Genetic Variation in the *FAS* Gene and Associations with Acute Lung Injury

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Rationale: Fas (CD95) modulates apoptosis and inflammation and is believed to play an important role in lung injury.

Objectives: To determine if common genetic variation in *FAS* is associated with acute lung injury (ALI) susceptibility, risk of death, and *FAS* gene expression.

Methods: We genotyped 14 single nucleotide polymorphisms (tagSNPs) in *FAS* in samples from healthy white volunteers (control subjects, $n = 294$) and patients with ALI (cases, $n = 324$) from the ARDSnet Fluid and Catheter Treatment Trial (FACTT). *FAS* genotypes associated with ALI in the discovery study were confirmed in a nested case-control validation study of critically ill patients at risk for ALI ($n = 657$). We also tested for associations between selected tagSNPs and *FAS* mRNA levels in whole blood from healthy control subjects exposed to media alone or LPS *ex vivo*.

Measurements and Main Results: We identified associations between four tagSNPs in *FAS* (*FAS*_{-11341A>T} [rs17447140], *FAS*_{9325G>A} [rs2147420], *FAS*_{21541C>T} [rs2234978], and *FAS*_{24484A>T} [rs1051070]) and ALI case status. Haplotype-based analyses suggested that three of the tagSNPs (*FAS*_{9325G>A}, *FAS*_{21541C>T}, and *FAS*_{24484A>T}) function as a unit. The association with this haplotype and ALI was validated in a nested case-control study of at-risk subjects ($P = 0.05$). This haplotype was also associated with increased *FAS* mRNA levels in response to LPS stimulation. There was no association between *FAS* polymorphisms and risk of death among ALI cases.

Conclusions: Common genetic variants in *FAS* are associated with ALI susceptibility. This is the first genetic evidence supporting a role for *FAS* in ALI.

Keywords: adult respiratory distress syndrome; Fas receptor; genetic predisposition; apoptosis

Acute lung injury (ALI) is a common complication of critical illness that is associated with considerable morbidity and mortality (1). Approximately 7% of patients admitted to an intensive care unit (ICU) will develop ALI and it is estimated that ALI leads to more than 74,000 deaths annually in the United States (1, 2). There are several clearly defined clinical risk factors for the development of ALI, including sepsis, pneumonia, and trauma (1, 3). However, our understanding of other factors that alter susceptibility to the development of ALI

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Multiple association studies have provided evidence for the role of common genetic variation in genes involved in inflammation, chemotaxis, and coagulation in the pathogenesis of acute lung injury (ALI).

What This Study Adds to the Field

We provide evidence linking common genetic variants in the *FAS* gene to ALI susceptibility as well as increased *FAS* mRNA expression.

and related poor outcomes remains incomplete. Recent reports using genetic approaches have provided some insight into host susceptibility to ALI and related outcomes. To date, genetic variants in more than 20 genes have been associated with ALI and ALI-related outcomes. However, only a minority of these associations have been confirmed in independent study populations highlighting the challenges involved in studying the genetics of ALI resulting from complex gene-by-environment interactions and phenotypic variability (4). Among the candidate genes that have been implicated in ALI, common variants in genes involved in the inflammatory response (Nuclear Factor of Kappa Light Chain Gene Enhancer in B Cells Inhibitor, Alpha [NFKBIA], lymphotoxin α [LTA]), vascular cell function (myosin light chain kinase [MYLK], angiotensin-converting enzyme [ACE]) and generation of reactive oxygen species (nicotinamide phosphoribosyltransferase [NAMPT]) have been linked to the development of ALI and related outcomes, such as death and duration of mechanical ventilation (5–10).

A growing body of literature implicates the dysregulation of inflammation and apoptotic pathways in the development of ALI (11, 12). Specifically, the Fas pathway has been investigated as a potential contributor to the inflammation and alveolar epithelial cell apoptosis observed in the lungs of patients with ALI (13, 14). The Fas pathway, activated by the binding of Fas ligand (FasL) to Fas on the surface of a cell, leads to an intracellular cascade resulting in inflammation and apoptosis of the Fas-bearing cell. Animal studies have demonstrated that activation of the Fas pathway in the lung leads to early inflammation, alveolar epithelial apoptosis, and fibrosis (15, 16). Human studies have shown that adverse outcomes in patients with ALI are associated with increased plasma and bronchoalveolar lavage fluid levels of soluble Fas ligand (17). Dysregulation of the expression of the gene coding for Fas, *FAS*, has also been implicated in the pathogenesis of other lung diseases, such as silicosis (18) and cystic fibrosis (19). These studies, taken together, suggest that *FAS* has both pleiotropic

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(ability of one gene to influence multiple phenotypes) and epistatic (ability of one gene to modify the effects of another) effects and may be important in a range of pathophysiologic processes in the lung. Given these findings we hypothesized that genetic variation in *FAS* could influence susceptibility and outcome in ALI.

In this study, we genotyped 14 single nucleotide polymorphisms (SNPs) tagging the common linkage disequilibrium (LD) bins (tagSNPs) in *FAS* in DNA from white patients enrolled in the National Heart, Lung, and Blood Institute ARDSnet Fluid and Catheter Treatment Trial (FACTT) ($n = 324$) and white healthy control subjects ($n = 294$). We tested for associations between these tagSNPs and the presence of ALI, altered risk of death, and altered expression of *FAS* in whole blood from healthy control subjects *ex vivo*. We then tested for the association between these tagSNPs and ALI in a nested case-control validation study of critically ill white subjects at risk for ALI ($n = 657$). Some of the results of these studies have been previously reported in the form of an abstract (20).

METHODS

Study Populations

Healthy control subjects. This group of healthy volunteers has been described previously (21). Nonsmoking individuals between 18 and 65 years of age were recruited from the metropolitan Seattle area. Exclusions to enrollment included recent antibiotic use, symptoms consistent with infection, a history of autoimmune disease, immunodeficiency, use of immunosuppressive medications, cancer, or pregnancy. Total and differential leukocyte counts were performed on each blood sample in the clinical laboratory at Harborview Medical Center, Seattle, Washington.

FACTT subjects. Patients with ALI enrolled in the multicenter Fluid and Catheter Treatment Trial (FACTT) (22, 23) who provided informed consent for genetic studies ($n = 474$) were included in this study. Eligible patients were intubated, receiving positive-pressure ventilation, and qualified for ALI as defined by American European Consensus Criteria (24). Major exclusion criteria included the presence of ALI for greater than 48 hours and the presence of irreversible conditions with an estimated 6-month mortality of greater than 50% (e.g., advanced cancer, severe liver disease).

