Immune function and Aging among Tsimane Foragers:

Rapid depletion of naïve CD4 cells is associated with increased natural killer cell counts in a subsistence population under high pathogen stress

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Prepared for Population Association of America 2012 Conference

Abstract

While the biology of human immunity must have evolved in response to much higher pathogen stress than exists today, almost nothing is known about immunosenescence among premodern populations. Tsimane forager-farmers of Bolivia experience high rates of acute and chronic infections of multiple types throughout life. Using flow cytometry and clinical exams on a sample of 500 Tsimane adults age 40+, we find that depletion of naïve T lymphocytes occurs more rapidly among the Tsimane than in populations with lower pathogen burden. Naïve T- and B-cell depletion are associated with increased natural killer cell (NKC) counts. We also find effects of sex, Body mass index, hematocrit and eosinophil counts on lymphocyte subsets. Our results provide initial evidence that high pathogen exposure results in more rapid immunosenescence, particularly for acquired immunity. Greater reliance on innate immunity with age might represent a compensatory response. Overall, however, Tsimane do maintain robust immune function through the eighth decade of life.

Introduction

During the last 150 years changes in public health infrastructure and medical technology have lowered exposure to infectious diseases (Barrett et al. 1998; Riley 2001). Those changes have been accompanied by increased food consumption, tobacco and other drug use, reduced exercise, and lower fertility, all of which may have complex effects on health outcomes. Yet the human immune system evolved under conditions of much higher pathogen loads than currently exist in the industrialized world. Throughout our evolutionary past, human populations were exposed to an array of pathogens, many of which were common to other primate species (Nunn et al. 2004). In addition, ancestral humans were probably exposed to a variety of viruses, bacteria, protozoa, and helminths due to the consumption of meat and fish in an omnivorous diet (Finch and Stanford 2004). Phylogenetic evidence for several pathogens, including smallpox, *Plasmodium falciparum*, and *Mycobacteria tuberculosis* suggests a pre-agricultural history of exposure (see review in Pearce-Duvet 2006). Sexually transmitted diseases are also likely have a long evolutionary history among humans (Donovan 2000). Antibodies to viral infections, such as herpes, Epstein-Barr and varicella have been documented in isolated Amazonian groups, along with cytomegalovirus, pneumonias, intestinal helminths, herpes, hepatitis B and arboviruses (Black et al. 1970; Salzano and Callegari-Jacques 1988).

Several lines of evidence suggest that there are significant differences in how the immune system develops and senesces when individuals are exposed to different levels of helminthic, bacterial, viral, and fungal pathogens throughout life. A history of diarrhea during the first year of life is associated with a greater immune response to vaccines, and lower baseline inflammatory markers later in life (McDade et al. 2001; McDade et al. 2010). T cell populations also vary between populations; relative to American children, children in Guinea-Bissau maintain a higher ratio of CD4+ to CD8+ T cells during early development (McDade 2003). Helminth infections can shift host CD4+ T cell populations toward a T_H2 biased phenotype, characterized by increased production of immunoglobulin E (IgE) and decreases in T_H1 and pro-inflammatory responses (Cooper et al. 2000; Maizels and Yazdanbakhsh 2003; Yazdanbakhsh et al. 2008). Blackwell et al (Blackwell et al. 2011) show that the age pattern of IgE varies significantly between populations, rising more quickly with age and reaching higher baseline levels in populations with higher helminth load.

Both 'innate' and 'adaptive' immunity senesce with age (Shanley et al. 2009), caused, in part, by cumulative effects of antigen exposure (Burkle et al. 2007). Cell-mediated immunity is particularly

vulnerable to senescence for a variety of reasons (Burkle et al. 2007; Vasto et al. 2007), including age-related reductions in the immuno-responsiveness of T cells (Linton and Toman 2001), reduction of overall T cell repertoire (Wack et al. 1998) and alterations to the proportional representation of different T cell subsets, with decreasing numbers of naïve (non-antigen experienced) T cells, and increasing numbers of memory and effector (antigen-experienced CD28-) T cells. These latter cells exhibit shortened telomeres (due to a history of waves of expansion and subsequent apoptosis), and presumably a reduced replicative capacity indicative of immunosenescence (Effros 2005; Effros and Pawelec 1997) due to cumulative antigen exposure (Fagnoni et al. 2000). Chronic viral antigenic stimulation, particularly cytomegalovirus (CMV) infection, may be responsible for age-related modifications of lymphocyte subsets including clonal expansion of viral antigen-specific CD8+ T cells expressing a memory phenotype (G. and E. 2011; 2002). This is thought to lead to a diminished capacity to respond to novel immunological insults with age.

