

A common intronic variant of *CXCR3* is functionally associated with gene expression levels and the polymorphic immune cell responses to stimuli

Jung-Won Choi, PhD,^{a,*} Choon-Sik Park, MD,^{b,*} Minyoung Hwang, BS,^c Hye-Young Nam, MS,^a Hun Soo Chang, PhD,^c Seong Gyu Park, PhD,^a Bok-Ghee Han, PhD,^a Kuchan Kimm, MD,^d Hyung Lae Kim, MD,^a Bermseok Oh, PhD,^a and Yeonjung Kim, PhD^a *Seoul and Bucheon, South Korea*

Background: CXCR3 is a chemokine receptor that plays important roles in mediating chemotactic signals and modulating the activation of lymphocytes. We have previously conducted a case-control study by using a candidate gene approach to investigate the association of *CXCR3* polymorphisms with the risk of asthma. Results from the epidemiologic study showed that a common nucleotide variant in the *CXCR3* intron (*rs2280964G>A*) was associated with disease susceptibility (1006 cases and 384 control subjects; odds ratio, 0.81; 95% CI, 0.69-0.94; *P* = .007).

Objective: The aim of our study was to evaluate the epidemiologic study and provide functional evidence for the association of *rs2280964G>A* with asthma by investigating the effects of intronic variant on chemokine-mediated phenotypes of human-derived T cells.

Methods: We used cell line-based *in vitro* and human primary T cell-based *ex vivo* studies to examine the functional consequences of the intronic polymorphism, focusing on the regulation of gene expression, splicing, and immune responsiveness toward activating signals.

Results: We present functional evidence indicating that the *rs2280964A* allele significantly correlates with decreased *CXCR3* gene expression, which would lead to variation in immune cell responses to chemokine-cytokine signals *in vitro* and *ex vivo* that includes a decrease in chemotactic activity.

Conclusion: These findings, in conjunction with those of our previous epidemiologic studies, might implicate a functional link between a common nucleotide variant of a chemokine receptor gene, *CXCR3*, and a cause for a complex-trait disease, asthma. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Chemokine receptor, *CXCR3*, single nucleotide polymorphism, asthma, functional study, case-control association study

Asthma is a complex-trait disease caused by an accumulation of complicated interactions among multiple genetic and environmental risk factors. Previous studies have established that the clinical symptoms result from chronic bronchial epithelial and submucosal inflammation led by IgE-mediated allergic reactions. During the events involved in the development of atopic reactions, T_H1 and T_H2 cells play critical roles in initiating and maintaining the inflammatory reaction triggered by antigen exposure. Together with the T_H cells, chemokines and their receptors regulate various inflammatory/immune responses by recruiting lymphocytes to inflammatory sites through chemotaxis. Additionally, chemokines also participate in T-cell activation and proliferation by developing and maintaining the T_H1/T_H2 cytokine responses.¹⁻⁴ Accordingly, it has been shown that chemokine receptor expression profiles on T cells in asthmatic patients were distinct from those of healthy subjects.⁵⁻⁹ These findings strongly suggest that certain chemokine receptors might modulate T_H responses during inflammatory reactions, including allergic reactions.

In an effort to assess the genetic contribution that chemokine receptor genes make to the pathogenesis of allergic bronchial inflammation, we previously conducted a case-control association study for *CXCR3* polymorphisms using hospital-based asthma cohort samples. The gene coding for the receptors for CXCL4/platelet factor 4, CXCL9/monokine induced by IFN- γ , CXCL10/IFN- γ inducible protein of 10 kd, and CXCL11/IFN-inducible T-cell α chemoattractant is expressed on many immune-related cell types (eg, natural killer cells, dendritic cells, monocytes, and eosinophils) but is predominantly expressed on activated T_H1-type cells.¹⁰⁻¹² CXCR3 has drawn much attention for its involvement in regulating chronic inflammation. In addition to the T_H1-related functions, a recent report suggested that CXCR3 has crucial regulatory roles in T_H1/T_H2 cytokine production by selectively mediating T_H1- or T_H2-shifting antigenic signals.^{13,14}

In our previous study¹⁵ we identified 4 nucleotide variants of the *CXCR3* gene locus in a Korean population (*n* = 1390) that map to chromosome Xq13 (Fig 1, A). Consistent with our hypothesis based on known CXCR3 functions, the case-control association study (1006 cases and 384 control subjects, Table I) revealed a common single nucleotide polymorphism (SNP) located in the intron *rs2280964* (referred to as *c.12+234G>A* in the previous report; minor allele frequency, 0.445) that was associated with

From ^athe Center for Genome Science, the National Institute of Health, Seoul; ^bthe Genome Research Center for Allergy and Respiratory Diseases, Soonchunhyang University, Bucheon Hospital; ^cthe Graduate School of Medicine, Korea University, Seoul; and ^dMerck Research Laboratories/WWL&ER/MSD Korea, Seoul.

*These authors contributed equally to this work.

