burden of infectious disease (lower right quadrant), characteristic of much of the modern developing world and potentially many populations throughout human evolution; such stimulation may result in some degree of Th1-biased reactivity (relative to the Th2 deviated state).

Immune deviation toward a Th1-biased phenotype provides an appealing explanation for the protective effect of early life infections against allergy and recent increases in the prevalence of allergic diseases: At the population level, as the incidence of Th1-stimulating intracellular infections in infancy has decreased dramatically and progressively over the course of the last century, the prevalence of Th2-mediated allergic

disease has increased; at the individual level, infants exposed to Th1-stimulating intracellular agents are at lower risk of subsequently developing Th2-mediated allergic disease. However, this hypothesis does not explain the increasing prevalence of autoimmune disease, and in fact seems to predict that most autoimmune disease should be decreasing in prevalence (as a larger fraction of the population retains a Th2-biased immune system, rendering them less susceptible to Th1-mediated autoimmune diseases). Further, the Th1/Th2 paradigm on which the immune deviation hypothesis rests has been criticized (Muraille and Leo, 1998; van Ooserhout and Motta, 2005; Rook, 2008) as an oversimplification of immune function which ignores the activity of other types of immune regulation and immune responses that do not fall into either the Th1 or Th2 category (e.g., Th17).

Although the immune deviation hypothesis fails to explain the increasing prevalence of autoimmune disease, closer examination of autoimmune and allergic disease risk at the individual level does provide some support. If failed deviation due to a dearth of early exposure to infectious agents results in enhanced risk of allergic disease, the lasting Th2 bias should also result in lower risk of autoimmune disease: allergy should protect against autoimmune disease and *vice versa.* Methodological issues and the rarity of many autoimmune diseases make testing such associations difficult. However, inverse associations between T1D, a relatively common autoimmune disorder, and allergic diseases are consistently reported (Stene et al., 2001; Cardwell et al., 2003; Thomsen et al., 2011); further, a large, registry-based study in Sweden documented an inverse association between allergen sensitization and 28 autoimmune diseases (Lindelöf et al., 2008). These results suggest that variation in Th1/Th2 balance is indeed important in explaining allergic and autoimmune disease risk at the individual level.

The immune deviation hypothesis has also been criticized on the basis of the inverse association between helminth infection and allergy (Yazdanbakhsh et al., 2001; Rook, 2008). Although the immune deviation hypothesis seems to predict a *positive* association between Th2-inducing helminth infections and allergic disease, an *inverse* association is consistently reported: At the population level, allergic diseases are rare where helminths are endemic (Yazdanbakhsh et al., 2001). At the individual level, allergic disease and allergic sensitization are consistently lower among those with chronic helminth infection (Yazdanbakhsh et al., 2001; Araujo et al., 2000; Nyan et al., 2001; Cooper et al., 2003). However, these findings reflect, in part, the ability of helminth parasites to induce suppressive immune regulatory mechanisms (Yazdanbakhsh et al., 2001) and represent the effect of *on-going* helminth infection, rather than helminth infection *during immune system development*. When considered separately, history of helminth infection seems to be positively associated with allergy, even as current helminth infection suppresses allergy (Gonzalez-Quintela et al., 2006; Wördemann et al., 2008). While preliminary, this evidence suggests that (Th2-inducing) helminth infections during immune system development can increase risk of later (Th2-mediated) allergy, supporting the immune deviation hypothesis. Overall, studies support a role for immune deviation in the hygiene hypothesis, but population-level observations of simultaneously increasing rates of allergy and autoimmunity demand further explanation.

Regulatory priming: Multiple mechanisms exist in the immune system to suppress, or down-regulate, immune responses. Natural suppressor, or regulatory, T cells

(T_{reg}) emerge from the thymus mature and antigen-primed (unlike naïve helper and cytotoxic T cells; Mills and McGuirk, 2004). Inducible suppressor/regulatory T cells include Tr1 and Th3 cells; these cells mature from naïve T cells in response to antigen stimulation (Belkaid and Rouse, 2005). Suppressor T cells down-regulate both Th1- and Th2-mediated immune responses. These cells may promote tolerance of resident microbes and minimize potentially damaging inflammation, or temper an immune response to pathogenic microbes in order to minimize collateral damage due to inflammation (Mills and McGuirk, 2004; Belkaid and Rouse, 2005). Much about the classification and action of natural and inducible regulatory T cells remains to be discovered; however, their role in preventing pathological responses to both autoantigens and allergens is clear (Mills and McGuirk, 2004; Belkaid and Rouse, 2005; Akdis et al., 2005).

It has been hypothesized that stimulation of the developing immune system by exposure to infectious agents in early life promotes the development of more robust suppressive regulatory mechanisms, lowering risk of subsequent development of allergic and autoimmune disease (Akdis et al., 2005; Yazdanbakhsh et al., 2002; Akbari et al., 2003), producing an inverse association between early life exposure to pathogens and later risk of immune-mediated diseases at both the population and individual levels.

The regulatory priming hypothesis may provide a means to explain the seemingly irreconcilable evidence that infections both increase and decrease risk of autoimmune disease: while an episode of a particular infectious disease (e.g., *S. pyogenes*) may increase risk of a particular autoimmune disease (e.g., ARF; through mechanisms discussed below), each episode of infectious disease during early life may also stimulate the development of robust suppressive regulatory mechanisms, decreasing risk of autoimmune and allergic diseases in general. Thus, infections may both increase risk of, and protect against, autoimmune disease. The regulatory priming hypothesis may also explain the seemingly disparate effects of routine and severe episodes of infection: while routine infections may serve to "prime" regulatory mechanisms, lowering risk of immune-mediated disease, severe episodes of infection may have a damaging effect on immune system development, resulting in *dysregulation* and elevated risk of immune-mediated disease.

The regulatory priming hypothesis adequately explains the increasing prevalence of allergic *and* autoimmune diseases: Individuals with limited exposure to infectious agents during immune system development develop weak immune regulatory mechanisms less capable of preventing autoreactive or allergic responses, resulting in elevated risk of autoimmune or allergic disease; at the population level, this results in increasing prevalence of both autoimmune and allergic diseases in the last century, as infectious diseases have declined in prevalence. However, the regulatory priming hypothesis fails to explain the observed inverse associations between autoimmune and allergic disease risk at the individual level.

Molecular mimicry: Infectious agents may induce autoimmune responses through molecular mimicry if lymphocytes involved in the immune response to a pathogen are capable of recognizing both microbial and self antigens. This is thought to be the case for the multiple autoimmune pathologies that follow GAS infection. For example, anti-streptococcal antibodies from ARF patients bind both the M protein of *S. pyogenes* and

human cardiac myosin; similarly, T lymphocytes from ARF patients bind both M protein and heart valvular tissue (Guilherme et al., 2005). Anti-streptococcal antibodies from ARF patients with Sydenham's chorea also demonstrate cross-reactivity between GAS M protein and gangliosides (Kirvan et al., 2006). Although strong evidence suggests that molecular mimicry underlies the multiple autoimmune pathologies that can result from GAS infection, molecular mimicry has proven to be a poor explanation for autoimmune disease more generally (Fujinami et al., 2006).

Bystander activation: Infectious agents may potentiate autoimmune responses by stimulating the innate immune system. Stimulation of an innate immune response is necessary for induction of an adaptive immune response: infection induces the expression of co-stimulatory molecules and cytokines by cells of the innate immune system, which allow proliferation of lymphocytes, initiating a specific immune response (Janeway et al., 2001). If the population of lymphocytes stimulated to proliferate by an innate immune response to an infectious agent includes both those that recognize pathogen, and autoreactive, "bystander", lymphocytes, autoimmune disease could result (Fujinami et al., 2006).

These hypothesized interactions between infections and the developing immune system are by no means mutually exclusive; a single intracellular infectious disease episode during infancy could stimulate deviation toward a Th1 bias (decreasing risk of allergic disease in general); stimulate the development of robust immune regulation (decreasing risk of allergic and autoimmune disease in general); induce production of cross-reactive antibodies or T cells (increasing risk of particular autoimmune diseases); and, potentiate proliferation of self-reactive T cells (increasing risk of autoimmune disease in general). A single intracellular infectious disease episode after immune system development is complete could induce production of cross-reactive antibodies or T cells (increasing risk of particular autoimmune diseases) and potentiate proliferation of self-reactive T cells (increasing risk of autoimmune disease in general).

The conflicting population- and individual-level evidence (summarized in Table 3) suggests that both the immune deviation and regulatory priming mechanisms are at work in immune system development. This begs the question: *Why?* Why should early life exposure to infectious agents have so much impact on later immune system function?

Evolutionary Explanations

Both the immune deviation and regulatory priming mechanistic hypotheses lend themselves to evolutionary hypotheses, involving adaptive priming of the immune system by early exposure to infectious agents. Immune deviation, or priming of the Th1/Th2 balance, may represent a predictive adaptive response in immune system development to minimize risk of ID morbidity and mortality, while regulatory priming may represent an adaptation to minimize immune-mediated damage. A broad, life-history perspective suggests that priming of immune system development by both these mechanisms may have evolved to balance the multiple benefits and risks associated with immune responses.

Predictive adaptive responses: Plasticity in immune system development may have evolved to respond adaptively to early life infections. Because Th1- and Th2-mediated reactivity are antagonistically regulated, it may be that an individual cannot be maximally protected against both intracellular and extracellular pathogens. Under the constraint of this trade-off between Th1- and Th2-mediated reactivity, the immune system may have evolved to respond to the local infectious disease ecology, as experienced in infancy, with a Th1 bias where intracellular pathogens predominate and a Th2 bias where helminths predominate. In other words, the Th1/Th2 balance may represent a *predictive adaptive response* in immune system development.

In addition to allergy and autoimmunity, early life conditions influence the development of multiple chronic diseases: for example, energetic stress *in utero* (as reflected by low birthweight) is associated with increased risk of cardiovascular disease (Barker et al., 1993) and diabetes (Hales et al., 1991; Whincup et al., 2008) in later life. Gluckman and Hanson's theory of predictive adaptive responses (PAR; Gluckman and Hanson, 2005) situates the epidemiology of these chronic diseases in evolutionary context: Early life conditions can be seen as signals to a developing system of stresses it is likely to face in the future; developmental adjustments in response to these signals, which augment an individual's ability to cope with such stresses, have the potential to enhance lifelong fitness, even at the expense of elevated chronic disease risk (Gluckman and Hanson, 2005). For example, energetic stress *in utero* increases risk of development of insulin resistance and hypertension (metabolic syndrome, or syndrome X; Gluckman and Hanson, 2005), predisposing to type 2 diabetes; this effect is reflected in the inverse association between birthweight and type 2 diabetes risk (Whincup et al., 2008). However, under conditions of resource scarcity, the suite of characteristics that accompany metabolic syndrome, including small lean body mass, reduced vascularization of muscle and gut tissue, reduced renal mass, a "hyper-responsive" hypothalamic-pituitary-adrenal axis and elevated cortisol levels, high blood pressure, and insulin resistance in tissues, may enhance survival; thus, natural selection might favor the development of these traits in response to signals of resource scarcity, such as limited *in utero* energy availability, despite their propensity to produce disease under conditions of plenty (Gluckman and Hanson, 2005).

The Th1/Th2 balance may represent a PAR in mammalian immune system development. The developing immune system may make adaptive adjustments to the Th1/Th2 balance that affect future immune responses, based on exposure to infectious agents during infancy. Immune deviation toward Th1-mediated reactivity in response to

stimulation from viral, bacterial, and protozoan agents could enhance Th1-mediated responses to these pathogens in environments where they predominate; while persistence of a Th2 bias or additional deviation toward Th2-mediated reactivity in response to early stimulation from helminths could enhance Th2-mediated responses to helminths in environments where they predominate. In this manner, a PAR in the Th1/Th2 balance could shape immune responses to the local ID ecology and increase the lifelong efficacy of the immune system, mitigating ID stress and enhancing fitness. This hypothesis suggests that early stimulation from intracellular pathogens should be associated with a Th1-biased suite of characteristics, including low risk of allergy, weak Th2-mediated responses to helminths, and enhanced Th1-mediated responses to intracellular pathogens. Similarly, early Th2-inducing helminth infections should produce a Th2-biased suite of characteristics, including a propensity for allergy, enhanced Th2-mediated responses to helminths, and weak Th1-mediated responses to intracellular pathogens.

Close examination of the epidemiology of allergy, helminth infection, and tuberculosis provides some support for the hypothesized Th2-biased suite of characteristics, developed among those with early exposure to Th2-stimulating helminths. As discussed above, although *chronic* helminth infection is clearly inversely associated with allergy due to helminths' ability to suppress host immune responses (including responses against themselves and those against allergens; Yazdanbakhsh et al., 2001), recent research suggests that *history* of helminth infection is positively associated with later Th2-mediated allergy (Wördemann et al., 2008). Further, the inverse association between allergy and helminth infection may reflect both suppressed Th2-mediated responses by chronic helminth infection *and* enhanced resistance to helminths associated with allergy: allergic individuals mount stronger Th2-mediated responses to helminths and are less susceptible to helminth infection (Lynch et a., 1998; Cooper et al., 2004). Finally, early helminth infection may be associated with impaired Th1-mediated immune responses to *Mycobacterium tuberculosis*. Helminth infection is common among native new world populations living in Amazonian South America, potentially inducing Th2-biased development. When exposed to *M. tuberculosis*, these populations tend to exhibit particularly high morbidity and mortality (Hurtado et al., 2003; Hurtado et al., 2004). Although much research remains to be done, this evidence suggests that where helminths dominate the local infectious disease ecology, Th2-biased development seems to enhance Th2-mediated immune responses to allergens and helminths and to weaken Th1-mediated immune responses to intracellular pathogens.

Preliminary evidence also supports the existence of a Th1-biased suite of characteristics (lower risk of Th2-mediated allergy and enhanced Th1-mediated responses to intracellular agents). The protection against allergy associated with early (predominantly Th1-inducing) infections is clearly consistent with this hypothesis. Only limited evidence is available to evaluate whether early exposure to intracellular pathogens results in stronger Th1-mediated reactivity to infectious agents. Two studies have documented positive associations between likely indicators of intracellular infections during infancy and later immune responses to pathogen: Among adolescents in the Philippines, diarrheal disease during infancy was positively associated with antibody responses to typhoid vaccination (McDade et al., 2001). Among children in Tanzania, large family size, hospitalization during infancy with an infectious disease, and BCG

vaccination were positively associated with delayed-type hypersensitivity to *Candida albicans* (Wander et al., 2012).

Overall, preliminary evidence supports the existence of a PAR in immune system development that produces Th1-biased individuals in response to early exposure to viruses, bacteria, and protists, and Th2-biased individuals in response to early exposure to helminths. Few studies have been undertaken to explicitly evaluate the hypothesized PAR; this area is ripe for future research.

Old friends: In an evolutionary interpretation of allergic disease, Rook (2008) argues that the low prevalence of "old friend" microbes—those with a long history of co-evolution with humans, resulting in mutualistic relationships (gut bacteria like lactobacilli) or low pathogenicity (helminths like *Enterobius* species, or pinworms)—in modern environments has resulted in inadequate priming of the immune system's suppressive regulatory mechanisms. Rook argues that, because immune responses mounted against commensal or low pathogenicity microbes may do more harm than good, the immune system evolved regulatory priming to achieve tolerance of these microbes, with the added benefit of tolerance of allergens and autoantigens (*ibid.*). Early and on-going stimulation of suppressive regulatory mechanisms is, in this hypothesis, the "normal" condition (under which humans evolved). It is only in modern, evolutionarily novel, environments that early stimulation from "old friends" has become minimal enough to result in weak regulation of the immune system and the resultant high prevalence of allergic and autoimmune disease. The old friends hypothesis finds support in the observation that helminths are capable of down-regulating both allergic and autoimmune responses; indeed, helminths are so effective in inhibiting autoimmune responses that deliberate infection with the helminth *Trichuris suis* (pig whipworm) has proven an effective therapy for autoimmune disease, including IBD (Summers et al., 2005; Reddy and Fried, 2007) and MS (Fleming et al., 2011).

Just as the immune deviation and immune regulation mechanistic hypotheses are both supported by epidemiological evidence and are both likely at work in immune system development, the "old friends" hypothesis is not inconsistent with a PAR in the Th1/Th2 balance. It is entirely possible, even likely, that *both* immune regulatory mechanisms (suppressive regulation and the Th1/Th2 balance) respond adaptively to early ID experience. Life history theory may provide the means to understand how simultaneous priming of these two regulatory systems can contribute to adaptive plasticity in immune system development.

Life history theory: asset allocation or risk minimization? Recent anthropological research has sought insight into immune function and immune system development from life history theory (LHT; e.g., McDade, 2003; McDade, 2005; Long and Nanthakumar, 2004; Muehlenbein and Bribiescas, 2005). LHT argues that organisms have evolved to optimize allocation of energy between current reproduction and future reproduction (in the form of growth and maintenance). Immune responses, as maintenance effort, consume energy that would otherwise be available for growth or reproduction (e.g., McDade et al., 2008). Thus, one might predict that under conditions of restricted energy availability, investment in immune system development may be adaptively suppressed, or shunted toward less energetically expensive responses (McDade, 2005), to result in lower

levels of energy expenditure on immune function, and thus maintenance, throughout the life course.

While potentially useful, the applicability of LHT to questions of immune function is currently limited by an almost exclusive focus on the *energetic* costs of an immune response. Such energetic costs can be empirically demonstrated (McDade, 2005; McDade, et al., 2008); nonetheless, they may represent the least of the costs of immunity. The immune system represents a tremendous hazard. It can cause collateral damage to host tissues during eradication of an infectious agent (for example, the extensive damage and life threatening state of shock induced by toxic shock syndrome and dengue hemorrhagic fever/dengue shock syndrome are due almost entirely to immune responses to pathogens, rather than to the pathogens themselves). The immune system mediates myriad pathologies, including autoimmune disease, allergic disease, and other immune-mediated disorders, such as sarcoidosis (a potentially fatal chronic disease involving the formation of nodules of inflammatory cells, called granuloma). Chronic systemic inflammation contributes to degenerative diseases, including cardiovascular disease (Pearson et al., 2003), diabetes (Duncan et al., 2003), and osteoporosis (Ginaldi et al., 2005). These hazards, of course, must be weighed against the hazard of infectious disease: the speed with which individuals with untreated HIV disease succumb to infections attests to the necessity of immune function for survival. In the face of this multitude of threats, the energy expended in mounting an immune response may prove to be a relatively small component of the force of natural selection. Instead, infectious disease stress and immune-mediated damage and disease are likely the more significant factors shaping the evolution of immune system developmental plasticity and immune responsiveness.