Papagno et al. (Papagno et al. 2004) show that acute HIV-1 infection induces massive activation of CD8+ T cells affecting many cell populations, not only those specific for HIV-1. Moreover, HIV disease progression correlates with increased proportions of highly differentiated CD8+ T cells, which exhibit characteristics of replicative senescence (expressing CD57+) and probably indicates a decline in T cell competence. They suggest that the differentiation of CD8+ and CD4+ T cells towards a state of replicative senescence may be part of the mechanism through which HIV-1-mediated immune activation exhausts the capacity of the immune system, explaining the progression from HIV to AIDS.

The Tsimane case may share certain similarities with HIV infection in terms of chronic activation of the immune system, except that instead of one dominant viral infection, the Tsimane suffer from acute and chronic infections of multiple types throughout life. At any given observation, over two-thirds of all Tsimane carry at least one species of intestinal parasite, the most common being hookworm (*Ancylostoma duodenale* or *Necator americanus*, prevalence 48%), roundworm (*Ascaris lumbricoides*, 21%) and whipworm (*Trichuris* sp., 4%). Pathogenic protozoan infections are also common, especially *Giardia lambia* (19%), and *E. histolytica* (5%). Roughly half of males and females have anemia, with children and adolescents showing the highest risk (56% females, 63% males). Controlling for age and sex, those with hookworm are almost twice as likely to be anemic, while *Trichuris* carriers are over three times more likely to be anemic.

This paper reports data on immune activation and lymphocyte subsets during aging among Tsimane forager-horticulturalists of Bolivia. The principal goal of this paper is to test the hypothesis that a) depletion of naïve T lymphocytes occurs more rapidly among the Tsimane than in populations with

lower pathogen burden; and b) naïve t-cell depletion is associated with increased natural killer cell counts. A second goal is to examine the effects of age, gender, body mass index, hematocrit and white blood cell composition on lymphocyte subsets. Jointly, those results will provide evidence about whether high pathogen exposure results in more rapid immunosenescence in acquired immunity and compensatory responses towards greater reliance on innate immunity.

Methods

Study population

The Tsimane are lowland forager-horticulturalists living in small villages composed of extended family clusters, located primarily in the Maniqui river system in the Ballivián and Yacuma Provinces of the Beni region of Bolivia. Approximately 9,000 Tsimane inhabit >80 villages in the forest and savanna regions between San Borja, the foothills of the Andes and San Ignacio de Mojos (VAIPO 1998). Most food the Tsimane consume comes from slash and burn agriculture, fishing, hunting, and gathering. Adults perform subsistence tasks within a cluster of kin-related households, although group fishing, cooperative hunting and field clearance are common. The Tsimane are largely isolated and have just begun an epidemiological and technological transition (Gurven et al. 2007b). There is no electricity, running water, or waste management.

Tsimane villages vary in their degree of market access and interaction with Bolivian nationals. Acculturation takes several principal forms: visits to the main market town, San Borja (population ~18,000), and the selling of agricultural produce, wage labor with loggers or colonists, debt peonage with river merchants and formal schooling. The Tsimane came into greater contact with outsiders as new roads were built in the 1970s, inviting a burst of logging and trading interests, and encroachment by lowland and highland colonists (Ellis, 1996; Chicchón, 1992). Schools now exist in about two-thirds of all Tsimane villages, having been established from two to twenty years ago, but most adults have little or no schooling.

Sampling, laboratory, and analysis methods

Adults age 40+ were brought to our medical clinic in the town of San Borja for extensive medical examinations. The acceptance rate varied between 80 and 90 percent of people present in the 50 villages from which these data were drawn. Fresh blood was used to obtain differential white blood cell counts. Total lymphocytes and hematocrit were obtained with an Autoread Plus (QBC Diagnostics). Cells were labeled in multiple sets per person with combinations of antibodies (eBioscience, Inc.) for CD4 (#12-0049-42, PE Conjugated, Clone RPA-T4), CD8 (#17-0088-42, APC, RPA-T8), CD19 (#17-0198-42, APC,

