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The IL1RN Promoter rs4251961 Correlates with IL-1 Receptor Antagonist Concentrations in Human Infection and Is Differentially Regulated by GATA-1

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IL-1R antagonist (IL-1Ra) is required for adequate host defense in invasive pneumococcal disease (IPD). The minor allele of an *IL1RN* gene (C/T) promoter polymorphism (rs4251961) has been shown to be associated with decreased IL-1Ra production in healthy adults. We genotyped 299 children with IPD, and examined 19 IL1RN haplotype-tagging single-nucleotide polymorphisms. Human embryonic kidney HEK293(T) cells were transfected with the promoter reporter plasmid pGL3p containing either allelic variant C (pGL3pCC) or T (pGL3pTT) with or without cotransfection with an expression construct overexpressing the globin transcription factor GATA-1. Plasma IL-1Ra concentrations were significantly higher in nonsurvivors compared with survivors ($p < 0.0005$), and the C allele of rs4251961 was associated with a significant increase in plasma IL-1Ra concentrations ($p = 0.01$) during the acute illness of IPD. These findings were validated in a cohort of 276 treatment-naive HIV-infected adults, with borderline significance ($p = 0.058$). Functional analyses demonstrated that the activity of the promoter constructs containing the T allele increased ∼6-fold as compared with basal activity, and that containing the C allele by ∼9-fold ($p < 0.001$) in the presence of GATA-1. Our findings suggest that the IL-1Ra single-nucleotide polymorphism rs4251961 plays a key role in the pathophysiology of IPD and in other human infections. *The Journal of Immunology*, 2011, 186: 000–000.

Invasive pneumococcal disease (IPD) is responsible for a significant number of deaths in children in sub-Saharan Africa (1, 2). In a trail of nine-valent pneumococcal vaccine in The Gambia, children aged 3–29 mo who received the vaccine had a 16% lower all-cause mortality than controls (3), indicating that there is a significant childhood mortality associated with IPD. The risk of IPD is highest in children under 2 y of age, with pneumonia, meningitis, and bacteremia being the commonest disease manifestations. A small number of genetic association studies in IPD has been published with conflicting or unconfirmed results. These studies have examined mannose-binding lectin polymorphisms (4), (5), the C-reactive protein dinucleotide repeat polymorphism (6), the IL-10 −1082 promoter polymorphism (rs1800896) (7), and the FcγR (FcγRIIA) R/R131 polymorphism (rs1801274) (8). More recently, larger studies have found protective effects in IPD for NF-κB inhibitor gene (NFKBIA) polymorphisms (9) and a variant of the adaptor protein Mal (Mal S180L) (rs8177374), which is involved in TLR signaling (10). Our group reported that polymorphic variation within the NOS2A gene does not influence invasive pneumococcal disease susceptibility or survival (11).

IL-1 signaling plays a critical role in the host response to pneumococcal infection. The effects of IL-1 are tightly controlled by two naturally occurring inhibitors, IL-1R antagonist (IL-1Ra) and IL-1R type II (IL-1RII). IL-1Ra possesses anti-inflammatory properties. IL-1R type I gene-deficient (IL-1R$^{-/-}$) mice are more susceptible to pneumococcal meningitis, and have higher mortality and enhanced growth of pneumococci compared with wild-type mice (12). Treatment with human rIL-1Ra results in an enhanced bacterial growth in murine pneumococcal pneumonia (13), and IL-1Ra$^{-/-}$ mice are resistant to pneumococcal bacterial challenge, with significantly lower bacterial loads in the lungs of IL-1Ra$^{-/-}$ mice compared with wild-type mice (14). Taken together, these data suggest that the regulation of IL-1Ra, IL-1R, and IL-1 is complex, and that there needs to be a delicate balance between beneficial effects of modulating bacterial clearance and the detrimental effects of exacerbating the inflammatory response.

The gene that encodes for IL-1Ra (IL1RN) and five other closely related cytokine genes form a gene cluster spanning ∼400 kb on chromosome 2 (Chr2q14.2). Rafiq et al. (15) reported their analysis of common variation across the IL-1RN gene, and found that the minor allele of rs4251961 (tagging the commonest haplotype)

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is associated with lowered IL-1Ra serum concentrations in otherwise healthy elderly individuals. The rs579543 single-nucleotide polymorphism (SNP), which is in strong linkage disequilibrium with the intron-2 VNTR variant, was associated with IL-1Ra levels in a regression model that included rs4251961. Additionally, they found that the IL1RN 1018 haplotype was associated with increased concentrations of IL-1β and IFN-γ. These findings were recently confirmed by a study that used three independent cohorts comprising elderly individuals, young adults, and adults with severe carotid disease (16). We hypothesized that the IL1RN gene may impact on disease severity and survival in invasive pneumococcal disease through modulation of IL-1Ra levels. We demonstrate that the C allele of rs4251961 is associated with higher plasma IL-1Ra concentrations during the acute illness and also provides supportive evidence of a functional role for this polymorphism. Using a separate cohort of treatment-naive HIV-infected adults, we validated the findings of the association between the C allele and elevated IL-1Ra concentrations in this population.