Immune system developmental plasticity may have evolved to minimize the competing risks of infection and immune-mediated damage and disease, through priming of both the Th1/Th2 balance and suppressive regulation. A PAR in the Th1/Th2 balance to promote deviation toward the response (Th1- or Th2-mediated) best suited to the local infectious disease ecology may minimize risk by enhancing the efficacy of the immune system and reducing infectious disease stress; and, by preventing the development of a Th1 bias, and the accompanying risk of autoimmune disease, except when early experience indicates such a bias would be beneficial—in the presence of high morbidity due to intracellular pathogens. Simultaneously, the development of vigorous suppressive regulatory mechanisms (contingent on priming by exposure to microbes, which have only recently come to be in short supply) may minimize risk of immune-mediated disease and collateral damage due to immune responses to pathogens.

These priming mechanisms may have independent effects on immune responses. The predicted combined effect of the two mechanisms is shown in Table 4. Table 4 considers multiple potential infectious disease conditions: little or no early ID (the evolutionarily novel condition experienced by many western populations), routine exposure to predominantly helminth pathogens (the condition experienced by many native Amazonian populations and a likely circumstance for many populations during much of human evolution), routine exposure to predominantly intracellular pathogens (another likely circumstance for many populations during much of human evolution), and severe ID in early life (likely to be due to a viral, bacterial, or protozoan infectious

agents, such as measles virus, *Streptococcus pneumoniae*, or *Plasmodium falciparum*, some of which were likely present during much of human evolution, many others of which are relatively new to humans). Each arrow in this table represents a testable hypothesis. For example, priming of both suppressive regulation and the Th1/Th2 balance are predicted to down-regulate immune responses to allergens in the presence of stimulation from routine infection with intracellular agents during infancy, explaining the consistent epidemiologic evidence demonstrating such an association. Those situations in which the effects of regulatory priming and priming of the Th1/Th2 balance are predicted to work in opposite directions may explain some of the contradictory evidence observed in epidemiologic studies. Future research into the early life predictors of allergy, autoimmune disease, and immune responses to pathogens should consider both pathways (regulatory priming and immune deviation) to provide better understanding of immune system developmental plasticity.

Table 4. Predicted Effect of Priming on Later Immune Responses

Early ID experience	Priming mechanism		Lasting effect on responses to antigens			
	Suppressive Regulation	T-helper type 1/ T-helper type 2	Autoantigens	Allergens	Helminths	Viruses, bacteria, protozoa
Very few or no intracellular infections during infancy	Weak regulation	Th2 bias	↑↓	↑↑	↑↑	↑↓
Few infections during infancy; predominantly intracellular	Weak regulation	Th1 bias	↑↑	↑↓	↑↓	↑↑
Routine infections during infancy; predominantly helminths	Strong regulation	Th2 bias	↓↓	↓↑	↓↑	↓↓
Routine infections during infancy; predominantly intracellular	Strong regulation	Th1 bias	↓↑	↓↓	↓↓	↓↑
Severe infection(s) during infancy (e.g., malaria, pneumonia)	Dysregulation	Th1 bias	↑↑	↑↓	↑↓	↑↑

Conclusions

That early life exposure to infectious agents plays a role in immune system development is clear in the epidemiology of allergy and autoimmune disease: Routine infections decrease risk of allergic disease and overall risk of autoimmune disease; at the same time, infection can potentiate an autoimmune response, resulting in particular infection-autoimmune disease associations. Multiple mechanisms are likely at work in producing these associations. Considering the epidemiology of allergy and autoimmune disease in an evolutionary context suggests evolutionary explanations for two seemingly purely mechanistic hypotheses: The immune deviation hypothesis—that Th1-stimulating intracellular infections induce a lasting Th1 bias, protecting against Th2-mediated allergic disease—suggests that a PAR exists in immune system development which produces a Th1-biased immune system under conditions of high risk of infection with intracellular pathogens and a Th2-biased immune system under conditions of high risk of infection with helminths. The regulatory priming hypothesis—that stimulation from microbes induces robust regulatory mechanisms that are protective against allergic and autoimmune disease—suggests that it is only in modern environments, where "old friend" symbiotic gut microbes and low virulence helminths have become scarce, that this adaptive mechanism is disrupted and inadequate regulation develops. Insight from LHT, and broad consideration of the costs of maintaining an immune system (such as allergic and autoimmune disease risk), suggest that both immune deviation and regulatory priming likely evolved in immune system development as means of minimizing the overall risk posed by infectious and chronic diseases. Additional research is needed to evaluate the effect of early exposure to infectious agents on protective immune responses to pathogens, and to tease apart the multiple means by which exposure to infectious agents during immune system development might affect immune responses of all kinds.

Immune System Evolution and Human Infectious Disease Ecologies

Both innate and adaptive immunity are *very* highly conserved: signaling pathways in innate immunity (e.g., Toll receptors and nuclear factor-κB) can be found in both plants and animals, and the capacity for adaptive immunity and immunological memory is found in jawed fishes and all higher vertebrates (Janeway et al., 2001); the relatively new Th2 system and IgE emerged in mammals (Palm et al., 2012). This highly conserved nature and the intricacy of immune responses likely results in a great deal of inertia in immune system evolution. This inertia renders it unlikely that the human immune system would have come to differ substantially from that of other mammals (and, indeed, mammals have proved to be excellent laboratory models for human immunobiology). In other words, infectious disease stress during human evolution may have played virtually no role in shaping human immune system evolution. However, much evidence suggests that infectious disease morbidity during human evolution was substantial and varied, adequate to provide at least maintenance selection on human immune function.

As discussed above, the substantial benefits of immune responses come with substantial costs (autoimmunity, allergy, and inflammation). Understanding the role of these trade-offs in mammalian and human evolution requires better understanding of the infectious disease ecologies of pre-agricultural humans (summarized below), as well as improved understanding of immune regulation and developmental adaptability (which this dissertation seeks to provide.

Conventional wisdom holds that hunter-gatherer populations were not dense enough nor large enough to sustain communicable disease of any import; the recent adoption of agriculture, animal husbandry, and settlement are blamed for most modern infectious diseases, and pathogens are downplayed as an force in human evolution (e.g., Haldane, 1932; Burnet and White, 1972; Cohen, 1989). Early humans are thought to have been subject to only two types of infectious disease: virulent, rabies-like zoonoses, catastrophic for an individual, but causing fairly random mortality and exerting little selective pressure; and avirulent, chronic infections, evolved to cause little disability in their host, with concomitantly little importance as a source of selection (Cohen, 1989). This characterization of infectious disease stress in human evolution is largely extrapolated from observations of modern infectious disease epidemiology and hunter-gatherer demography. Empirical data with which to test this conjecture are only recently available and come primarily in the form of pathogen population genetics and phylogenies; their analysis frequently contradicts the conventional wisdom (for review, see Pearce-Duvet, 2006).

Pathogen population genetic analyses confirm the hypothesized ancient association between humans and many low virulence pathogens, including herpesviruses, (Van Blerkom, 2003; McGeoch et al., 2006), papillomaviruses (HPV, Ong et al., 1993; Bernard, 1994), hepatitis B virus (HBV, Van Blerkom, 2003), and *Helicobacter pylori* (Linz et al., 2007), and the post-agricultural emergence of some highly virulent pathogens, including *Yersenia pestis* (Achtman et al., 2004), measles virus (Pearce-

Duvet, 2006), poliovirus (Suzuki, 2006; Jiang et al., 2007), and variola virus (Van Blerkom, 2003). However, phylogenetic analyses have also indicated that many virulent pathogens have a longer history with humans than previously thought, substantially predating agriculture. For example, the *Mycobacterium tuberculosis* complex (MTBC), which causes tuberculosis, was thought to have evolved the ability to infect humans following close contact with *M. bovis* associated with cattle domestication. However, *M. bovis* has proven to be one of the most recently derived species of the MTBC. The most deeply rooted species (deemed *M. prototuberculosis*) is an obligate human pathogen that exhibits tremendous genetic diversity (accumulated over the course of 2.6 to 2.8 million years), suggesting that the MTBC has been parasitizing humans throughout our evolution (Fabre et al., 2004; Gutierrez et al., 2005; Brudey et al., 2006; but see Smith, 2006). So, too, has *Plasmodium falciparum*, once thought to have a recent origin from an avian strain of malaria (Joy et al., 2003; but see Hartl, 2004). Schistosomes (Despres et al., 1992; Lockyer et al., 2003), *Taenia* tapeworms (Hoberg et al., 2001), and trypanosomes (Welburn et al., 2001; MacLeod et al., 2001) likely came into contact with hominids entering new habitats, and evolved the ability to infect humans well over one million years ago. *P. vivax* (Escalante et al., 2005; Mu et al., 2005), *Salmonella typhi* (Kidgell et al., 2002), and *Shigella* species (Pupo et al., 2000) emerged more recently, but well before agriculture. Overall, these analyses suggest that many virulent pathogens, causing acute, life-threatening infections, have a long history with humans and likely played a role in human evolution.

While the importance of heirloom pathogens as a source of selection is often downplayed, infection with such low virulence pathogens can substantially affect fitness. For example, HBV causes liver cancer (Beasley et al., 1981), and HPV cervical and other cancers (Palefsky et al., 1991; Walboomers et al., 1999; Harwood et al., 2000), all potential sources of premature mortality; and helminth infestation significantly impairs growth and learning (Awasthi et al., 2003). The mortality and impairment caused by these infectious diseases likely rendered them important sources of natural selection.

It is clear that infectious diseases have always been a fact of life for human populations: evidence suggests that pathogens of multiple types and varying virulence have been a source of substantial stress and potentially significant natural selection throughout human evolution. The summary above suggests that the infectious disease ecologies of human populations both before and after the advent of agriculture were, and remain, complicated, varying substantially across time and space. This suggests that adaptability in the face of infectious disease stress was paramount to humans' survival and ultimate global distribution. The importance of *adaptability*, in the face of variable infectious disease threats and the substantial fitness costs to both infectious diseases and immune responses, may have selected for mechanisms in immune development that respond adaptively to early exposure to infectious agents.

Immune System Development

Immune system maturation provides ample opportunity for priming by exposure to infectious agents. Pregnancy is associated with suppression of immune responses, in particular T-helper type 1-mediated immune responses; this likely protects the fetus from potentially damaging inflammation and attack by specific maternal immune responses (Holt and Jones, 2000; West, 2002). At birth, the immune system is both immature and naïve; it undergoes processes of maturation and education throughout the first years of life (Holt and Jones, 2000; Field, 2005; M'Rabet et al., 2008). These processes are shaped by exposure to pathogenic and non-pathogenic microbes; innocuous environmental antigens; and, immune cells, regulatory mediators, and other substances in breast milk (M'Rabet et al., 2008). Maturation and education of the immune system should result in both the capacity to mount protective immune responses to pathogens and development of tolerance of non-threatening antigens, including self, allergens, and non-pathogenic microbes. Studies comparing immune characteristics of human newborns to normal adults have identified ways in which the newborn immune system is particularly immature, which may provide clues to the targets of factors shaping immune system development.

Newborn leukocytes exhibit poor antigen presentation: Leukocytes are present in lower numbers at birth than in adulthood; newborns' leukocytes' expression of surface receptors (e.g., for complement) is low, and opsonization and phagocytosis are impaired (West, 2002; M'Rabet et al., 2008). Newborns' monocytes and macrophages exhibit impaired Toll-like receptor signaling and lower cytokine production than adults' (in particular, lower Th1 cytokine production; Holt and Jones, 2000; M'Rabet et al., 2008). Newborns' antigen presenting cells (APC, in particular, dendritic cells, DC, which act as professional antigen presenting cells with the primary function of antigen presentation) exhibit low functionality: DC express MHC and costimulatory molecules at low rates (Holt and Jones, 2000); and, APC signaling cascades are altered, resulting in defective interaction between APC and T cells (M'Rabet et al., 2008). In addition, strong down-regulation of newborn APC by suppressive regulatory T cells has been observed (*ibid*). As a result, newborn APC stimulate poor lymphocyte responses.

Newborn lymphocytes' limited functionality is likely attributable to poor antigen presentation: T cells make up a lower percentage of the total lymphocyte population of newborns than adults; antigenically naïve T cells make up a substantially larger proportion of these cells (West, 2002) and memory T cells make up a substantially lower proportion (Holt and Jones, 2000). Newborns' T cell expression of some surface molecules (which interact with antigen and immune response mediators) is lower than among adults (West, 2002). Newborns' T cells are capable of proliferation and cytokine production similar to that of adults upon *in vitro* stimulation, but exhibit lower cytotoxicity (West, 2002). In some experimental conditions, newborn T cells exhibit poor cytokine production relative to adults; this is particularly true for Th1 cytokines (Holt and Jones, 2000). It is likely antigen presentation (e.g., by DC), rather than T cell capacity to respond to antigen, that is deficient in *in vivo* newborn T cell responses (when paired with adult antigen presenting cells *in vitro*, newborn T cells respond in ways comparable to adult T cells; Holt and Jones, 2000; Field, 2005).

At birth, circulating levels of immature B cells are high. These cells produce largely immunoglobulin M (IgM) antibody and exhibit defective class switching (i.e., IgM to IgG production, as part of the process of affinity maturation) and decreased reactivity to stimulation (Holt and Jones, 2000; West, 2002). Antibody production is also low at birth, resulting in low levels of circulating IgM, IgA, and IgE antibody; higher levels of IgG are largely of maternal origin. Natural killer lymphocytes are present in limited number at birth, and these cells exhibit limited reactivity to stimulation and low cytotoxic capacity (West 2002).

Newborns exhibit Th2-skewed cytokine production: At birth circulating levels of cytokines interleukin (IL)-2, IFN-γ, IL-4, IL-6 are lower than among adults (West, 2002). More generally, circulating cytokines and stimulated cytokine production by immune cells is low overall and exhibits a skew toward Th2 cytokines.

Due to these differences, a newborn's immune system provides only limited protection against infectious disease. For the first two years of life susceptibility to infection is increased, and immune cell function, antibody production, and cytokine levels change rapidly (West, 2002; M'Rabet et al., 2008). Even after improved immunocompetence at two years of age is achieved, change in immune parameters continues, and critical windows likely extend beyond this period. For example, circulating Th1 cytokine levels reach those comparable to adults by ~5 years of age, and a memory T cell population comparable to adults is achieved by ~15 years of age; in addition, the rate of maturation and age at which adult measures of immune function are reached can vary tremendously between individuals (Holt and Jones, 2000).

Breastfeeding plays a critical role in immune system development—breastmilk provides protection against infection that extends well beyond the period of breastfeeding (Field, 2005). Breastmilk contains multiple antimicrobial substances (e.g., antibodies, lactoferrin, lysozymes, complement) and immune cells (primarily macrophages and neutrophils, as well as some lymphocytes; *ibid*.). These factors protect both mother (breast tissue) and infant from infection; in addition, they may also play a role in "directing" immune system development. Maternal macrophages secrete immunoregulatory factors which may activate neonatal T and B cells; maternal T cells may compensate for the limited activity of immature T cells in the infant, and may promote their maturation (Field, 2005; M'Rabet et al., 2008). Concentrations of cytokines in breastmilk can vary tremendously, and their role is poorly understood; breastmilk cytokines may interact with the infant immune system in the intestines to affect its development (Field, 2005).

Both the Th1/Th2 balance and suppressive regulation may be particular targets of stimuli in immune system development. APC, in particular DC, which present antigen to the adaptive immune system and likely influence the Th1/Th2 nature of the incipient immune response, seem to be exceptionally immature at birth—they may retain a particularly high degree of plasticity and sensitivity to environmental exposures (Holt and Jones, 2000). High levels of regulatory cytokines (IL-10 and TGF-β) in breastmilk are thought to promote the development of tolerance for both dietary and microbiota antigens (Field, 2005).

Summary

Much of what is known about immune system development comes from epidemiological examination of immune-mediated disorders (allergy and autoimmune disease). This body of literature points very consistently to an inverse association between early exposure to infectious agents and subsequent immune-mediated disease, prompting proposal of the *hygiene hypothesis*—that early stimulation from infectious agents plays a causative role in preventing immune-mediated pathology through interaction with the developing immune system. An evolutionary perspective on the hygiene hypothesis suggests that immune system development may be responsive to early exposure to infectious agents as a means to 1) promote immunocompetence by shaping immune responses to the local infectious disease ecology; and/or, 2) minimize risk of immune-mediated damage and disease. Examination of mechanisms of immune regulation and immune system development identify ample opportunity for "priming" of immune responses to pathogens (*via* the Th1/Th2 balance) and suppressive regulation (to minimize immune-mediated damage and disease) in early life. This dissertation takes a first step toward understanding such adaptive priming in immune system development, capitalizing on the extensive body of literature investigating the epidemiology of allergic disease to evaluate the effect of early exposure to infectious agents on immunocompetence. To this end, this project sought to answer three questions:

1) *How is immunocompetence best characterized?* Conceptually, immunocompetence can be thought of as the capacity to mount a protective immune response against a pathogen antigen. However, there exists no "gold standard" means of operationalizing this definition. This work compared two putative biomarkers of immunocompetence, delayed-type hypersensitivity to *Candida albicans* antigen and anti-Epstein-Barr virus antibody, against known predictors of immunocompetence and each other to evaluate their utility as indicators of immunocompetence among children in Kilimanjaro, Tanzania.

2) *Is the hygiene hypothesis evident in the epidemiology of allergy among children in Kilimanjaro?* In the infectious disease ecologies of Europe, North America, and Australia, early infections (primarily gastrointestinal and respiratory infections, attributable to viral and bacterial pathogens) protect against allergy. Little work has evaluated the epidemiology of allergy in the more complex infectious disease ecologies of Africa (characterized by high morbidity due to viral, bacterial, protozoan, and helminth pathogens). This work evaluated whether indicators of early exposure to infectious agents are associated with lower risk of allergic disease among children in Kilimanjaro.

3) *Do patterns in immunocompetence mirror those of allergy among children in Kilimanjaro?* Having selected an appropriate biomarker of immunocompetence, and evaluated the role of infectious agents in immune system development with respect to allergic disease risk, this project turns to the question of immunocompetence. Indicators of early exposure to infectious agents were evaluated as predictors of immunocompetence among children in Kilimanjaro.

MATERIALS AND METHODS

Study Population

This project was carried out in two adjoining villages in the Machame area of Kilimanjaro, Tanzania (the study villages are largely similar and children were not expected to differ in any systematic way). Machame is inhabited almost exclusively by members of the Chagga ethnic group. The majority of Chagga in Machame are farmers, cultivating bananas as both a subsistence and cash crop; many farms also cultivate coffee, intercropped with bananas. In addition, most households have farmland holdings outside of Machame—lower elevation plots used only during the rainy season for cash crops (e.g., corn and beans). Most households also raise animals, for family consumption (e.g., milk cows) or for sale (e.g, meat cows, hogs). Banana farming and cattle husbandry are particularly central to Chagga identity. Many households rely on additional, non-agricultural, sources of income, such as wage labor (ranging from unskilled to highly skilled) and small business (e.g., cafes, butchers, and market stalls).

Households often contain multiple generations; related nuclear families often maintain distinct households within a cluster of family houses. Even when nuclear families maintain their own household distinct from any other, grandparents and other relatives often live nearby. Thus, multiple related adults usually available to provide care for a child. While mothers generally responsible for the majority of childcare, a variety of other individuals (fathers, grandparents, aunts, older siblings, and occasionally, unrelated live-in paid childcare providers) also provide care. Household composition among the Chagga can be fluid, with adults and children moving between households for a variety of reasons.