SJ25C1), CD56 (#12-0567-42, PE, CMSSB), CD45RA (#45-0458-42, PerCP-Cy5.5, HI100), CD69 (#11-0699-42, FITC, FN50), CD28 (#45-0289-42, PerCP-Cy5.5, CD28.2), and CD57 (#11-0577-42, FITC, TB01). Flow cytometry performed on an Accuri C6 Cytometer (BD Accuri Cytmeters) to determine both absolute numbers and differential proportions of lymphocyte sub-types. FCS datafiles were exported from CFlow Plus (BD Accuri Cytmeters) and imported into R 2.14.1. Cells were gated for lymphocytes using *lymphGate* from the Bioconductor package for R (http://www.bioconductor.org/). Florescence gates were determined using a combination of Bioconductor *rangeGate* and custom R scripting. We categorized cells as T-Helper or CD4 cells (CD4+CD8-), cytotoxic T-lymphocytes (CTLs) (CD8+CD4-), β-cells (CD19+), and natural killer cells (NKC) (CD56+). T cells were further classified as naïve (CD45RA+), activated (CD69+), senescent (CD28-CD57+) and memory (neither naïve nor senescent). Determinants of lymphocyte sub-sets were analyzed using multiple linear regression in SPSS 18.0 (SPSS Inc.).

Results

Over 90% of Tsimane present some complaint or symptom of morbidity based on a sample of 2,828 medical exams (Fig. 1). Gastrointestinal illness and respiratory infections are the most frequent diagnoses, with some 30-40% of infants and young children suffering from each. Throughout adulthood 30-40% and 20-30% continue to suffer from these two macro-categories, respectively. There is also substantial co-morbidity. The probabilities of being diagnosed with gastrointestinal, respiratory and other diseases are significantly correlated with one another in every age group. Some 75% (508/673) of adults report being sick enough that they could not get out of bed at least once in the past three months. Mean sickness duration (which included fevers, influenza, gastrointestinal infection, physical pains and swellings, accidents and injury) was 8.7±20.9 days, with 91% cases longer than 3 days The cumulative toll of high levels of childhood infection is reflected in stunted growth and short adult height (average height is 5’4” for men and 4’11” for women).

This high level of pathogen exposure is reflected in chronic inflammation throughout life and higher white blood cell counts. C-reactive protein, an acute inflammatory response, is elevated across the lifespan among the Tsimane compared with other populations (Figure 2). Tsimane also have higher white blood cell counts, particularly neutrophils, eosinophils and lymphycotes, and appear to show a greater age-related decline than Americans (Figures 3 and 4).

Figure 1. Probability of sickness among Tsimane during medical checkups (n=2,828)

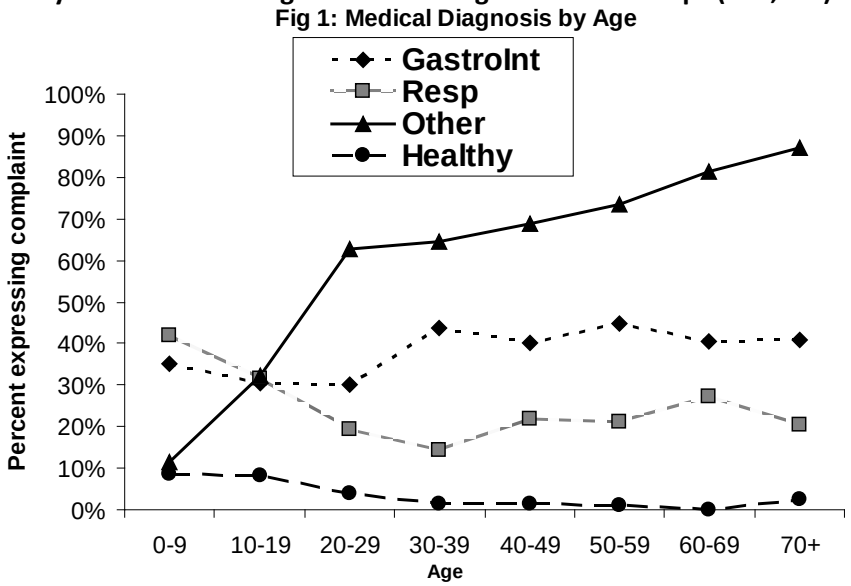


Figure 2. Acute Inflammation over the life course: Tsimane vs. other populations. NHANES data is from the 2005-2006 data round. InCHIANTI data is from Ferrucci et al, 2005.