Materials and Methods

Study populations

Derivation cohort: Malawian children with invasive pneumococcal disease. This study was conducted at Queen Elizabeth Central Hospital (Blantyre, Malawi), a government-funded teaching and referral center, which serves a population of approximately 1 million. Children presenting with signs and symptoms of meningitis or pneumonia or with confirmed pneumococcal bacteremia were prospectively recruited into the study. All children who met the study criteria between April 2004 and October 2006 were included in this study, and are therefore representative of children with IPD in Malawi. Ethical approval for this study was granted from the College of Medicine Research Committee, Malawi, and the Liverpool School of Tropical Medicine Local Research Ethics Committee.

Cases. Cases were children with confirmed invasive pneumococcal disease, as follows: pneumococcal pneumonia, pneumococcal meningitis, or pneumococcal bacteremia confirmed by either culture, Ag test, or PCR.

Confirmed pneumococcal pneumonia. There was radiological evidence of pneumonia (focal or lobar consolidation) plus one or more of the following: blood or lung aspirate culture positive for pneumococci, lung aspirate positive for pneumococcal polysaccharide Ag, or pneumococcal DNA.

Confirmed pneumococcal meningitis. There was abnormal CSF cell count, $>10/\mu l$ plus one or more of the following: CSF culture positive for pneumococci, CSF Gram stain consistent with pneumococci, CSF positive for pneumococcal polysaccharide Ag, and CSF positive for pneumococcal DNA.

Controls were healthy afebrile, aparasitemic children from the same villages as the index cases, and were as closely age matched as possible. For each index case, at least three healthy, age-matched children were selected from the neighborhood.

Validation cohort: antiretroviral naive HIV-infected Malawian adults. One thousand antiretroviral naive Malawian adult patients commencing triple therapy with Nevirapine, Stavudine, and Lamivudine were recruited between March 2007 and August 2009. Patients were followed up for 26 wk as part of a prospective clinical cohort examining nevirapine hypersensitivity. Careful clinical assessment of all patients were undertaken every 2 wk, and laboratory parameters including CD4$^+$ count and liver function tests were monitored. HIV viral loads were not available, but all of these patients were not yet on treatment and therefore would have had significant viremia. Ethical approval for this study was granted from the College of Medicine Research Committee, Malawi, and the Liverpool School of Tropical Medicine Local Research Ethics Committee.

Sample collection

Derivation cohort: Malawian children with invasive pneumococcal disease. Both blood and buccal samples for genotyping were collected onto Whatman FTA Elute cards (Whatman, Maidestone, U.K.) and stored in airtight containers until extraction. Blood samples were collected by venepuncture (cases) or finger prick (controls). The venepuncture samples were transferred by syringe onto the Whatman card, and the finger-prick sample was transferred directly from the patient to the Whatman card. Buccal swabs were collected from inside the cheek onto a swab, which was

then transferred from the swab onto the indicating circle on the Whatman card. Samples for cytokine determination were taken into an EDTA tube and separated within 1 h, and the plasma stored at −80°C until analysis.

Validation cohort: antiretroviral naive HIV-infected Malawian adults. Blood samples were collected on day 0 of commencing treatment (i.e., prior to starting antiretroviral treatment). For cytokine determination, 5 ml blood was collected in plain bottles and centrifuged within 2 h. The serum supernatant was aliquoted and immediately frozen at −80°C. For genotyping, 5 ml blood anticoagulated with EDTA was collected and stored at −80°C until analysis.

Cytokine determination

Derivation cohort: Malawian children with invasive pneumococcal disease. Cytokine determination was performed in plasma using a commercial 27-plex Bioplex Cytokine kit (Bio-Rad) that utilizes Luminex 100 technology in the Bio-plex Protein Array System (Bio-Rad), according to the manufacturer's instructions.

Validation cohort: antiretroviral naive HIV-infected Malawian adults. IL-1Ra serum levels were determined based on the Quantikine colorimetric sandwich ELISA system (R&D Systems, Abingdon, U.K.) following manufacturer's instructions. Briefly, standard controls and samples were run in duplicates, and 100 μl diluent RD1S and 100 μl sample/standard were applied to each well and incubated for 2 h at room temperature. Plates were then washed five times, and 200 μl conjugate was added to each well and incubated at room temperature. Following another series of wash steps, 200 μl substrate was added to each well, the plates were then protected from light and rested for another 30 min, and 50 μl stop solution was added in the end. Samples were read at 450 nm and adjusted for both the negative control and the background (λ correction at 540 nm) using a Fluostar Ω multidetection microplate reader (BMG Labtech, Offenburg, Germany).