Common infectious diseases among children in Kilimanjaro include respiratory and gastrointestinal infections, as well as malaria. The infectious disease ecology of Kilimanjaro includes viral, bacterial, protozoan, fungal, and helminth infectious agents. Children are exposed to infectious agents through multiple aspects of life in Kilimanjaro; some examples include: bed-sharing with siblings, other children, and adults; interaction with household animals; contact with contaminated soil and water (rivers and irrigation ditches); consumption of contaminated food or water (e.g., unboiled drinking water); and, contact with insect vectors. Piped, treated drinking water was not available in the study area at the time of data collection. Children in Kilimanjaro are routinely vaccinated in the first year of life with BCG (against tuberculosis), oral polio vaccine, diphtheria-pertussis-tetanus vaccine, and measles vaccine; vaccination coverage is > 90% for all scheduled doses of vaccination (National Bureau of Statistics Tanzania and ICF Macro, 2011).

In comparison to Tanzanian children overall, Kilimanjaro children seem to enjoy better health and nutrition by multiple indicators. At 58 per 1000 live births, under-five mortality in the northern zone (which includes Kilimanjaro) is the lowest in the country by a substantial margin (other zones range from 84 to 109; National Bureau of Statistics Tanzania and ICF Macro, 2011). Low birth weight (< 2500 g; 1.2% of live births) and stunting (height-for-age Z-score <-2; 27.6%) are less common in Kilimanjaro region than

Tanzania as a whole (6.7% low birth weight, 42.0% stunting; *ibid.*). However, 2010 DHS data suggest that Kilimanjaro children experience infectious diseases at rates comparable to or higher than the rest of the country, with the highest prevalence of acute respiratory infection (10.1%; other regions ranged from 0.6% to 9.3%) and rates of diarrheal disease, recent fever, and anemia comparable to the country as a whole. Total fertility in Kilimanjaro, and Tanzania as a whole, seems to be declining; the TFR for the northern zone is 4.6 (*ibid.*).

Data Collection

This research project was conducted in collaboration with Nshara Community Medical Centre, a small health clinic located in Machame. NCMC hosted the investigator as a volunteer from 2001-2002, and hosted pilot research for this project in 2008, and the main data collection, reported here, in 2010. NCMC facilitated meetings with community leadership (to inform them of the study and disseminate preliminary results), and provided facilities and staffing for data collection.

Data collection commenced with a census of the study area, which was conducted by requesting the names of all children between 2 and 7 years of age from village political leadership (neighborhood chairmen, who enlisted the help of record-keepers to provide the requested lists). A random sample of ~400 were then selected (for a target sample of 300 participating children) using a random number generator (Excel; Microsoft). The randomly selected children were sorted by village political units and invited to participate: fieldworkers employed in the role of community liaisons approached the parents of selected children in their homes, evaluated children's eligibility (verifying that the child fell within the study age range of 2 to 7 years of age, and that the child was living with at least 1 parent and had been living in the study area for at least 6 months), and invited families of eligible children to participate. If parents were interested, a date (within 1 week) for participation was scheduled, parents were provided a copy of the consent form to review prior to participation, and, if the child had previously received mebendazole (a broad-spectrum antihelminthic; administered in chewable tablets) with no evidence of drug allergy, a 500 mg dose of mebendazole was administered to the child (or left with the child's parent to administer with the next meal).

At the outset of participation, the contents of the consent form were reviewed a second time with parents, and they were encouraged to ask questions. After all parents' questions had been addressed, those who chose to participate signed a consent form (in Swahili). At this time, a study identification number was assigned to each participating child; all data collection forms were labeled only with this number. A total of 314 children participated in the project.

Data collection was conducted over the course of 4 weeks in spring of 2010. Children and their primary caregiving parents participated in 2 days of data collection. Data were collected at a healthcare facility belonging to NCMC. Data collection was conducted by the investigator and 4 field assistants. Field assistants were residents of the study area and medical personnel (2 nurse/midwives, 1 nurse's assistant, 1 physician) who were trained in data collection techniques prior to data collection. Written informed consent was obtained from parents of all participating children. Procedures and data collection protocols were approved by the Institutional Review Board of the University of Washington and the Tanzanian National Institute for Medical Research (NIMR) and research permission obtained from the Tanzania Commission for Science and Technology (COSTECH).

On the first data collection visit, participating children's primary caregivers completed a questionnaire (in Swahili; English version provided in the Appendix), providing household and family demographic information, as well as information about the child's current health and medical history. Each child's finger was pricked with a

sterile lancet to obtain capillary whole blood; blood was immediately tested for HIV (SD BioLine HIV-1/2 3.0 rapid HIV-1/2 test) and hemoglobin concentration (Hb; HemoCue Hb 201^{+} hemoglobin system). Additional blood was allowed to fall freely only filter paper (Whatman #903 Protein Saver Cards) and stored as dried blood spots (DBS). The Candin (Allermed Laboratories, Inc., San Diego, CA) skin test for delayed-type hypersensitivity (DTH) to *C. albicans* antigen was administered intradermally on each child's forearm by injecting 0.1 mL under the most superficial layers of the skin with a 27 gauge tuberculin syringe.

On the second data collection visit, ~24 h after the first, the site of the Candin skin test was evaluated for the presence of an induration; the size of any induration measured in millimeters (mm) across two perpendicular diameters. The presence of a bacille Calmette-Guérin (BCG) vaccination scar on the child's shoulder was recorded. Weight was measured with a digital scale and height measured with an anthropometer. Mid-upper arm circumference (MUAC) was measured using a body tape. Triceps skinfold (TSF) thickness was measured using a Lange Skinfold Measurement Caliper (Graham-Field). All anthropometric measurements and Candin skin test evaluations were performed by the lead author without knowledge of the child's interview data to maintain measurement consistency and avoid bias. Upon completion of all data collection, the results of all point-of-care testing (HIV and Hb) and measurements (DTH and anthropometry) were explained to each child's parent, and parents were given the opportunity to ask questions. Initial review of the consent form was conducted in the public areas of the data collection facility; all subsequent data collection and discussion with parents was conducted individually to ensure privacy.

Children experiencing symptoms of infectious disease at the time of data collection, HIV positive children, and anemic children were immediately referred to NCMC for additional care. Further, parents were invited to return for a referral to NCMC for care if they detected signs of illness after their participation in the project was complete, but while data collection in the study community was still on-going. With parents' permission, the diagnoses and treatments children received for these (immediate and subsequent) referral visits were recorded.

Laboratory analysis

Dried blood spots were allowed to dry at room temperature for < 24 h, and were then frozen until shipped on dry ice to the University of Washington, where they were frozen until assay. DBS were analyzed in the Biological Anthropology and Biodemography Laboratory at the UW for: C-reactive protein (CRP) and α_1-acid glycoprotein (AGP, or orosomucoid), two acute phase reactants and indicators of infection; anti-Epstein-Barr virus antibody (EBV Ab), a potential biomarker of cell-mediated immunocompetence; and total immunoglobulin E (IgE), a mediator of immune responses to helminthic pathogens and allergens, and a potential biomarker of allergic disease morbidity.

C-reactive protein was evaluated with an in-house assay, described in detail elsewhere (Brindle et al., 2010). Briefly, specimens, calibrators (Fitzgerald, cat. no. 30-AC10, prepared in washed erythrocytes and preserved on filter paper), and controls were eluted in buffer, and then applied to microtiter plates coated with mouse anti-human CRP monoclonal antibody (Biodesign Clone C5, cat. no. M86005M), followed by signal antibody (Biodesign mouse anti-human CRP monoclonal antibody; Clone C6, cat. no. M86284B). Horseradish peroxidase (HRP)-conjugated streptavidin (Invitrogen Corporation, cat. no. 43-8323) and substrate solution were added to develop a color signal proportional to CRP concentration.

α_1-Acid glycoprotein was assessed with a commercially-available enzyme immunoassay kit, modified for use with DBS specimens. After eluting, specimens, calibrators (GenWay Biotech human orosomucoid antigen, cat. no. 10-288-22927F), and controls were applied to microtiter plates coated with chicken anti-human orosomucoid antibody (GenWay Biotech, cat. no. 15-288-22927F). HRP-conjugated chicken anti-human orosomucoid signal antibody (GenWay Biotech, cat. no. 27-288-22927F) and substrate solution were added to develop a color signal proportional to AGP concentration.

Anti-Epstein-Barr virus antibody was assessed with a commercially-available enzyme immunoassay kit, modified for use with DBS specimens (DiaSorin ETI-VCA-G Catalog No P001606A; McDade et al., 2000b). Specimens and controls were eluted in buffer, and eluent, calibrator, and kit controls were applied to microtiter plates coated with Epstein-Barr viral capsid antigen (VCA p18). Horseradish-peroxidase (HRP)-conjugated signal antibody (affinity-purified goat anti-human IgG) and substrate solution were added to develop a color signal proportional to EBV Ab concentration.

Total immunoglobulin E (IgE) was assessed with a commercially-available enzyme immunoassay kit, modified for use with DBS specimens (Tanner and McDade, 2007). Briefly, DBS specimens were eluted in buffer. Eluent, calibrator (Bethyl Laboratories, Human IgE Calibrator RC80-108), and controls were applied to microtiter plates coated with affinity purified Human IgE Coating Antibody (Bethyl Laboratories A80-108A), followed by signal antibody (Bethyl Laboratories, HRP Conjugated Human IgE Detection Antibody, A80-108P). Substrate solution was added to develop a color signal proportional to IgE concentration.

All specimens, calibrators, and controls were assayed in duplicate. Concentrations were estimated from optical density using a four-parameter logistic model

in Gen5 Software (BioTek, Winooski, VT). For all plates, an extrapolation factor of 1 was used to estimate concentrations of unknown specimens (i.e., only concentrations that fell within the range of the standard curve were estimated; those over the upper limit of detection were re-analyzed at higher dilution), with the exception of one plate of the EBV assay, containing ~15 specimens, for which an extrapolation factor of 2 was used, to estimate values of specimens that remained higher than standard curve after multiple dilutions.

Data analysis

Outcomes were parameterized as follows: DTH to *C. albicans* was defined as an induration size ≥ 5 mm in mean diameter (and anergy to *C. albicans* as induration < 5 mm). EBV Ab and total IgE were evaluated as continuous variables. Diagnosed allergic disease was categorized based on parents' report of physicians' diagnosis of hay fever, eczema, asthma, or food allergy.

We identified four indicators of early exposure to infectious agents (predictors of interest): *Family size* was characterized as small (≤ 3 cohabiting siblings) or large (> 3 cohabiting siblings) based on the number of siblings living in the household at the time of participation in the project. *House construction materials* were categorized as earth or cement based on parents' report. *Hospitalization with an infectious disease during infancy* (before the first birthday) was assessed based on parents' report of the timing and diagnosis of any hospitalizations in the child's medical history. *BCG* was considered present if a BCG vaccination scar was observed during physical examination.

Additional variables were defined as follows: Age was calculated from the child's reported date of birth. Anthropometric measurements were used to define categorical variables for malnutrition. Z-scores were calculated (EPI INFO; Centers for Disease Control and Prevention, Atlanta, GA) for weight for height (WHZ), weight for age (WAZ), and height for age (HAZ). WHZ < -2 defined wasting and HAZ < -2 defined stunting. Elevated CRP and AGP values were used to identify acute infection (based on Wander et al., in press). Breastfeeding and age at weaning were described based on parents' report. Anemia was defined based on Hb values following WHO guidelines (Nestel, 2002).

Data analysis was performed using Stata 11.2 software (Statacorp; College Station, TX). Logistic and linear regressions were used to evaluate the predicted relationships. Significance was defined as $p \leq 0.05$. Age and sex were included as *de facto* control variables in most models; additional control variables were retained in models if their inclusion substantially altered the estimated effect of a predictor of interest (>20% change in the regression coefficient or odds ratio; this threshold was chosen to allow control for substantial confounding while preserving statistical power by limiting the number of variables included in a model).

COMPARISON OF DELAYED-TYPE HYPERSENSITIVITY TO CANDIDA ALBICANS AND EPSTEIN-BARR VIRUS ANTIBODY AS BIOMARKERS OF IMMUNOCOMPETENCE AMONG CHILDREN IN KILIMANJARO, TANZANIA

Authors and institutions:
Katherine Wander[1]

[1]Department of Anthropology, University of Washington, Seattle, WA 98195

Corresponding author:
Katherine Wander
Department of Anthropology
Box 353100
University of Washington
Seattle, WA 98195
Phone: 425-443-4230
Fax: 206-543-3285
Email: kwander@u.washington.edu

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Abstract

Immunocompetence is the immune system's ability to mount protective responses against infectious agents. There is much interest in anthropology in understanding variability in immunocompetence. Lack of a gold standard biomarker of immunocompetence, as well as a focus on remote field settings and young research subjects, has complicated anthropological research into immunocompetence. We evaluated the utility of two biomarkers of immunocompetence, delayed-type hypersensitivity (DTH) to *Candida albicans* and anti-Epstein-Barr virus antibody (EBV Ab), among 267 2-7 year old children in rural Kilimanjaro, Tanzania. DTH to *C. albicans* largely conformed to expectations for a biomarker of immunocompetence (positively associated with age and adiposity; inversely associated with clinically apparent infections); EBV Ab did not. There were no significant associations between DTH to *C. albicans* and EBV Ab. We conclude that DTH to *C. albicans* is a useful biomarker of immunocompetence among East African children; however, the utility of EBV Ab in this population is limited.

Introduction

One of the most important factors in susceptibility to infectious disease (ID) is *immunocompetence*: the immune system's ability to mount protective responses against infectious agents. Immune responses rely on many components. Innate immunity physically isolates (via inflammation) and damages or destroys (via complement cascade and phagocytes) infectious agents; innate immunity also presents (via dendritic cells) antigens to the adaptive immune system (Janeway et al., 2001). Adaptive immunity is responsible for recognizing and "remembering" novel pathogen antigens. Humoral immunity targets pathogens directly (via antibody, produced by B-cells); cell-mediated immunity (CMI) recognizes and destroys host cells displaying evidence of infection with a pathogen (via cytotoxic T-cells) (*ibid.*). CMI is the component of the immune response that is most readily perturbed; compromised CMI increases risk of ID (Fine et al., 1994; Shell-Duncan and Wood, 1997). For these reasons, CMI is often the focus of immunocompetence research.

The concept of immunocompetence is difficult to operationalize, with no widely-accepted "gold standard" measure. Profound immunodeficiency, such as that associated with advanced HIV disease, is manifest as very low numbers of immune cells (white blood cells; leukopenia); however, the absence of leukopenia does not confer immunocompetence. This measure is most useful in a clinical setting, to identify severe immunosuppression; its utility in population-based research is limited. Similarly, measures of on-going immune responses are of limited utility in evaluating immunocompetence. An elevated white blood cell count, for example, suggests that an immune response is on-going; however, its lack does not constitute evidence that an immune response is not possible, but rather that one is not needed. A biomarker of immunocompetence should identify individuals with the *capacity* to mount a cell-mediated immune response.

Recent anthropological research has focused on the study of immunocompetence (Shell-Duncan, 1993; 1995; 1997; Shell-Duncan and Wood, 1997; McDade, 2001; 2002; McDade et al., 2000a; 2008; Gurven et al., 2008; Muehlenbein et al., 2010; Wander et al., 2012). Such research is inherently population-based, and is often conducted in remote and challenging field settings (where infrastructure is poor, and access to healthcare is limited). In addition, children are often the subjects of immunocompetence research (e.g., Shell-Duncan, 1993; 1995; 1997; Shell-Duncan and Wood, 1997; Wander et al., 2012), as they are particularly vulnerable to infectious diseases, often due to immature or compromised CMI. The practical constraints of population-based research, remote settings, and young research subjects further complicate the use of biomarkers in immunocompetence research. We evaluated the appropriateness of two biomarkers of immunocompetence, delayed-type hypersensitivity to *Candida albicans* and anti-Epstein-Barr virus antibody, for use in population-based research among children in rural East Africa.

Delayed-type hypersensitivity (DTH) reactions can be evaluated with a simple skin test. A small amount of pathogen antigen is introduced under the skin; if a test subject has previously responded to the antigen, *and* is immunocompetent, local activation of a secondary (or memory) immune response by CMI will be evident as an

induration at the test site after 24-72 hours (Christou et al., 1985). DTH tests are useful in two scenarios: if immunocompetence can be assumed, DTH is useful in evaluating exposure to a pathogen; if exposure to a pathogen can be assumed, DTH is useful in evaluating immunocompetence. For example, in the US, most people are generally assumed to be immunocompetent, and DTH to tuberculin PPD is used to screen for *Mycobacterium tuberculosis* exposure (Lee and Holzman, 2002). DTH testing with an antigen to which exposure can be assumed, such as the ubiquitous fungal pathogen, *C. albicans*, is used to evaluate immunocompetence (e.g., Edelman et al., 1973; Segerstrom, 2008). *C. albicans* is the causative agent of "thrush" (oral candidiasis) and "yeast infection" (vaginal candidiasis); among severely immunocompromised individuals, it can cause life-threatening systemic infection (candidemia). Occasionally (when neither immunocompetence nor exposure can be assumed), DTH responses to tuberculin PPD and *C. albicans* are employed simultaneously; for example, to screen for tuberculosis exposure among HIV-infected individuals (e.g., Huebner et al., 1994).

Delayed-type hypersensitivity is an appealing measure of immunocompetence because it allows direct observation of a subject's cell-mediated immune response to a pathogen antigen—the essence of the concept of immunocompetence. The drawbacks of employing DTH to operationalize immunocompetence are 1) interpretation: previous exposure to the recall antigen (e.g., *C. albicans*) must be assumed, but cannot be proven; and 2) practicality: DTH testing can be expensive, requires injection of the test antigen under the skin with a syringe, and poses a small risk of anaphylaxis to test subjects. These characteristics make DTH testing difficult to employ in population-based research, and are particularly problematic in remote field settings, and among young subjects.

The high prevalence of another pathogen, Epstein-Barr virus (EBV), allows an alternative means of operationalizing the concept of immunocompetence. By adulthood, ~90% of people have acquired chronic EBV infection; this infection is kept largely in check by the activity of cytotoxic T lymphocytes (Rickinson and Moss, 1997). When CMI is compromised, EBV replication increases, inducing increased production of anti-EBV immunoglobulin G antibody (EBV Ab). Thus, elevated EBV Ab is associated with immunosuppression (Glaser et al., 1991; 1993; McDade et al., 2000a), and has been used as a biomarker of immunocompetence.

