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A Genome-wide In Vitro Bacterial-Infection Screen Reveals Human Variation in the Host Response Associated with Inflammatory Disease

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Recent progress in cataloguing common genetic variation has made possible genome-wide studies that are beginning to elucidate the causes and consequences of our genetic differences. Approaches that provide a mechanistic understanding of how genetic variants function to alter disease susceptibility and why they were substrates of natural selection would complement other approaches to human-genome analysis. Here we use a novel cell-based screen of bacterial infection to identify human variation in *Salmonella*-induced cell death. A loss-of-function allele of *CARD8*, a reported inhibitor of the proinflammatory protease caspase-1, was associated with increased cell death in vitro ($p = 0.013$). The validity of this association was demonstrated through overexpression of alternative alleles and RNA interference in cells of varying genotype. Comparison of mammalian *CARD8* orthologs and examination of variation among different human populations suggest that the increase in infectious-disease burden associated with larger animal groups (i.e., herds and colonies), and possibly human population expansion, may have naturally selected for loss of *CARD8*. We also find that the loss-of-function *CARD8* allele shows a modest association with an increased risk of systemic inflammatory response syndrome in a small study ($p = 0.05$). Therefore, a by-product of the selected benefit of loss of *CARD8* could be increased inflammatory diseases. These results demonstrate the utility of genome-wide cell-based association screens with microbes in the identification of naturally selected variants that can impact human health.

Introduction

Genome-wide association studies of clinical phenotypes have successfully identified common variants that correlate with an increased risk for the development of human diseases, including macular degeneration (MIM #610698),^{1–3} diabetes (MIM #125853),^{4,5} and inflammatory bowel disease (MIM #266600).^{6,7} Such studies challenge researchers to experimentally validate these associations and to elucidate the molecular and cellular mechanisms whereby the genetic variants alter disease susceptibility. Dense genotyping data have also made possible a second kind of genome-wide study, screens for signatures of natural selection.^{8,9} These studies use sequence analysis to highlight chromosomal regions that might have been targets of natural selection, but they provide no information about which traits have been under selection and how the identified regions have affected the fitness of humans now or in the past. Thus, although genome-wide association studies and scans for selection have identified loci important for disease and understanding our evolutionary past, there is a growing need for approaches that (1) provide mechanistic information for how variants impact disease pathogenesis and (2) identify genetic variation in traits subject to natural selection.

One set of traits that has certainly been shaped by natural selection is the host response to microorganisms.^{10,11} Pathogens that have caused significant epidemics and/or increased in frequency as human populations have increased

in density are likely to have left lasting effects both on gene frequencies and human health. Although *Salmonellae* infection has been greatly reduced by modern waste disposal and water treatment, this Gram-negative enteric pathogen still causes major outbreaks, is currently the most common cause of bacterial gastroenteritis in the United States, and is estimated to cause 1.3 billion cases of disease annually worldwide.^{12,13} *Salmonellae*, particularly the human-specific species *S. typhi*, which causes more severe systemic illness, was probably a significant menace during times of increasing population density in particular; for example, one such time was the rise of agriculture (4000–10000 years ago¹⁴), and a more recent example is the Industrial Revolution. Polymorphisms that have increased in frequency as a result of past epidemic selection could still affect human health because genes involved in protection against pathogens are likely to be important to all inflammatory diseases, including autoimmunity.¹⁵ By examining variation in human cell-based measures of infectious-disease susceptibility and severity, we can begin to link variation affecting human disease and variation identified as being the subject of natural selection.

Here we present a screen for identifying genetic variation involved in cellular phenotypes of bacterial infection. In contrast to clinical association studies involving naturally infected human populations, this approach is not restricted to pathogens that currently infect large populations, and it reduces variation due to differences in pathogen exposure

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and availability of treatment. In this paper, we apply this approach to rapid cell death induced by *Salmonella*. In macrophages and dendritic cells, this has been shown to be a proinflammatory cell death dependent on caspase-1 (MIM *147678).^{16,17} Caspase-1 knockout mice are more susceptible to *S. typhimurium*¹⁸ and other bacterial infections,^{19,20} demonstrating the relevance of this intermediate phenotype to bacterial infection at the organismal level. Using our screening approach, we have identified a nonsynonymous *CARD8* allele (MIM *609051) that increases susceptibility to *Salmonella*-induced cell death. In addition to demonstrating the validity of this association through overexpression and RNA interference, we provide evidence of past natural selection on this gene, suggesting a link between evolution and a trait still important for multiple aspects of human health.

Material and Methods

Cells

HapMap lymphoblastoid cells (LCLs) were provided by the Coriell Institute. A total of 173 LCLs from CEU subjects (Centre d'Etude du Polymorphisme Humain Utah residents with ancestry from northern and western Europe) and YRI subjects (from Yoruba in Ibadan, Nigeria) were assayed because some were not available from Coriell at the time of order. Cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin-G, and 100 µg/ml streptomycin.

Bacterial Strains

S. typhimurium 14028s was obtained from ATCC. *prgH* and *ssaT* deletions were constructed with lambda red²¹ and verified by PCR. The *fliC/fliB* mutant was previously described.²² For green fluorescent protein (GFP) tagging, strains were electroporated with pMMB67GFP, the kind gift of James Bliska.²³ All GFP-tagged strains were grown in LB media with 100 µg/ml ampicillin.

Infection Assay

Cells were passaged at least twice after being received from Coriell. Three days prior to assay, cells were split to 150,000/ml. The day of assay, cells were washed with RPMI supplemented with 1% FBS and resuspended in RPMI 1640 (without phenol red) supplemented with 10% FBS. Overnight, bacterial cultures were subcultured 1:33 and grown for 2 hr 40 min at 37°C. Bacterial invasion was conducted for 1 hr at a multiplicity of infection (MOI) of 30. We chose the timing of infection and MOI to allow for a high-intermediate level of cell death and invasion with a normal distribution of variation. After infection, we added gentamicin for 1 hr to obtain a concentration of 50 µg/ml. We then split each infected culture into three subcultures to allow for measurement at 3.5, 24, and 48 hr (time from beginning of invasion). Seventy-five minutes prior to measurement, we added isopropyl β-D-1-thiogalactopyranoside (IPTG) to obtain a concentration of 1.5 mM. A portion of the supernatant was kept for cytokine measurement. Cells were stained with 7-AAD (eBioscience), and cell death and GFP-expressing bacteria were measured on a Guava Easycyte Plus flow cytometer. Each LCL was assayed on three sequential passages separated by 3 days. Percent *Salmonella*-induced cell death was background subtracted for uninfected cell death. Pilot measure-

ments of LDH release gave results similar to those obtained for 7-AAD staining (not shown). As an additional measure of caspase-1 activation, IL1β was measured by ELISA (R&D Systems), but no increase over baseline was detected even with prestimulation with LPS. This is possibly due to the lack of expression of the appropriate TLRs for increasing pro-IL1β expression.

For inhibition with Ac-YVAD-FMK (Calbiochem), cells were incubated with the indicated concentration for 30 min prior to infection. For measurement of caspase-1 activity with FAM-YVAD-FLICA (Immunochemistry Technologies, LLC), cells were stained for 1 hr according to the manufacturer's instructions.

Overexpression and RNAi

For transient overexpression, 5×10^5 LCLs were transfected with the Amaxa Nucleofection Kit V according to the manufacturer's instructions. Cells were assayed 6–8 hr after transfection. Tests with GFP showed that more than 50% of viable, transfected cells showed expression. HA-CARD8 (A) and HA-CARD8 (T) expression vectors were the kind gift of Jose Fernandez-Luna.²⁴ For detection of overexpressed *CARD8*, immunoblotting was performed with a rabbit *CARD8* antibody used at a 1:1000 dilution (Abcam).

For stable knockdown, LCLs were transduced with pGIPz-based lentiviruses containing nonsilencing or *CARD8* directed shRNA (*CARD8*-1:V2LHS_96279 and *CARD8*-2: V2LHS_96282; Open Biosystems). Lentiviruses were packaged with the Lenti-X system (Clontech). LCLs were selected for at least 2 weeks prior to assay with puromycin (0.1–0.5 µg/ml, depending on the sensitivity of the particular LCL). Knockdown was verified by two-step real-time RT-PCR with validated probes from Applied Biosystems (Hs00209095_m1) and normalized to an amplification of 18S rRNA.