Systemic inflammatory response validation cohort. The Harborview Medical Center systemic inflammatory response syndrome (SIRS) cohort includes a DNA and clinical database of prospectively enrolled ICU subjects meeting criteria for SIRS (25), excluding those with major trauma, an intracranial hemorrhage, HIV or immunosuppression, or current diagnosis of cancer, and has been described previously (26). Genomic DNA is purified from peripheral blood samples and comprehensive phenotyping including the subsequent occurrence of ALI has been performed through both manual chart abstraction and automated query of electronic medical records. Clinical data including demographics, admission diagnosis, comorbidities, admission Acute Physiology and Chronic Health Evaluation III score, presence of ALI (chest X-ray scored by three independent readers), presence of infection, organ dysfunction, death, or discharge disposition has been acquired from the electronic medical record. Patients are classified as having SIRS, sepsis, septic shock, and ALI using consensus conference criteria. Clinical risks for ALI are manually abstracted, collected from the medical record, and include infection/sepsis, witnessed gastric aspiration, and multiple transfusions. All studies described were approved by the University of Washington, Division of Human Subjects Research.

Identification of Genotypes and Genotyping

We selected 14 tagSNPs characterizing common genetic variation in the *FAS* gene using LD SELECT (27) as implemented on the genome variation server (<http://gvs.gs.washington.edu/GVS>). *FAS* tagSNPs were genotyped by the 5' nuclease assay (TaqMan) using the ABI PRISM Sequence Detection System (SDS; Applied Biosystems, Foster City, CA) in all three study populations as previously described (28).

Additional detail on the methods for selecting and genotyping these TagSNPs is provided in an online data supplement.

Statistical Analysis

Observed genotype frequencies were compared with expected frequencies to test for deviations from Hardy-Weinberg equilibrium using Fisher exact test. To minimize the risk of confounding due to racial differences in SNP frequency, our primary analysis was restricted to whites (29). Given the lack of independence in our tests of association, a Bonferroni correction would have resulted in an overly conservative threshold for statistical significance. Therefore, we used a false discovery rate (FDR) threshold of 0.1 (indicating a 10% chance that a significant result represents a false positive) to control per family-wise error (30). All analyses were performed using STATA X (Stata Corp., College Station, TX).

We compared genotypic frequencies for the 14 *FAS* tagSNPs in the healthy control subjects and the FACTT ALI population using Fisher exact test. We performed a subgroup analysis to determine if the strength of the associations were increased in the subgroup of patients with pneumonia comparing the genotype frequencies for the same tagSNPs in healthy control subjects and subjects with ALI with pneumonia using a codominant model and Fisher exact test. We also performed exploratory haplotype analysis to determine if the associated genotypes represented a common haplotype conferring increased risk for ALI using HAPLOVIEW (<http://www.broad.mit.edu/haploview/haploview>) to generate haplotypes (31). We then performed logistic regression to determine the association between haplotype copy number and ALI case status using HAPSTAT (<http://www.bios.unc.edu/~lin/hapstat/>).

FAS Genotypes and ALI Mortality

We tested for associations between the 14 *FAS* tagSNPs and 60-day mortality using logistic regression with recessive and codominant models. We adjusted for age, sex, ALI risk factor, and FACTT treatment arm allocation as covariates that were determined *a priori*. We made no adjustment for severity of illness because we hypothesize that genetic variation in *FAS* may participate, causally, in the development of organ dysfunction and, in turn, outcomes in ALI.

Measurement of mRNA Levels

To determine the association between an identified *FAS* ALI risk haplotype and levels of *FAS* mRNA, we selected the 3 healthy white control subjects carrying two copies of the *FAS* ALI risk haplotype, 9 subjects carrying one copy of the *FAS* ALI risk haplotype and one copy of the most common haplotype, and 10 subjects carrying two copies of the most common haplotype, who were matched for age and sex. Levels of *FAS* mRNA were measured in whole blood incubated with media alone or *Escherichia coli* 0111:B4 LPS (10 ng/ml, List Biological Laboratories, Inc., Campbell, CA) as previously described (32). Additional detail on the methods for measuring *FAS* mRNA is provided in the online data supplement.

To determine if *FAS* expression is elevated in the peripheral blood monocytes of subjects with ALI, we also compared *FAS* RNA levels in peripheral blood monocytes purified from whole blood obtained from 8 subjects with ALI and 10 healthy control subjects. Additional details on the characteristics of these subjects and the methods for monocyte purification are included in the online data supplement. Monocytes were purified using negative immunoselection and total RNA was isolated from each monocyte preparation using the ABI Prism 6100 (Life Technologies, Carlsbad, CA). RNA quality was assessed by capillary electrophoresis (Bio-Rad, Hercules, CA). Approximately 20 ng of total RNA was converted to biotin-labeled cRNA using the two-cycle cDNA preparation kit followed by the GeneChip IVT labeling kit (Affymetrix, Santa Clara, CA) per the manufacturer's protocol. Biotin-labeled cRNA was then hybridized with the Affymetrix U133 plus 2.0 microarray. Raw intensity values were normalized and $\log_{10}$ transformed using DCHIP (33). Normalized expression values for all probes for *FAS*, *CD14* (a monocyte marker), and *ACTB* (a cytoskeletal housekeeping gene) were extracted. Expression levels measured in monocytes from healthy control subjects and patients with ALI were then compared using Student *t* test.

TABLE 1. DEMOGRAPHICS OF SUBJECTS WITH ACUTE LUNG INJURY GENOTYPED VERSUS SUBJECTS WITH ACUTE LUNG INJURY NOT GENOTYPED FROM FLUID AND CATHETER TREATMENT TRIAL AND HEALTHY CONTROL SUBJECTS

Characteristic	FACTT (ALI) Subjects Genotyped (n = 474)	FACTT (ALI) Subjects Not Genotyped (n = 526)	P Value*	Healthy Control Subjects (n = 477)
Patient age, mean $\pm$ SD	50 $\pm$ 16	50 $\pm$ 16	0.68	33 $\pm$ 13
Male patients, no. (%)	255 (53.8)	280 (53.2)	0.91	188 (39.5)
Patient race, no. (%)				
White (non-Hispanic)	324 (68.4)	312 (59.3)	0.01	294 (61.6)
Black	93 (19.6)	123 (23.4)	0.13	40 (8.4)
Other	57 (12.0)	91 (17.3)	0.06	143 (30.0)
Hospital LOS, median (IQR)	15 (9–26)	16 (9–26)	0.61	
ICU LOS, median (IQR)	10 (6–18)	10 (6–18)	0.63	
60-d Mortality, no. (%)	118 (24.9)	150 (28.5)	0.2	
ARDS risk factor, no. (%)				
Aspiration	113 (23.8)	94 (17.9)	0.02	
Pneumonia	304 (64.1)	345 (65.6)	0.63	
Sepsis	203 (42.8)	223 (42.4)	0.89	
Trauma	45 (9.5)	41 (7.8)	0.34	
Multiple transfusions	13 (2.7)	19 (3.6)	0.44	
Other	41 (8.6)	43 (8.2)	0.79	
APACHE III score, mean $\pm$ SD	95 $\pm$ 30.7	93 $\pm$ 31.1	0.40	
Comorbidities, no. (%)				
Diabetes	86 (18.1)	87 (16.5)	0.48	
HIV or AIDS	22 (4.6)	48 (9.1)	0.01	
Cirrhosis	15 (3.2)	17 (3.2)	0.89	
Solid tumors	8 (1.7)	7 (1.3)	0.64	
Leukemia/lymphoma	9 (1.9)	26 (4.9)	0.01	
Immunosuppression	35 (7.4)	43 (7.7)	0.66	

Definition of abbreviations: ALI = acute lung injury; APACHE = Acute Physiology and Chronic Health Evaluation; ARDS = adult respiratory distress syndrome; FACTT = Fluid and Catheter Treatment Trial; ICU = intensive care unit; IQR = interquartile range in days; LOS = length of stay in days.