HIV determination

Derivation cohort: Malawian children with invasive pneumococcal disease. HIV status was assessed in children ≥18 mo using at least two of the following tests: Unigold (Trinity Biotech, Wicklow, Ireland), Serocard (Trinity Biotech), or Determine-HIV (Abbott Laboratories). In children <18 mo, and those with discordant Ab tests, HIV status was determined using Amplicor HIV-1 DNA (Roche Diagnostics).

Validation cohort: antiretroviral naive HIV-infected Malawian adults. HIV status was assessed in duplicate per subject using second-generation Determine-HIV (Abbott Laboratories) and Unigold (Trinity Biotech) as confirmatory tests.

Selection of haplotype-tagging SNPs

SNP information within the 16.1-kb IL1RN gene and 3 kb of 5′ and 3′ untranslated flanking sequence was taken from HapMap (http://www.hapmap.org) using the Yoruba population from Ibadan. Haplotype-tagging SNPs with a minor allele frequency >5% were selected using Tagger software v.4.0, as implemented in Haploview v.3.32. The minimum pairwise correlation (r^2) to select haplotype-tagging SNPs was 0.8.

Genotyping

Derivation cohort: Malawian children with invasive pneumococcal disease. DNA was extracted from the Whatman cards according to the manufacturer's instructions. PCR were carried out on PTC-225 Peltier Thermal Cyclers (MJ Research, Waltham, MA) in 384-well microtitre plates using 10 ng genomic DNA with a final reaction volume of 10 μl. Genotyping was performed using Sequenom technology (San Diego, CA). Five replication samples and two blank controls were used as quality controls. All laboratory work was performed under the ISO 9001:2000 quality management requirements. Genotyping was performed twice, initially on cases, only then on both cases and controls together. The case data results were the same in both runs. When the cases and controls were run together, only 10 SNPs were run in the replication.

Validation cohort: antiretroviral naive HIV-infected Malawian adults. Genomic DNA was extracted from 200 μl peripheral blood anticoagulated with EDTA using the QIASymphony SP Instrument (Qiagen, Hilden, Germany) following the manufacturer's instructions for the Fixed200 DNA Blood protocol. The genotyping assay containing sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest and the TaqMan minor groove binding probes (based on VIC and FAM fluorescent dyes) was ordered at ×40 concentration directly from Applied Biosystems (Life Technologies, Warrington, U.K.). Approximately 20 ng DNA was amplified by real-time PCR using ABI Prism 7900HT Real-Time System (Life Technologies), and the following PCR conditions were used: incubation period at 50°C for 2 min, activation at 95°C for 10 min,

followed by 40 cycles of 10 s at 95°C and 1 min at 60°C. Genotyping was performed for 280 samples and duplicates; positive and negative controls without DNA template were included with the runs. Postread allelic discrimination analysis was performed with SDS Software v2.2.2 (Life Technologies), and alleles were clustered according to their genotype groups.

Prediction of transcription factor binding sites

Bioinformatics screening for transcription factor binding sites was performed using the latest versions of publicly available suites Poly Matrix Search, Transcription Factor Search, and Transcription Element Search Software based on TRANSFAC, CBIL-GibbsMat, JASPAR, and IMD matrices. Analyses were carried out on the 50-bp sequence encompassing the promoter SNP rs4251961 using both allelic variants T and C, and a minimum matrix similarity of 0.85 was applied.

Several predicted binding sites for the globin transcription factors (GATA-1, GATA-2, and GATA-3) were identified in the sequence. One in particular, in the reverse strand, was predicted directly over the SNP and corresponded to a GATA motif, which serves as a tag for several DNA-binding complexes, including GATA-1 (17). It is theoretically possible that the presence of genetic polymorphisms could alter the affinity or specificity of the protein-binding complex direct differential expression of target genes dependent on genotype; therefore, we decided to test whether the alleles of the promoter SNP rs4251961 could differentially regulate expression in the presence of GATA-1.