Computational Analysis

Descriptive statistics, linear regression, and IC₅₀ calculation were performed with GraphPad Prism 5. Linkage disequilibrium at the *CARD8* locus was examined with HaploView.²⁵ Genome-wide association analysis was conducted with PLINK, developed by Shaun Purcell.²⁶ Analysis was carried out with QFAM-parents and QFAM-total with adaptive permutation under default settings. The QFAM procedures implemented in PLINK use linear regression to test for association while employing permutation of within- and between-family components separately to control for family structure. Although protection from stratification is not as complete as with a strictly within-family test (such as the TDT), consideration of parental phenotypes in QFAM results in a significant increase in power.²⁷

After we pruned for frequencies and genotyping quality, 2,869,783 SNPs remained for association testing. Because of our small sample size, very low p values would only be obtained if the SNP had a very large genotypic effect. Because we did not know what effect size to expect and the low heritability of the trait suggested a large fraction of nongenetic variation for this phenotype, we used a relatively modest p value filter of 0.01 for our most powerful test, QFAM-parents on combined CEU-YRI. This filter alone gives 23,840 SNPs. The use of this less stringent filter is expected to result in greater sensitivity but would also result in a greater number of false positives. To reduce false positives, we therefore ran the QFAM-parents analysis on CEU and YRI populations separately; we reasoned that for SNPs present in both populations, we should see some evidence of association in both populations separately with the same direction of effect. Because these separate population analyses employ even fewer

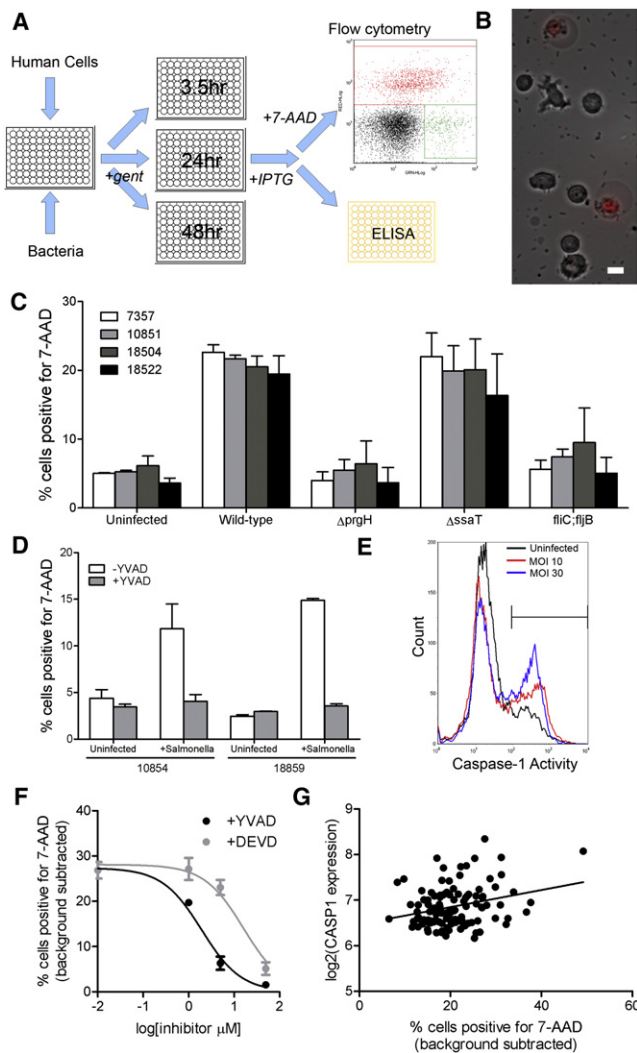


Figure 1. Hi-HOST Reveals that LCLs Undergo *Salmonella*-Induced Cell Death

(A) A schematic diagram showing the steps involved in carrying out the Hi-HOST assay. LCLs are infected in 96-well plates with bacteria tagged with an inducible GFP plasmid. After waiting an hour to allow for invasion, one adds gentamicin for an additional hour to kill extracellular bacteria and then splits LCLs into plates for each time point. Adding IPTG 75 min prior to each time point induces GFP expression. Supernatants are collected so that cytokine release can be measured, and cells are stained with 7-AAD and analyzed by flow-cytometry so that viability and intracellular bacteria can be measured.

(B) Phase image of LCLs overlaid with 7-AAD fluorescence (red) 2 hr after infection with *S. typhimurium*. The scale bar represents 10 μm .

(C) Effects of bacterial mutations on *Salmonella*-induced cell death. Four different LCLs were infected at an MOI of 30 with wild-type, ΔprgH , ΔssaT , or $\text{fliC};\text{fljB}$ *S. typhimurium*. Cell death is greatly reduced in all LCLs with the prgH and $\text{fliC};\text{fljB}$ mutants, and the observed variation among the four LCLs is similar to that in uninfected cells. Data shown are the means and standard deviation from two sets of experiments.

(D) Inhibition of cell death with the caspase-1 inhibitor, Ac-YVAD-cmk. Two LCLs were treated with 50 μM Ac-YVAD-cmk beginning 30 min prior to infection and assayed for cell death. Data shown are the means and standard deviation from two sets of experiments.

(E) Fluorescence-labeled inhibitor of caspase (FLICA) measurement of caspase-1 2 hr after *S. typhimurium* infection. The fraction of cells labeled by FAM-YVAD-fmk increases with *Salmonella* infection.

trios and consequently have less power, a less stringent p value filter of 0.1 was used. Finally, the QFAM-total filter ($p < 0.05$) allowed us to retain SNPs that showed an association even when we did not control for stratification.

Filtering of PLINK results with these p-value filters resulted in 2136 SNPs. SNPs that are truly modifying the cell-death phenotype will be correlated with the phenotype, as reflected in the PLINK p value, but they also will have a functional affect on the genes in which they are located or on genes that are nearby. Therefore, we further filtered our candidate SNP list to include only SNPs with a high probability of having a functional affect. In the complete Hapmap dataset, 34,895 SNPs were nonsynonymous (16,610), transcriptional regulatory (15,873), over splice sites (2787), or within microRNAs (34). Applying both p value and SNP-characteristic filters resulted in 20 SNPs for the cell-death phenotype. These filtering steps were carried out with the GWAS Analyzer database tool (our unpublished data). GWAS Analyzer serves as a database to store and integrate the HapMap genotypes and pedigrees, the phenotypic data generated by high-throughput human in vitro susceptibility testing (Hi-HOST, Figure 1A), and the p values generated by association analysis carried out with PLINK. In addition to allowing for easy retrieval of data queried by phenotype, SNP, or LCL ID, the web-based interface allows for filtering of PLINK results on the basis of p value and SNP characteristics.

Expression levels in LCLs were obtained from the GENEVAR dataset.²⁸ For *CASP1*, levels of probe GI-15431333-A were examined. For *CARD8*, levels of probe GI_7662403-S were examined.

We determined the presence or absence of *CARD8* in mammalian genomes by using the UCSC Genome Browser²⁹ and Ensembl release 49.³⁰ Individual genomes were queried with “*CARD8*” in UCSC Genome Browser and Ensembl. If no hits were obtained, individual genomes were searched with the human 431 amino acid *CARD8* isoform via protein BLAST of peptides and *ab initio* peptides in Ensembl. The phylogeny used in the analysis was obtained from Prasad et al., 2008.³¹ Animal group size was determined from internet resources, including Wikipedia and the Animal Diversity Web. Assignments of group size have the caveat that current social structure might not reflect the structure during most of the organisms’ evolutionary histories, and this might be especially true of domesticated and commensal organisms. Humans were classified as a “small group” because we have lived in small hunter-gatherer groups throughout most of our evolution. Correlation of animal group size and *CARD8* was done via Pagel’s method³² as implemented in Mesquite.³³ Tests were conducted with the 15 species with higher confidence ($>5\times$ coverage to call absence; presence requiring at least half of protein sequence found) and also on all 24 mammalian species. A few species are

Data shown are for LCL 7357 but are representative of several LCLs tested.