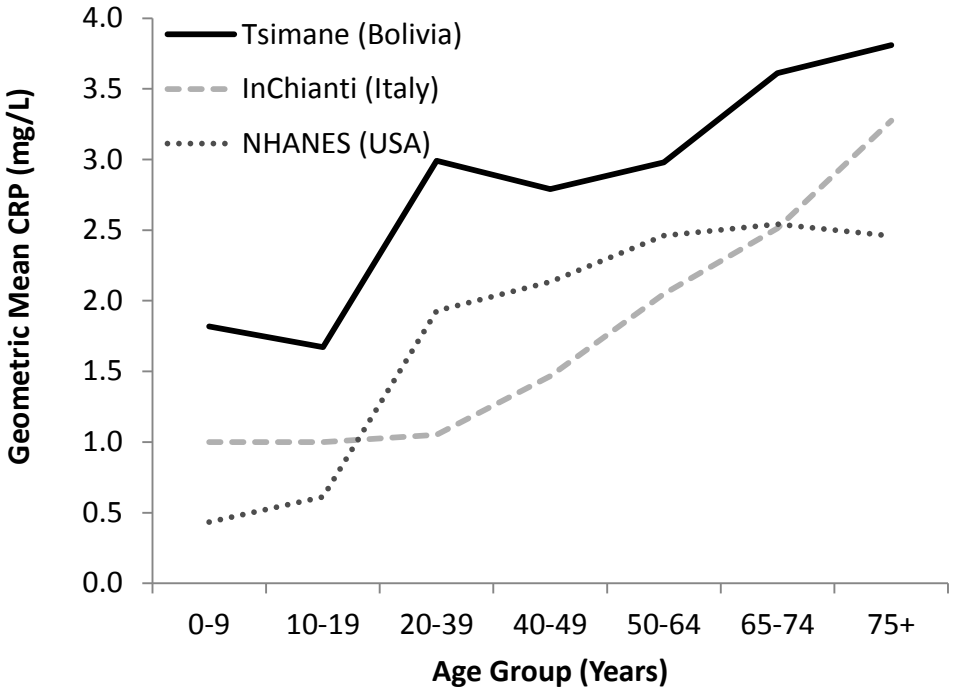


Figure 3. Percentage of Individuals with elevated (>10,800 cells/ul) white blood cells: Tsimane vs NHANES

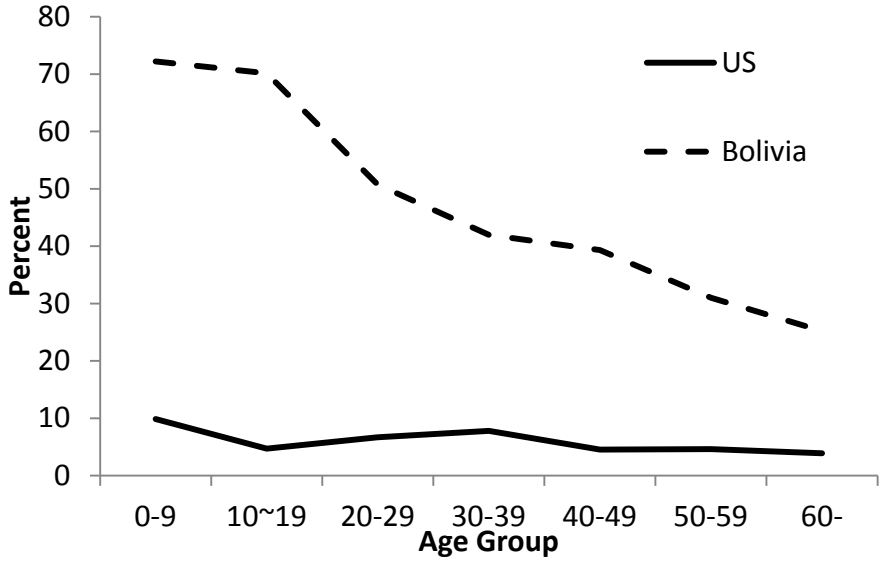
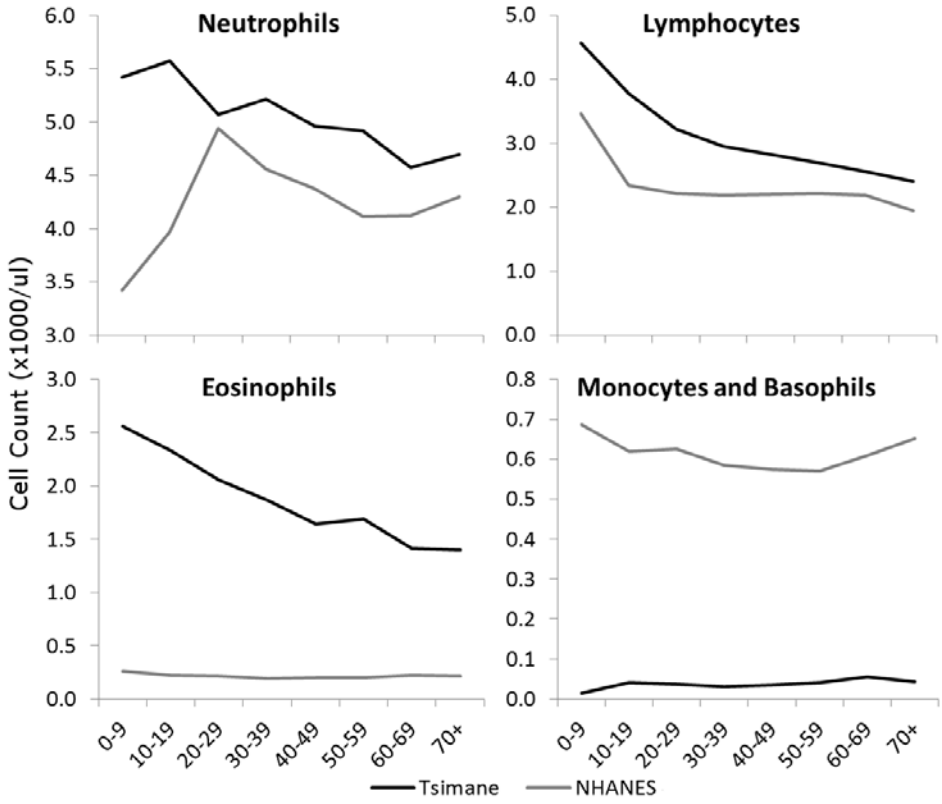