Cloning of reporter gene constructs

A short (309-bp) region of the IL1RN gene that included the site of the variant allele (C or T) was amplified from a genomic DNA of known genotype by high fidelity PCR in 25 μl using 0.6 U PFU Taq polymerase (Promega). The following primers were used: IL1RN forward, 5′-aaACGCGTagcaagggcctgtgtaaatg-3′ and IL1RN reverse, 5′-aaCTCGA-Gagcttcatgtctctgcattctg-3′ (note capitals representing restriction sites for MluI and XhoI at the 3′ of each primer, respectively). After an initial denaturation at 94°C for 3 min, PCR was performed using 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Products of the correct length were purified using a Qiaquick gel extraction kit (Qiagen), according to the manufacturer's instructions, and were ligated using 1 U T4 DNA ligase (Promega) into pGL3p and pGL3b (Promega) expression constructs linearized at the multiple cloning site by overnight digestion with MluI and XhoI enzymes. Plasmids were transformed into competent Dh5α (Invitrogen), maxipreps (Qiagen) were prepared according to the manufacturer's instructions, and sequences were confirmed by DNA sequencing. The outsourced pMT2-GATA overexpression construct (Addgene plasmid 13626) (18) was prepared identically to the other overexpression constructs used in this study. All constructs were diluted to 1 ug/μl before use in the reporter gene assays detailed below.

Reporter gene assays

Reporter gene assays were carried out using a dual luciferase assay system (Promega; catalogue E1910) after initial cotransfection of an internal control renillin reporter construct and a pGL3 reporter construct into HEK293(T) cells, according to the manufacturer's instructions. The following samples were examined: pGL3b, pGL3p, pGL3p-TT, pGL3p-CC, pGL3p plus GATA1, pGL3p-TT plus GATA1, and pGL3p-CC plus GATA1. The assays were carried out in sextuplicate in a 96-well format. The luciferase activity was normalized relative to renillin activity. Results were then expressed as fold induction of luciferase activity of the construct relative to luciferase activity of pGL3p alone.

Statistical analysis

Cytokine parameters were compared between two groups using the Mann-Whitney U test, and correlations were examined using Spearman's correlation coefficient. Linkage disequilibrium between markers was calculated using the software HelixTree (Golden Helix, Bozeman, MT). Haplotype blocks were designated using an algorithm based on the confidence interval method (19), which was implemented in Haploview version 4.1 (Broad Institute). Association analysis was performed using least squares multiple regression analysis in Stata v.8.2 (2001).

Results

Study participant characteristics

Derivation cohort: Malawian children with invasive pneumococcal disease. **Cases.** We genotyped 299 children with IPD, 166 (56%) of

whom were male, with a median age of 2.3 y (interquartile range [IQR] 0.7–6.1 y). There were 65 deaths (22%). HIV status was available on 294 children, of whom 168 (57%) were HIV infected. There were 221 children with pneumococcal meningitis (74%), 41 (14%) with pneumococcal pneumonia (with or without bacteremia), and 37 (12%) with pneumococcal bacteremia only.

Controls. We genotyped 933 healthy controls, 476 (51%) of whom were male, with a median age of 4.0 y (IQR 2.3–7 y). HIV status was not determined in the controls. In a subset of 81 consecutive controls that were tested anonymously, the HIV positivity rate was 5%.

Validation cohort: antiretroviral naive HIV–infected Malawian adults. **Cases.** We genotyped 280 treatment-naive HIV-infected adults, 99 (35%) of whom were male, with a median age of 36 y (IQR 30–43 y). Median CD4 count was 202 cells/mm^3 (IQR 114–326 cells/mm^3), and median body mass index 20.5 (IQR 18.8–22.7).

Plasma IL-1Ra is elevated in nonsurvivors with IPD

Plasma TNF-α, IL-1Ra, IL-6, and IL-10 were all significantly higher in cases compared with controls ($p < 0.0005$). In cases, median plasma IL-1Ra concentrations were significantly higher in nonsurvivors than survivors (17,206, IQR 8,937–44,569 versus 6,853, IQR 3,090–14,704 pg/ml), in meningitis than pneumonia
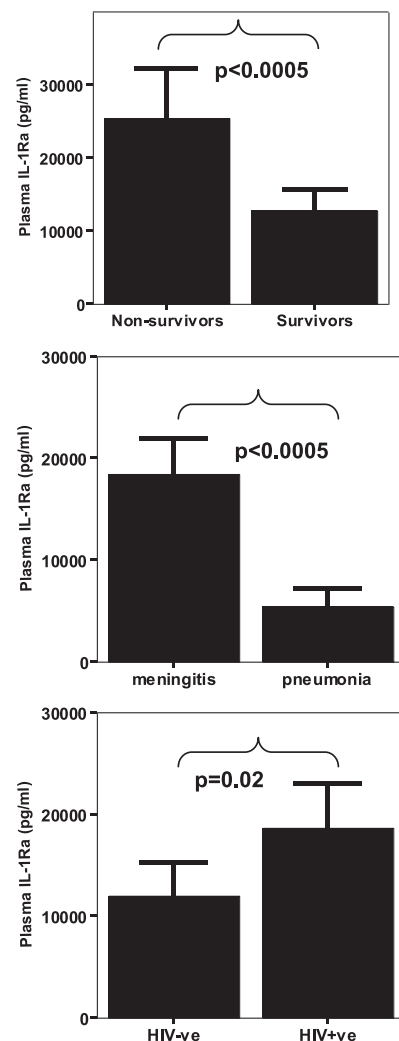

FIGURE 1. Plasma IL-1Ra levels in survivors/nonsurvivors, meningitis/pneumonia, and HIV-infected/HIV-uninfected children (error bars are mean ± 2.0 SE of mean).