* P values represent t tests for continuous variables and χ^2 tests for categorical variables.

RESULTS

Characteristics of Patients

The characteristics of the FACTT subjects are shown in Table 1. The healthy control subjects (n = 477) were younger (mean age $\pm$ SD, 33 $\pm$ 13 vs. 50 $\pm$ 16) and were less likely to be male (39.5 vs. 53.8%). Characteristics of the 474 FACTT subjects who consented to genetic studies were similar to FACTT subjects who did not consent, with the exception that those who consented were more likely to be white and have had aspiration as a risk factor for ALI and were less likely to have HIV or leukemia/lymphoma. When the comparison was limited to only white subjects, the only significant differences were that

the genotyped subjects were slightly younger (mean age 50 vs. 53, $P = 0.05$) and were less likely to have leukemia or lymphoma (1.5 vs. 7.3%, $P = 0.001$). The similarity between subjects consenting and not consenting to genetic analyses suggests that the subgroup of the FACTT study genotyped in our study is representative of the entire trial population.

FAS Genotype Frequencies in Patients with ALI and Healthy Control Subjects

We genotyped 14 tagSNPs in the *FAS* gene with a 98% success rate (Table 2). We confirmed the accuracy of our genotyping assays in the three SNPs that showed evidence for deviation from Hardy-Weinberg equilibrium (HWE) in our control

TABLE 2. GENOTYPE FREQUENCIES IN HEALTHY CONTROL SUBJECTS VERSUS ACUTE LUNG INJURY CASES FROM FLUID AND CATHETER TREATMENT TRIAL

FAS Polymorphism	dbSNP No.	Healthy Control Genotype Counts (%) (n = 294)				χ^2 P Value for HWE in Healthy Control Whites	FACTT Genotype Counts (%) (n = 324)				χ^2 P Value for HWE in FACTT Whites	Fisher Exact Test for Genotype Freq. (P Value)*
FAS _{20077C>T}	rs11202914	TT 55 (18.8)	CT 120 (41.0)	CC 118 (40.3)	0.02		TT 60 (18.8)	CT 154 (48.1)	CC 106 (33.1)	0.76		0.14
FAS _{9019T>A}	rs17447091	AA 10 (3.5)	AT 57 (19.7)	TT 233 (76.9)	0.01		AA 10 (3.1)	AT 59 (18.5)	TT 250 (78.4)	0.01		0.93
FAS _{11341A>T}	rs17447140	TT 77 (26.8)	AT 122 (42.5)	AA 88 (30.7)	0.01		TT 63 (19.8)	AT 179 (56.1)	AA 77 (24.1)	0.03		0.004
FAS _{9736A>G}	rs6586160	GG 16 (5.5)	AG 110 (37.8)	AA 165 (56.7)	0.67		GG 23 (7.3)	AG 101 (31.9)	AA 193 (60.9)	0.06		0.25
FAS _{9029T>A}	rs6586161	AA 2 (0.8)	AT 58 (22.4)	TT 199 (76.8)	0.06		AA 7 (2.2)	AT 73 (22.7)	TT 242 (75.2)	0.61		0.46
FAS _{6947G>A}	rs7910825	AA 49 (16.9)	AG 144 (49.7)	GG 97 (33.5)	0.72		AA 67 (21.7)	AG 162 (52.4)	GG 80 (25.9)	0.38		0.08
FAS _{1624T>A}	rs10509561	AA 28 (9.6)	AT 147 (50.5)	TT 116 (39.9)	0.06		AA 49 (15.3)	AT 148 (46.1)	TT 124 (38.6)	0.66		0.11
FAS _{2956A>G}	rs7097467	GG 2 (0.7)	AG 56 (19.1)	AA 235 (80.2)	0.50		GG 4 (1.2)	AG 62 (19.3)	AA 256 (79.5)	0.91		0.84
FAS _{9325G>A}	rs2147420	AA 41 (14.2)	AG 144 (50.0)	GG 103 (35.8)	0.41		AA 54 (17.1)	AG 178 (56.5)	GG 83 (26.4)	0.01		0.04
FAS _{16308A>G}	rs9658750	GG 11 (4.1)	AG 63 (23.6)	AA 193 (72.3)	0.05		GG 10 (3.3)	AG 68 (22.2)	AA 229 (74.6)	0.09		0.73
FAS _{19710T>C}	rs982764	CC 26 (8.9)	CT 127 (43.3)	TT 140 (47.8)	0.71		CC 28 (8.9)	CT 118 (37.3)	TT 170 (53.8)	0.25		0.34
FAS _{21541C>T}	rs2234978	TT 18 (6.2)	CT 115 (39.8)	CC 156 (54.0)	0.60		TT 29 (9.3)	CT 150 (48.1)	CC 133 (42.6)	0.15		0.02
FAS _{23206T>G}	rs1977389	GG 56 (19.2)	GT 139 (47.8)	TT 96 (33.0)	0.65		GG 47 (14.7)	GT 155 (48.4)	TT 118 (36.9)	0.73		0.27
FAS _{2448A>T}	rs1051070	TT 2 (0.7)	AT 41 (14.6)	AA 238 (84.7)	0.87		TT 5 (1.6)	AT 70 (21.9)	AA 244 (76.5)	0.99		0.04

Definition of abbreviations: FACTT = Fluid and Catheter Treatment Trial; HWE = Hardy-Weinberg equilibrium; SNP = single nucleotide polymorphism.

* Bolded values correspond to a false discovery rate < 0.1.