Like DTH, interpretation of EBV Ab as an biomarker of immunocompetence is limited by the necessary assumption that subjects have experience with the pathogen: while low levels of EBV Ab indicate immunocompetence, extremely low levels of EBV Ab cannot be interpreted, as they may indicate a subject is uninfected with EBV, and whether immunosuppressed or not, is not producing EBV Ab. In addition, EBV Ab interpretation relies on an indirect mechanism—it is not the cell-mediated immune response *per se* that is observed, but a consequence of its failure—elevated antibody production—which is itself an aspect of immune function. EBV Ab is appealing as a biomarker of immunocompetence for its practicality: it can be easily, accurately, and cheaply measured in whole blood stored as dried blood spots (DBS), lending it to use in population-based research in a wide range of field settings and among subjects of all ages.

We compared these two biomarkers of immunocompetence (DTH to *Candida albicans* and EBV Ab) among children in rural Kilimanjaro. First, we evaluated each

biomarker independently for associations with known predictors of cell-mediated immunocompetence (age, sex, nutritional status, and acute ID morbidity; Table 5), to assess the extent to which each conformed to expectations for a biomarker of immune function. We then evaluated associations between the two biomarkers.

Table 5. Expected Predictors of Cell-Mediated Immune Function

Predictor	Cell-mediated immune function	DTH to *Candida*	EBV antibody
Age	↑	↑	↓
Male sex	↓	↓	↑
Adequate nutrition	↑	↑	↓
Acute infection	↓	↓	↑

Materials and Methods

Participants: This project was carried out in the Machame area of Kilimanjaro, Tanzania. 314 2-7 year old children participated in the project, randomly sampled from a census of all 2-7 year old children in the study area. Children were eligible to participate if they were living with at least 1 parent and had been living in the study area for at least 6 months.

Data collection: Data collection was conducted over the course of 4 weeks in spring of 2010. Children and their primary caregiving parents participated in 2 days of data collection. Data were collected at a healthcare facility belonging to Nshara Community Medical Centre (NCMC). Data collection was conducted by the lead author and 4 field assistants. Field assistants were residents of the study area and medical personnel (2 nurse/midwives, 1 nurse's assistant, 1 physician) who were trained in data collection techniques prior to data collection. Written informed consent was obtained from parents of all participating children. Procedures and data collection protocols were approved by the Institutional Review Board of the University of Washington and the Tanzanian National Institute for Medical Research (NIMR) and research permission obtained from the Tanzania Commission for Science and Technology (COSTECH).

On the first data collection visit, participating children's primary caregivers completed a questionnaire (in Swahili), providing household and family demographic information, as well as information about the child's medical history. Each child's finger was pricked with a sterile lancet to obtain capillary whole blood; blood was immediately tested for HIV (SD BioLine HIV-1/2 3.0 rapid HIV-1/2 test) and hemoglobin concentration (HemoCue Hb 201^{+} hemoglobin system). Additional blood was dropped on filter paper (Whatman #903 Protein Saver Cards) and stored as dried blood spots (DBS). The Candin (Allermed Laboratories, Inc., San Diego, CA) skin test for DTH to *C. albicans* antigen was administered intradermally on each child's forearm by injecting 0.1 mL under the most superficial layers of the skin with a 27 gauge tuberculin syringe. Children experiencing symptoms of ID, HIV positive children, and anemic children were referred to NCMC for additional care. With parents' consent, NCMC's diagnosis and treatment was recorded for children referred to NCMC.

On the second data collection visit, ~24 h after the first, the site of the Candin skin test was evaluated for the presence of an induration; the size of any induration measured in millimeters (mm) across two perpendicular diameters. Weight was measured with a digital scale and height measured with an anthropometer. Mid-upper arm circumference (MUAC) was measured using a body tape. Triceps skinfold (TSF) thickness was measured using a Lange Skinfold Measurement Caliper (Graham-Field). All anthropometric measurements and Candin skin test evaluations were performed by the lead author to maintain measurement consistency.

Laboratory analysis: DBS specimens were allowed to dry at room temperature for < 24 h, and were then frozen until shipped on dry ice for analysis in the Biological Anthropology and Biodemography Laboratory at the University of Washington. EBV Ab was evaluated using a kit modified for use with DBS (DiaSorin ETI-VCA-G Catalog No. P001606A; McDade et al., 2000b). Specimens and controls were eluted in buffer, and eluent, calibrator, and kit controls were applied to microtiter plates coated with Epstein-

Barr viral capsid antigen (VCA p18). Horseradish-peroxidase (HRP)-conjugated signal antibody (affinity-purified goat anti-human IgG) and substrate solution were added to develop a color signal proportional to EBV Ab concentration.

Dried blood spot specimens were also analyzed for C-reactive protein (CRP) and α_1-acid glycoprotein (AGP, or orosomucoid), two biomarkers of inflammation used to identify acute infection. CRP was assessed with an in-house DBS assay (Brindle et al., 2010). Specimens and controls were eluted in buffer, and eluent, calibrator (Fitzgerald, cat. no. 30-AC10), and controls were applied to microtiter plates coated with mouse anti-human CRP monoclonal antibody (Biodesign Clone C5, cat. no. M86005M), followed by signal antibody (Biodesign mouse anti-human CRP monoclonal antibody; Clone C6, cat. no. M86284B). HRP-conjugated streptavidin (Invitrogen Corporation, cat. no. 43-8323) and substrate solution were added to develop a color signal proportional to CRP concentration.

α_1-Acid glycoprotein was assessed with a commercially-available enzyme immunoassay kit, modified for use with DBS specimens. After eluting, specimens, calibrators (GenWay Biotech human orosomucoid antigen, cat. no. 10-288-22927F), and controls were applied to microtiter plates coated with chicken anti-human orosomucoid antibody (GenWay Biotech, cat. no. 15-288-22927F). HRP-conjugated chicken anti-human orosomucoid signal antibody (GenWay Biotech, cat. no. 27-288-22927F) and substrate solution were added to develop a color signal proportional to AGP concentration.

All specimens, calibrators, and controls were assayed in duplicate. Concentrations were estimated from color signal optical density using a four-parameter logistic model in Gen5 Software (BioTek, Winooski, VT). An extrapolation factor of 1 was used to estimate concentrations of all unknown specimens, with the exception of one run of the EBV assay (~15 specimens), for which an extrapolation factor of 2 was used, to estimate values of specimens that remained higher than standard curve after multiple dilutions. Intra-assay coefficients of variation (CV; for the assay runs included in this analysis) were 3.6% for EBV, 16.2% for CRP, and 10.6% for AGP. Inter-assay CVs were 12.0% at low and 6.7% at high concentrations of EBV Ab; 11.9% at low and 10.2% at high concentrations of CRP; 18.6% at low and 9.8% at high concentrations of AGP.

Data analysis: An induration ≥ 5mm defined positive DTH to *C. albicans*; induration size was also evaluated as a continuous variable. EBV Ab was natural logarithm-transformed and evaluated as a continuous variable. Age was calculated from the child's reported date of birth. Infections were identified through biomarkers: elevated CRP (> 1.1 mg/L) or AGP (> 0.76 g/L) based on Wander et al., in press. Biomarkers were also used to describe the following categories of infection: healthy (normal CRP and normal AGP), chronic illness (normal CRP, elevated AGP), current infection (elevated CRP and elevated AGP), and recent/new infection (elevated CRP and normal AGP), based on the differences in time-course between CRP and AGP in acute infection (while both are elevated in response to infection, elevations in CRP, as well as declines to background levels, typically precede those in AGP by several days; modified from Thurnham et al., 2005). Finally, clinically-apparent ID were characterized from diagnoses given by NCMC to children referred for care during data collection.

Data analysis was performed using Stata 11.2 software (Statacorp; College Station, TX). Linear regression was used to assess predictors of induration size and EBV Ab. Logistic regression was used to assess predictors of positive DTH. Significance was defined as $p \leq .05$.

Results

Complete information for DTH to *Candida* and EBV Ab was available for 267 children (inadequate sample was available to characterize EBV for 13 children; 26 children were EBV negative; 8 children exhibited Candin skin test administration problems). Characteristics of these children are shown in Table 6. DTH induration distribution is shown in Figure 1; EBV Ab distribution is shown in Figure 2. EBV Ab exhibited a right-skewed distribution.

Table 6. Sample Characteristics

Sex		
Female	144	53.93%
Male	123	46.07%
Age in years, mean (SD) [a]	4.5	(1.61)
Triceps skinfold in mm, mean (SD) [b]	11.81	(3.28)
Elevated CRP or AGP [c]	153	61.20%
Stage of infection [d]		
Healthy	97	39.92%
Chronic illness	28	11.52%
Current ID	62	25.51%
Recent/new ID	56	23.05%
Infectious disease diagnosis upon referral	55	20.60%
Delayed-type hypersensitivity to *Candida albicans*		
Positive DTH (induration $\geq$ 5 mm)	142	53.18%
Induration in mm, mean (SD)	5.76	(4.29)
Anti-Epstein-Barr virus antibody, geometric mean (AU [e])		24 675

[a] N = 254; [b] N = 226; [c] N = 250; [d] N = 243; [e] AU = arbitrary units

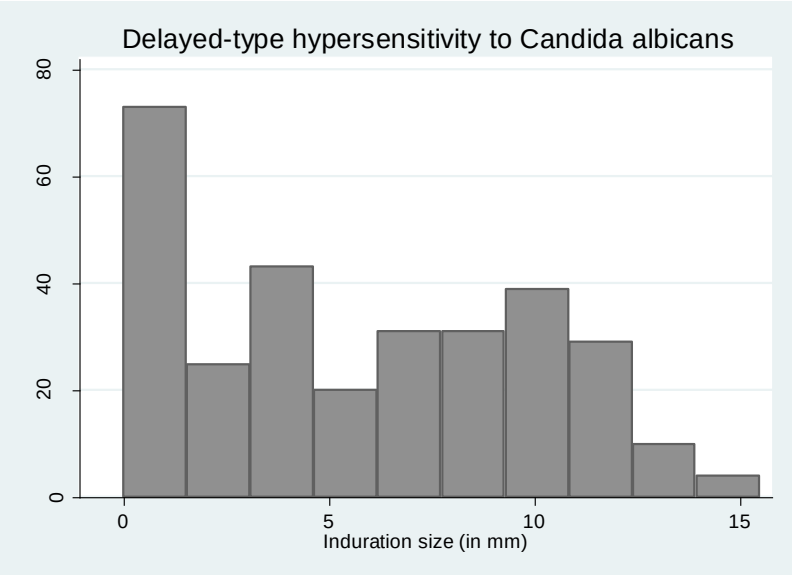

Figure 2. Delayed-Type Hypersensitivity to *Candida albicans* Distribution of Induration Size

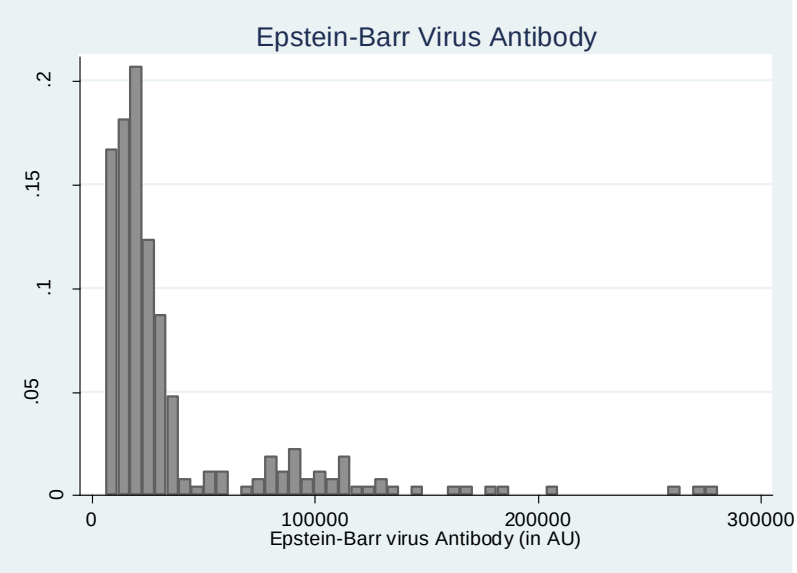

Figure 3. Distribution of Anti-Epstein-Barr Virus Antibody Concentration

Age and TSF were positively and significantly associated with DTH to *Candida albicans* (Table 7a) and induration size (Table 7b). Male sex and elevated biomarkers of inflammation (indicative of acute infection) were not significantly associated with DTH or induration size. The use of alternative biomarker definitions of acute infection (i.e., CRP > 2.0 mg/L or 5.0 mg/L; AGP > 1.2 g/L) did not alter these results (data not shown). DTH also did not vary consistently by stage of infection (data not shown). Controlling for

age and sex, ID diagnosis upon referral was inversely associated with both DTH (OR: 0.55; 95% CI: 0.29, 0.98) and induration size (β: -1.17; 95% CI: -2.45, 0.070).

Table 7. Predictors of Delayed-Type Hypersensitivity to *Candida*

a. Positive DTH (induration ≥ 5mm)

Predictor	Odds ratio	95% CI	p-value
Age	1.27	1.04, 1.55	0.017
Male sex	1.29	0.74, 2.24	0.369
Triceps skinfold	1.11	1.01, 1.22	0.029
Elevated CRP or AGP	0.87	0.51, 1.50	0.623

b. Induration size (in mm)

Predictor	β	95% CI	p-value
Age	0.60	0.20, 0.99	0.004
Male sex	0.24	-0.90, 1.38	0.677
Triceps skinfold	0.24	0.04, 0.43	0.017
Elevated CRP or AGP	-0.48	-1.60, 0.64	0.403

Male sex was associated with a significant decrease in EBV Ab of 36%; age, TSF, and elevated biomarkers of inflammation were not significantly associated with EBV Ab (Table 8). EBV was not associated with stage of infection or ID diagnosis upon referral (data not shown). The use of alternative biomarker definitions of acute infection did not alter these results (data not shown).

Table 8. Predictors of Anti-Epstein-Barr Virus Antibody

Predictor	β	exp(β)*	95% CI	p-value
Age	0.01	1.01	-0.07, 0.10	0.760
Male sex	-0.46	0.63	-0.69, -0.23	0.000
Triceps skinfold	-0.03	0.97	-0.07, 0.00	0.083
Elevated CRP or AGP	-0.01	0.99	-0.24, 0.22	0.916

*Proportional change in EBV Ab associated with each predictor

There was no significant association between EBV Ab and positive DTH or induration size (Table 9).

Table 9. Association between Delayed-Type Hypersensitivity to *Candida* and Anti-Epstein-Barr Virus Antibody (N = 267)

Correlation between induration and EBV Ab:
Pair-wise correlation = -0.0028 p = 0.9639

T-test of EBV Ab by DTH (positive or negative) to *Candida*
t = -0.3137 p = 0.7540 (265 degrees of freedom)

Discussion

We evaluated two indicators of immunocompetence, DTH to *C. albicans* and EBV Ab, among 2-7 year old children in Kilimanjaro, Tanzania. DTH to *C. albicans* was significantly positively associated with age and adiposity (TSF), was inversely associated with some indicators of infection (infectious disease diagnosis upon referral), and was not associated with male sex. EBV Ab was significantly inversely associated with male sex and was not associated with age, adiposity, or acute infection.

Immunocompetence increases with age during early childhood (Kniker et al., 1985). Male sex, protein-energy malnutrition, and acute infection are risk factors for CMI failure (Washburn et al., 1965; Neumann et al., 1975; Pinner et al., 1996; Zaman et al., 1997; Shell-Duncan, 1997). Based on these well-established predictors of immunocompetence, the associations we expected to observe in our data are summarized in Table 5. Associations between DTH to *C. albicans* and known predictors of immunocompetence were largely consistent with our expectations. DTH to *C. albicans* and induration size increased significantly with age and with adiposity, and decreased with clinically-apparent infection. DTH and induration size were not significantly associated with male sex, perhaps due to the young age of children sampled (2-7 years old; excess ID mortality among males seems to begin in adolescence; Owens, 2002). Overall, DTH to *C. albicans* conforms to expectations for a biomarker of immunocompetence.

Associations between EBV Ab and known predictors did *not* conform to our expectations of a biomarker of immunocompetence. We expected a positive association (or no association) between EBV Ab and male sex, but instead observed a significant *inverse* association; EBV Ab was not significantly associated with other expected predictors (age, adiposity, or acute infection) of immunocompetence. We observed no significant association between EBV Ab and DTH to *C. albicans*.

Delayed-type hypersensitivity to *C. albicans*, assessed with the Candin skin test, appears to be a useful biomarker of immunocompetence among East African children. It remains possible that DTH positivity reflects variation in exposure to *C. albicans*, rather than in immunocompetence; however, our results are inconsistent with this interpretation: it is more likely that immunocompetence increases with age and adiposity than it is that exposure to *C. albicans* increases with age or adiposity; nor is it likely that exposure to *C. albicans* is rarer among children with clinically-apparent infections.

Anti-Epstein-Barr virus antibody appears to be much less useful as a biomarker of immunocompetence among East African children. There are multiple possible explanations for this finding. First, the age of children in our sample may complicate interpretation of EBV Ab. The utility of EBV Ab as a biomarker of immunocompetence is contingent on the assumption of near-universal chronic EBV infection. The high rate of seropositivity (~92%) indicates a very high prevalence of EBV in our study population. However, a subset of the prevalent infections we observed may have been in the acute, rather than chronic, stage. In this case, EBV Ab production more likely reflects the acute stage of EBV infection than compromised CMI. It is also possible that EBV Ab does not perform as predicted for a biomarker of immunocompetence because it is affected by other physiological processes, obscuring any effect of cell-mediated immunocompetence

on EBV Ab production. The majority of studies employing EBV Ab as a biomarker of immunocompetence explore the effect of psychosocial stress on immune function. Stress may promote EBV proliferation directly (Kupfer and Summers, 1990; Schuster et al., 1991; Glaser et al., 1995), as well as through compromised CMI, potentially over-estimating the effect of stress on CMI. Our results do not support the use of EBV Ab as a biomarker of immunocompetence among East African children. Further research is needed to evaluate EBV Ab among older subjects in this population, and to further evaluate the association between CMI and EBV Ab production in the absence of psychosocial stress.

EARLY LIFE EXPOSURE TO INFECTIONS AGENTS PREDICTS DIAGNOSED ALLERGIC DISEASE AND TOTAL IMMUNOGLOBULIN E AMONG CHILDREN IN KILIMANJARO, TANZANIA

Authors and institutions:
Katherine Wander[1]

[1]Department of Anthropology, University of Washington, Seattle, WA 98195

Corresponding author:
Katherine Wander
Department of Anthropology
Box 353100
University of Washington
Seattle, WA 98195
Phone: 425-443-4230
Fax: 206-543-3285
Email: kwander@u.washington.edu

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Abstract

Objective: To examine the effect of early exposure to infectious agents on risk of allergic disease among 2-7 year old children in rural Kilimanjaro, Tanzania.

Methods: A questionnaire and physical examination were used to characterize diagnosed allergic disease and variables reflecting early exposure to infectious agents (family size, house construction materials, BCG vaccination, hospitalization history). Whole blood stored as dried blood spots was evaluated for total immunoglobulin E (IgE); total IgE was natural-logarithm transformed for data analysis. Logistic and linear regression were used to evaluate indicators of early exposure to infectious agents as predictors of diagnosed allergic disease and total IgE.