(F) Dose-response curves of inhibition of cell death in LCL 7357 via the caspase inhibitors Ac-YVAD-cmk and Ac-DEVD-cmk. Cells were treated with 0, 1, 5, or 50 μM of each inhibitor 30 min prior to infection and assayed for cell death. The percentage of cell death in uninfected cells was subtracted from the amount measured at 3 hr after the initial infection. The IC_{50} for YVAD is 1.9 μM , and that for DEVD is 16.1 μM . Similar values were obtained when an additional LCL (18853) was assayed (not shown). Data points are the means and standard error of the mean (SEM) for three separate experiments.

(G) Linear regression of *Salmonella*-induced cell death versus \log_2 (*CASP1* expression) in combined CEU-YRI parents shows that higher levels of *CASP1* are correlated with increased levels of cell death (Pearson $r = 0.28$; $p = 0.002$).

intermediate, having groups larger than the family-sized packs of dogs (alpha pair and previous year's offspring) but less than the size of wild horse herds, for example. Specifically, the animals with intermediate-sized groups are (1) chimpanzees, which form troops of approximately 10 but then come together occasionally in dynamic "fusion-fission" groups, (2) European rabbits, which form small colonies that range in size from a single territorial breeding pair and offspring to larger groups of 6–10 adults, and (3) elephants, which form small herds of related females and offspring while males are primarily solitary after maturity. When we re-ran our analysis and switched the categorization of these three species with intermediate-sized groups to the "large group/herd" category, we obtained a p value of 0.04 for the 25-mammal tree. For the 15-mammal tree (which does not include rabbits because of their low genome coverage), we obtained a p value of 0.006.

The branch-site test of positive selection³⁴ was carried out with PAML³⁵ for the five complete primate *CARD8* sequences aligned by MEGA4.³⁶ Human Genome Diversity Project (HGDP) allele frequencies were obtained with SPSmart.³⁷

Case-Control Studies for Sepsis and Systemic Inflammatory Response Syndrome

Healthy control subjects were recruited from the Seattle area and enrolled as described.³⁸ The systemic inflammatory response syndrome (SIRS) cohort was enrolled from the intensive care units (ICUs) of Harborview Medical Center (Seattle, WA). Inclusion criteria included admission to an ICU for more than 24 hr and presence of three of four criteria for SIRS.³⁹ Exclusions included admission for major trauma, chronic treatment with anti-inflammatory medications, history of cancer, massive transfusion, presence of an advanced directive against resuscitation, and an age of <18 or >90 years. The trauma cohort employed in the sepsis study has been previously described.⁴⁰ In brief, patients admitted to the Harborview Medical Center ICU after sustaining major traumatic injury were enrolled, and DNA samples were collected. All subjects were followed for the presence of SIRS criteria and for the development of a microbiologically documented or clinically suspected infection. For both cohorts, we limited our analyses to subjects of European descent to minimize confounding due to population-specific allele frequencies. These studies were approved by the Division of Human Subjects Research, University of Washington (Seattle, WA). Genotyping for rs2043211 was conducted with a Taqman-based allelic discrimination assay (C_11708080_1) from Applied Biosystems. We achieved high-quality genotypic calls on >98% of the subjects tested, and genotype frequencies did not deviate from Hardy-Weinberg equilibrium.

Analysis was conducted with the Cochran-Armitage trend test^{41,42} as implemented in the COIN package for R⁴³ and described in the MAXTest vignette (see [Web Resources](#)). On the basis of the genotypic medians for cell death in parental CEU and YRI LCLs, scores of 0, 0.75, and 1 were assigned for the AA, AT, and TT genotypes for an additive model with partial dominance of the T allele.

Results

Hi-HOST Reveals Variation in Cell Death after *Salmonella* Infection

In order to identify genetic variation that influences susceptibility to bacterial infection, we developed a high-

throughput assay, Hi-HOST (Figure 1A), to measure multiple infection parameters. Standard cell-based infection assays typically involve bacterial enumeration through lysis of host cells and counting of bacterial colony-forming units. The method is not amenable to large-scale phenotyping and is not sensitive enough to detect small differences. Hi-HOST uses bacteria tagged with inducible GFP and flow cytometry to allow for rapid and accurate measurement of bacterial invasion, intracellular survival and replication, host cell death, and host cytokine production from a single set of infection assays. HapMap LCLs (Epstein-Barr virus (EBV)-transformed B cells) were used for phenotyping because they have been genotyped at more than 3 million SNPs.⁴⁴ The utility of these cells for the examination of human variation in gene expression,^{28,45} chemotherapeutic sensitivity,⁴⁶ and HIV invasion⁴⁷ has been demonstrated. Importantly, *Salmonella typhimurium* infects B cells both in vitro and in vivo⁴⁸, demonstrating the physiological relevance of this infection model.

One outcome of *S. typhimurium* infection measured in Hi-HOST is *Salmonella*-induced cell death. A caspase-1-dependent, proinflammatory form of cell death termed "pyroptosis" rapidly occurs in *Salmonella*-infected macrophages and dendritic cells⁴⁹, and we found that a similar process occurs in LCLs. In LCLs, *S. typhimurium* triggered cell death within 2 hr of the start of infection, and the dying cells underwent a characteristic rounding and enlargement, consistent with the osmotic changes and proinflammatory release of cellular contents seen in pyroptosis (Figure 1B). Similar to what is reported for macrophages,^{16,22,50} *Salmonella*-induced cell death in LCLs was completely abolished when cells were infected with a SPI-1 type-III secretion mutant ($\Delta prgH$) and partially abolished when infected with a flagellin mutant (*fliC*;*fliB*) (Figure 1C). Furthermore, pharmacological inhibition of caspase-1 by the peptide-based inhibitor Ac-YVAD-cmk also prevented cell death (Figure 1D), and a fluorescent derivative of this inhibitor labeled a greater fraction of cells after infection (Figure 1E). On the basis of published in vitro IC₅₀ values, YVAD is highly specific to caspase-1. The IC₅₀ of this drug measured with the canonical proapoptotic effector caspase-3 is at least 10,000× greater, and even the related inflammatory caspase-5 has an IC₅₀ 200× greater.⁵¹ To further examine the specificity of cell death in LCLs, we measured the effect of an additional caspase inhibitor, DEVD. This peptide strongly inhibits caspase-3, but it also inhibits caspase-1 with an IC₅₀ only 20× greater than that of YVAD.⁵¹ Dose-response curves of YVAD and DEVD in inhibiting *Salmonella*-induced cell death show that the in vivo IC₅₀ of DEVD is about 8× higher than that of YVAD (Figure 1F). This profile of being strongly inhibited by YVAD and moderately so by DEVD is unique to caspase-1.⁵¹

Finally, mining of publicly available genome-wide expression analysis of the HapMap LCLs uncovered a clear correlation between the level of *CASP1* expression and the