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Reprint requests: Yeonjung Kim, PhD, and Bermseok Oh, PhD, Center for Genome Science, National Institute of Health, Seoul, 122-701, Korea. E-mail: kimye@ngri.go.kr. 0091-6749/\$34.00

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Abbreviations used

EMSA: Electrophoretic mobility shift assay
 GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
 LCL: Lymphoblastoid cell line
 PMA: Phorbol 12-myristate 13-acetate
 SNP: Single nucleotide polymorphism

an atopic asthma risk. An increase in the *rs2280964A* genotype frequency correlated with decreased susceptibility to asthma, which was more evident in the atopic male population (odds ratio, 0.61; 95% CI, 0.46-0.82; $P = .0009$).

In this study we report several lines of functional evidence indicating that the intronic SNP of the chemokine receptor gene *CXCR3* might lead to variation in not only the gene expression level but also in the immune cell responses to stimuli both *in vitro* and *ex vivo*. Based on these observations and previous results from the epidemiologic study, we suggest that *rs2280964*-associated phenotypic polymorphisms can contribute to individual susceptibility to allergic asthma.

METHODS**Subjects**

EBV-transformed lymphoblastoid cell lines (LCLs) and blood samples from human subjects were obtained from the Genome Research Center for Allergy and Respiratory Diseases, Soonchunhyang University, Bucheon; Seoul Hospitals; and the Center for Genome Science, National Institute of Health. Informed consent was obtained from each subject before sample collection, and the institutional review boards of the 2 institutions approved the study. To assess *CXCR3* transcript levels and chemotaxis in blood samples, primary CD3⁺ cells were prepared from the PBMCs of subjects with appropriate genotypes, as previously described.¹⁶ The donors were randomly selected from affected or unaffected subjects from the cohort population (Table 1).

Reporter gene plasmid construction and expression assay

The *CXCR3* genomic sequence was amplified by using standard PCR methods. DNA extracted from an *rs2280964G*- or *A*-carrying individual was used as a template for the reactions. Each PCR product was thoroughly sequenced before insertion upstream of the *bla* (*M*) reporter gene coding for β -lactamase (GeneBLAzer; Invitrogen, Carlsbad, Calif). Plasmid DNA of the reporter constructs was prepared for transfection of Jurkat T or HEK293 cells. β -Lactamase reporter assays for the transiently transfected cells were carried out according to the manufacturer's instructions. Each assay was done with 10 replicates, and the data presented (Fig 2, B) are representative of 2 independent experiments. More detailed information is available in this article's Methods section in the Online Repository at www.jacionline.org.

Establishment of *rs2280964G*- or *rs2280964A*-*CXCR3*-transfected Jurkat T-cell lines

The entire *CXCR3* gene locus (−1833 to +1560 with respect to A of start codon as +1) was amplified and cloned by using AccuPrime pfx DNA Polymerase and the TOPO XL PCR cloning kit (Invitrogen). The genetic region was subsequently subcloned into the pEM7/bsd vector (Invitrogen) for transfection of Jurkat T cells. Transfected cells were grown in complete RPMI 1640 for 48 hours before being transferred into blasticidin S-HCl (5 μ g/mL, Invitrogen)-containing complete RPMI 1640 for selection. After 4 weeks of incubation in the selective culture, 2 clones from each transfection were isolated with a serial dilution method and were separately cultured for 2 weeks before assessing phenotypes. More detailed information is available in this article's Methods section available in the Online Repository.