Results: Among 284 children, diagnosed allergic disease was associated with significantly higher total IgE. Controlling for age and sex, large family size (> 3 siblings) was inversely associated with diagnosed allergic disease (OR: 0.24; 95% CI: 0.07, 0.85), while hospitalization with an infectious disease during infancy (OR: 6.26; 95% CI: 1.84, 21.27) and earth housing materials (OR: 2.03; 95% CI: 1.21, 3.41) were positively associated with diagnosed allergic disease. Earth housing materials (β: 0.51; 95% CI: 0.33, 0.75) were positively associated with total IgE.

Conclusions: Large family size was inversely associated with allergic disease; hospitalization with an infectious disease during infancy and earth housing materials were positively associated with allergic disease. The inverse association between family size and allergy likely reflects a protective effect of early life intracellular infections. The positive association between hospitalization and allergy likely reflects a disruptive effect of severe episodes of early infection on immune system development, while the positive association between earth housing materials and allergy may reflect increased risk of allergy resulting from early life helminth infections.

Keywords: Hygiene hypothesis, allergic disease, immunoglobulin E

Introduction

Among children in the United States, Europe, and Australia, infections during early life are associated with lower allergic disease risk (including atopic rhinitis, atopic dermatitis, and allergic asthma). Exposure to other children (presumably a source of infectious disease transmission) through larger family size (Strachan, 1989; Jarvis et al., 1997; Matricardi et al., 1998; Ball et al., 2000; for review, see von Mutius, 2001; Bach, 2002; Matricardi, 2010), higher birth order (Matricardi et al., 1998), or day care attendance (Krämer et al., 1999; Haby et al., 2000; Infante-Rivard et al., 2001) is inversely associated with allergic disease risk. Episodes of routine infection in early life (Illi et al., 2001; Njå et al., 2003; Williams et al., 2004; von Mutius et al., 1999; Calvani et al., 2002) and seropositivity to common viruses and bacteria (including hepatitis A virus, herpes simplex virus type 1, cytomegalovirus, *Toxoplasma gondii*, and *Helicobacter pylori;* Matricardi et al., 2000, 2002; Chen and Blaser, 2007; Janson et al., 2007) are also inversely associated with allergic disease. This evidence underpins the *hygiene hypothesis* (Strachan, 1989), which argues that exposure to infectious agents during immune system development plays a causative role in preventing subsequent allergic disease.

Comparatively little research has explored the epidemiology of allergy in developing world populations. However, recent research suggests the prevalence of allergic disease, including asthma, is increasing in Africa and other areas of the developing world; in some populations, rates of allergic disease are comparable to those in the US and Europe (Ait-Khaled et al., 2007). Thus, it has become important to investigate the epidemiology of allergy in developing countries. This is especially important with respect to the hygiene hypothesis, as the infectious disease ecologies of the developing world likely differ dramatically from the US and Europe, with higher morbidity due to viral, bacterial, and protozoan agents, and the presence of helminth pathogens, which are largely absent in industrial/developed settings.

We examined associations between early life exposure to infectious agents and allergy among 2-7 year old children in rural Kilimanjaro, Tanzania. We identified four factors likely to capture variation in early exposure to infectious agents: family size (children likely contract infections from older siblings during infancy); earth house construction materials (children living in earth, rather than cement, houses, likely contract infections, e.g., soil-derived helminths, from the environment at a higher rate); BCG vaccination (exposure to an attenuated strain of *Mycobacterium tuberculosis* as vaccination against tuberculosis); and hospitalization in infancy with an infectious disease. We examined associations between these indicators of early exposure to infectious agents and two outcomes: reported diagnoses of allergic disease, and total immunoglobulin E (IgE), the antibody associated with atopic responses.

Materials and Methods

Participants: This project was carried out in the Machame area of Kilimanjaro, Tanzania. 314 2-7 year old children participated in the project, randomly sampled from a census of all 2-7 year old children in the study area. Children were eligible to participate if they were living with at least 1 parent and had been living in the study area for at least 6 months.

Data collection: Data collection was carried out over the course of 4 weeks in spring of 2010. Data were collected at a healthcare facility belonging to Nshara Community Medical Centre (NCMC). Data collection was conducted by the lead author and 4 field assistants. Field assistants were residents of the study area and medical personnel (2 nurse/midwives, 1 nurse's assistant, 1 physician) who were trained in data collection techniques prior to data collection. Written informed consent was obtained from each participating child's primary caregiving parent. Procedures and data collection protocols were approved by the Institutional Review Board of the University of Washington and the Tanzanian National Institute for Medical Research (NIMR) and research permission obtained from the Tanzania Commission for Science and Technology (COSTECH).

Upon recruitment (between 1 and 7 days prior to data collection), 500 mg of mebendazole (a broad-spectrum antihelminthic; administered in chewable tablets) was administered to participating children. Mebendazole was not administered if parents reported a child had never before received mebendazole, to minimize risk of adverse reactions (N = 18 children).

Participating children's primary caregivers completed a questionnaire (in Swahili), providing household and family demographic information, as well as information about the child's recent health, history of hospitalization, and physicians' diagnoses of allergic disease (hay fever, food allergy, asthma, eczema). Each child's finger was pricked with a sterile lancet to obtain capillary whole blood; blood was immediately tested for HIV (SD BioLine HIV-1/2 3.0 rapid HIV-1/2 test) and hemoglobin concentration (HemoCue Hb 201^{+} hemoglobin system). Children experiencing symptoms of infectious disease, HIV positive children, and anemic children were referred to NCMC for care. Additional blood was dropped on filter paper (Whatman #903 Protein Saver Cards) and stored as dried blood spots (DBS) for later assay. Weight was measured with a digital scale. Height was measured with an anthropometer. Mid-upper arm circumference was measured with a body tape. Triceps skinfold thickness was measured with a Lange Skinfold Measurement Caliper (Graham-Field). The presence of a bacille Calmette-Guérin (BCG) vaccination scar on the child's shoulder was recorded.

Laboratory analysis: DBS were allowed to dry at room temperature for < 24 h and were then frozen until shipped on dry ice to the Biological Anthropology and Biodemography Laboratory at the University of Washington for analysis. Total immunoglobulin E (IgE) was evaluated using an enzyme immunoassay kit modified for use with DBS samples (Tanner and McDade, 2007). Briefly, DBS specimens were eluted in buffer. Eluent, calibrator (Bethyl Laboratories, Human IgE Calibrator RC80-108), and controls were applied to microtiter plates coated with affinity purified Human IgE Coating Antibody (Bethyl Laboratories A80-108A), followed by signal antibody (Bethyl

Laboratories, HRP Conjugated Human IgE Detection Antibody, A80-108P). Substrate solution was added to develop a color signal proportional to IgE concentration.

Dried blood spot specimens were also analyzed for C-reactive protein (CRP) and α_1-acid glycoprotein (AGP, or orosomucoid), two acute phase reactants and indicators of infection. CRP was assessed with an in-house DBS assay (Brindle et al., 2010). Specimens and controls were eluted in buffer, and eluent, calibrator (Fitzgerald, cat. no. 30-AC10), and controls were applied to microtiter plates coated with mouse anti-human CRP monoclonal antibody (Biodesign Clone C5, cat. no. M86005M), followed by signal antibody (Biodesign mouse anti-human CRP monoclonal antibody; Clone C6, cat. no. M86284B). Horseradish peroxidase (HRP)-conjugated streptavidin (Invitrogen Corporation, cat. no. 43-8323) and substrate solution were added to develop a color signal proportional to CRP concentration.

α_1-Acid glycoprotein was assessed with a commercially-available enzyme immunoassay kit, modified for use with DBS specimens. After eluting, specimens, calibrators (GenWay Biotech human orosomucoid antigen, cat. no. 10-288-22927F), and controls were applied to microtiter plates coated with chicken anti-human orosomucoid antibody (GenWay Biotech, cat. no. 15-288-22927F). HRP-conjugated chicken anti-human orosomucoid signal antibody (GenWay Biotech, cat. no. 27-288-22927F) and substrate solution were added to develop a color signal proportional to AGP concentration.

All specimens, calibrators, and controls were assayed in duplicate. Concentrations were estimated from color signal optical density using a 4-parameter logistic model in Gen5 Software (BioTek, Winooski, VT). Intra-assay coefficients of variation (CVs; for the assay runs included in this analysis) were 8.5% for IgE; 16.2% for CRP; and, 10.6% for AGP; inter-assay CVs were 15.0% at low and 19.0% at high concentrations of IgE; 11.9% at low and 10.2% at high concentrations of CRP; 18.6% at low and 9.8% at high concentrations of AGP.

Data analysis: The outcomes of interest were diagnosed allergic disease (physician's diagnosis of allergic disease, as reported by primary caregivers) and total IgE. We identified four variables reflecting early exposure to infectious agents (predictors of interest): *family size* was characterized as small (≤ 3 cohabiting siblings) or large (> 3 cohabiting siblings) based on the number of siblings living in the household at the time of participation in the project; *house construction materials* were categorized, based on parents' report, as cement or earth; *hospitalization with an infectious disease during infancy* was assessed based on parents' report of the timing and diagnosis of any hospitalizations in the child's medical history; *BCG vaccination* was considered present if a BCG vaccination scar was detected during physical examination. Potential control variables were defined as follows: Age was calculated from the child's reported date of birth. Anthropometric measurements were used to define categorical variables for malnutrition. Z-scores were calculated (EPI INFO; Centers for Disease Control and Prevention, Atlanta, GA) for weight for height (WHZ), weight for age (WAZ), and height for age (HAZ). WHZ < -2 defined wasting and HAZ < -2 defined stunting. Elevated CRP and AGP values were used to identify acute infection (based on Wander et al., in press). Breastfeeding and age at weaning were described based on parents' report. Anemia was defined from hemoglobin values following WHO guidelines (Nestel, 2002).

Data analysis was performed using Stata 11.2 software (Statacorp; College Station, TX). Logistic regression was used to assess predictors of diagnosed allergic disease. IgE values were natural logarithm-transformed for regression analysis; linear regression was used to assess predictors of total IgE. Significance was defined as $p \leq .05$. Control variables were retained in a model if their inclusion altered the effect of any predictor of interest by > 20% (chosen to allow control of substantial confounding while limiting the inclusion of control variables in models to preserve power); age and sex were retained as control variables in all models of early life predictors of allergic disease outcomes.

Results

Complete information (excluding anthropometry and biomarkers) was available for 284 children: age was unavailable for 17 children; number of siblings living in the household was unavailable for 3 children; information regarding physician's diagnosis of allergic disease was unavailable for 9 children; and data from 1 HIV-positive child were excluded from analysis. Complete information including total IgE was available for 266 children; complete information including biomarkers of acute infection was available for 262 children; and, complete information including anthropometry was available for 248 children (without TSF) and 217 children (with TSF).

Sample characteristics are described in Table 10. Family size ranged from 0 to 6 siblings (in addition to the participating child), with 8.45% of children from "large" families (> 3 siblings). Most participating children (93.59%) exhibited a discernible BCG vaccination scar. 14 children (4.93%) were hospitalized in the first year of life with an infectious disease (pneumonia, malaria, or diarrhea; based on parents' report). Diagnosed allergic disease was relatively common, reported for 96 children (33.80%); eczema was the most common allergic disease reported (18.66%), and asthma the least common (2.14%). Geometric mean total IgE was 803 IU/mL; total IgE exhibited a right-skewed distribution (Figure 1).

Table 10. Sample Characteristics

Sex		
Female	153	53.87 %
Male	131	46.13%
Age in years, mean (SD)	4.47	(1.65)
House materials		
Cement	144	50.70%
Earth	140	49.30%
Electricity in the home [a]	57	20.58%
Coffee farming household	110	38.73%
Anthropometry		
Wasting [b]	1	0.38%
Stunting [c]	77	29.17%
Triceps skinfold in mm, mean (SD)	11.70	(3.24)
Acute infection [d]	153	58.4%
Siblings living in the household, mean (range)	1.71	(0, 6)
Large family (> 3 siblings)	24	8.45%
Hospitalization in first year of life for infectious disease [e]	14	4.93%
BCG vaccination scar	263	93.59%
Currently breastfeeding	21	7.39%
Mean age at weaning in years (SD) [f]	1.90	(0.76)
Anemic (at the time of data collection)	90	31.91%
Allergic disease		
Hay fever	37	13.03%
Eczema	53	18.66%
Food allergy	19	6.71%
Asthma	6	2.14%
Any allergy	96	33.80%
Total IgE geometric mean	803 IU/ml	

[a] Information available for 277 children
[b] Weight for height Z-score < -2
[c] Height for age Z-score < -2
[d] Elevated biomarkers of inflammation (CRP or AGP); based on Wander et al., in press
[e] Parents' report of physician's diagnosis of pneumonia, malaria, or diarrhea for a hospitalization during the child's first year of life
[f] Among children not currently breastfeeding; information available for 259 children

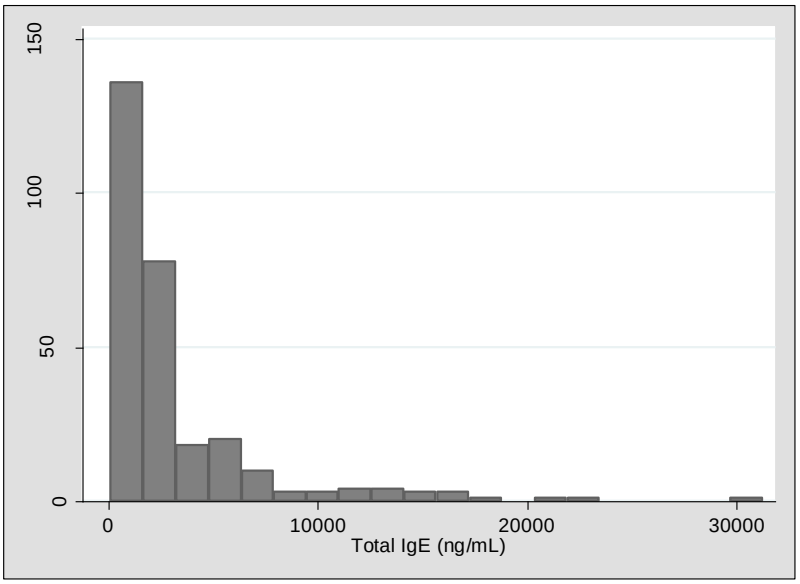

Figure 4. Distribution of Total Immunoglobulin E

Chi-squared tests of the associations between diagnosed allergic disease and reported allergy symptoms (in the previous year) are described in Table 11.

Table 11. Symptoms of Allergy and Diagnosed Allergic Disease

	Diagnosis:		χ^2 (p-value)
	Hay fever	No hay fever	
Sneezing	2.38%	5.51%	0.74 (0.390)
Runny nose	2.38%	7.35%	1.44 (0.230)
Itchy eyes	11.90%	4.78%	3.42 (0.064)
Watery eyes	9.52%	3.32%	3.51 (0.061)
Red eyes	9.52%	3.33%	3.49 (0.062)
	Eczema	No eczema	
Itchy rash	25.45%	3.15%	34.01 (0.000)
Dry rash	54.55%	3.94%	102.75 (0.000)
Swollen rash	7.27%	0.79%	9.99 (0.002)
Recurring rash	16.36%	0.79%	31.95 (0.000)
	Asthma	No asthma	
Wheeze	16.67%	0.00%	50.50 (0.000)
Cough	0.00%	5.96%	0.38 (0.538)
Shortness of breath	83.33%	4.97%	59.50 (0.000)
Chest tightness	100.00%	22.52%	19.35 (0.000)

The results of linear regression evaluating the association between natural-logarithm transformed total IgE and reported physician's diagnosis of allergy is described

in Table 12. Controlling for age and sex, diagnosed allergic disease was associated with a significant increase in total IgE of 30% (β: 0.26, 95% CI: 0.02, 0.49).

Table 12. Allergic Disease and Total Immunoglobulin E (IgE)

Predictor	β	exp(β)[a]	95% CI	p-value
Male sex	0.16	1.17	0.09, 0.23	0.000
Age	0.06	1.06	-0.16, 0.28	0.606
Diagnosed allergic disease	0.26	1.30	0.02, 0.49	0.035

[a] Proportion change in IgE associated with change in predictor variable

The results of logistic regression evaluating early life predictors of diagnosed allergic disease are described in Table 13. Controlling for age and sex, large family size was associated with significantly lower odds of allergic disease (OR: 0.24; 95% CI: 0.07, 0.85). Hospitalization with an infection during infancy (OR: 6.26; 95% CI: 1.84, 21.27) and living in a house constructed from earth (OR: 2.03; 95% CI: 1.21, 3.41) were associated with significantly higher odds of allergic disease. BCG vaccination scar was not significantly associated with allergic disease diagnosis (data not shown).

Table 13. Early Life Predictors of Allergic Disease

Predictor	Odds Ratio	95% CI	p-value
Male sex	0.83	0.49, 1.40	0.484
Age	0.94	0.81, 1.10	0.434
Large family size	0.24	0.07, 0.85	0.027
Hospitalization in the first year of life with an infectious disease	6.26	1.84, 21.27	0.003
Earth housing materials	2.03	1.21, 3.41	0.008

The results of linear regression evaluating early life predictors of (natural logarithm-transformed) total IgE are described in Table 14. Living in a house constructed from earth (β: 0.51; 95% CI: 0.33, 0.75) was associated with a significant increase (of 67%) in total IgE; family size and hospitalization during infancy were not significant predictors of total IgE; BCG vaccination scar was not significantly associated with total IgE (data not shown).

Table 14. Early Life Predictors of Total Immunoglobulin E (IgE)

Predictor	β	exp(β)[a]	95% CI	p-value
Male sex	0.01	1.01	-0.20, 0.22	0.901
Age	0.17	1.19	0.11, 0.23	0.000
Large family size	0.15	1.16	-0.22, 0.51	0.427
Hospitalization in the first year of life with an infectious disease	0.35	1.42	-0.20, 0.89	0.210
Earth housing materials	0.51	1.67	0.33, 0.75	0.000

[a] Proportion change in IgE associated with a change in predictor variable

Discussion

We document a positive and significant association between total IgE and diagnosed allergic disease among 2-7 year old children in rural Kilimanjaro, Tanzania. We demonstrated that diagnosed allergic disease was significantly less common among children from large families, and significantly more common among children with a history of hospitalization for infection in infancy and among children living in houses constructed of earth. Earth housing materials were also positively associated with total IgE; however, total IgE was not associated with family size or hospitalization history.