Table I.   IL1RN SNPs and allele frequencies in cases and controls

SNP	rs Number	Polymorphism	Nucleotide Position	MAF Cases	HWE p Value	Call Rate	Previously Reported MAF	MAF Controls
IL1RNsnp1	rs315931	CA 5′ near gene	113586314	0.41	0.21	0.97	0.50	ND
IL1RNsnp2	rs17042917	GA 5′ near gene	113587134	0.08	0.22	0.98	0.19	ND
IL1RNsnp3	rs4251961	TC 5′ near gene	113590938	0.07	0.94	1.00	0.09	0.07
IL1RNsnp4	rs2637988	GA intron 1	113593250	0.48	0.52	0.99	0.35	0.47
IL1RNsnp5	rs928940	TG intron 1	113593966	0.42	0.33	0.99	0.47	0.43
IL1RNsnp6	rs3213448	GA intron 1	113595768	0.24	0.33	0.98	0.30	ND
IL1RNsnp7	rs4251991	TG intron 1	113596520	0.24	0.60	0.99	0.29	0.25
IL1RNsnp8	rs315936	CT intron 1	113597418	0.17	0.68	0.94	0.18	0.16
IL1RNsnp9	rs315935	AG intron 1	113597836	0.19	0.49	0.99	0.18	0.17
IL1RNsnp10	rs315934	AG intron 1	113600177	0.08	0.87	1.00	0.10	0.08
IL1RNsnp11	rs3181052	GA intron 2	113602520	0.16	0.69	0.98	0.22	0.16
IL1RNsnp12	rs1794066	AG intron 2	113602821	0.41	0.92	1.00	0.48	0.43
IL1RNsnp13	rs380092	TA intron 4	113605371	0.18	0.66	0.99	0.17	ND
IL1RNsnp14	rs452204	GA intron 4	113605532	0.45	0.81	0.97	0.60	ND
IL1RNsnp15	rs315952	CT exon 5 syn	113606775	0.45	0.76	0.99	0.48	ND
IL1RNsnp16	rs9005	GA 3′UTR	113607883	0.13	0.53	1.00	0.18	0.13
IL1RNsnp17	rs315949	GA 3′ near gene	113609245	0.27	0.59	0.99	0.28	ND
IL1RNsnp18	rs315943	GA 3′ near gene	113610809	0.32	0.07	0.92	0.34	ND
IL1RNsnp19	rs2902452	CA 3′ near gene	113611987	0.12	0.32	0.98	0.22	ND

HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency; ND, not done; 3′UTR, untranslated region.

(10,157, IQR 5,066–21,406 versus 4,041, IQR 1,938–6,309 pg/ml), and in HIV-infected than HIV-uninfected children (9,162, IQR 4,732–23,119 versus 6,446, IQR 3,023–14,833 pg/ml) (Fig. 1). Plasma IL-1Ra correlated positively with blood pneumococcal bacterial loads ($r = 0.48$, $p < 0.0005$) and negatively with white blood count ($r = -0.38$, $p < 0.0005$) after adjusting for HIV status. Median plasma IL-6 and IL-10 concentrations were also significantly higher in nonsurvivors (IL-6: 4,168, IQR 881–9,454 versus 1,055, IQR 402–3,277 pg/ml, and IL-10: 161, IQR 113–536 versus 108, IQR 59–201 pg/ml, $p < 0.0005$), in meningitis (IL-6: 1,852, IQR 632–6,094 versus 492, IQR 236–1,072 pg/ml, and IL-10: 133, IQR 77–262 versus 82, IQR 29–170 pg/ml, $p < 0.005$), and in HIV-infected children (IL-6: 1,845, IQR 568–7,719 versus 1,021, IQR 350–2,435 pg/ml, and IL-10: 137, IQR 81–296 versus 112, IQR 52–180 pg/ml, $p < 0.01$).