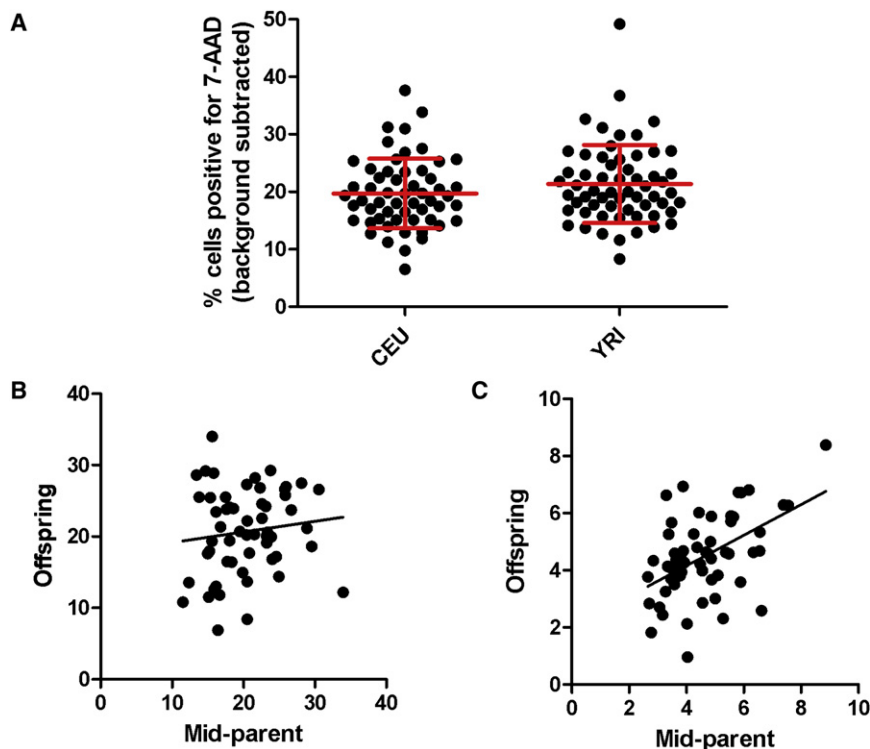


Figure 2. Variation in *Salmonella*-Induced Cell Death among CEU and YRI LCLs

(A) Scatter plots of cell death in CEU and YRI parental LCLs. Each dot represents the mean percentage of each LCL that stained positive for 7-AAD (uninfected cell death subtracted from background) assayed in three separate experiments. Mean and standard deviation of each population is displayed in red.

(B) Parent-offspring regression for cell death in combined CEU-YRI populations. Heritability is estimated at 14.9%.

(C) Parent-offspring regression for *S. typhimurium* viable invasion, measured as the percentage of LCLs containing GFP-*Salmonella* at 3.5 hr, in combined CEU-YRI populations. Heritability is estimated at 54%.

amount of LCL cell death (Figure 1G). Cells with more *CASP1* mRNA had higher levels of *Salmonella*-induced cell death. This was true when LCLs were examined together (Pearson $r = 0.28$, $p = 0.002$) or as separate populations (for CEU, $r = 0.31$, $p = 0.02$; for YRI, $r = 0.32$, $p = 0.01$). We also attempted to measure the amount of activated caspase-1 by immunoblotting with human-specific p10 and p20 subunit antibodies. Although pro-CASP1 was readily detectable, we were unable to detect specific cleavage products generated upon *S. typhimurium* infection (not shown). The fraction of caspase-1 that is processed in LCLs could be a very small fraction of the total caspase-1, or the cleaved protein might undergo rapid turnover. Thus, evidence based on morphology, bacterial mutants, pharmacologic inhibition, and gene expression all indicates that *Salmonella*-induced cell death in LCLs is most likely dependent on the proinflammatory protease caspase-1.

The amount of *Salmonella*-induced cell death in 173 CEU and YRI LCLs demonstrated significant variation (range = 6.5% to 49%; Figure 2A and Table 1). Measurements were highly repeatable: analysis of variance (ANOVA) of three replicates performed on different passages for all LCLs demonstrated that 89% and 92% of the total variance was attributable to differences among individuals in CEU and YRI populations. The large interindividual variation could be due to genetic or environmental factors. The family structure of the CEU and YRI populations allows for estimation of narrow-sense (additive) heritability by parent-offspring regression.⁵² The heritability of *Salmonella*-induced cell death was 14.9% in the combined CEU-YRI dataset (Figure 2B). Heritability of

of *S. typhimurium* as measured in Hi-HOST is 54% (Figure 2C).

Family-Based Association Analysis Identifies SNPs that Correlate with Variation in Cell Death

Next, using family-based association analysis as implemented in PLINK^{26,27}, we tested the hypothesis that common genetic variation contributed to heritability of *Salmonella*-induced cell death. The procedures employed use linear regression of phenotype on genotype and, subsequently, adaptive permutation to control for family structure. Our relatively modest set of 173 LCLs does not

traits can vary between populations, but similar values were obtained when the CEU and YRI data were plotted individually (20.8% and 12.8%; not shown). For comparison, the heritability of viable invasion

Table 1. Population Statistics of Hapmap LCLs Assayed for *Salmonella*-Induced Cell Death

	CEU	YRI	Combined	p Value ^a (CEU versus YRI)
Mean	19.70%	21.40%	20.56%	0.17
Standard Dev	6.06%	6.79%	6.47%	0.4
p value normality test ^b	0.07	0.25		
Repeatability ^c	89%	92%		
Heritability ^d	20.80%	12.80%	14.90%	

Mean, standard deviations, and normality test values are for parents only so that the independence of samples is maintained.

^a p value from unpaired two-tailed t test.

^b D'Agostino & Pearson normality test. A single outlier, 18523, was four standard deviations above the mean and was left out of this analysis.

^c Repeatability is defined as the between-individual component of variance calculated from ANOVA of three separate assays for each LCL.

^d Additive heritability estimated from the slope of parent-offspring linear regression of trios.

Table 2. SNPs Associated with *Salmonella*-Induced Cell Death

Marker Name	QFAMpboth p Value	QFAMpCEU p Value	QFAMpYRI p Value	QFAMtboth p Value	SNP Type	Functional Effect ^a	Within Gene	Gene Expression Affected ^b
rs12912	0.000002	0.01472	0.00003	0.002218	genomic	Expression CEU, YRI, ASN		Hs. 397369 ^c
rs243327	0.000018	0.02666	0.0005328	0.01004	intronic	Expression CEU, YRI, ASN	C16orf75	C16orf75
rs268671	0.00004508	0.0008454	0.02074	0.0457	nonsynonymous	nonsynonymous	PRX	
rs243324	0.00009442	0.02393	0.00195	0.01683	intronic	Expression CEU, YRI, ASN	C16orf75	C16orf75
rs2943512	0.000329	0.01142	0.01701	0.0004216	nonsynonymous	nonsynonymous	MUC5B	
rs7099565	0.0007904	0.04287	0.01149	0.02011	nonsynonymous	nonsynonymous	TRUB1	
rs4802073	0.001498	0.02872	0.02316	0.03941	intronic	Expression CEU	C19orf47	C19orf47
rs753852	0.001523	0.01223	0.04502	0.03563	intronic	Expression CEU, YRI, ASN	ANKRD11	SPG7
rs683866	0.002736	0.03405	0.0455	0.01078	nonsynonymous	nonsynonymous	KIAA0367	
rs9630856	0.002853	0.07961	0.02528	0.005746	intronic	Expression CEU	SERPINB2	SERPINB10
rs530978	0.003101	0.08761	0.0245	0.04131	nonsynonymous	nonsynonymous	KIAA0367	
rs4680	0.003585	0.06614	0.04163	0.01608	nonsynonymous	nonsynonymous	COMT	
rs6509365*	0.00421*	0.083*	0.02272*	0.04947*	intronic*	Expression CEU, YRI, ASN*	CARD8*	CARD8*
rs188384	0.0052	0.05263	0.02943	0.01565	intronic	Splice site SNP	CCDC50	
rs3177676	0.007466	0.01323	0.0865	0.01018	nonsynonymous	nonsynonymous	C1orf105	
rs1014390	0.007859	0.0328	0.07482	0.03516	intronic	Expression YRI, Expression CEU, YRI, ASN	JMJD6	MXRA7
rs6509366*	0.008496*	0.09732*	0.04492*	0.03857*	intronic*	Expression CEU, YRI, ASN*	CARD8*	CARD8*
rs1729659	0.009015	0.05243	0.09547	0.02354	synonymous	Expression CEU, YRI, ASN	KIAA1841	LOC339804
rs557352	0.009863	0.09281	0.06316	0.04339	nonsynonymous	nonsynonymous	KIAA0367	
rs9807444	0.009885	0.07428	0.08632	0.01725	genomic	Expression CEU, YRI, ASN		TUBB6
rs2043211*	0.01336*	0.1283*	0.0531*	0.03723*	nonsynonymous*	Expression CEU, YRI, ASN*	CARD8*	CARD8*

SNPs were filtered with PLINK results from QFAM-parents CEU+YRI < 0.01, QFAM-parents CEU < 0.1, QFAM-parents YRI < 0.1, and QFAM-total CEU+YRI < 0.05. rs2043211 falls just outside the initial filtering criteria but is in LD with the other two asterisked CARD8 SNPs.