There are limitations to both total IgE and parents' reports of diagnosed allergic disease as indicators of atopic disease. IgE mediates allergic responses (Gould et al., 2003) and both total (Croner et al., 1982; Droste et al., 1996; Sherrill et al., 1999) and allergen-specific (Droste et al., 1996; Pastorello et al., 1995) IgE have been reported to be elevated among those with atopic disease. In addition, IgE mediated immune responses to helminth parasites, and may be associated with acute or chronic helminth morbidity. To limit variation in IgE due to helminth infection, we restricted analyses of total IgE to children who received mebendazole, a broad spectrum anti-helminth, upon recruitment into the project; however, mebendazole does not work against all helminths, and may not have been taken in time to completely eradicate helminth infections (between 1 and 7 days prior to data collection). As such, total IgE may have been affected not only by allergic disease morbidity, but also by acute or chronic helminth infection. Parents' report of diagnosed allergic disease may be limited by their memory and understanding of physicians' diagnoses. Physicians' diagnoses are likely imperfect, as well, due to limited availability of diagnostic testing for atopic sensitization (e.g., skin prick testing). Nonetheless, diagnosed allergic diseases were associated with both recent allergic disease symptoms and total IgE, suggesting that, despite potential misclassification due to these limitations, both diagnosed allergic disease and total IgE capture real allergic disease.

We also acknowledge limitations in the variables used as indicators of early exposure to infectious agents. Both family size and earth house construction are indirect indicators, and thus may not perfectly reflect variation in early ID experience. Hospitalizations during infancy with infectious diseases are subject to the same potential misclassification due misdiagnosis by physicians and misreporting by parents as allergic disease diagnoses. None of these sources of misclassification is likely to introduce systematic bias, but should instead be expected to bias associations toward the null hypothesis.

We demonstrated a significant inverse association between family size and allergic disease akin to that consistently reported in the US and Europe (Strachan, 1989; Jarvis et al., 1997; Matricardi et al., 1998; Ball et al., 2000), but no significant association between family size and IgE. We also demonstrated a significant *positive* association between hospitalizations for infectious disease during infancy and diagnosed allergic disease, and a significant *positive* association between living in a house constructed from earth (compared to cement) and allergy (both diagnosed allergic disease and total IgE). The hygiene hypothesis finds some support in the positive association between family size and reported allergy diagnosis, but seems contradicted by the positive effects of hospitalization with an infection during infancy (a direct indicator of early life infectious

disease exposure) and earth housing materials (an indirect indicator of enhanced exposure to pathogens) on allergy. Consideration of the multiple mechanisms by which exposure to infectious agents may affect immune system development may help to reconcile these seemingly contradictory results.

Two hypotheses have been proposed to explain the effect of early exposure to infectious agents on allergic disease risk; these hypotheses are not mutually exclusive, and may indeed be considered complementary (Romagnani, 2006). The immune deviation hypothesis invokes the T-helper type 1/T-helper type 2 (Th1/Th2) balance. Th1-mediated immune responses are associated with viral, bacterial, and protozoan (predominantly intracellular infectious agents) infections and are thought to cause most, but not all, autoimmune diseases; Th2-mediated responses are associated with helminth infections and cause atopic diseases (Bach, 2002, 2005; Romagnani, 2006). Th1- and Th2-mediated immune responses are antagonistically regulated, such that the chemical mediators that promote Th1-mediated activity also inhibit Th2-mediated activity and *vice versa* (Pastorello et al., 1995; Janeway et al., 2001; Szabo et al., 2003). Infants are born with a bias toward Th2-mediated responses (Holt and Jones, 2000); exposure to intracellular infectious agents during immune system development has been hypothesized to protect against atopic disease by inducing Th1-mediated responses and shifting the immune system toward a lasting Th1 bias, inhibiting later Th2-mediated reactivity, including responses to allergens (Martinez and Holt, 1999).

The regulatory priming hypothesis suggests that stimulation of the developing immune system by exposure to infectious agents in early life promotes the development of more robust suppressive immune regulatory mechanisms. These mechanisms (including natural and inducible regulatory T cells) are capable of inhibiting both Th1- and Th2-mediated responses, and their role in preventing pathological responses to both autoantigens and allergens is clear (Mills and McGuirk, 2004). Early life infections may promote the later activity of suppressive regulatory T cells, lowering subsequent allergic disease risk.

Consideration of both of these mechanisms may help to explain our seemingly contradictory results. The protective effect of family size we observed may reflect either, or both, mechanisms at work in immune system development: children from large families likely experienced more frequent intracellular infections (e.g., respiratory infections acquired from siblings), hypothesized to decrease risk of allergic disease by promoting deviation toward a Th1 phenotype and/or stimulating more robust suppressive regulation.

The increased risk of diagnosed allergy associated with hospitalization for infection during infancy may capture and effect exclusive to severe infectious disease episodes. Severe infections have occasionally been shown to increase risk of allergic and autoimmune diseases, despite a protective effect of infections overall. For example, lower respiratory tract infections (LRTI, which include pneumonia, bronchitis, and croup) in infancy have been associated with asthma (Njå et al., 2003; Nafstad et al., 2005), and neonatal infections (McKinney et al., 1999) and some of the more severe childhood diseases (measles, mumps, rubella, chickenpox) increase risk of type 1 diabetes (Tenconi et al., 2007). It is possible that, while routine infections during infancy promote the development of robust suppressive regulation, severe infections result in *dysregulation*,

increasing risk of allergy and leading to the positive association between hospitalization for infection during infancy and childhood allergic disease we observed.

The increased risk of diagnosed allergy and elevated total IgE associated with living in a home constructed of earth may reflect the effect of helminth morbidity on immune system development. In rural Kilimanjaro, the variable earth housing materials likely reflects enhanced exposure to helminth pathogens. Many helminths, such as hookworm, are contracted directly from the soil, and children living in houses constructed from earth, with earth floors, may be at higher risk. Helminth morbidity may also be higher among children living in earthen houses due to a general effect of poverty (e.g., limiting resources available to purchase shoes, which can protect against soil-dwelling helminths, or limiting access to regular anti-helminth treatment). Helminths can have multiple and complex effects on the expression of allergy and total IgE, but generally, chronic helminth infection is expected to suppress manifestation of allergic disease (Yazdanbakhsh et al., 2002). However, efforts to disentangle effects of helminth morbidity *during immune system development* from the suppressive effects of helminth infection at the time of assessment for allergy have documented an *increased* risk of allergic disease (Wördemann et al., 2008) or sensitization (Gonzalez-Quintela et al., 2005) associated with early helminth infection (but see Rodrigues et al., 2008), even in the presence of decreased risk associated with current helminth infection (Wördemann et al., 2008). These studies suggest that early promotion of Th2-mediated reactivity due to early exposure to helminth infection increases risk of subsequent allergic disease. In our sample, children living in earth houses may have experienced more frequent episodes of helminth infection, or a higher burden of chronic helminth infection, during immune system development. This enhanced exposure to helminths may have promoted Th2-biased development, or counterbalanced the Th1-biasing effect of early life intracellular infections, resulting in higher risk of diagnosed allergic disease and higher total IgE.

In summary, we document some support for the hygiene hypothesis among children in rural Kilimanjaro: large family size was inversely associated with diagnosed allergic disease. We also report some unexpected results, which suggest that the epidemiology of allergy, especially with respect to the hygiene hypothesis, may be more complicated in the disease ecologies of East Africa than in the US and Europe. We found suggestive evidence of allergy-promoting effects of severe episodes of infectious disease during infancy, and early exposure to helminths: hospitalization during infancy with and infectious disease was positively associated with diagnosed allergic disease, and a home constructed of earth were positively associated with both total IgE and diagnosed allergic disease. The more complex infectious disease ecology of Kilimanjaro may provide an important opportunity to expand understanding of the role of exposure to infectious agents in immune system development.

Much research remains to be done to understand the epidemiology of allergic disease in rural African populations, especially the respective roles of intracellular and helminth infectious agents. The relatively high rate of allergic disease reported among our sample (~34%) speaks to the urgency of additional research. In particular, projects that better capture sensitization to allergens and allergic disease morbidity (e.g., via skin prick testing and evaluation of allergen-specific IgE), as well as research designed to

directly assess early exposure to infectious agents (e.g., via prospective monitoring of infants' infectious disease morbidity) are needed.

EXPANDING THE HYGIENE HYPOTHESIS: EARLY EXPOSURE TO INFECTIOUS AGENTS PREDICTS DELAYED-TYPE HYPERSENSITIVITY TO *CANDIDA* AMONG CHILDREN IN KILIMANJARO

Authors and institutions:
Katherine Wander[1]
Kathleen O'Connor[1, 2]
Bettina Shell-Duncan[1]

[1]Department of Anthropology, University of Washington, Seattle, WA 98195
[2]Center for Studies in Demography and Ecology, University of Washington, Seattle, WA 98195

Corresponding author:
Katherine Wander
Department of Anthropology
Box 353100
University of Washington
Seattle, WA 98195
Phone: 425-443-4230
Fax: 206-543-3285
Email: kwander@u.washington.edu

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Abstract

Background: Multiple lines of evidence suggest that infections in early life prevent the development of pathological immune responses to allergens and autoantigens (the *hygiene hypothesis*). Early infections may also affect later immune responses to pathogen antigen.

Methods: To evaluate an association between early infections and immune responses to pathogen antigen, delayed-type hypersensitivity (DTH) to *Candida albicans* was evaluated among 283 2-7 year old children in Kilimanjaro, Tanzania. A questionnaire and physical examination were used to characterize variables reflecting early exposure to infectious agents (family size, house construction materials, BCG vaccination, hospitalization history). Logistic regression was used to evaluate the association between early exposure to infectious agents and DTH to *C. albicans*.

Results: Triceps skinfold thickness (OR: 1.11; 95% CI: 1.01, 1.22) and age (OR: 1.27; 95% CI: 1.04, 1.55) were positively associated with DTH to *C. albicans*. Adjusted for age and sex, large family size (OR: 2.81; 95% CI: 1.04, 7.61), BCG vaccination scar (OR: 3.10; 95% CI: 1.10, 8.71), and hospitalization during infancy with an infectious disease (OR: 4.67; 95% CI: 1.00, 21.74) were positively associated with DTH to *C. albicans*.

Conclusions: Early life infections were positively associated with later DTH to *C. albicans*. This result supports an expansion of the hygiene hypothesis to explain not only pathological immune responses to allergens, but also appropriate immune responses to pathogens. Immune system development may be responsive to early infections as an adaptive means to tailor reactivity to the local infectious disease ecology.

Keywords: Hygiene hypothesis, immunocompetence, ecological immunity

Introduction

The *hygiene hypothesis* (Strachan, 1989) proposes that infections during early life shape the development of the immune system, preventing subsequent development of allergic disease. Since its initial publication, a large body of epidemiological evidence has accumulated in support of the hygiene hypothesis. Exposure to other children (presumably a source of transmission of infectious agents) through larger family size (Strachan, 1989; Jarvis et al., 1997; Matricardi et al., 1998; Ball et al., 2000; von Mutius, 2001; Bach, 2002; Matricardi, 2010), higher birth order (Matricardi et al., 1998), or day care attendance (Krämer et al., 1999; Ball et al., 2000; Haby et al., 2000; Infante-Rivard et al., 2001), is inversely associated with allergic disease; episodes of routine infection in early life (Illi et al., 2001; Njå et al., 2003; Williams et al., 2004; von Mutius et al., 1999; Calvani et al., 2002) and seropositivity to common viruses and bacteria (including hepatitis A virus, herpes simplex virus type 1, cytomegalovirus, *Toxoplasma gondii*, and *Helicobacter pylori*) are also associated with lower risk of allergic disease (Matricardi et al., 2000; Matricardi et al., 2002; Chen and Blaser, 2007; Janson et al., 2007). Although inconsistent findings have been reported, overall evidence strongly supports the hypothesis that early exposure to infectious agents mitigates subsequent allergic disease risk (for further review, see von Mutius, 2001; Bach, 2002; Matricardi, 2010).

The hygiene hypothesis has been expanded to explain risk of autoimmune disease, as well. Although some infections can potentiate autoimmune responses (e.g., *Streptococcus pyogenes* pharyngitis results in acute rheumatic fever in ~3% of untreated cases; Guilherme et al., 2005), in general, early life infections seem to be protective. For example, early day care attendance (Kaila and Taback, 2001) and older siblings or larger family size (Stene et al., 2001; Cardwell et al., 2005; Cardwell et al., 2008; D'Angeli et al., 2010) are inversely associated with type 1 diabetes; large family or household size are also inversely associated with multiple sclerosis (Ponsonby et al., 2005; Montgomery et al., 2004) and inflammatory bowel disease (Amre et al., 2006; Montgomery et al., 2002; Hampe et al., 2003; Koloski et al., 2008); and, *H. pylori* infection is inversely associated with inflammatory bowel disease (Koloski et al., 2008).

Despite the importance of immune responses to pathogens in combating infectious disease, and the clear evidence from the epidemiology of allergy and autoimmune disease that early infections impact later immune responsiveness, relatively few studies have investigated the impact of early exposure to infectious agents on later immune responses to *pathogens*. We hypothesize that immune system development responds adaptively to stimulation from infectious agents during infancy with enhanced immune responses to pathogen antigen during childhood. To test this hypothesis, we evaluated the effect of indicators of early infectious disease morbidity on delayed-type hypersensitivity (DTH) to *Candida albicans*, a ubiquitous fungal pathogen, among children in Kilimanjaro, Tanzania, a population subject to high infectious disease stress.

Delayed-type hypersensitivity testing introduces a small amount of pathogen antigen intradermally (under the most superficial layers of the skin). Local activation of an immune response by memory T cells results in the formation of induration and erythema at the site of the injection. *C. albicans* is the causative agent of oral candidiasis ("thrush") and vaginal candidiasis ("yeast infection"); among profoundly

immunocompromised individuals, *C. albicans* can cause candidemia, a potentially fatal systemic infection. Most individuals can be assumed to have formed immunological memory of *C. albicans*; immunocompetent individuals mount a cell-mediated DTH response upon skin testing with *C. albicans*, visible as an induration at the test site. Thus, DTH to the recall antigen *C. albicans* provides a means to evaluate cell-mediated reactivity to pathogen antigen. We evaluated family size and other indicators of elevated exposure to infectious agents during infancy as predictors of DTH to *C. albicans*.

Materials and Methods

Participants: This project was carried out in two adjoining villages in the Machame area of Kilimanjaro, Tanzania (the study villages are largely similar and children were not expected to differ in any systematic way). Infectious disease is quite common among children in Kilimanjaro, including viral, bacterial, protozoan, fungal, and helminth infectious agents. Children are exposed to infectious agents through multiple aspects of life in Kilimanjaro; some examples include bed-sharing with siblings and other children, interaction with household animals, contact with contaminated soil and water (rivers and irrigation ditches), and contact with insect vectors. Piped, treated drinking water was not available in the study area at the time of data collection. 314 2-7 year old children participated in the project, randomly sampled from a census of all 2-7 year old children in the study area. Children were invited to participate if they were living with at least 1 parent and had been living in the study area for at least six months.

Data collection: Data collection was conducted over the course of four weeks in spring of 2010. Children and their primary caregiving parents participated in two days of data collection. Data were collected at a healthcare facility belonging to Nshara Community Medical Centre (NCMC). Data collection was conducted by the lead author and 4 field assistants. Field assistants were residents of the study area and medical personnel (2 nurse/midwives, 1 nurse's assistant, 1 physician) who were trained in data collection techniques prior to data collection. Written informed consent was obtained from parents of all participating children. Procedures and data collection protocols were approved by the Institutional Review Board of the University of Washington and the Tanzanian National Institute for Medical Research (NIMR) and research permission obtained from the Tanzania Commission for Science and Technology (COSTECH).

On the first data collection visit, participating children's primary caregivers completed a questionnaire (in Swahili), providing household and family demographic information, as well as information about the child's medical history. Each child's finger was pricked with a sterile lancet to obtain capillary whole blood; blood was immediately tested for HIV (SD BioLine HIV-1/2 3.0 rapid HIV-1/2 test) and hemoglobin concentration (Hb; HemoCue Hb 201^{+} hemoglobin system). Additional blood was dropped on filter paper (Whatman #903 Protein Saver Cards) and stored as dried blood spots (DBS). The Candin (Allermed Laboratories, Inc., San Diego, CA) skin test for DTH to *C. albicans* antigen was administered intradermally on each child's forearm by injecting 0.1 mL under the most superficial layers of the skin with a 27 gauge tuberculin syringe. Children experiencing symptoms of infectious disease, HIV positive children, and anemic children were referred to NCMC for additional care.

On the second data collection visit, ~24 h after the first, the site of the Candin skin test was evaluated for the presence of an induration; the size of any induration measured in millimeters (mm) across two perpendicular diameters. The presence of a bacille Calmette-Guérin (BCG) vaccination scar on the child's shoulder was recorded. Weight was measured with a digital scale and height measured with an anthropometer. Mid-upper arm circumference (MUAC) was measured using a body tape. Triceps skinfold (TSF) thickness was measured using a Lange Skinfold Measurement Caliper (Graham-Field). All anthropometric measurements and Candin skin test evaluations were

performed by the lead author without knowledge of the child's interview data to maintain measurement consistency and avoid bias.

Laboratory analysis: DBS were allowed to dry at room temperature for < 24 hours, and were then frozen until shipped on dry ice to the University of Washington, where they were frozen until assay (~4 months). DBS were analyzed in the Biological Anthropology and Biodemography Laboratory at the UW for C-reactive protein (CRP) and α_1-acid glycoprotein (AGP, or orosomucoid), two acute phase reactants and indicators of infection. CRP was assessed with an in-house DBS assay described elsewhere (Brindle et al., 2010). AGP was assessed with a commercially available kit (GenWay Biotech), modified for use with DBS samples.

Data analysis: The outcome of interest, DTH to *C. albicans*, was defined as an induration size ≥ 5 mm in mean diameter (and anergy to *C. albicans* as induration < 5 mm). We identified four indicators of early exposure to infectious agents (predictors of interest): *Family size* was characterized as small (≤ 3 cohabiting siblings) or large (> 3 cohabiting siblings) based on the number of siblings living in the household at the time of participation in the project. *House construction materials* were categorized as earth or cement based on parents' report. *Hospitalization with an infectious disease during infancy* (before the first birthday) was assessed based on parents' report of the timing and diagnosis of any hospitalizations in the child's medical history. *BCG* was considered present if a BCG vaccination scar was observed during physical examination. Potential control variables were defined as follows: Age was calculated from the child's reported date of birth. Anthropometric measurements were used to define categorical variables for malnutrition. Z-scores were calculated (EPI INFO; Centers for Disease Control and Prevention, Atlanta, GA) for weight for height (WHZ), weight for age (WAZ), and height for age (HAZ). WHZ < -2 defined wasting and HAZ < -2 defined stunting. Elevated CRP and AGP values were used to identify acute infection (based on Wander et al., 2012). Breastfeeding and age at weaning were described based on parents' report. Anemia was defined based on Hb values following WHO guidelines (Nestel, 2002).

Data analysis was performed using Stata 11.2 software (Statacorp; College Station, TX). Logistic regression was used to test the predicted relationship between early life infectious disease variables and DTH. Significance was defined as $p \leq 0.05$. Age and sex were included in all models; additional control variables were retained in the model of early life predictors of DTH to *C. albicans* if their inclusion substantially altered the estimated effect of a predictor of interest (>20% change in odds ratio; this threshold was chosen to allow control for substantial confounding while preserving statistical power by limiting the number of variables included in the model).