TABLE 3. ACUTE LUNG INJURY CASES VERSUS HEALTHY CONTROL SUBJECTS BY *FAS* HAPLOTYPE

	<i>FAS</i> Haplotype*			Case/ Control Frequency	OR (95% CI) [†]	<i>P</i> Value
	<i>FAS</i> _{9325G>A}	<i>FAS</i> _{21541C>T}	<i>FAS</i> _{2448A>T}			
1	0	0	0	0.54/0.61	ref	
2	1	0	0	0.13/0.14	1.05 (0.74–1.49)	0.78
3	1	1	0	0.20/0.18	1.31 (0.97–1.77)	0.08
4	1	1	1	0.13/0.08	1.73 (1.16–2.57)	0.007

Definition of abbreviations: CI = confidence interval; OR = odds ratio.

* Only haplotypes with an overall frequency > 0.01 are shown. Zero indicates the presence of the common allele, whereas 1 indicates the presence of the rare allele.

[†] OR associated with each copy of the haplotype using an unadjusted, additive logistic regression model.

population by performing genotyping in 23 Centre d'Etude du Polymorphisme Humain (CEPH) European individuals previously genotyped by Perlegen (<http://genome.perlegen.com>). For each of the three SNPs in the 23 individuals, we obtained identical genotyping results to those reported in dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP>). Queries of existing databases of copy number variations and polymorphisms (<http://projects.tcag.ca/variation>) found no evidence of additional genetic variation that could alter apparent frequencies of these genotypes. These findings confirm the accuracy of our genotyping and, thus, all tagSNPs were carried forward to statistical analysis.

To identify genetic variation that might alter host susceptibility to ALI we compared tagSNP genotype frequencies in white patients with ALI from FACTT and healthy control subjects. We identified four tagSNPs that were associated with ALI case status in whites (Table 2). In the case of *FAS*_{-11341A>T}, heterozygotes were overrepresented among the subjects with ALI, whereas homozygotes for the minor allele were overrepresented among subjects with ALI for *FAS*_{9325G>A}, *FAS*_{21541C>T}, and *FAS*_{2448A>T}. To determine if these associations were being driven by cases of primary lung injury due to pneumonia, we performed a subgroup analysis limiting the patients in FACTT to those with pneumonia (*n* = 207), the most common predisposing risk for ALI in FACTT. In spite of the large reduction in sample size, the association between all of these polymorphisms and case status remained significant (*P* ≤ 0.05) (see Table E2 in the online supplement). This suggests that genetic variation may alter susceptibility to severe pneumonia, which, in turn, leads to ALI.

To determine if the four genotypes that were associated with the presence of ALI might function as a haplotype to confer increased risk for ALI, we performed a haplotype-based analysis. We found that the three minor allele genotypes overrepresented in ALI can be found on a single *FAS* ALI risk haplotype (*FAS* haplotype 4) (Figure E1). Moreover, the *FAS* haplotype 4 (*FAS*_{-6947A}, *FAS*_{2448A}, *FAS*_{21541T}) is inferred to be relatively common (9.1% overall), and carriers of this haplotype were overrepresented in ALI cases (odds ratio [OR], 1.73; 95% CI, 1.16–2.57; *P* = 0.007) (Table 3). These findings suggest that these three genotypes individually associated with ALI are actually representative of a single haplotype and that a functional polymorphism found on this haplotype alters susceptibility to critical illness with ALI. The fourth genotype associated with ALI in the 5' upstream region of *FAS* (*FAS*_{-11341A>T}) was not present on this haplotype. This suggests that this variant functions independently of *FAS* haplotype 4 to alter susceptibility.

FAS Genotype and Outcome in Patients with ALI

We next tested for associations between tagSNPs in *FAS* and 60-day mortality. The overall 60-day mortality among genotyped subjects with ALI was 24.9% and was similar to the overall mortality previously reported in the FACTT (23). We found no association between *FAS* genotypes and 60-day mortality in the ALI cohort in either the unadjusted or adjusted logistic regression models (Table E3).

FAS Genotype Frequencies in Subjects with ALI and At-Risk Control Subjects

We sought to validate the associations observed in our study of ALI cases from FACTT and healthy control subjects with the four *FAS* tagSNPs by genotyping these SNPs in an independent cohort of ICU subjects with SIRS at risk for development of ALI. The characteristics of these subjects are shown in Table 4. Frequencies of all four *FAS* tagSNPs were consistent with HWE in the SIRS cohort (Table 4). In our primary analysis adjusted for age, sex, and ALI risk factor, none of the four SNPs demonstrated a significant association with susceptibility to ALI, although carriers of the T allele at *FAS*_{21541C>T} demonstrated a trend toward higher susceptibility, as was the case in our discovery case control study (OR, 1.28; 95% CI, 0.97–1.68; *P* = 0.08) (Table 5). It has been suggested that chest radiographs in patients with hypoxemic respiratory failure might be insensitive to subtle underlying lung injury, and interrater reliability in the diagnosis of ALI has been shown to be only

TABLE 4. CHARACTERISTICS OF SUBJECTS IN THE SYSTEMIC INFLAMMATORY RESPONSE SYNDROME VALIDATION NESTED CASE-CONTROL STUDY

Characteristic	Control Subjects (<i>N</i> = 682)	ALI Cases (<i>N</i> = 283)	Total (<i>N</i> = 965)
Patient age, mean ± SD	54 ± 15	56 ± 16	54 ± 15
Male patients, no. (%)	426 (62.5)	199 (70.3)	625 (64.8)
Patient race, no. (%)			
White (non-Hispanic)	463 (67.9)	194 (68.6)	657 (68.1)
Black	108 (15.8)	35 (12.4)	143 (14.8)
Asian/Pacific islander	57 (8.4)	25 (8.8)	82 (8.5)
Other	54 (7.9)	29 (10.2)	83 (8.6)
Hospital LOS, median (IQR)	12 (7–21)	18 (11–34)	14 (7–25)
Hospital mortality, no. (%)	60 (8.8)	70 (24.7)	130 (13.5)
ALI risk factor, no. (%)			
Aspiration	44 (6.5)	57 (20.1)	101 (10.5)
Pneumonia	120 (17.6)	112 (39.6)	232 (24.0)
Sepsis	412 (60.4)	245 (86.6)	657 (68.1)
Multiple Transfusions	4 (0.6)	9 (3.2)	13 (1.4)
Other	46 (6.7)	15 (5.3)	61 (6.3)

Definition of abbreviations: ALI = acute lung injury; IQR = interquartile range in days; LOS = length of stay in days.