SNP rs4251961 is associated with IL-1Ra levels

Derivation cohort: Malawian children with invasive pneumococcal disease. The mean genotype call rate was >98%, and all SNPs were

in Hardy-Weinberg equilibrium. Differences in allele frequencies were observed when comparing the Malawi population with HapMap frequencies reported for the Nigerian Yoruba tribe (http://www.hapmap.org) (Table I). Haplotype block prediction based on the tagging SNPs suggests two blocks that span between SNPs 3–5 (3028 bp) and SNPs 6–12 (7053 bp) (Fig. 2). Linear regression analysis comparing allele frequencies against IL-1Ra levels showed a significant association with SNP rs4251961 ($p = 0.0006$), which remained significant after Bonferroni correction ($p = 0.01$) (Table II). This SNP sits within haplotype block 1 and showed moderate linkage disequilibrium with SNPs rs2637988 ($r^2 = 0.54$) and rs928940 ($r^2 = 0.49$), although these two SNPs showed no association.

Validation cohort: antiretroviral naive HIV–infected Malawian adults. The mean genotype call rate was >98.9%. Although allele frequency for the replication cohort (8.7%) was similar to what was found in the derivation cohort (7%), and in line with frequencies reported by HapMap for a sub-Saharan population (9.2%), genotype distribution was not consistent with Hardy-

FIGURE 2.   Linkage disequilibrium (LD) plot of IL1RN gene showing R-squared values and haplotype blocks. The LD plot and haplotype blocks were generated using standard settings of the Haploview software (available from http://www.broadinstitute.org/haploview).
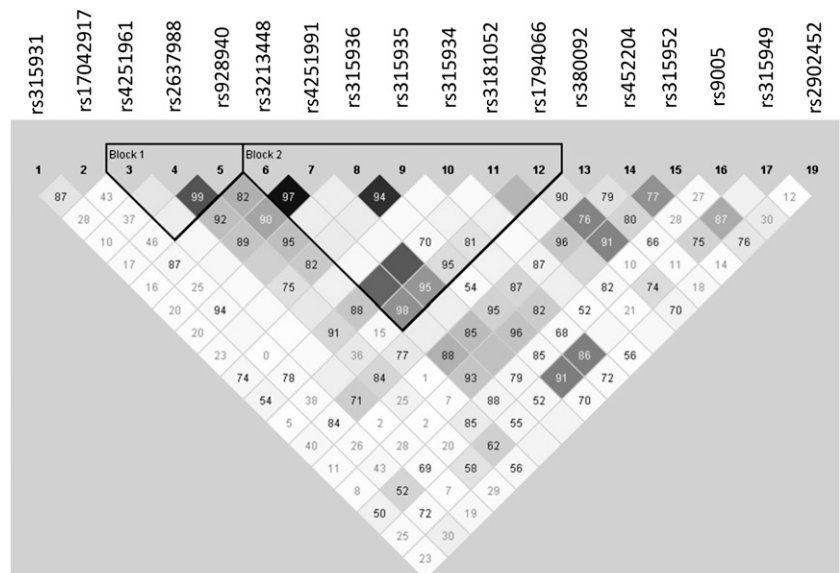
Table II. Summary of IL1RN SNPs in relation to blood IL-1Ra concentrations in IPD

SNP	Homozygous Wild Type		Presence of Minor Allele		*p* Value	95% CI	Bonferroni *p* Value
	n	Cytokine Mean (pg/ml)	*n*	Cytokine Mean (pg/ml)			
IL1RNsnp1	58	17,777	111	14,289	0.7	−8,467.0; 5,471.7	1
IL1RNsnp2	144	15,869	27	14,768	0.4	−12,781.5; 5,188.5	1
IL1RNsnp3[a]	150	13,898	24	27,024	0.0006	7,508.0; 24,894.9	0.012
IL1RNsnp4	49	14,982	123	16,190	0.4	−3,861.8; 10,248.3	1
IL1RNsnp5	62	16,349	110	15,534	0.4	−9,713.9; 4,319.6	1
IL1RNsnp6	95	14,472	76	17,029	0.5	−4,161.3; 8,987.1	1
IL1RNsnp7	98	14,629	75	17,219	0.6	−4,734.6; 8,375.9	1
IL1RNsnp8	115	17,507	50	12,416	0.07	−14,472.1; 609.4	1
IL1RNsnp9	114	16,580	59	14,137	0.3	−10,554.6; 3,204.1	1
IL1RNsnp10	147	15,442	27	17,161	0.7	−7,115.4; 10,690.5	1
IL1RNsnp11	118	14,523	54	18,430	0.5	−4,530.8; 9,325.6	1
IL1RNsnp12	60	15,657	114	15,736	0.9	−6,955.4; 6,901.3	1
IL1RNsnp13	116	15,435	57	15,851	0.5	−4,800.6; 9,105.7	1
IL1RNsnp14	50	14,021	118	15,626	0.7	−5,920.3; 8,850.3	1
IL1RNsnp15	52	17,501	121	14,743	0.4	−10,193.1; 3,981.3	1
IL1RNsnp16	133	16,008	41	14,740	0.5	−10,367.1; 5,376.7	1
IL1RNsnp17	95	16,205	77	14,807	0.5	−8,847.9; 4,516.0	1
IL1RNsnp18	80	14,678	80	14,730	0.9	−7,396.0; 6,501.7	1
IL1RNsnp19	131	15,628	40	15,543	0.5	−10,165.1; 4,797.1	1

[a]The only polymorphism found to be significantly associated with cytokine levels.
CI, confidence interval.