Results

Complete information (excluding anthropometry and biomarkers analysis) was available for 283 children (age was unavailable for 17 children; information regarding siblings living in the household was unavailable for 2 children; information regarding BCG vaccination scar was missing for 2 children; and data from 10 children were excluded from analysis: 1 was HIV positive; 9 exhibited Candin skin test administration problems). Complete information including biomarkers of inflammation was available for 264 children; complete information including anthropometry was available for 240 children (43 children refused TSF measurement). Children from the two study villages were largely similar: significant differences were observed in only two variables (HAZ and house construction materials). As such, data from villages were pooled for all analyses.

Sample characteristics are described in Table 14. 54.42% of children were DTH positive to *C. albicans*, indicating cell-mediated immunocompetence. DTH to *C. albicans* did not substantially differ among the children for whom age was unknown (52.94% DTH positive) or TSF was not measured (46.51% DTH positive). Just over half (53.00%) of the sample was female.

Family size ranged from 0 to 6 siblings (in addition to the participating child), with 8.13% of children living in "large" families (>3 siblings). Just under half (48.76%) of participating children lived in houses constructed from earth. Most (93.29%) exhibited a discernible BCG vaccination scar. For those children hospitalized in the first year of life (17), 13 (4.59% of participating children) reported an infectious disease diagnosis (pneumonia, 10; malaria, 2; diarrhea, 1); the other four reported diagnoses were ambiguous or non-infectious in nature (chest tightness, stomachache, intussusception, and jaundice).

Table 15. Sample Characteristics

Sex	Female	150	53.00%
	Male	133	47.00%
Age in years, mean (SD)		4.50	(1.65)
House materials	Cement	145	51.24%
	Earth	138	48.76%
Coffee farming household		110	38.87%
Anthropometry	Wasting [a]	1	0.37%
	Stunting [b]	74	27.72%
	Triceps skinfold in mm, mean (SD)	11.73	(3.24)
Siblings living in the household, mean (range)		1.71	(0, 6)
Large family (> 3 siblings)		23	8.13%
BCG vaccination scar present		264	93.29%
Hospitalization in first year of life		17	6.01%
Hospitalization in first year of life for infectious disease [c]		13	4.59%
Fever (at the time of data collection)		7	2.47%
Elevated AGP or CRP [d] (at the time of data collection)		150	57.25%
Currently breastfeeding		21	7.42%
Mean age at weaning in years (SD) [e]		1.90	(0.74)
Anemic (at the time of data collection) [f]		89	31.56%
DTH to *Candida albicans* [g]		154	54.42%

[a] Weight for height Z-score < -2; information available for 270 children
[b] Height for age Z-score < -2; information available for 270 children
[c] Parents' report of physician's diagnosis of pneumonia, malaria, or diarrhea for hospitalization before 1 year of age
[d] C-reactive protein (CRP) > 1.1 mg/L or α_1-acid glycoprotein (AGP) > 0.76 g/L, indicative of acute infection (Wander et al., in press); information available for 264 children
[e] Among those children who are not currently breastfeeding
[f] Information available for 282 children
[g] Candin skin test; induration ≥ 5mm in diameter indicates positive delayed-type hypersensitivity (DTH)

Logistic regression results for known risk factors for immunosuppression and DTH to *C. albicans* are shown in Table 15. TSF (a measure of adiposity that is reduced in children undergoing protein energy malnutrition) and age were both positively associated with DTH to *C. albicans*. Infection, as indicated by elevated acute phase reactants (CRP and AGP), fever (data not shown), or reported recent symptoms (data not shown) was not a significant predictor of DTH to *C. albicans*; use of alternative biomarker definitions of acute infection did not alter these results.

Table 16. Predictors of Delayed-Type Hypersensitivity (DTH) to *Candida*

Variable	OR	95% CI	p-value
Male sex	1.29	0.74, 2.24	0.369
Age in years	1.27	1.04, 1.55	0.017
Triceps skinfold thickness in mm	1.11	1.01, 1.22	0.029
Elevated CRP or AGP [a]	0.87	0.51, 1.50	0.623

[a] C-reactive protein (CRP) > 1.1 mg/L or α_1-acid glycoprotein (AGP) > 0.76 g/L, indicative of acute infection (Wander et al., 2012)

Logistic regression results for early life factors and DTH to *C. albicans* are show in Table 16. Adjusted for age and sex, large family size, BCG vaccination scar, and hospitalization in infancy with an infectious disease diagnosis were significantly positively associated with DTH to *C. albicans*. Large family size was associated with 2.8-fold higher odds of DTH to *C. albicans*; a BCG vaccination scar with 3.1-fold higher odds of DTH to *C. albicans*; and, hospitalization with an infectious disease diagnosis during infancy with 4.7-fold higher odds of DTH to *C. albicans*. Housing materials were not a significant predictor of DTH to *C. albicans* (data not shown). When family size was defined by the number of other children, rather than siblings, living in the household, results were similar (OR for greater than three other children: 2.13; 95% CI: 0.99, 4.58). Consideration in separate models, adjusted for age and sex, did not substantially alter the magnitude or significance of these associations (although the association between family size and DTH was of marginal significance; data not shown), nor did additional adjustment for on-going acute infection (elevated CRP or AGP), TSF, HAZ, WHZ, breastfeeding/age at weaning, or anemia (data not shown).

Table 17. Early Life Predictors of Delayed-Type Hypersensitivity (DTH) to *Candida*

Variable	OR	95% CI	p-value
Male sex	1.03	0.64, 1.67	0.899
Age in years	1.11	0.96, 1.29	0.155
Large family size (>3 siblings living in the home)	2.81	1.04, 7.61	0.041
BCG vaccination scar	3.10	1.10, 8.71	0.032
Hospitalization during infancy with an infectious disease [a]	4.67	1.00, 21.74	0.050

[a] Pneumonia, malaria, or diarrhea, according to parents' report

Conclusions

We document positive associations between three factors reflecting early life exposure to infectious agents—family size, BCG vaccination scar, and reported hospitalization with an infectious disease diagnosis in infancy—and DTH to *C. albicans*, an indicator of cell-mediated immunocompetence.

Just over half (54.42%) of participating children exhibited DTH to *C. albicans*; this is comparable to observations among children in other East African populations (e.g., Shell-Duncan and Wood, 1997). To verify DTH to *C. albicans* as an indicator of cell-mediated immunocompetence, we assessed its association with known risk factors for suppressed cell-mediated immunity: male sex (Washburn et al., 1965; Pinner et al., 1996), young age (Kniker et al., 1985), protein-energy malnutrition (Neumann et al., 1975; Zaman et al., 1997; Shell-Duncan, 1997), and acute infection (Shell-Duncan, 1997). DTH to *C. albicans* did not differ significantly by sex, potentially due to the young age of the participating children (excess male infectious disease morbidity and mortality may not begin until adolescence; Owens, 2002). As expected, age and adiposity (TSF) were positively associated with DTH. Acute infection was not associated with DTH. With the exception of acute infection, these results are consistent with expectations for a marker of cell-mediated immunity and support the interpretation of DTH to *C. albicans* as an indicator of cell-mediated immunocompetence.

Delayed-type hypersensitivity to *C. albicans* is dependent on both immunological memory of *C. albicans* and intact cell-mediated immunity. In employing the Candin skin test, we assume that *C. albicans* is ubiquitous in the environment and that virtually all subjects have had the opportunity to form immunological memory of *C. albicans* antigen. If this assumption is faulty, it is possible that significant predictors of DTH to *C. albicans* reflect variability in exposure to *C. albicans*, rather than variability in immunocompetence. We are confident in our interpretation of DTH to *C. albicans* as a marker of immunocompetence for two reasons. First, it varies with known predictors of cell-mediated immunity (age and adiposity). Second, it is unlikely that the common factor between the predictors of interest (large family size, BCG vaccination scar, and history of hospitalization with an infection during infancy) is enhanced exposure to *C. albicans*. Exposure to infectious agents in early life, with lasting effects on later cell-mediated DTH, is a more likely explanation for the independent effects of these three variables.

In keeping with the hygiene hypothesis (Strachan, 1989; Jarvis et al., 1997; Matricardi et al., 1998; Ball et al., 2000; Stene et al., 2001; von Mutius, 2001; Bach, 2002; Montgomery et al., 2002, 2004; Hampe et al., 2003; Cardwell et al., 2005, 2008; Ponsonby et al., 2005; Amre et al., 2006; Koloski et al., 2008; D'Angeli et al., 2010; Matricardi, 2010), we interpret family size as an indirect indication of the infectious disease experience of early childhood: siblings are likely an important source of infectious disease transmission, and those from larger families likely experienced more frequent infections. Thus, the association between large family size and DTH to *C. albicans* can be interpreted as a positive effect of early life infections on later cell-mediated immunocompetence. We recognize limitations in family size as a predictor variable. To obtain accurate information about family size, we inquired about household composition at the time of the study, and categorized family size based on *currently*

cohabiting siblings. However, it is the presence of other children in the household *during infancy* that is of interest. Household composition at the time of data collection is unlikely to perfectly reflect household composition during the period of interest, as household composition in this society can be fluid, with children and adults moving between households for a variety of reasons. We chose to base the family size variable on currently cohabiting siblings (rather than all currently cohabiting children) as a means to count only those children most likely to have been a part of the household during the participating child's infancy. The family size variable, although subject to the limitations discussed here, also has an important advantage: it allowed us to demonstrate a parallel between the epidemiology of immune-mediated disease (inversely associated with siblings) and DTH to *C. albicans* (positively associated with siblings).

Bacille Calmette-Guérin vaccination scars and reported hospitalizations we interpret as two direct measures of exposure to infectious agents. BCG vaccine is usually administered in the first week of life and exposes infants to an attenuated strain of *Mycobacterium tuberculosis*. We use BCG vaccination scar, rather than parents' report or medical records (maternal-child health clinic cards, which were often unavailable), in hopes of most accurately distinguishing those few children who were not exposed to BCG as infants. The positive association between BCG vaccination scar and DTH may reflect an effect of BCG vaccination on immune system development, or may reflect immune characteristics already in place during infancy (i.e., malnourished newborns or those with a genetic predisposition to anergy may fail to form a scar after BCG vaccination *and* may fail to exhibit DTH to *C. albicans* at age 2-7 yo). This limits interpretation of the positive association between BCG scar and DTH we observed. However, such confounding does not explain the other early life predictors of DTH to *C. albicans* we report. Indeed, such an explanation would predict an *inverse* association between hospitalization and DTH, rather than the positive association we observed, as children with innate predisposition to anergy would be more susceptible to severe infectious disease during infancy.

Although parents' report of many direct measures of early exposure to infectious agents may be unreliable (e.g., routine infectious disease episodes, vaccinations), we chose to inquire into hospitalizations during the first year of life, as these were likely to be more serious, and therefore memorable, events for parents. Relying on parents' memory of the hospitalization and accurate reporting of the physician's diagnosis may well introduce classification error in both the hospitalization timing and diagnosis; however, any misclassification of hospitalizations due to inaccurate reporting by parents is unlikely to be biased with respect to DTH to *C. albicans*, and thus was unlikely to unduly affect our results.

In summary, by both indirect and direct measures, we demonstrate that exposure to infectious agents in early life is positively associated with DTH to *C. albicans* (indicative of intact cell-mediated immunity) during childhood. This result is consistent with findings from allergy and autoimmune disease epidemiology suggesting that early infections influence immune-mediated disease risk. Our results suggest expansion of the hygiene hypothesis beyond allergy and autoimmune disease: In addition to inhibiting pathological immune responses to allergens and autoantigens, stimulation by early infections may promote cell-mediated immune responses to pathogen antigen. This effect

may represent an adaptive mechanism in immune system development—"priming" by exposure to infectious agents during infancy may enhance cell-mediated immune responses to infectious agents encountered later in childhood, mitigating infectious disease risk.

Our data cannot directly evaluate the mechanisms that may underlie this priming; however, our results are consistent with the hypothesis that early exposure to infectious agents stimulates deviation toward T-helper type 1 (Th1)-mediated responses (including protective responses against viral, bacterial, and protozoan infectious agents and many pathological responses against autoantigens) and away from T-helper type 2 (Th2)-mediated responses (including protective responses against helminthic agents and pathological responses against allergens; Martinez and Holt, 1999). DTH responses are largely, but not exclusively, dependent on the Th1 mediator interferon-γ, and thus reflect Th1-mediated reactivity to pathogen antigen (Black, 1999). Early stimulation from viral, bacterial, and protozoan agents may stimulate deviation toward stronger Th1 responses (increasing DTH reactivity to *Candida*) and away from Th2 responses (decreasing risk of allergy). However, recent evidence suggests that priming of regulatory mechanisms that act against both Th1- and Th2-mediated responses may underlie the inverse association between early exposure to infectious agents and allergy (as well as autoimmune disease) (Wills-Karp et al., 2001; Maziels, 2005). As such, early exposure to infectious agents may impact allergic disease risk and DTH via distinct mechanisms. It is likely that infectious agents interact with the developing immune system in multiple and complex ways; this area is ripe for additional research.

DISCUSSION

This work set out to explore the evolutionary context of the *hygiene hypothesis* (that exposure to infectious agents during immune system development shapes future immune reactivity to decrease risk of allergic disease) by expanding the hypothesis to explain not only variation in allergy, but immune reactivity to *pathogens*, or immunocompetence. To this end, data were collected among 2-7 year old children in Kilimanjaro, Tanzania, to address three questions: 1) *How is immunocompetence best characterized?*; 2) *Is early exposure to infectious agents inversely associated with allergy among children in Kilimanjaro?*; and, 3) *Is early exposure to infectious agents positively associated with immunocompetence among children in Kilimanjaro?* In summary, results suggest that:

1. DTH to Candida albicans *is a useful biomarker of cell-mediated immunocompetence:* Immunocompetence, or the capacity to mount an appropriate and protective immune response to pathogen antigen, is a difficult concept to operationalize. No single, "gold standard" measure of immunocompetence exists. We evaluated two putative biomarkers of immunocompetence, DTH to *Candida* and EBV Ab. DTH to *Candida* was positively associated with age and adiposity, as expected for a biomarker of immunocompetence. EBV Ab did not conform to expectations for a biomarker of immunocompetence, and was not significantly associated with DTH to *Candida*. These results support the use of DTH to *Candida*, but not EBV Ab, as a biomarker of immunocompetence among East African children.

2. Some aspects of early exposure to infectious agents are inversely associated with allergy among children in Kilimanjaro, others are positively associated with allergy; this likely reflects the complexity of the infectious disease ecology of rural Kilimanjaro: Large family size, likely reflecting more frequent infections during infancy, acquired from siblings, was associated with lower risk of diagnosed allergy. This is consistent with observations in the US and Europe, and consistent with the hygiene hypothesis. Earth housing materials, a variable that potentially captures elevated helminth morbidity during immune system development, was associated with higher risk of diagnosed allergy and higher total IgE. This is consistent with studies suggesting that early helminth morbidity may increase risk of allergy (likely by promoting Th2-mediated reactivity), and may be consistent with hypothesized priming of the Th1/Th2 balance during immune system development. Hospitalization in infancy with an infection was associated with higher risk of diagnosed allergy. This is consistent with evidence from western populations that severe episodes of infection are associated with asthma; this effect seems to be exclusive to severe episodes of infectious disease (which may act to disrupt, rather than prime, immune system development, resulting in dysregulation of pathological immune responses). The positive associations between some indicators of early exposure to pathogens and allergy risk seem, on their face, inconsistent with the hygiene hypothesis. However, closer examination reveals that these findings complicate, rather than contradict, the hygiene hypothesis—it is likely that some aspects of early exposure to infectious agents (e.g., viral and bacterial respiratory infections, acquired from siblings) decrease risk of allergy, while others (e.g., higher helminth morbidity resulting from dwelling in a house constructed of earth, or severe bouts of infection

resulting in hospitalization) increase risk of allergy. This increasing complexity should perhaps not be surprising, given the tremendously complex infectious disease ecology of Kilimanjaro, compared to that of the US and Europe.

3. Early exposure to infectious agents is positively associated with DTH to Candida albicans *among children in Kilimanjaro, supporting expansion of the hygiene hypothesis to explain variation in immunocompetence as well as allergy and autoimmune disease:* The impact of exposure to infectious agents during immune system development seems to extend beyond allergy and autoimmune disease risk, to affect immune responses to pathogens, as well. Three indicators of exposure to infectious agents in early life, large family size, BCG vaccination scar, and hospitalization in infancy with an infection, were positively associated with DTH to *Candida*. This suggests that both routine episodes of infection (reflected in the family size variable) and severe episodes of infection (reflected in the hospitalization variable) may act to prime immune reactivity to pathogen antigen during immune system development. The only early infection exposure we examined that was not associated with DTH to *Candida* was earth housing materials, which we interpret as an indicator of primarily helminth morbidity during early life. These results are consistent with the hypothesis that the Th1/Th2 balance of the developing immune system responds adaptively to early exposure to infectious agents, deviating toward a Th1 bias in the presence of strong stimulation from viral, bacterial, and protozoan (largely intracellular) infectious agents: DTH to *Candida* (interferon-γ dependent and Th1-mediated) is positively associated with early exposure to intracellular agents but not with early exposure to helminth agents.

On its face, the finding that the prevalence of allergy is as high in Kilimanjaro (~30% of 2-7 year olds) as in many western, industrialized populations is inconsistent with the hygiene hypothesis—the tremendously high burden of infectious disease experienced by children in Kilimanjaro should, according to the hygiene hypothesis, protect them against allergy. Rates of allergic disease in Kilimanjaro should be lower than in western populations, roughly in proportion to the difference between populations in infectious disease burden. Although this population-level comparison seems to contradict the hygiene hypothesis, individual-level analyses provide evidence that the hygiene hypothesis is at work in Kilimanjaro—early exposure to infectious agents shapes allergy risk, with routine exposure to infectious agents protecting against allergy. The complexities of allergy epidemiology in Kilimanjaro, due to the regions complex infectious disease ecology, expand understanding of the role of pathogens in immune system development and subsequent allergic disease risk. Our novel finding that early exposure to infectious agents also predicts DTH to pathogen antigen (*Candida*) also supports and expands on, the hygiene hypothesis.

As discussed at the outset, adaptive priming of two regulatory mechanisms—the Th1/Th2 balance and suppressive regulation—may be at work in immune system development to minimize the intertwined risks of susceptibility to infection and immune-mediated damage and disease. Understanding such adaptive priming requires examination of the effects of early exposure to multiple types of infectious agents (i.e., intracellular; helminth) on later immune responses to multiple types of antigens (i.e., Th1 responses to intracellular agents and autoantigens; Th2 responses to helminths and

allergens). This project took the first step to provide such an examination. Table 17 presents a summary of findings regarding early exposure to infectious agents and later immune reactivity. Limitations to both predictors and outcomes have been acknowledged, and further research will be necessary to verify and expand on these findings. However, some comparison of these results with the expectations laid out in the introduction (Table 4) is possible.