TABLE 5. GENOTYPE FREQUENCIES IN AT-RISK CONTROL SUBJECTS VERSUS ACUTE LUNG INJURY CASES AMONG WHITES IN THE SYSTEMIC INFLAMMATORY RESPONSE SYNDROME COHORT

FAS Polymorphism	At-Risk Control Genotype Counts (%) (n = 463)						χ^2 P Value for HWE	Entire Cohort			Restricted Phenotypes*		
								OR for ALI†	95% CI	P Value	OR for ALI†	95% CI	P Value
FAS _{-11341A>T}	TT 122 (27.1)	AT 226 (50.2)	AA 102 (22.7)	TT 51 (27.1)	AT 98 (52.1)	AA 39 (20.7)	0.69	0.93	0.72–1.22	0.61	0.97	0.71–1.30	0.83
FAS _{9325G>A}	AA 86 (18.9)	AG 221 (48.7)	GG 147 (32.4)	AA 36 (19.1)	AG 97 (51.3)	GG 56 (29.6)	0.93	0.98	0.76–1.28	0.89	1.07	0.79–1.44	0.67
FAS _{21541C>T}	TT 40 (8.8)	CT 174 (38.3)	CC 240 (52.9)	TT 21 (11.1)	CT 84 (44.4)	CC 84 (44.4)	0.35	1.28	0.97–1.68	0.08	1.39	1.01–1.90	0.04
FAS _{24484A>T}	TT 4 (0.9)	AT 85 (18.7)	AA 365 (80.4)	TT 3 (1.6)	AT 34 (18.2)	AA 150 (80.2)	0.99	0.99	0.65–1.53	0.88	1.18	0.72–1.94	0.51

Definition of abbreviations: ALI = acute lung injury; CI = confidence interval; HWE = Hardy-Weinberg equilibrium; OR = odds ratio.

* Analysis excluding subjects with non-ALI hypoxic respiratory failure (defined as $\text{PaO}_2/\text{FiO}_2 < 300$ without a qualifying chest X-ray).

† Additive model, adjusted for age, sex, and ALI risk factors.

moderate (34, 35). Thus, to eliminate potential misclassification bias based on these issues, we performed a secondary analysis restricting the control subjects to only those without hypoxemia ($\text{PaO}_2/\text{FiO}_2$ ratio < 300). Elimination of control subjects with a $\text{PaO}_2/\text{FiO}_2$ ratio < 300 who had not had a chest radiograph with qualifying bilateral opacities revealed a significant association between FAS_{21541C>T} and development of ALI (OR, 1.39; 95% CI, 1.01–1.90; $P = 0.04$). We then performed the same haplotype analysis that was performed for the discovery case-control study, which revealed an association between a haplotype carrying this risk allele (FAS haplotype 3) and ALI in the SIRS validation study ($P = 0.05$) (Table 6). When the subjects with hypoxic respiratory failure but no ALI were excluded from the haplotype analysis, the association with FAS haplotype 3 increased slightly and remained significant ($P = 0.05$) (Table 6).

FAS ALI Genotypes and FAS Gene Expression

To determine if the FAS ALI risk haplotype is associated with altered gene expression of FAS in primary leukocytes, we compared FAS mRNA levels in whole blood from healthy volunteers who carried two copies of the FAS ALI risk haplotype to carriers of one copy of the FAS ALI risk haplotype and those carrying two copies of the most commonly occurring haplotype, matched for age and sex. Given that FAS expression can be markedly up-regulated following an inflammatory stimulus and in patients with sepsis (36–39), we tested FAS gene expression after stimulation with bacterial LPS, a potent innate immune agonist, relative to FAS expression in the resting state. Despite the small number of healthy subjects in our cohort who carried copies of FAS haplotype 4 ($n = 3$), we found that FAS mRNA levels increased with each additional copy of FAS haplotype 4 after incubation with bacterial LPS (Figure 1).

FAS Expression in ALI

To investigate whether regulation of FAS gene expression in the peripheral blood compartment might have relevance in ALI, we measured gene expression of FAS, CD14, and ACTB, using an oligonucleotide microarray, in peripheral blood monocytes purified from 8 subjects with recently diagnosed ALI (within 24 h of onset) and 10 healthy control subjects. Four of five probes specific for the predominant and reference FAS transcript (NCBI RefSeq: NM_000043.3) present on the microarray showed increased expression in monocytes from patients with ALI relative to control subjects (Table 7). In contrast, expression of the monocyte marker CD14, and of all seven probes for ACTB (β -actin), was not significantly different between the two groups.

DISCUSSION

FAS is a key mediator of apoptosis and inflammation and its activation has been shown to cause lung injury *in vitro* and in murine models (12, 13, 16). Selectively blocking Fas has been shown to attenuate lung injury and activation via Fas has been shown to cause lung injury in animal models (16). However, the effect of modulating Fas/FasL pathway activation on the development of ALI in humans remains unknown. In humans, rare genetic deficiency in FAS expression leads to a T-cell lymphoproliferative disorder (40, 41) and common variants in FAS have been associated with systemic sclerosis (42) and lymphoid malignancies, such as non-Hodgkin lymphoma (43). To our knowledge, our study is the first to report the potential role for genetic variation in FAS in ALI susceptibility. Using a two-stage case-control followed by nested case-control study design in two independent populations, we identified associations between common genetic variation in FAS and ALI

TABLE 6. FAS HAPLOTYPE AND ACUTE LUNG INJURY CASE STATUS AMONG WHITES IN THE SYSTEMIC INFLAMMATORY RESPONSE SYNDROME COHORT

FAS Haplotype*				Restricted Phenotypes†						
FAS _{9325G > A}	FAS _{21541C > T}	FAS _{24484A > T}	Frequency, Cases:Control Subjects	OR†	95% CI	P Value	Frequency, Cases:Non-hypoxic Respiratory Failure Control Subjects	OR†	95% CI	P Value
1	0	0	0.55:0.56	ref	0.55:0.59	Ref				
2	1	0	0.11:0.16	0.75	0.51–1.10	0.13	0.11:0.15	0.83	0.67–1.02	0.36
3	1	0	0.23:0.17	1.33	1.00–1.82	0.05	0.23:0.17	1.40	1.18–1.67	0.05
4	1	1	0.11:0.10	1.10	0.72–1.61	0.72	0.11:0.08	1.38	1.01–1.74	0.16

Definition of abbreviations: ALI = acute lung injury; CI = confidence interval; OR = odds ratio.

* Only haplotypes with a frequency $> 1\%$ in the control subjects are shown. Zero indicates the presence of the common allele, whereas 1 indicates the presence of the rare allele.

† Adjusted for age, sex, and ALI risk factor.

‡ Analysis excluding subjects with non-ALI hypoxic respiratory failure (defined as $\text{PaO}_2/\text{FiO}_2 < 300$ without a qualifying chest X-ray).

Figure 1. *FAS* gene expression in blood leukocytes by *FAS* haplotype 4 copy number. Bars represent the mean fold change ($\pm$ SD) in *FAS* mRNA level measured using duplicate reverse transcription polymerase chain reaction measurements of Δ Ct (cycle threshold) values normalized to a control mRNA (glyceraldehyde-3-phosphate dehydrogenase) in whole blood incubated with LPS relative to whole blood incubated with media. The *P* value reflects an unadjusted additive linear regression model.

susceptibility. In our first case-control study using ALI cases from FACTT and healthy control subjects, we demonstrated an association between four *FAS* genotypes and the presence of ALI at an FDR threshold of 0.1. We went on to test whether these polymorphisms might function as a haplotype and found that three of the individually associated SNPs can be found on a common haplotype and that this haplotype is associated with ALI case status. This finding suggests that the true ALI risk alleles reside on the haplotype tagged by these SNPs.