Figure 4. Differential White Blood Cell Counts by Age: Tsimane vs. NHANES



Comparing Tsimane over age sixty with Tsimane age 40-60 (Table 1), older Tsimane have lower counts of Naïve CD4 cells (t=-2.92, p=0.004), higher counts of senescent CD4 cells (t=2.30, p=0.022), and lower counts of total lymphocytes (t=-2.07, p=0.039). By percentage, a lower percentage of both CD4

(t=-2.89, p=0.004) and CD8 (t=-1.96, p=0.050) cells are naïve in older Tsimane and a greater percentage of CD4 cells are senescent (t=2.88, p=0.004). Overall, a greater percentage of total lymphocytes are natural killer cells in older Tsimane (t=4.43, p<0.001).

Table 1. Comparison of lymphocyte subset counts by age group

	40-60 Mean +/-SD	60+ Mean +/-SD	p
CD4+	596+/-251	565+/-284	.248
Naïve CD4+	107+/-139	69+/-79	.004
Memory CD4+	515+/-275	512+/-224	.955
Senescent CD4+	9+/-11	16+/-34	.022
CD8+	428+/-217	413+/-204	.471
Naïve CD8+	247+/-158	225+/-136	.166
Memory CD8+	101+/-102	91+/-100	.536
Senescent CD8+	87+/-87	99+/-92	.398
Natural Killer Cells	615+/-339	655+/-335	.248
β-Cells	348+/-183	321+/-170	.153
Total Lymphocytes	2195+/-704	2049+/-642	.039

p-values are from a two-tailed independent samples t-test with equal variance assumed

Table 2. Comparison of lymphocyte subset percentages by age group

	40-60 Mean % +/-SD	60+ Mean % +/-SD	p
CD4+	27.7+/-7.8	27.5+/-8.6	.783
Naïve CD4+	17.6+/-19.4	12.2+/-14.1	.004
Memory CD4+	78.0+/-24.3	84.0+/-15.0	.105
Senescent CD4+	1.4+/-1.7	2.5+/-4.2	.004
CD8+	19.5+/-6.9	19.9+/-8.1	.572
Naïve CD8+	56.8+/-15.8	53.5+/-16.7	.050
Memory CD8+	27.8+/-10.9	32.6+/-12.9	.777
Senescent CD8+	18.1+/-10.8	19.4+/-13.3	.429
Natural Killer Cells	27.8+/-10.9	32.6+/-12.9	<.001
β-Cells	16.0+/-6.0	15.7+/-7.8	.628

p-values are from a two-tailed independent samples t-test with equal variance assumed. Naïve, Memory, and Senescent subsets are percent of CD4+ or CD8+ rather than percent of total lymphocytes

Table 3. Association between lymphocyte sub-type populations (cells x 10^3/ul) and age and sex

	CD4 (helper T)		CD8 (cytotoxic T)		CD19 (Beta)		CD56 (NKC)	
Parameter	**B**	**P**	**B**	**P**	**B**	**P**	**B**	**P**
Intercept	726.7	<0.01	502.2	<0.01	432.9	<0.01	668.9	<0.01
Age (years)	-3.0	<0.01	-1.7	0.03	-2.0	<0.01	0.48	0.69
Sex=female	51.2	0.03	36.7	0.05	35.8	0.03	-123.0	<0.01
Adjusted R^2		0.03		0.01		0.03		0.03
N		527		527		508		528