Weinberg equilibrium ($p = 0.0094$). This was largely due to the small number of observations within the homozygote variant group ($n = 6$), and to address this problem we retested a subset of our samples containing controls and all heterozygous and homozygous variant samples to confirm genotype calls. In our replication cohort, linear regression analysis comparing allele frequencies against IL-1Ra levels also showed that the presence of the minor allele of SNP rs4251961 (C allele) predicted higher levels of IL-1Ra in adults with borderline significance ($p = 0.058$) (Table III). Age and gender were not associated with the outcomes.

GATA-1 binding increases expression in the C allele

Adjacent to the sequence of the IL1RN promoter containing the SNP is a strong consensus binding site for the GATA transcription factor, AGATAG. As the binding site for a transcription factor is usually longer than the core consensus binding site, the SNP has the potential to modify the affinity with which GATA might recognize this region. To test the hypothesis that GATA-1 might differentially regulate the IL1RN promoter depending upon the polymorphism (C/T) rs4251961, human embryonic kidney, HEK293(T) cells were transfected with the promoter reporter plasmid pGL3p containing either allelic variant C (pGL3pCC) or T (pGL3pTT) with or without cotransfection with an expression construct overexpressing GATA-1. The results (Fig. 3) showed that neither allelic variant on its own induced a significant increase in activity when compared with the basal activity of the reporter plasmid. However, after the addition of the plasmid overexpressing GATA-1, the activity of the promoter constructs containing the T allele increased ~6-fold as compared with basal activity, and that containing the

C allele by ~9-fold. For both alleles, the increase in activity in the presence of GATA-1 as compared with without was statistically significant ($p < 0.001$). In addition, the increased activity of the promoter construct containing the C allele over that containing the T allele in the presence of GATA-1 was significantly different ($p < 0.005$).

The results indicate that the element containing the SNP affects the regulatory properties of the promoter fragment. If we were to extrapolate to the endogenous gene, then it could allow for differential levels of protein expression in response to challenges that modulated activity of GATA factors. However, as demonstrated in this study, the function of the promoter is determined by the challenge the cell is receiving. In this case, we have overexpressed the GATA transcription factor. We predict this factor would be a target for a relevant signal transduction cascade correlated with an environmental challenge that leads to IPD. Other variants in IL-1Ra or transcriptional regulators of IL-1Ra could modulate the activity of this polymorphism. Nevertheless, we have demonstrated that the clinically significant variant demonstrates differential transcriptional properties. We have demonstrated such differential transcriptional properties for other genetic variants associated with clinical predisposition to a disease or disorder (20–23).

Discussion

Our study has shown that the C allele of IL1RN promoter polymorphism (C/T) rs4251961 is associated with a significant increase in plasma IL-1Ra concentrations ($p = 0.01$) during the acute episode of IPD. We did not observe any significant association

Table III. Summary of IL1RN SNP rs4251961 in relation to blood IL-1Ra concentrations in HIV-infected adults

SNP	Homozygous Wild Type		Presence of Minor Allele		*p* Value	95% CI
IL1RNsnp3 (MAF = 8.66%)	*n*	Cytokine Mean (pg/ml)	*n*	Cytokine Mean (pg/ml)		
rs4251961	276	524.4	31	690.0	0.058	−5.57, 336.8

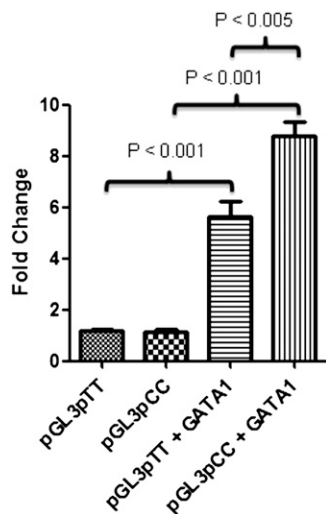CI, confidence interval; MAF, minor allele frequency.