In addition to *CARD8*, three other genes on the list (*SERPINB2*, *COMT*, and *KIAA0367*) have been reported to be involved in caspase function or cell death.

^a Functional SNP filtering criteria that were fulfilled by the SNP.

^b Genes whose expression is correlated with the SNP on the basis of the GENEVAR data.²²

^c Hs. 397369 is found on Illumina arrays but is no longer matched with a gene in NCBI (Build 36) or Ensembl (release 49).

have sufficient power to identify true positives solely on statistical grounds. We therefore took a multistep approach, which we felt would maximize the probability of identifying true positives. Four sets of analyses were run within PLINK: (1) family-based association with parental phenotypes (QFAM-parents), which reduces false positives by controlling for between-family stratification, on the combined CEU-YRI data; (2) QFAM-parents on the CEU data alone; (3) QFAM-parents on YRI; and (4) total association taking into account family structure but not controlling for stratification (QFAM-total). QFAM-parents on CEU-YRI data has greater power than the same analysis on CEU and YRI data separately, but the individual population analysis allows for determining whether the same SNP correlates with cell death in two different populations. The QFAM-total test does not control for stratification, but the size of the genotypic effect is better reflected in p values obtained from this test than from the QFAM-parents test. By filtering on the basis of uncorrected p values from these

tests, we obtained a set of 2136 SNPs that correlated with *Salmonella* cell death in both populations, controlled for between-family stratification, and resulted in a large effect on the phenotype. A second filtering step included only those SNPs that have a functional effect on the gene in which the SNP is located or on a nearby gene. This included SNPs that cause nonsynonymous changes, correlate with transcript levels, or overlap with splice sites or microRNAs. This functional-candidate-variant approach, similar to that used by Stranger et al., 2007²⁸, reduced our candidate list from 2136 to 20 SNPs (Table 2; complete results are available for further analysis and data mining with a newly developed web-based tool, the GWAS Analyzer). Because the statistical tests were chosen prior to analysis but p value filters were set post-hoc to give a manageable number of candidates, we then focused on performing additional experiments on SNPs from our candidate list to determine whether this approach was uncovering true positives.

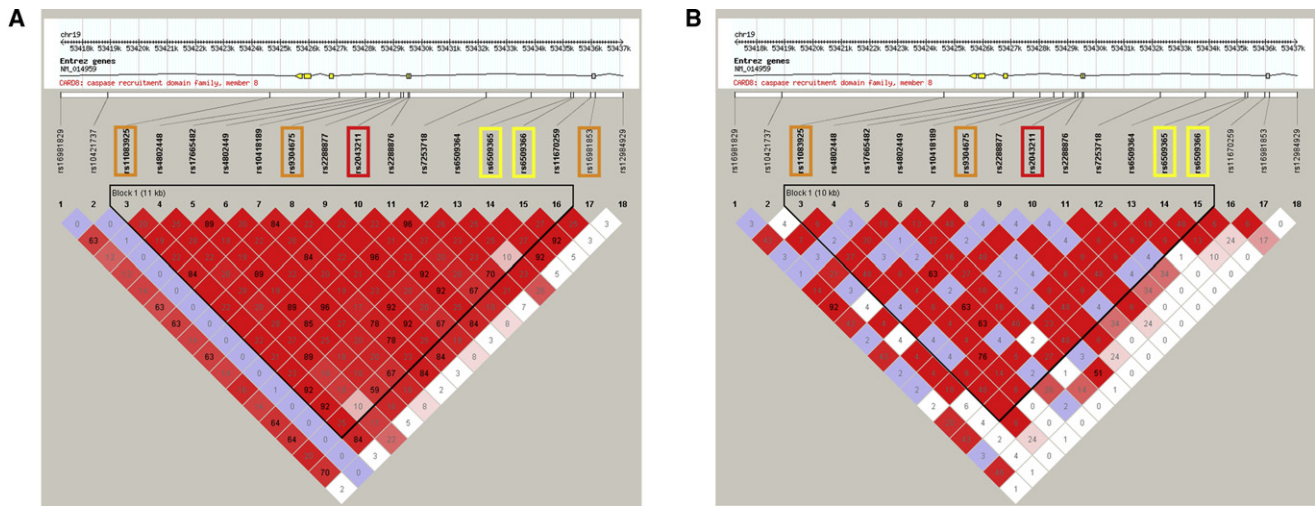


Figure 3. Linkage Disequilibrium around rs2043211 in CEU and YRI Populations

(A) CEU and (B) YRI populations. Boxes are colored on the basis of D'/LOD ; red represents high LD. Numbers within boxes are r^2 values. The two SNPs identified by the screen (rs6503965 and rs6503966) are highlighted in yellow, rs2043211 is highlighted in red, and other SNPs in strong LD with rs6503966 ($r^2 > 0.8$) are highlighted in orange. Only 20 kb of the *CARD8* gene (41.4 kb) is shown. Images were exported from HaploView.²⁵

A *CARD8* Loss-of-Function Allele Is Unable to Inhibit *Salmonella*-Induced Cell Death

Two of the 20 candidate SNPs are within *CARD8*. Previous work has demonstrated that stable expression of *CARD8* in THP-1 monocytes causes caspase-1 inhibition on the basis of decreased IL1 β ELISA secretion and that this effect is probably through a direct physical interaction with caspase-1.⁵³ Other reported functions of *CARD8* include regulation of apoptosis⁵⁴ and NF- κ B-inhibitory properties.^{53,55} Because the two candidate SNPs are both within an intron, we analyzed the linkage disequilibrium (LD) of *CARD8* to determine whether a more obvious causal variant was being inherited together with these SNPs. The two SNPs are in strong LD with each other and several other *CARD8* SNPs (Figure 3). One of these SNPs, rs2043211, is predicted to replace an early residue with a stop codon (C10X) and cause a nonsense mutation in some *CARD8* isoforms. Consistent with this, there is a strong correlation between the derived nonsense allele and reduced expression levels of *CARD8* from the GENEVAR dataset²⁸ ($p = 3.72 \times 10^{-5}$; Figure 4A), which we suspect is due to nonsense-mediated mRNA decay.⁵⁶

Cells with the derived rs2043211 allele exhibit modestly increased levels of *Salmonella*-induced cell death ($p = 0.013$; Figure 4B). This is consistent with the predicted loss-of-function nature of the polymorphism and the reported activity of *CARD8* as an inhibitor of caspase-1: cells with the derived allele do not have as much functional *CARD8*, resulting in more active caspase-1 and more cell death. Although this is strong correlative evidence of the effect of rs2043211 on *Salmonella*-induced cell death, we performed experimental verification of the effect of the SNP on the phenotype to prove its functional significance.