Although this finding suggested a role for *FAS* polymorphisms in ALI susceptibility, the use of healthy volunteers as control subjects in this study did not allow for the differentiation of genetic effects altering susceptibility to ALI from those altering susceptibility to critical illness that, in turn, leads to ALI. Indeed, in a secondary analysis in which we limited our comparison to only ALI cases from FACTT with pneumonia, the associations between these same *FAS* genotypes and the ALI population remained highly significant for all four genotypes, suggesting that variants in *FAS* might alter the risk of developing severe pneumonia and, subsequently, ALI.

To further clarify the influence of *FAS* genetic variants on the development of ALI, we performed a nested case-control

study in a cohort of critically ill patients with SIRS at risk for development of ALI. The use of at-risk control subjects in this cohort provides support for the assertion that genetic variation in *FAS* influences susceptibility to ALI. First, we found that one of the genotypes associated with ALI in the initial case-control analyses, *FAS*_{21541C>T}, was also associated with higher risk of ALI when the control subjects were restricted to those without hypoxemic respiratory failure. We performed this secondary analysis restricting the control group in an effort to minimize misclassification of borderline cases of ALI as control subjects. Our findings suggest that such misclassification bias exists and could lead to false-negative results in gene association studies of ALI, as has recently been argued by Shah and colleagues (44). We also found that a haplotype containing *FAS*_{21541T} and *FAS*_{9325A} was associated with development of ALI in the SIRS cohort. The difference in the haplotypes demonstrating maximal association with ALI susceptibility in the initial (haplotype 3: *P* = 0.08; haplotype 4: *P* = 0.007; Table 3) and validation studies (haplotype 3: *P* = 0.05; haplotype 4: *P* = 0.16; Table 6) may be explained by differences in the study design, differences in underlying ALI risk factors (e.g., proportion of pneumonia and sepsis cases), and differences in underlying population structure. Future efforts in larger populations will be needed to identify the variant(s) in *FAS* in linkage disequilibrium with these genotyped variants that are most likely to play a direct functional role in ALI susceptibility. Of note, we did not observe any association between *FAS* genotypes and mortality in either the initial or validation study. This suggests that common genetic variation in *FAS* has a greater influence on susceptibility to ALI as opposed to severity. However, this finding might also reflect the relative infrequency of mortality among our ALI cases.

Query of existing databases revealed no protein-altering variants in significant linkage disequilibrium with the variants we identified as associated with ALI susceptibility. Therefore, we sought to identify effects of the identified risk variants on *FAS* gene expression. We tested for an effect of genetic variation in *FAS* on expression of *FAS* in whole blood from healthy volunteers in the presence or absence of a potent inflammatory stimulus, LPS. Our findings in this model demonstrated that the *FAS* ALI risk haplotype (*FAS* haplotype 4) was associated with increased *FAS* expression after innate immune stimulation with LPS. We have also demonstrated that *FAS* mRNA expression in peripheral blood monocytes of

TABLE 7. DIFFERENCES IN RNA EXPRESSION FOR *FAS*, *ITGAM*, *CD14*, AND *ACTB* IN MONOCYTES FROM HEALTHY CONTROL SUBJECTS AND SUBJECTS WITH ACUTE LUNG INJURY

Gene	Probeset ID*	Probe Intensity Values†		Two-Sided <i>t</i> Test <i>P</i> Value
		Control, Mean (95% CI) (<i>N</i> = 10)	ALI Mean (95% CI) (<i>N</i> = 8)	
<i>FAS</i>	204780_s_at	8.74 (8.55–8.93)	9.42 (9.21–9.63)	<0.0001
<i>FAS</i>	204781_s_at	8.92 (8.73–9.11)	9.58 (9.46–9.70)	<0.0001
<i>FAS</i>	215719_x_at	6.32 (5.82–6.82)	7.07 (6.66–7.48)	0.02
<i>FAS</i>	216252_x_at	6.01 (5.59–6.43)	6.26 (5.58–6.93)	0.47
<i>FAS</i>	233820_at	3.72 (3.46–3.99)	4.19 (3.84–4.53)	0.02
<i>CD14</i>	201743_at	12.76 (12.66–12.86)	12.90 (12.72–13.07)	0.13
<i>ACTB</i>	224594_x_at	13.23 (13.16–13.29)	13.27 (13.09–13.44)	0.58
<i>ACTB</i>	AFFX-HSAC07/X00351_5_at	10.06 (9.51–10.61)	10.52 (9.81–11.22)	0.25
<i>ACTB</i>	AFFX-HSAC07/X00351_M_at	12.09 (11.69–12.48)	12.24 (11.89–12.60)	0.52
<i>ACTB</i>	213867_x_at	13.19 (13.07–13.30)	13.27 (13.11–13.42)	0.35
<i>ACTB</i>	200801_x_at	13.32 (13.25–13.39)	13.35 (13.18–13.52)	0.70
<i>ACTB</i>	224594_x_at	13.23 (13.16–13.29)	13.27 (13.09–13.44)	0.58
<i>ACTB</i>	AFFX-HSAC07/X00351_3_at	13.18 (13.12–13.25)	13.17 (13.02–13.32)	0.86

Definition of abbreviations: ALI = acute lung injury; CI = confidence interval.

* Probeset IDs for the Affymetrix U133 plus 2.0 microarray.

† Raw intensity values were normalized and log₁₀ transformed using DCHIP.

subjects with ALI is increased compared with healthy control subjects. To our knowledge, this is the first report of increased *FAS* gene expression in peripheral leukocytes of subjects with ALI and links our expression data from healthy subjects to the relevant clinical phenotype. Interestingly the *FAS* tagSNP, *FAS*_{21541C>T} (rs2234978) that was most significantly associated with ALI in the validation cohort creates a putative miRNA binding site (<http://compbio.uthsc.edu/miRSNP/help.php>). Future studies will investigate the possibility that this site may alter post-transcriptional control via miRNA.

Our findings build on existing data demonstrating that Fas activation promotes alveolar inflammation and apoptosis resulting in lung injury. In animal models, it has been shown that genetic deficiency of Fas renders mice resistant to experimental lung injury (15, 16). Furthermore, treatment of rabbits with Fas ligand leads to alveolar epithelial apoptosis and lung injury (16). In humans, elevated levels of soluble Fas and Fas ligand in bronchoalveolar lavage fluid are associated with ALI/adult respiratory distress syndrome (17). Our data suggest that a genetic predisposition to greater Fas expression might increase susceptibility to ALI in the presence of clinical risk factors for ALI. Future studies will be needed to clarify the effects of the *FAS* variants on *FAS* gene and protein expression, the timing and tissue specificity of these effects, and whether these effects may be causal in the development of ALI in humans.