Table 3 shows the linear relationship of age and sex to each of the four lymphocyte subsets. The data show that the three lymphocyte subsets that are associated with acquired immunity, CD4+, CD8+, and β-cells, all decrease significantly with age, whereas NK cells remain fairly constant with age. In addition, females have significantly higher levels than males of the first three subtypes, whereas males show higher counts of NK cells. There were no significant interactions between age and sex. Table 4 examines additional predictors of the four lymphocyte subsets. There is a general tendency for indicators of health and energy surplus (hematocrit and BMI) to be positively associated with T lymphocytes and β-cells. Other white blood cells, especially eosinophils, are also associated with lymphocyte counts.

Table 4. Association between lymphocyte sub-type populations and current condition, controlling for age and sex (significant effects in bold)

	CD4 (helper T)		**CD8 (cytotoxic T)**		**CD19 (Beta)**		**CD56 (NKC)**	
Parameter	**B**	**P**	**B**	**P**	**B**	**P**	**B**	**P**
Intercept	139.0	0.43	105.1	0.46	-158.8	0.22	64.2	0.78
Age	-1.4	0.15	-0.3	0.70	-1.0	0.171	1.2	0.36
Sex=female	**61.0**	**0.02**	**42.6**	**0.04**	**61.0**	**<0.01**	**86.1**	**0.01**
BMI	**12.6**	**<0.01**	**5.9**	**0.04**	**6.6**	**<0.01**	-1.2	0.79
Eosinophil count	**0.04**	**<0.01**	**0.05**	**<0.01**	**0.03**	**<0.01**	**0.05**	**<0.01**
Neutrophil count	-0.01	0.66	**-0.01**	**0.02**	0.01	0.519	**0.02**	**0.05**
Hematocrit (% RBC)	3.1	0.30	3.3	0.17	**6.9**	**<0.01**	**9.4**	**0.02**
Adjusted R^2		0.06		0.07		0.08		0.06
N		491		491		474		491

Eosinophils, BMI and self-reported health are significantly and positively associated with CD8+ CTLs; neutrophil counts show a mild negative relationship to CTLs. In addition, the effect of age becomes non-significant, because part of the decrease is captured by age-related decreases in eosinophils and BMI. Similarly, eosinophils and BMI are positively associated with CD4+ cells, but we find no effects of neutrophils. β-cells are also positively associated with eosinophil counts and BMI; however, they are also linked to hematocrit levels as well. Finally, NK cells are positively associated with hematocrits and eosinophils.

To examine whether the age-related decline in CD4+ cells was primarily in memory or naïve cells, Table 5 disaggregates CD4+ cells into naïve and memory forms. It shows that the age-related decline in CD4+ cells is due almost entirely to decreases in the naïve forms. Moreover, only the memory

forms are affected by BMI, and the depletion of naïve forms is due to another process, such as exposure to new pathogens.

Table 5. Predictors of naïve and memory helper T cell populations (n=481)

	Naïve CD4		Memory CD4	
Parameter	**B**	**P**	**B**	**P**
Intercept	114.2	0.04	123.5	0.26
Age	-1.7	<0.01	-0.14	0.88
Sex=female	11.2	0.32	39.4	0.07
BMI	2.7	0.11	10.8	<0.01
Eosinophil count	0.01	0.98	0.04	<0.01
Adjusted R^2		0.03		0.05

Table 6 presents tests of the hypothesis that reductions in antigen specific cellular immunity are related to increases non-specific cellular responses, by examining levels of NKCs in relation to naïve, senescent, and memory cell types. Model 1 uses the full dataset and Model 2 uses a subset of 201 individuals for which we not only have the percentage of T cells that are naïve, but also those that are senescent. Model 1 shows while the total number of T cells is positively associated with NK cells, the percentages that are naïve and the β-cell count are negatively correlated with NK counts. Model II shows that the percent of T-cells that are senescent positively predict NK cells. β-cell counts were no longer significant in Model 2. These results would be expected if there was substitution between innate and acquired immunity.