To measure the effects of the alternative rs2043211 alleles, we carried out transient transfections with two

different *CARD8* alleles. Overexpression of ancestral *CARD8*, but not the derived nonsense allele, reduced the fraction of cells that underwent cell death (Figure 4C). As a second measure of caspase-1 activation in these cells, we used a fluorescently labeled inhibitor of caspase-1 (FLICA⁵⁷). Infection with *S. typhimurium* caused a clear increase in the mean fluorescence of the population of cells labeled by the fluorescent caspase-1 inhibitor. Increased fluorescence is indicative of greater amounts of active caspase-1 within *Salmonella*-infected cells. This increase in mean fluorescence is blocked in a dose-dependent manner by expression of the ancestral rs2043211 allele (Figure 4D). In contrast, transfection of the derived allele, containing the stop codon, is unable to block the *Salmonella*-dependent increase in measured caspase-1 activity. Therefore, *CARD8* appears to inhibit caspase-1 activation in LCLs, consistent with the reported inhibition of IL1 β secretion from monocytes.⁵³

To examine the role of *CARD8* in a loss-of-function experiment, we stably transduced cells of varying rs2043211 genotypes with two different RNAi vectors directed against *CARD8*. If *CARD8* is an inhibitor of *Salmonella*-induced cell death, as the overexpression experiments suggest, reducing expression of the gene should result in an increase in cell death. Furthermore, this effect should only be seen in cells with the ancestral allele, because those with the derived allele are predicted to already have impaired *CARD8* function. The effectiveness of knockdown, as assessed by quantitative RT-PCR, was variable among the cell lines. However, increased cell death (compared to that of cells transduced with a nonsilencing shRNA vector) was detected only in cells that had the ancestral *CARD8* genotype and displayed a large reduction of *CARD8* transcripts (Figure 4E). No effect on cell death was detected in cells with the derived, nonsense

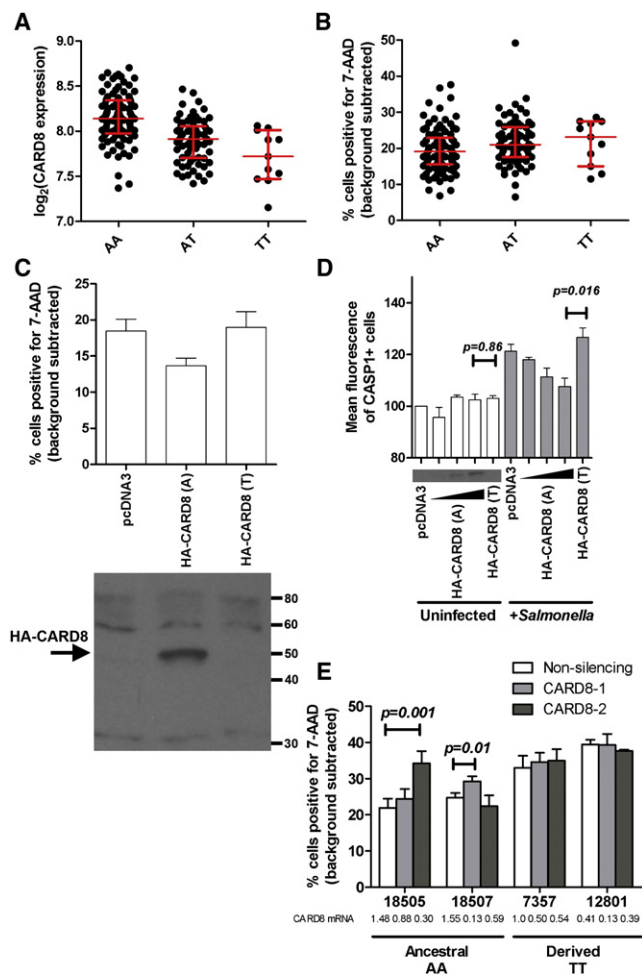


Figure 4. A Polymorphism in *CARD8* Causes Increased Cell Death in Response to *Salmonella*

(A) The derived allele of rs2043211 correlates with decreased *CARD8* expression levels. A is the ancestral allele, and T is the derived nonsense allele (designated based on the + strand of chromosome 19, as is the convention for the HapMap Project; *CARD8* coding sequence is on the - strand). Expression levels for LCLs are from Stranger et al., 2007.²⁸ All LCLs are displayed, and when family structure is controlled for in PLINK (QFAM-parents)²⁶, a *p* value of 3.72×10^{-5} is obtained for the correlation between rs2043211 genotype and *CARD8* expression. Median and interquartile ranges are displayed in red.

(B) The derived allele of rs2043211 correlates with increased cell death. All LCLs were assayed for *S. typhimurium* cell death in three separate experiments, and mean values for each are shown. Background-subtracted values reflect the subtraction of uninfected cell death. All LCLs are displayed, and when family structure is controlled for in PLINK (QFAM-parents), a *p* value of 0.013 is obtained for the correlation between rs2043211 genotype and *Salmonella*-induced cell death. Median and interquartile ranges are displayed in red.

(C) Overexpression of the ancestral *CARD8* allele inhibits cell death. LCL 7357 was transfected with 2 μ g empty vector or ancestral or derived alleles of *CARD8*. 6 hr after transfection, cells were infected with *S. typhimurium* at an MOI of 30, and cell death was measured by 7-AAD staining 2 hr after the initial infection. Only the ancestral allele inhibits the amount of detected cell death. The *p* value from repeated-measures ANOVA is 0.002. Similar results were obtained for LCL 18507 (not shown). Expression of HA-CARD8 was verified with a rabbit *CARD8* antibody. Error bars show the SEM for three separate experiments.

(D) Overexpression of the ancestral *CARD8* allele reduces caspase-1 activity in *Salmonella*-infected cells. LCL 7357 was transfected

with 2 μ g empty vector or ancestral or derived alleles of *CARD8*. For *CARD8* ancestral dose-response measurements, 0.5, 1, and 2 μ g were used. Western blot inset demonstrates increasing HA-CARD8 protein detected with *CARD8* antibody. 6 hr after transfection, cells were infected with *S. typhimurium* at an MOI of 30, and caspase-1 activation was measured by FLICA. No difference was noted in caspase-1 activation for uninfected cells, but infected cells show that the ancestral allele inhibits caspase-1 activation. The *p* values on the graph are from two-tailed *t* tests. Error bars are SEM for assays conducted four separate times (except HA-CARD8 [A] 0.5 μ g and 1 μ g, which were assayed twice).

Evidence for Adaptive Evolution of *CARD8*

The role of *CARD8* in the host response to infection prompted us to test the hypothesis that *CARD8* has been subjected to adaptive evolution. Others have noted that *CARD8* is absent in several mammalian species, including mice and cows.⁵⁸ Because group size and infectious-disease burden have been correlated in mammals that live in larger groups⁵⁹, we hypothesized that loss of *CARD8* could have been selected for in these animals. Animals that live in herds or large colonies demonstrate a significant correlation with absence and presumed evolutionary loss of *CARD8* (Figure 5A; *p* value = 0.002 for Pagel's correlation method^{32,33}). Notably, this correlation is robust despite some ambiguity in the categorization of animal group size and the determination of *CARD8* presence or absence as a result of low coverage of some genomes (Figure S1 in the Supplemental Data; see also Material and Methods). The correlation suggests that loss of *CARD8* might have been selected for; a stronger caspase-1 inflammatory response would be more effective in combating a greater infectious disease burden.

In sequenced primates, *CARD8* is uniformly present, consistent with a small-group lifestyle. The two exceptions to this social structure in our analysis are rhesus monkeys,

with 2 μ g empty vector or ancestral or derived alleles of *CARD8*. For *CARD8* ancestral dose-response measurements, 0.5, 1, and 2 μ g were used. Western blot inset demonstrates increasing HA-CARD8 protein detected with *CARD8* antibody. 6 hr after transfection, cells were infected with *S. typhimurium* at an MOI of 30, and caspase-1 activation was measured by FLICA. No difference was noted in caspase-1 activation for uninfected cells, but infected cells show that the ancestral allele inhibits caspase-1 activation. The *p* values on the graph are from two-tailed *t* tests. Error bars are SEM for assays conducted four separate times (except HA-CARD8 [A] 0.5 μ g and 1 μ g, which were assayed twice).

(E) RNAi against *CARD8* causes increased cell death in LCLs with the ancestral allele. Each LCL was stably transduced with lentivirus expressing nonsilencing shRNA or two different *CARD8* shRNAs. Knockdown was quantified in stably transduced lines via real-time RT-PCR, and expression levels normalized to LCL 7357 stably transduced with nonsilencing shRNA are shown. Each LCL was assayed in three separate experiments, and means with SEM are displayed. The *p* values on the graph are from paired two-tailed *t* tests. All other comparisons of nonsilencing versus *CARD8* RNAi constructs give *p* > 0.05.