Our study has several clear strengths. First, our LD bin tagSNP approach provided a comprehensive analysis of common genetic variation in *FAS* and its role in host susceptibility to ALI. Second, we used well-characterized cases of ALI enrolled in the multiinstitutional NHLBI ARDSnet FACTT study. The rigorous case definitions used for FACTT ensured a gold-standard definition of ALI in this study. Second, we used an independent cohort of critically ill patients at risk for development of ALI to clarify that *FAS* genotypes are likely to play a role in ALI susceptibility. The findings in the two different case-control studies greatly reduce the chances that our findings represent type I error and imply that the effect of *FAS* variants on ALI susceptibility can be generalized to the white American population. Of note, we observed no significant associations between any of the *FAS* tagSNPs and ALI in the smaller subset of blacks, though power was limited to detect such a difference.

This study has several important limitations. First, given reductions in ALI-related mortality over the past decade, our sample size had limited statistical power to identify modest effects of *FAS* polymorphisms on this important ALI-related clinical outcome. Second, differences in the designs used in the discovery and validation studies preclude a good overall estimate of the effect of *FAS* genotypes on risk for ALI. Additional nested case-control studies will be needed to achieve better estimates of the overall effect of these genotypes. Furthermore, our study was limited to whites and, therefore, the extent to which our findings can be generalized to other populations is unknown. Our studies linking *FAS* genotypes with altered gene expression of *FAS* were performed in whole blood and, thus, primarily reflect modulation of expression in peripheral leukocytes. It is unknown whether these effects will also be observed in alveolar epithelium. Future studies in large, deeply phenotyped populations at risk for ALI will be needed to better understand the epidemiologic and biologic effects of *FAS* variants on ALI susceptibility.

In conclusion, we have provided the first evidence linking common variants in the *FAS* gene to ALI susceptibility. In a two-stage discovery/validation study, the commonly occurring *FAS* haplotype including *FAS*_{21541T} and *FAS*_{9325A} was significantly associated with ALI susceptibility and increased *FAS* mRNA expression in peripheral leukocytes in response to innate

immune stimulation. Further studies are needed to determine how this haplotype modifies *FAS* expression and how genetic differences in Fas expression alter the risk of ALI in humans.

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References

1. Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, Stern EJ, Hudson LD. Incidence and outcomes of acute lung injury. *N Engl J Med* 2005;353:1685–1693.
2. Goss CH, Brower RG, Hudson LD, Rubenfeld GD. Incidence of acute lung injury in the United States. *Crit Care Med* 2003;31:1607–1611.
3. Hudson LD, Milberg JA, Anardi D, Maumder RJ. Clinical risks for development of the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1995;151:293–301.
4. Gao L, Barnes KC. Recent advances in genetic predisposition to clinical acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L713–L725.
5. Christie JD, Ma SF, Aplenc R, Li M, Lanken PN, Shah CV, Fuchs B, Albelda SM, Flores C, Garcia JG. Variation in the myosin light chain kinase gene is associated with development of acute lung injury after major trauma. *Crit Care Med* 2008;36:2794–2800.
6. Zhai R, Zhou W, Gong MN, Thompson BT, Su L, Yu C, Kraft P, Christiani DC. Inhibitor kappab-alpha haplotype GTC is associated with susceptibility to acute respiratory distress syndrome in Caucasians. *Crit Care Med* 2007;35:893–898.
7. Zhai R, Gong MN, Zhou W, Thompson TB, Kraft P, Su L, Christiani DC. Genotypes and haplotypes of the VEGF gene are associated with higher mortality and lower VEGF plasma levels in patients with ARDS. *Thorax* 2007;62:718–722.
8. Marshall RP, Webb S, Bellingan GJ, Montgomery HE, Chaudhari B, McNulty RJ, Humphries SE, Hill MR, Laurent GJ. Angiotensin converting enzyme insertion/deletion polymorphism is associated with susceptibility and outcome in acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2002;166:646–650.
9. Gong MN, Zhou W, Williams PL, Thompson BT, Pothier L, Boyce P, Christiani DC. -308GA and TNFB polymorphisms in acute respiratory distress syndrome. *Eur Respir J* 2005;26:382–389.
10. Ye SQ, Simon BA, Maloney JP, Zambelli-Weiner A, Gao L, Grant A, Easley RB, McVerry BJ, Tudor RM, Standiford T, et al. Pre-B-cell colony-enhancing factor as a potential novel biomarker in acute lung injury. *Am J Respir Crit Care Med* 2005;171:361–370.
11. Perl M, Lomas-Neira J, Chung CS, Ayala A. Epithelial cell apoptosis and neutrophil recruitment in acute lung injury—a unifying hypothesis? What we have learned from small interfering RNAs. *Mol Med* 2008;14:465–475.
12. Martin TR, Hagimoto N, Nakamura M, Matute-Bello G. Apoptosis and epithelial injury in the lungs. *Proc Am Thorac Soc* 2005;2:214–220.
13. Matute-Bello G, Lee JS, Liles WC, Frevert CW, Mongovin S, Wong V, Ballman K, Sutlief S, Martin TR. Fas-mediated acute lung injury requires fas expression on nonmyeloid cells of the lung. *J Immunol* 2005;175:4069–4075.
14. Altemeier WA, Zhu X, Berrington WR, Harlan JM, Liles WC. Fas (CD95) induces macrophage proinflammatory chemokine production via a myd88-dependent, caspase-independent pathway. *J Leukoc Biol* 2007;82:721–728.
15. Hagimoto N, Kuwano K, Miyazaki H, Kunitake R, Fujita M, Kawasaki M, Kaneko Y, Hara N. Induction of apoptosis and pulmonary fibrosis in mice in response to ligation of Fas antigen. *Am J Respir Cell Mol Biol* 1997;17:272–278.