Table 6. Association between NKC count and indicators of innate and acquired immunity controlling for age, sex, and current condition for Tsimane age 40+

	Model 1		Model 2	
Parameter	**B**	**P**	**B**	**P**
Intercept	92.4	0.64	-133.8	0.67
Age	1.9	0.12	0.6	0.77
Sex=female	-109.7	<0.01	-96.6	0.05
Eosinophil count	0.04	<0.01	0.1	<0.01
Hematocrit (% RBC)	8.1	0.03	14.5	0.02
Total cell count	0.3	<0.01	0.1	0.04
% T cells naïve	-2.2	0.02	-3.5	<0.01
% T cells senescent			9.1	<0.01
β-cell count	-0.4	<0.01		
Adjusted R^2		0.16		0.22
N		497		201

Discussion and Conclusions

In response to higher rates of pathogen exposure Tsimane increase immune activity during childhood, as evidenced by the high lymphocyte levels. Cell-mediated immunity is activated at earlier ages as well. After age 40, all three lymphocyte subtypes associated with acquired immunity decline substantially. For β- and T-helper cells, the decline is about 20% from age 40 to age 80.

Some of this decline appears to be explained by decreasing energy reserves, as both BMI and hematocrit decrease with age. Overall, there appears to be a positive relationship between lymphocyte counts and proxies of condition. This would suggest that higher counts reflect a greater budget for immune defense rather than compromised health. Moreover, there are strong positive correlations among lymphocyte subtypes and other white blood cells within individuals, suggesting that some people are better able to support elevated immune activity.

There is strong evidence of depletion of naïve CD4+ T-helper subsets, suggesting a more rapid immunosenesence in cell-mediated immunity among the Tsimane compared to populations with lower pathogen load. Virtually the entire decline is due to decreases in naïve cell types. At the same time, there is greater relative reliance on natural killer cells with age, and this may be, in part, an adaptive response to an aging immune system. Controlling for total number of T lymphocytes, NKC counts are negatively associated with the percentage that are naïve, but positively associated with the percentage that are senescent. The decline in naïve T-cell subsets likely compromises the ability of older Tsimane adults to respond effectively to novel pathogens, and may therefore be responsible for increasing mortality rates from infection at late ages (Gurven et al. 2007a).

This pattern is strikingly different from what is found in other countries with lower diseases burdens. In particular, adult Tsimane have lower levels of naïve CD4+ T-helper cells than other published populations (Figure 5). Of particular interest, Borkow, et al (2000) reported on naïve T-cells in Ethiopian immigrants to Israel, and showed an association between time in