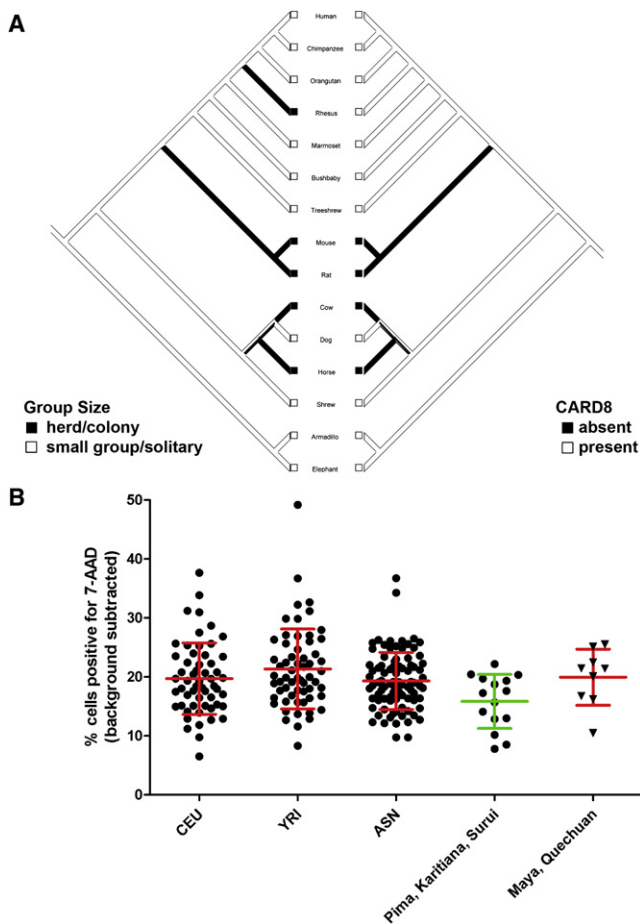


Figure 5. Evidence of Natural Selection Involving *CARD8* and Cell Death in Mammals and Humans

(A) Absence of *CARD8* in a 15-mammal phylogenetic tree correlates with large group size. A mirror tree of 15 mammalian species displays color-coded group size on the left and the presence of *CARD8* on the right. Details regarding putative orthologs can be found in Table S1. From Pagel's test of independent evolution³², a p value of 0.002 is obtained from 1000 simulations. This tree contains only species where the presence or absence of *CARD8* could be determined with high confidence ($>5\times$ coverage to call absence; presence requiring at least half of protein sequence found). An additional mirror tree of 24 mammalian species is presented as Figure S1. Image was exported from Mesquite.³³

(B) Reduced *Salmonella*-induced cell death in humans from hunter-gatherer versus agricultural populations. Cells from Native American individuals were grouped into populations that had traditionally lived as hunter-gatherers (Pima, Karitiana, and Surui) and those that had lived in denser agricultural communities (Maya and Quechuan) at the time of contact with Europeans. The aggregated hunter-gatherer populations exhibit on average 4.2% less cell death (a 21% reduction) than the CEU, YRI, ASN, and agricultural Native American groups. Error bars show means and standard deviations for each population. One-way ANOVA gives a p value of 0.017 for the means' being different among the populations. The data suggest adaptation of inflammatory caspase activation in response to changes in human societies.

which live in large overlapping troops that can number in the hundreds, and humans, who underwent a relatively recent transition from small groups of hunter-gatherers to large, agriculture-based societies. Comparison of dN/dS ratios in the five primates with complete *CARD8* sequences

demonstrates evidence of positive selection only in the rhesus monkey lineage (p value = 3.8×10^{-3} according to the branch-site test of positive selection³⁴). *CARD8* in rhesus monkeys might be undergoing positive selection for loss of function, although proof of this would require functional assays of the rhesus monkey *CARD8* gene.

This signature of selection is not present in human *CARD8*, but the introduction and increase in frequency of rs2043211 could be a step toward loss of the gene. Gene frequencies for the strongly linked SNP rs6509366 (see Figure 3) from the HGDP-CEPH Diversity Panel⁶⁰ demonstrate that the two geographic regions that acquired agriculture most recently (~4000 years ago¹⁴) have the lowest frequency: 0.18 in America and 0.211 in Africa versus 0.347 for the complete set. Furthermore, the two lowest population frequencies in this dataset are found in a hunter-gatherer population in America (Pima, 0.04) and Africa (San, 0.1). Do these population differences in *CARD8* allele frequency indicate that phenotypic variation in caspase-1 activation is subject to natural selection in humans? If so, LCLs from hunter-gatherer populations should display a reduced level of caspase-1 activation. We therefore assayed publicly available Native American LCLs for cell death in response to *Salmonella*. Although each population has undergone its own unique demographic history, we grouped the 24 LCLs as primarily hunter-gatherer (Pima, Karitiana, and Surui) or agricultural (Maya and Quechuan) for our analysis. The Maya of Mexico and Quechuan-speaking people of the Andes (including the Inca) practiced high-density terraced farming methods for many crops, including corn, cotton, and potatoes. Additionally, the Inca domesticated several animal species, including llamas and guinea pigs. The mean cell death for the LCLs derived from primarily agricultural Native Americans was very similar to the mean cell death for CEU, YRI, and ASN LCLs. In contrast, the mean cell death for the hunter-gatherer LCLs is approximately 4% less than that for CEU, YRI, ASN, or agricultural Native American populations (a relative 21% decrease; p value = 0.017 for one-way ANOVA; Figure 5B). Although we assayed nearly all the publicly available LCLs derived from Native American populations, the total number is still small, and confirming the significance of these results will require replication with additional cells or populations. Still, the result suggests population differentiation toward a more optimal level of caspase-1 activation depending on the subsistence method of the population.

Variation in *CARD8* Is Associated with Acute Inflammatory Disease

Although a more robust inflammatory response is probably adaptive to survival under conditions with a higher infectious-disease burden, an enhanced ability to fight off bacterial infection may not come without a price. Despite this advantage, the *CARD8* nonsense allele has not reached fixation. We hypothesized that losing *CARD8* inhibition of caspase-1 could be detrimental if it led to an inappropriately

Table 3. Association between *CARD8* rs2043211 and Risk of SIRS

SIRS Cases versus Healthy Controls	AA	AT	TT	p Value ^a
SIRS (n = 319)	135 (42.3%)	149 (46.7%)	35 (11.0%)	0.050
Healthy Controls (n = 567)	278 (49.0%)	238 (42.0%)	51 (9.0%)	
HapMap CEU parents (n = 60)	29 (48.3%)	26 (43.3%)	5 (8.3%)	

Genotypes are relative to the + strand of chromosome 19. *CARD8* coding sequence is on the – strand.

For comparison, the genotypes of the 60 HapMap CEU parents is shown but was not used in the case-control study.

^a p value is for the Cochran-Armitage trend test using a genotypic model with scores of 0, 0.75, and 1 for the three genotypes.

robust immune response. Acutely, this could manifest as a higher incidence or severity of inflammation in the setting of a severe infection. To test this, we genotyped individuals who were admitted to an ICU and met criteria for SIRS³⁹ and compared their *CARD8* genotype frequencies with those of healthy controls (Table 3). Patients with SIRS have physiological changes in response to an inflammatory stimulus that may be infectious (for example, pneumonia) or noninfectious (for example, pancreatitis). We tested for an association between *CARD8* genotype and SIRS by using a genotypic model dictated by the effect of the *CARD8* allele on cell death in LCLs. Because the median genotypic values for AA, AT, and TT in the parental CEU and YRI populations were 19.2, 20.4, and 20.8, we used an additive model with partial dominance of the T allele (genotypic scores of 0, 0.75, and 1) in the Cochran-Armitage trend test. We found that genotypes with the T allele were associated with the presence of SIRS (p value = 0.050). This value was just slightly lower than the values obtained for simple additive (scores of 0, 0.5, 1) and T-dominant (scores of 0, 1, 1) models, which gave p values of 0.058 and 0.055, respectively. The overrepresentation of the ancestral *CARD8* allele in the healthy controls suggests that a functional *CARD8* gene might be protective against SIRS.