16. Matute-Bello G, Liles WC, Frevert CW, Nakamura M, Ballman K, Vathanaprida C, Kiener PA, Martin TR. Recombinant human Fas ligand induces alveolar epithelial cell apoptosis and lung injury in rabbits. *Am J Physiol Lung Cell Mol Physiol* 2001;281:L328–L335.
17. Albertine KH, Soulier MF, Wang Z, Ishizaka A, Hashimoto S, Zimmerman GA, Matthay MA, Ware LB. Fas and Fas ligand are up-regulated in pulmonary edema fluid and lung tissue of patients with acute lung injury and the acute respiratory distress syndrome. *Am J Pathol* 2002;161:1783–1796.
18. Otsuki T, Miura Y, Nishimura Y, Hyodoh F, Takata A, Kusaka M, Katsuyama H, Tomita M, Ueki A, Kishimoto T. Alterations of Fas and Fas-related molecules in patients with silicosis. *Exp Biol Med (Maywood)* 2006;231:522–533.
19. Kumar V, Becker T, Jansen S, van Barneveld A, Boztug K, Wolf S, Tummler B, Stanke F. Expression levels of Fas are regulated through an evolutionary conserved element in intron 2, which modulates cystic fibrosis disease severity. *Genes Immun* 2008;9:689–696.
20. Glavan BJ, Holden TD, Goss CH, Black TR, Martin TR, Wurfel MM. Genetic variation in the *fas* gene and associations with outcomes in acute lung injury [abstract]. *Am J Respir Crit Care Med* 2009;179:A3863.
21. Wurfel MM, Gordon AC, Holden TD, Radella F, Strout J, Kajikawa O, Ruzinski JT, Rona G, Black RA, Stratton S, *et al.* Toll-like receptor 1 polymorphisms affect innate immune responses and outcomes in sepsis. *Am J Respir Crit Care Med* 2008;178:710–720.
22. Wheeler AP, Bernard GR, Thompson BT, Schoenfeld D, Wiedemann HP, deBoisblanc B, Connors AF Jr, Hite RD, Harabin AL. Pulmonary-artery versus central venous catheter to guide treatment of acute lung injury. *N Engl J Med* 2006;354:2213–2224.
23. Wiedemann HP, Wheeler AP, Bernard GR, Thompson BT, Hayden D, deBoisblanc B, Connors AF Jr, Hite RD, Harabin AL. Comparison of two fluid-management strategies in acute lung injury. *N Engl J Med* 2006;354:2564–2575.
24. Bernard GR, Artigas A, Brigham KL, Carlet J, Falke K, Hudson L, Lamy M, Legall JR, Morris A, Spragg R. The American-European Consensus Conference on ARDs. Definitions, mechanisms, relevant outcomes, and clinical trial coordination. *Am J Respir Crit Care Med* 1994;149:818–824.
25. Levy MM, Fink MP, Marshall JC, Abraham E, Angus D, Cook D, Cohen J, Opal SM, Vincent JL, Ramsay G. 2001 SCCM/ESICM/ACCP/ATS/SIS international sepsis definitions conference. *Intensive Care Med* 2003;29:530–538.
26. Ko DC, Shukla KP, Fong C, Wasnick M, Brittnacher MJ, Wurfel MM, Holden TD, O'Keefe GE, Van Yserloo B, Akey JM, *et al.* A genome-wide in vitro bacterial-infection screen reveals human variation in the host response associated with inflammatory disease. *Am J Hum Genet* 2009;85:214–227.
27. Carlson CS, Eberle MA, Rieder MJ, Yi Q, Kruglyak L, Nickerson DA. Selecting a maximally informative set of single-nucleotide polymorphisms for association analyses using linkage disequilibrium. *Am J Hum Genet* 2004;74:106–120.
28. Livak KJ. Allelic discrimination using fluorogenic probes and the 5' nuclease assay. *Genet Anal* 1999;14:143–149.
29. Cardon LR, Bell JL. Association study designs for complex diseases. *Nat Rev Genet* 2001;2:91–99.
30. Storey JD, Tibshirani R. Statistical significance for genomewide studies. *Proc Natl Acad Sci USA* 2003;100:9440–9445.
31. Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, *et al.* The structure of haplotype blocks in the human genome. *Science* 2002;296:2225–2229.
32. Lee JS, Wurfel MM, Matute-Bello G, Frevert CW, Rosengart MR, Ranganathan M, Wong VW, Holden T, Sutlief S, Richmond A, *et al.* The Duffy antigen modifies systemic and local tissue chemokine responses following lipopolysaccharide stimulation. *J Immunol* 2006;177:8086–8094.
33. Li C, Hung Wong W. Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application. *Genome Biol* 2001;2:RESEARCH0032.
34. Rubenfeld GD, Caldwell E, Granton J, Hudson LD, Matthay MA. Interobserver variability in applying a radiographic definition for ARDs. *Chest* 1999;116:1347–1353.
35. Meade MO, Cook RJ, Guyatt GH, Groll R, Kachura JR, Bedard M, Cook DJ, Slutsky AS, Stewart TE. Interobserver variation in interpreting chest radiographs for the diagnosis of acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2000;161:85–90.
36. Darville MI, Eizirik DL. Cytokine induction of fas gene expression in insulin-producing cells requires the transcription factors NF-kappaB and C/EBP. *Diabetes* 2001;50:1741–1748.
37. Liu D, Darville M, Eizirik DL. Double-stranded ribonucleic acid (RNA) induces beta-cell Fas messenger RNA expression and increases cytokine-induced beta-cell apoptosis. *Endocrinology* 2001;142:2593–2599.
38. Papathanassoglou ED, Moynihan JA, McDermott MP, Ackerman MH. Expression of Fas (CD95) and Fas ligand on peripheral blood mononuclear cells in critical illness and association with multiorgan dysfunction severity and survival. *Crit Care Med* 2001;29:709–718.
39. Marsik C, Halama T, Cardona F, Wlassits W, Mayr F, Pleiner J, Jilma B. Regulation of Fas (apo-1, CD95) and Fas ligand expression in leukocytes during systemic inflammation in humans. *Shock* 2003;20:493–496.
40. Rieux-Laucat F, Le Deist F, Hivroz C, Roberts IA, Debatin KM, Fischer A, de Villartay JP. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science* 1995;268:1347–1349.
41. Drappa J, Vaishnav AK, Sullivan KE, Chu JL, Elkon KB. Fas gene mutations in the Canale-Smith syndrome, an inherited lymphoproliferative disorder associated with autoimmunity. *N Engl J Med* 1996;335:1643–1649.
42. Broen J, Gourh P, Rueda B, Coenen M, Mayes M, Martin J, Arnett FC, Radstake TR. The Fas -670A>G polymorphism influences susceptibility to systemic sclerosis phenotypes. *Arthritis Rheum* 2009;60:3815–3820.
43. Wang SS, Purdue MP, Cerhan JR, Zheng T, Menashe I, Armstrong BK, Lan Q, Hartge P, Krickler A, Zhang Y, *et al.* Common gene variants in the tumor necrosis factor (TNF) and TNF receptor superfamilies and NF-kB transcription factors and non-Hodgkin lymphoma risk. *PLoS ONE* 2009;4:e5360.
44. Shah CV, Lanken PN, Localio AR, Gallop R, Bellamy S, Ma SF, Flores C, Kahn JM, Finkel B, Fuchs BD, *et al.* An alternative method of acute lung injury classification for use in observational studies. *Chest* (In press)