FIGURE 3. Relative activity of IL-1RN promoters containing either the allelic variant C or T of polymorphism rs4251961. Promoter constructs based on pGL3p and containing a 309-bp region of the IL1RN gene that included the site of the variant allele C (pGL3pCC) or T (pGL3pTT) were transfected either alone or in the presence of a plasmid (pMT2-GATA) that overexpresses GATA-1 into HEK293(T) cells. Luciferase levels were normalized relative to renillin activity from a cotransfected control. Results are shown as mean fold increase in luciferase activity relative to that seen for pGL3p alone $\pm$ SD from the mean ($n = 6$).

between this polymorphism and disease susceptibility, nor did we find any direct association between the polymorphism and survival, as the frequency of the C allele is low (7%). To our knowledge, this is the first study to report an association between this polymorphism and IL-1Ra levels in IPD. We have also demonstrated that GATA can differentially activate both alleles with increased expression in the C allele in a reporter gene construct. We validated our findings in a cohort of treatment-naive HIV-infected adults and found an increase in plasma IL-1Ra concentrations ($p = 0.06$) during routine clinic visits.

Our data differ from those of Rafiq et al. (15) and Reiner et al. (16), who demonstrated that the C allele was associated with reduced IL-1Ra concentrations in elderly individuals, young adults, and adults with severe carotid artery disease. The subjects in Rafiq's and Reiner's studies (15, 16) were healthy adults with measurements made after an overnight fast. In contrast, our subjects were children and adults in sub-Saharan Africa, with a severe acute illness, or a chronic viral infection, both of which are associated with a high mortality. It is of note that the minor allele frequency is significantly higher in the Italian (44%) and United States populations (37%) than in our African population. HapMap data (http://hapmap.ncbi.nlm.nih.gov) show minor allele frequencies for African populations and populations of African descent of between 12 and 25% (Supplemental Fig. 1). A recent study examined the association of several cytokine polymorphisms with cytokine concentrations in amniotic fluid and showed that genetic regulation of cytokine concentrations differs by ethnicity (24). In that study, the C allele of rs4251961 was associated with increased amniotic fluid IL-1β concentrations. The authors conclude that conditions associated with increased inflammation leading to preterm birth are more common in African Americans than Caucasians and speculate that this may reflect recent evolutionary history of differential exposure to infectious agents. As the mortality from IPD in this cohort is high (22%), this may select against the mutant allele, which is associated with higher IL-1Ra levels, which itself is associated with mortality. Our study

did not demonstrate a direct association with mortality in the IPD cohort, because the frequency of the minor allele is low in this African population, and a sample size of several thousand would have been required to demonstrate this effect. We have previously reported a study describing a variable number tandem repeat polymorphism of the IL-1Ra gene in children in the United Kingdom with meningococcal disease (25). That study did not find any significant association between the presence of the A2 allele and disease susceptibility or severity, but there was a trend suggesting that the A2 allele was associated with more severe disease.

Our data are consistent with that of Kafka et al. (14) suggesting that IL-1Ra may have an adverse biological influence on the innate host immune response to *Streptococcus pneumoniae* infection. In primary murine listeriosis, overexpression of IL-1Ra reduces survival by limiting leukocytosis, WBC accumulation at sites of infection, and macrophage activation (26). IL-1Ra overexpression appears to cause a defect in WBC migration from the bone marrow into the bloodstream or to the actual sites of infection. It appears that IL-1Ra overproduction from its endogenous promoter causes a dysregulation of the cytokine network that compensates for the reduced IL-1 signaling by elevating the production of other proinflammatory cytokines. In support of these data, we have demonstrated the following: 1) high levels of IL-1Ra are associated with mortality in IPD; 2) a significant inverse correlation between plasma IL-1Ra and peripheral blood WBC; and 3) the minor allele is associated not only with IL-1Ra levels, but also that of other cytokines.

Our study is strengthened by the validation cohort of otherwise healthy treatment-naive adults with HIV infection. Although the results are of borderline significance, we have confirmed the association between IL-1Ra levels and the rs4251961 polymorphism in a separate cohort with infection within the same study population. The lack of significant association with susceptibility and survival could be explained by the relatively small numbers for a genetic association study. We did not explore the effects of variations in virulence genes or serotype of the infecting organism or genetic influences on HIV progression, as both these factors may influence disease severity and survival.

In conclusion, we report novel data that demonstrate that plasma IL-1Ra levels are significantly elevated in nonsurvivors with IPD, and that there is a robust association between the C allele of the rs4251961 polymorphism and IL-1Ra levels in the acute phase of IPD. We confirmed our results by finding increased IL-1Ra concentrations in HIV-infected adults with the C allele of the rs4251961 polymorphism. The demonstration of differential effects of the polymorphism on the reporter gene also confirms a functional role for this polymorphism. Our study provides new insight suggesting that IL-1Ra plays an important role in the pathophysiology of IPD, and may be a potential therapeutic target for adjunctive therapies. Further studies investigating the role of common genetic polymorphisms in the IL-1Ra pathway in susceptibility to other infectious diseases associated with a high mortality would be an important next step.

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Disclosures

The authors have no financial conflicts of interest.

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