Table 18. Summary of Observed Effect of Early Life Exposure to Infectious Agents

Predictor	Cell-mediated immunity	Allergic disease morbidity
Large family size	↑	↓
Earth house construction materials	—	↑[a]
Hospitalization in infancy with an infection	↑	↑
BCG vaccination scar	↑	—

[a] Both diagnosed allergic disease and total immunoglobulin E

Table 18 compares the results of this project with expectations from Table 4. The early conditions (*Very few or no intracellular infections during infancy*; and, *Few infections during infancy; predominantly intracellular*) and expected outcomes (immune responses to *Autoantigens* and *Helminths)* this project was not able to address have been removed from the table. Variables indicating early exposure to infectious agents (predictors of interest) from this project are listed with the early infectious disease condition to which they most likely correspond (e.g., *Earth house materials* likely reflects elevated helminth morbidity during early life), and the observed effects are depicted in the columns to the far right. The direction of the arrows represents the observed association (e.g., *Earth house materials* were positively associated with immune responses to *allergens*) and the color represents the hypothesis (Suppressive Regulation; T-helper type 1/T-helper type 2; Either/Both) supported by the observation.

Table 19. Observed Effects of Priming on Later Immune Responses

Early ID experience	Priming mechanism		Lasting effect on responses to antigens	
	Suppressive Regulation	T-helper type 1/ T-helper type 2	Allergens	Viruses, bacteria, protozoa
Routine infections during infancy; predominantly helminths *Earth house materials*	Strong regulation	Th2 bias	↑	
Routine infections during infancy; predominantly intracellular *Large family size* *BCG vaccination scar*	Strong regulation	Th1 bias	↓	↑
Severe infection(s) during infancy (e.g., malaria, pneumonia) *Hospitalization in infancy*	Dysregulation	Th1 bias	↑	↑

These results provide stronger evidence for priming of the Th1/Th2 balance than suppressive regulation, but do not contradict either hypothesis. The summary in Table 18 suggests that routine exposure to intracellular infectious agents in early life (large family size and BCG vaccination scar) promotes Th1-mediated responses to pathogen (cell-mediated immunocompetence, or DTH to *Candida*) and inhibits Th2-mediated responses to allergen (diagnosed allergic disease), while exposure to helminths in early life (earth housing materials) promotes Th2-mediated responses to allergens (diagnosed allergy and total IgE) and is unrelated to Th1-mediated responses to pathogens. Severe episodes of infection in early life (resulting in hospitalization in infancy) are associated with both Th1-mediated responses to pathogen (DTH to *Candida*) and Th2-mediated responses to allergen (diagnosed allergy): this may represent lower levels of suppressive regulation (leading to higher immune reactivity overall), or may reflect both a Th1-biasing effect of early morbidity due to intracellular agents (leading to stronger DTH) *and* a dysregulatory effect of severe infection (leading to stronger Th2-mediated responses and allergy).

Priming of the Th1/Th2 balance, promoting a lasting Th1 bias in response to stimulation from viruses, bacteria, and protozoa and a lasting Th2 bias in response to stimulation from helminths, may shape immune responses to the local infectious disease ecology, enhancing immunocompetence and minimizing susceptibility to infection. This mechanism may explain the unexpected pairing of high rates of infectious *and* allergic disease among Kilimanjaro children—the high burden of viral, bacterial, and protozoan

infections during immune system development, which have the effect of decreasing the prevalence of childhood allergy, may be counterbalanced in this setting by the high burden of helminth infection during immune system development, which may have the effect of promoting Th2-mediated reactivity and increasing the prevalence of childhood allergy. Thus, the high overall burden of infectious disease, due to a high burden of helminths in particular, may result in a high prevalence of allergic disease among children, even as most infectious diseases (routine viral and bacterial infections) protect children against allergy.

While *much* additional research is needed, these findings are consistent with multiple adaptive mechanisms working in immune system development. In addition to seeking to elucidate how priming of Th1/Th2 regulation and suppressive regulation act independently in immune system development, future research should explore the ways in which these mechanisms may work in synergy to balance the multiple threats of infection and immune-mediated damage and disease.

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APPENDIX: QUESTIONNAIRE

IMMUNOCOMPETENCE AND THE HYGIENE HYPOTHESIS			
Participant ID:		Date (day 1 data collection):	Mo: Day: Year:
Completed by:		Caregiver's relationship to enrolled child:	
Previous mebendazole tx:	Yes No	History of hypersensitivity following mebendazole tx:	Yes No
Mebendazole administered:	Yes No	Aching, vomiting, diarrhea:	Present Absent
Village:		Neighborhood:	
Balozi:		Current school:	
Primary healthcare facility:		Grade:	NA 1 2 3 4 5 6 7
Ethnic group:		Religion:	Muslim Christian Other
Sex:	Male Female	Age:	Years: Months:
Date of birth (reported):	Mo: Day: Year:	Clinic card available:	Yes No
Date of birth (clinic card):	Mo: Day: Year:	Birthweight (clinic card):	
Previous DTH skin testing:	Yes No	History of anaphylaxis following skin testing:	Yes No
Currently breastfeeding:	Yes No	If "No", age at weaning:	
Birth order (out of all live births):		Number of living siblings (total):	
Age of immediate younger living sibling:		Age of immediate older living sibling:	
Date of birth of immediate younger living sibling:	Mo: Day: Year:	Date of birth of immediate older living sibling:	Mo: Day: Year:
Number of children living in the household (for at least six months):		Number of siblings living in the household (for at least six months):	
Physician's diagnosis of allergic rhinitis:	Yes No	Physician's diagnosis of food allergy:	Yes No
Physician's diagnosis of asthma:	Yes No	Food allergy (1):	
Physician's diagnosis of eczema:	Yes No	Food allergy (2):	
Symptoms of allergic rhinitis within the last year:		Symptoms of atopic dermatitis in the last year:	
Sneezing without fever:	Yes No	Itchy skin rash:	Yes No
Runny nose without fever:	Yes No	Dry, flaking:	Yes No
Sinus pain:	Yes No	Swollen:	Yes No
Sore throat:	Yes No	Oozing:	Yes No
Itchy eyes:	Yes No	Recurring:	Yes No
Watery eyes:	Yes No	Location (most recent):	
Red eyes:	Yes No	Duration (most recent):	
Symptoms vary by season:	Yes No	Itch or rash following contact with an irritant:	Yes No
Worst season/month:		Irritant:	
Symptoms of asthma within the last year:		Hospitalizations:	
Wheezing:	Yes No	Age:	
Coughing without fever:	Yes No	Condition:	
Shortness of breath:	Yes No	Age:	
Trouble breathing:	Yes No	Condition:	
Cheat tightness:	Yes No	Age:	
Symptoms worse at night:	Yes No	Condition:	
Symptoms during cold:	Yes No	Age:	
Symptoms after exercise:	Yes No	Condition:	

Illness in the last two weeks:		Physician visit in the last two weeks:	
Coughing:	Yes No	Hospital:	Yes No
Sneezing:	Yes No	Clinic:	Yes No
Runny nose:	Yes No	Pharmacy:	Yes No
Sore throat:	Yes No	Diagnosis given (1):	
Fever:	Yes No	Diagnosis given (2):	
Diarrhea:	Yes No	Diagnosis given (3):	
Vomiting:	Yes No	Medication prescribed (1):	
Stomachache:	Yes No	Medication prescribed (2):	
Headache:	Yes No	Medication prescribed (3):	
Weakness or fatigue:	Yes No	Medication prescribed (4):	
Consumes fresh milk:	Never Very rarely Monthly Weekly Daily	Other milk products:	Never Very rarely Monthly Weekly Daily
Agriculture (Yes if anyone in the child's household participates in the activity):		Domesticated animals (Yes if they are owned by anyone in the child's household *and* kept on the property):	
Coffee farming:	Yes No	Domestic cats:	Yes No
Bean farming:	Yes No	Domestic dogs:	Yes No
Banana farming:	Yes No	Domestic chickens/ducks:	Yes No
Corn farming:	Yes No	Goat/sheep husbandry:	Yes No
Pesticide used on coffee:	Yes No	Cattle husbandry:	Yes No
Name:		Number of cattle:	
Pesticide used on corn:	Yes No	Meat cattle:	Yes No
Name:		Milk cows:	Yes No
Housing materials:	Earth Wood Cement	Electricity in the home:	Yes No
Number of rooms in house:		Number of buildings:	
Grandparents part of household:	Yes No	Aunts/uncles part of household:	Yes No
Mother's occupation:	Housewife Farmer Employed by an individual Employed by a company Fundi Business owner Other	Father's occupation:	None Farmer Employed by an individual Employed by a company Fundi Business owner Other
Other:		Other:	

CURRICULUM VITAE

EDUCATION

The University of Washington
Master of Arts, 2006; Doctor of Philosophy, 2012: Biocultural Anthropology
Dissertation: *Immunocompetence and the Hygiene Hypothesis*
Master of Public Health, 2009: Epidemiology
Thesis: *The Evolutionary Epidemiology of Iron Deficiency*

The Ohio State University
Bachelor of Arts, 2001: Anthropology (minor: Evolution, Ecology, and Organismal Biology)

TEACHING AND RESEARCH INTERESTS

Epidemiology, infectious disease ecology, immune function, growth and development, nutrition, female circumcision, East Africa, West Africa

RESEARCH EXPERIENCE

2011-present	Research Assistant, *Contingency and Change in the Practice of Female Genital Cutting: Dynamics and Decision-Making in Senegambia:* Preparing study results for dissemination, including meeting and conference presentations and peer-reviewed journal articles (Principal Investigator: Bettina Shell-Duncan).
2007-present	Principal Investigator, *Immunocompetence and the Hygiene Hypothesis:* Evaluating the role of early life infectious disease exposure in immune system development among children in Kilimanjaro, Tanzania.
2008-2009	Research Assistant, *Contingency and Change in the Practice of Female Genital Cutting: Dynamics and Decision-Making in Senegambia:* Described trends and changes in the practice of female genital cutting (FGC), evaluated the role of motivational balance and contingency in the process of change in FGC, and tested hypotheses explaining the perpetuation of FGC among women in Senegal and The Gambia (Principal Investigator: Bettina Shell-Duncan).
2005-2007	Principal Investigator, *C-Reactive Protein Across the Menstrual Cycle:* Evaluated the effect of changes in concentration of endogenous hormones, menses, and ovulation on circulating concentration of C-reactive protein, a biomarker of inflammation.
2004-2005	Research Assistant, *Contingency and Change in the Practice of Female Genital Cutting: Dynamics and Decision-Making in Senegambia:* Assessed the decision-making process surrounding FGC, the applicability of a stage of change model to FGC, and identified appropriate stages of change among men and women in Senegal and The Gambia (Principal Investigator: Bettina Shell-Duncan).

TEACHING EXPERIENCE

Fall, 2011	Presenter, *Pedagogy in Anthropology (Managing a classroom: authority, rapport, and credibility):* Training for new anthropology teachers in managing introductory-level classrooms, including lecture, discussion section, and laboratory settings; covers effective communication in the classroom, establishing authority and minimizing discipline problems; focuses on pedagogical issues unique to anthropology.
Spring, 2011	Teaching Assistant, *Biocultural Anthropology 201, Principles of Biological Anthropology:* Foundational course in biocultural anthropology; covers evolution and adaptation of the human species, evidence from fossil record and living populations of monkeys, apes, and humans, and the role of natural selection in shaping human evolutionary past, present, and future.
Winter, 2011	Teaching Assistant, *Biocultural Anthropology 369, Origins and Evolution of Human Diseases:* Elective course for advanced anthropology undergraduates; surveys the interaction between human hosts and infectious agents, covering host-pathogen coevolution, the emergence of infectious and chronic diseases, and multiple sources of evidence routinely used in the study of modern and ancient human disease.
Fall, 2010	Co-Instructor, *Biocultural Anthropology 473, Biological Adaptability of Human Populations:* Core course in biocultural anthropology; covers human adaptation to environmental stressors, adaptability across the lifecourse, and the application of evolutionary principles to understanding human health and disease.
Winter, 2010	Teaching Assistant, *Biocultural Anthropology 201, Principles of Biological Anthropology*
Fall, 2009	Teaching Assistant, *Biocultural Anthropology 101, Human Biological Diversity:* Survey course in biocultural anthropology; explores human biological variation, introduces the theory of evolution through natural selection.
Summer, 2009	Mentor, *Anthropology 499 (independent study):* Mentored an advanced undergraduate anthropology major in the conduct of a literature review of inflammation, biomarkers of inflammation, and variation in background levels of inflammation.

CONSULTANCIES

Spring, 2010:	UNICEF, *Analytical report on Female Genital Mutilation/Cutting:* Participated in the formulation of an analysis plan for a UNICEF publication on the current global state of female genital cutting (FGC).
Fall, 2009:	World Health Organization, *Contingency and Change in the Practice of Female Genital Cutting: Dynamics and Decision-Making in Senegambia:* Presented study findings to representatives from WHO, government agencies, and non-governmental organizations in Senegal

and The Gambia; discussed the relevance of the study findings for interventions aimed at eliminating FGC in the Senegambia.

PUBLICATIONS

Wander K. In prep. Comparison of delayed-type hypersensitivity to *Candida albicans* and Epstein-Barr virus antibody as biomarkers of immunocompetence among children in Kilimanjaro, Tanzania. Target journal: *Am J Phys Anthropol.*

Wander K. In prep. Early life exposure to infectious agents predicts diagnosed allergic disease and total immunoglobulin E among children in Kilimanjaro, Tanzania. Target journal: *Trop Med Int Health.*

Wander K. Submitted. The Hygiene Hypothesis in Evolutionary Context. *Evol Anthropol.*

Wander K, O'Connor KA, Shell-Duncan B. 2012. Expanding the Hygiene Hypothesis: Early Exposure to Infectious Agents Predicts Delayed-Type Hypersensitivity to *Candida* among Children in Kilimanjaro. *PLoS ONE* 7(5): e37406.

Wander K, Brindle E, O'Connor KA. In press. Sensitivity and specificity of C-reactive protein and α_1-acid glycoprotein for episodes of acute infection among children in Kilimanjaro, Tanzania. *Am J Hum Biol.*

Shell-Duncan B, Wander K, Hernlund Y, Moreau A. 2011. Dynamics of change in the practice of female genital cutting in Senegambia: Testing predictions of social convention theory. *Soc Sci Med* 73:1275-1283.

Shell-Duncan B, Hernlund Y, Wander K, Moreau A. 2010. Contingency and Change in the Practice of Female Genital Cutting: Dynamics of Decision Making in Senegambia. Summary Report to the World Health Organization. http://csde.washington.edu/~bsd/FGC/

Wander K, Shell-Duncan B, McDade TW. 2009. Evaluation of iron deficiency as a nutritional adaptation to infectious disease: an evolutionary medicine perspective. *Am J Hum Biol* 21(2):172-179.

Wander K, Brindle E, O'Connor KA. 2008. C-reactive protein across the menstrual cycle. *Am J Phys Anthropol* 136(2):138-146.

PRESENTATIONS

Shell-Duncan B, Wander K. Theoretical and Empirical Dimensions of Behavior Change with Respect to Female Genital Cutting: The Senegambia Study. Biocultural Anthropology Seminar Series, University of Washington, October 2011.

Wander K. Immunocompetence and the hygiene hypothesis. Annual Meeting of the American Association of Physical Anthropologists, April 2011. *Am J Phys Anthropol* 144(S52):304.

Wander K, Shell-Duncan B, Hernlund Y. Legislating change?: Community responses to the law banning female genital cutting in Senegal. Annual Meeting of the Society for Applied Anthropology, March 2011.

Shell-Duncan B, Hernlund Y, Wander K. Women's business?: Reassessing the role of men in the perpetuation and abandonment of female genital cutting. Annual Meeting of the Society for Applied Anthropology, March 2011.

Wander K. Immunocompetence and the hygiene hypothesis. Biocultural Anthropology Seminar Series, University of Washington, November 2010.

Wander K. How well do C-reactive protein and α_1-acid glycoprotein identify acute infection among children in Kilimanjaro, Tanzania? Biomarker Methods Research Group, University of Washington, November 2010.

Wander K. Family size and immunocompetence: Insight from evolutionary medicine. Annual Meeting of the Human Biology Association, April 2009. *Am J Hum Biol* 21(2):245-275.

Wander K, Brindle E, O'Connor KA. C-reactive protein across the menstrual cycle. Annual Meeting of the Human Biology Association, March 2006. *Am J Hum Biol* 18(2):250-284.

Hernlund Y, Shell-Duncan B, Wander K. "One blade per girl": AIDS education and the inadvertent medicalization of female genital cutting in The Gambia. Annual Meeting of the Society for Applied Anthropology, March 2006.

AWARDS AND HONORS

2004	Top Scholar Award, Graduate School Fund for Excellence & Innovation and the Graduate Opportunities & Minority Achievement Program, University of Washington

FELLOWSHIPS

Pending	National Institutes of Health National Research Service Award Postdoctoral Fellowship
2009-2010	Daris R. Swindler Memorial Fellowship
2005-2008	National Science Foundation Graduate Research Fellowship

GRANTS

2010	Doctoral Dissertation Improvement Grant, National Science Foundation
2009	Dissertation Fieldwork Grant (and Osmundsen Initiative supplement), Wenner-Gren Foundation
2007	Lewis and Clark Fund for Exploration and Field Research, American Philosophical Society
2007	Pre-dissertation Research Fund, Department of Anthropology, University of Washington

SERVICE

Committee Membership

2009-present	Member, Anthropology Department Resources Committee
2006-2008	Member, Anthropology Graduate Activities Fund Committee
2005-2008	Member, Anthropology Department Development Committee
2005-2006	Chair, Anthropology Graduate Activities Fund Committee

Journal Article Review
American Journal of Human Biology
Archives of Gynecology and Obstetrics

PROFESSIONAL AFFILIATIONS

American Association of Physical Anthropologists; Human Biology Association; Society for Applied Anthropology

OTHER AFFILIATIONS

Center for Studies in Demography and Ecology (UW); American Association of University Women; Sigma Xi

LANGUAGES

Swahili: excellent speaking and moderate writing skills

COMPUTER SKILLS

Stata; SAS; Atlas ti

REFERENCES

Bettina Shell-Duncan
Professor of Anthropology and Chair, Department of Anthropology
Adjunct Professor of Global Health, Department of Global Health
University of Washington, Box 353100
Seattle, Washington 98195
bsd@u.washington.edu

Kathleen O'Connor
Associate Professor of Anthropology, Department of Anthropology
University of Washington, Box 353100
Seattle, Washington 98195
oconnork@u.washington.edu

Steven Goodreau
Associate Professor of Anthropology, Department of Anthropology
University of Washington, Box 353100
Seattle, Washington 98195
goodreau@u.washington.edu

Jonathan Mayer
Professor of Geography, Epidemiology, and Global Health, Department of Geography
Adjunct Professor of Medicine, Family Medicine, and Health Services
University of Washington, Box 353550
Seattle, Washington 98195
jmayer@u.washington.edu