Discussion

The experimental validation and evolutionary analysis of the *CARD8* SNP demonstrate the utility of the Hi-HOST approach in identifying functional genetic variation in cellular phenotypes that have probably been the target of natural selection. Natural selection modifies traits at the level of organisms within populations. However, an organism's quantitative traits are the aggregate of many intermediate phenotypes at the organ, tissue, cellular, and molecular levels. Thus, adaptation of populations might be manifest in the variation of cellular phenotypes, such as cell death in response to pathogens, that play a role in the overall survival of individuals. Hi-HOST is a useful approach in understanding the variation of important cellular phenotypes, and the relevance of variants identi-

fied in this way can be put into the broader context of human health through clinical association studies.

Our data support the idea that populations acquire a level of caspase-1 response adapted to their environment and that one way this is achieved is through the presence or absence of functional *CARD8*. It is likely that each species has undergone fine-tuning through natural selection to strike the optimal balance between the risks of too much or too little caspase-1 inflammatory response. Too little response could be ineffective at warding off bacterial infections, whereas too much response could predispose a species to SIRS or chronic autoimmune disease. For many human populations, this balance probably underwent a shift as the risk of death from highly transmissible human "crowd diseases" (such as *S. typhi*) has grown to outweigh the risk of overreacting to bacterial strains incidental to trauma.

Animal models provide further evidence for the consequences of too little or too much caspase-1 response. *CASP1* knockout mice have increased susceptibility to bacterial infection.¹⁸ Conversely, these mice are resistant to endotoxic shock from LPS challenge⁶¹, and the caspase-1 inhibitor YVAD has been shown to decrease inflammation and mortality in rat models of endotoxic shock.^{62,63} Surprisingly, the mechanism by which caspase-1 inhibition protects against sepsis is apparently independent of its pro-inflammatory cleavage of IL-1 β and IL-18. Unlike *CASP1*^{-/-} mice, IL-1 β and IL-1 β /IL-18 double-knockout mice are not protected from death due to *E. coli* sepsis.⁶⁴ A key difference noted among the mice in these studies was the presence of extensive B lymphocyte apoptosis in wild-type and IL-1 β knockout mice but not in *CASP1*^{-/-} mice. The authors suggest that prevention of lymphocyte apoptosis might be protective during sepsis. This echoes the ideas of Hotchkiss et al.⁶⁵, who have documented apoptosis of B cells during sepsis in humans⁶⁶, and the finding that prevention of lymphocyte cell death in a mouse sepsis model improves survival.⁶⁷ We suggest that the higher incidence of SIRS in individuals with the derived, nonsense *CARD8* allele might be mediated by increased caspase-1 activity, which could result in more B lymphocyte cell death. Therefore, the cell-death read-out in the LCLs we used could have further relevance as an in vitro model of lymphocyte cell death during systemic infection.

Our data suggest that individuals carrying the *CARD8* nonsense allele are at higher risk of developing SIRS, possibly as an acute consequence of an excessive caspase-1 response. The association between *CARD8* and risk of SIRS is weak, but a more informative evaluation could perhaps be made if we examined subcategories of SIRS patients. One obvious distinction is between sepsis (SIRS with a verified or strongly suspected source of infection) versus SIRS without infection. In a cohort of patients admitted to a level-one trauma center after sustaining major traumatic injury, we compared *CARD8* genotype frequencies in patients meeting criteria for SIRS with or without clinically suspected or microbiologically confirmed infection (i.e.,

a comparison of sepsis versus SIRS only). We found no significant association between *CARD8* genotypes and the development of sepsis in these trauma patients ($p = 0.53$; Table S2). A possibly more useful criterion in defining subgroups of SIRS that may show a stronger association with *CARD8* genotype could be made if we had a clearer understanding of which SIRS etiologies lead to caspase-1 activation. Sepsis, for example, is probably highly variable in caspase-1 activation depending on the causative bacteria. It will be interesting to determine in future, larger studies whether a more significant association and larger effect can be detected if cases in the study are restricted to those with measurable caspase-1 activation (perhaps on the basis of IL-1 β levels) or to etiologies known to result in caspase-1 activation.

Other published evidence suggests that *CARD8* genotype could also have chronic consequences on human health. An inappropriately strong caspase-1 response could result in autoimmune disease. Consistent with this, anakinra, a drug that blocks IL-1 (which is cleaved into its active form by caspase-1) is used to treat rheumatoid arthritis.⁶⁸ rs2043211 does not show an association with the incidence of rheumatoid arthritis or seven other common diseases (including Crohn's disease) on the basis of mining of the Wellcome Case-Control Consortium data.⁶⁹ However, two recent studies show that the *CARD8* nonsense allele is associated with increased severity of rheumatoid arthritis (MIM #180300).^{24,70} Decreased inhibition of NF- κ B has been proposed as the mechanism of action for this association²⁴, but our results show that loss of caspase-1 inhibition may be an additional mechanism. Interestingly, *CARD8* is not the only potential inhibitor of caspase-1 to have undergone a loss-of-function mutation during human evolution. *CASP12* (MIM *608633) has also acquired a nonsense mutation, and in this case, the allele has increased to fixation in European and Asian populations.⁷¹ Therefore, the loss of regulators of inflammatory responses may be a common evolutionary mechanism for protecting against negative infectious disease outcomes.

The *CARD8* validation experiments provide evidence that our p value and functional SNP filters are useful in prioritizing polymorphisms identified by association studies and has prompted us to pursue validation of the other SNPs associated with the cell-death phenotype. One of the 20 SNPs (rs9630856) lies in *SERPINB2* (MIM *173390) and correlates with the expression of the adjacent *SERPINB10* (MIM *602058) gene (GENEVAR dataset²⁸). *SERPINB2* is a serine-protease inhibitor that has been implicated in cell death in response to bacteria^{72–74}, and one member of the *SERPINB* family, CrmA, has even been demonstrated to directly inhibit caspase-1.^{74,75} Another associated SNP is the V158T polymorphism in COMT (catechol-O-methyl-transferase [MIM +116790]; rs4680), an enzyme that inactivates catecholamines and has been linked to cognitive function and schizophrenia.⁷⁵ Not only does this SNP correlate with cell death (QFAM-

parents of CEU and YRI p value = 0.0036), but the more active valine variant correlates with lower caspase-1 expression (QFAM-parents of CEU and YRI p value = 0.016). Others have shown in lymphocytes that catecholamines can function in an autocrine fashion to increase cell death, partially through transcriptional regulation of caspases.⁷⁶ We hypothesize that the greater COMT activity demonstrated by the valine variant might decrease catecholamine levels and thus result in less *CASP1* transcription and less cell death upon *Salmonella* infection. Thus, not only can the Hi-HOST approach identify SNPs important in human phenotypic variation, but it might also lead to the discovery of new branches intersecting the pathways being screened. Although not all of the 18 additional candidate SNPs are likely to be validated, it is plausible that a significant fraction regulate *Salmonella*-induced cell death. Our ongoing studies are aimed at discovering additional SNPs that truly regulate this process and the numerous other Hi-HOST phenotypes.

Therefore, this work provides proof-of-principle that in vitro human phenotyping with microbes could develop as functional tests of susceptibility and outcomes for acute and chronic inflammatory disease. The approach is adaptable for any invasive pathogen, and we have applied it to other Gram-negative bacteria, including *Yersinia spp.* (not shown). Such tests may be practically important in the future in combination with individual genotyping, particularly for diseases attributable to multiple complex genotypes.

Supplemental Data

Supplemental Data include one figure and two tables and can be found with this article online at <http://www.ajhg.org/>.

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Web Resources

The URLs for data presented herein are as follows:

Animal Diversity Web, <http://animaldiversity.ummz.umich.edu/>
Data and the GWAS Analyzer software, <http://nwrce.org/gwas-analyzer>
GENEVAR dataset, <http://www.sanger.ac.uk/humgen/genevar/>
MAXTest vignette, <http://cran.r-project.org/web/packages/coin/vignettes/MAXtest.pdf>

MEGA, <http://www.megasoftware.net/>
Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim>
PAML, <http://abacus.gene.ucl.ac.uk/software/paml.html>
PLINK, <http://pngu.mgh.harvard.edu/~purcell/plink/>
SPSmart, <http://spsmart.cesga.es/>
Wikipedia, <http://www.wikipedia.org/>

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