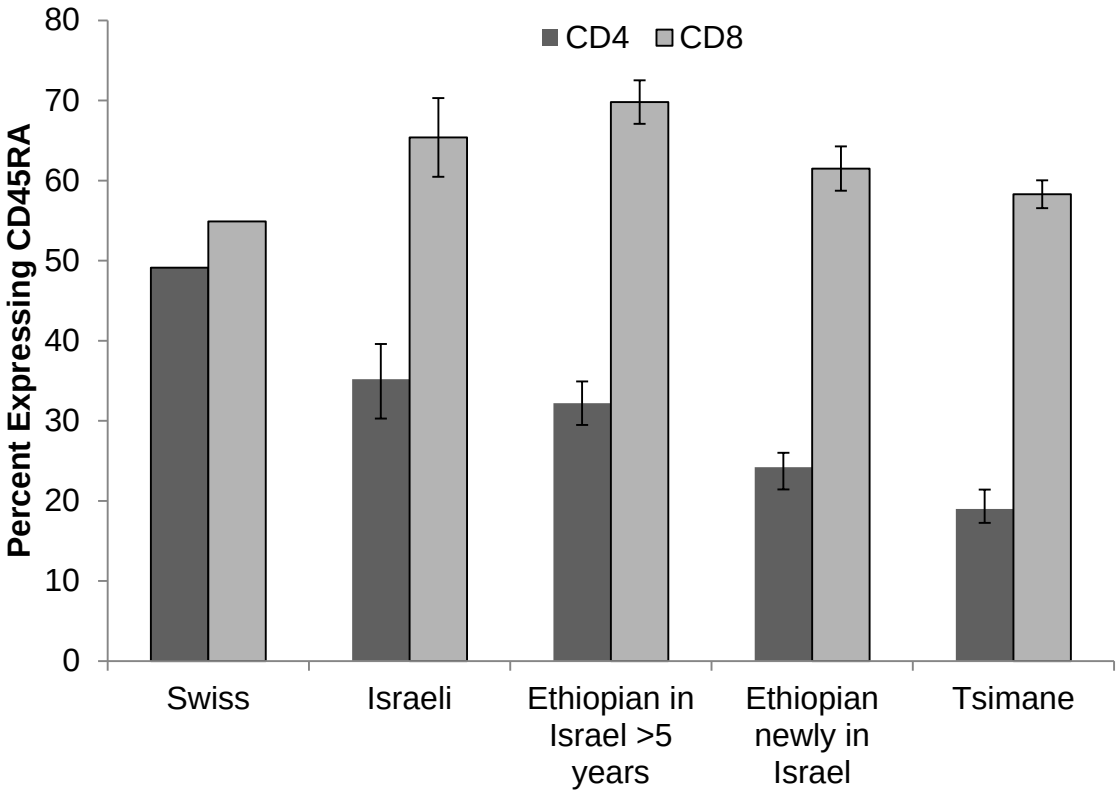


Figure 5. Percent of CD4 and CD8 positive cells expressing CD45RA across populations. Swiss data are from Bisset et al, 2004, European Journal of Haematology. Israeli and Ethiopian values are from Borkow, et al, 2000, Journal of Clinical Investigation. Swiss values are for adults fifty and under. Tsimane values are for adults thirty-fifty. Borkow, et al, did not report the age of subjects. Error bars are two standard errors around the mean. Note that Swiss values were not reported with standard errors.

Israel and naïve cells. More recent immigrants had lower naïve T-cell counts (Figure 5).

NK and β-cells are at much higher levels among the Tsimane than in other populations (Figure 6). In fact, NK cells among the Tsimane are about three times the Swiss. B cells are almost twice as high as in other populations. At the same time, the Tsimane have much lower total CD4+ counts than the other populations. It is striking that the Swiss have almost four times the number of CD4+ cells as NK cells, whereas the are about equal in the Tsimane.

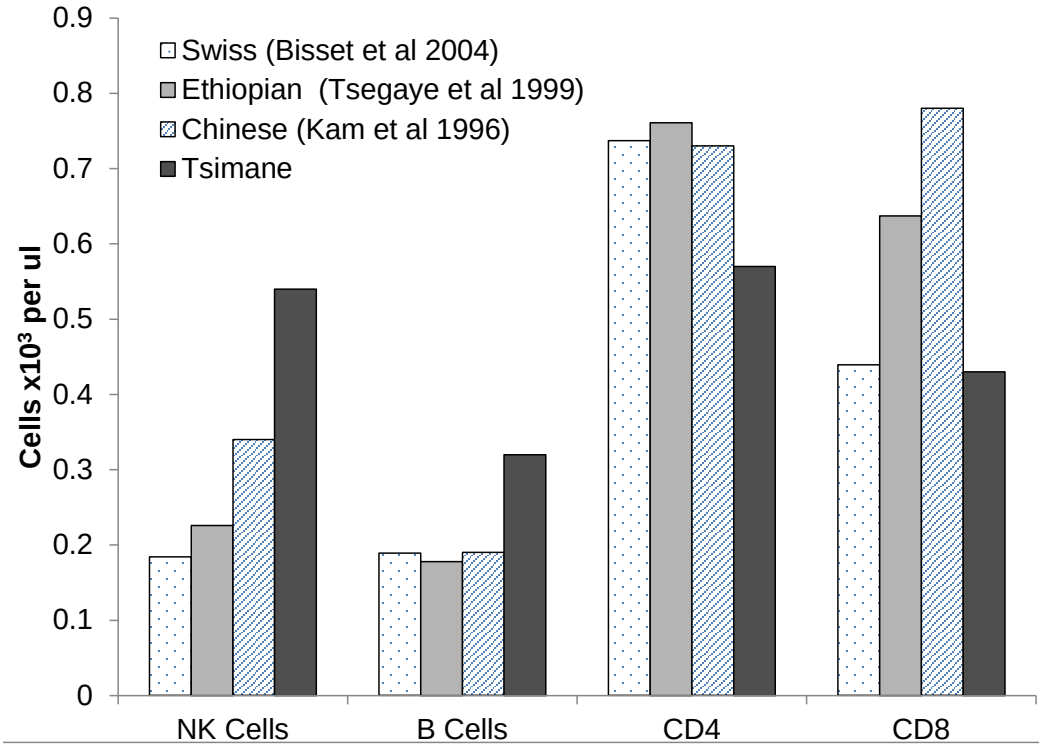


Figure 6. Comparison of lymphocyte counts across populations.

In spite of a very high pathogen load and what appears to be a more rapid rate of immunosenesence, it is also clear that Tsimane immune systems function well through the eighth decade of life. We do not observe a precipitous decline in cell numbers (most of which occurs before adulthood), and in fact, age explains a very small part of the variance in lymphocyte counts. This would suggest that a long lifespan, well past the age at which reproduction ceases, has a deep evolutionary history.

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