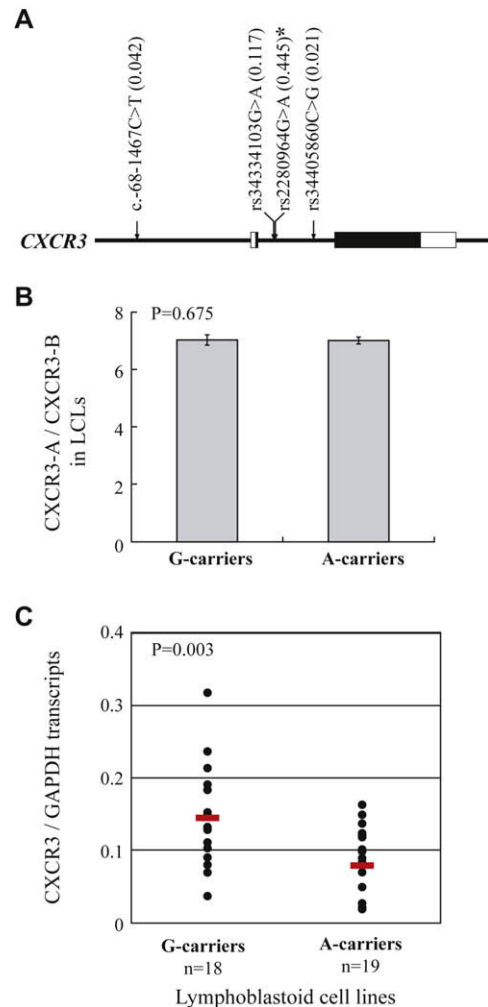


FIG 1. The asthma-associated intron variant *rs2280964* differentially regulates *CXCR3* expression levels. **A**, Map of the *CXCR3* gene locus located on the X chromosome. The black box shows the coding region, and the white box shows the untranslated regions. The SNP is marked with an asterisk. **B**, Comparison of *CXCR3*-A and *CXCR3*-B splicing variant levels in LCLs from unaffected male subjects (indicated as G or A carriers for *CXCR3* on the X chromosome). Columns represent mean values $\pm$ SDs of 3 independent experiments. The significance of differences in mean *CXCR3*-A/*CXCR3*-B values was calculated by using the Student *t* test. **C**, *CXCR3* transcript levels in LCLs. Each dot represents a mean value of a duplicated assay. Mean values of the group are indicated with red bars. Statistical significance was calculated by using the Student *t* test.

Quantitative estimation of *CXCR3* gene copy number in stably transfected cell lines

Copy numbers of *CXCR3* were determined by using quantitative real-time PCR with intron-specific oligonucleotides as primers. Data presented in Fig 3, C, are from 2 independent experiments, each of which was carried out in duplicate with 2 separate clones selected from transfectants. For more detailed information on these experiments, see this article's Methods section available in the Online Repository.

Quantitative determination of *CXCR3* transcript levels

CXCR3 transcript levels were measured by using real-time RT-PCR. TaqMan chemistry (Applied Biosystems, Foster City, Calif) was used to examine transcript levels in LCLs, and SYBR Green Dye (Applied Biosystems) was used

TABLE I. Clinical profiles of previous case-control study subjects

Clinical profiles	Healthy control subjects	Asthmatic subjects
No. of subjects	384	1006
Age (y), mean (range)	44.8 (6-83)	48.7 (4-86)
Sex (male/female)	164/220	395/611
Current smoker (%) [*]	29.11	34.27
FVC (% predicted)	93.0 ± 11.9	83.5 ± 18.2
FEV ₁ (% predicted) [*]	102.1 ± 14.8	80.1 ± 22.7
PC ₂₀ methacholine (mg/mL) [*]	23.4 ± 4.8	6.2 ± 8.6
Log (total IgE [IU/mL]) [*]	1.71 ± 0.63	2.13 ± 0.65
Blood eosinophils (%)	2.55 ± 2.10	5.37 ± 5.37
Positive skin prick test responses (%) [*]	34.85	56.87

A positive skin prick test response was defined by the presence of an immediate skin reaction (>3 mm in diameter) to 1 or more of the tested allergens.

^{*}P < .001 between healthy control subjects and asthmatic groups.

to assess transcript levels in stably transfected Jurkat T and primary CD3⁺ cells or CXCR3-A and CXCR3-B splice variant levels. The data shown in Figs 1, 3, 4, 5, and E1 are representative of 3 independent assays, each done in 2 or 5 replicates. More detailed information on primer sequences and experimental procedures is available in this article's Methods section available in the Online Repository.

Quantification of CXCR3 proteins on the cell surface

CXCR3 surface protein levels were assessed by using flow cytometry. The data shown in Fig 3, B, represent results from 3 independent experiments each done in triplicate. See this article's Methods section available in the Online Repository for more information on antibodies and experimental conditions.

Assay for calcium flux

pEM7- or *rs2280964G>A*-CXCR3-carrying Jurkat T cells were prestimulated with IFN- γ or IL-4 (at 20 ng/mL; R&D Systems, Minneapolis, Minn) for 20 hours. By using a Fluo-4 NW Calcium assay kit (Molecular Probes, Eugene, Ore), the measurement of calcium flux was initiated on CXCL11 treatment (2 μ g/mL) and was continued for 7.5 minutes at 15-second intervals. Detailed information on the assay procedure is described in this article's Methods section available in the Online Repository. The results shown in Fig 4, A, are representative of 3 independent experiments showing similar results, each done in duplicate.

Assay for chemotactic activity

An assay to determine the level of chemotaxis was conducted by using calcein AM (Invitrogen) in conjunction with the HTS Transwell-96 insert system (Corning, Corning, NY). Before measuring, stably transfected cells were prestimulated with IFN- γ (at 100 ng/mL for CXCL11) or IL-4 (at 10 ng/mL for CXCL11) for 24 hours. To test for chemotaxis of primary cells from donors, isolated CD3⁺ cells were preincubated with either IFN- γ (100 ng/mL), IL-4 (10 ng/mL), or phorbol 12-myristate 13-acetate (PMA; 100 ng/mL) plus PHA (5 μ g/mL) in the presence of CXCL11 (1 μ g/mL) for 24 hours. For detailed information on the experiment, see this article's Methods section available in the Online Repository. The data shown in Fig 4, B, represent 3 independent assays with similar results (each assay done in duplicate).

Quantitative analyses of the cytokine mRNA production

CXCR3-transfected cells were preincubated with IFN- γ or IL-4 (5 ng/mL) for 24 hours before 1-hour ligand treatment (CXCL11 at 200 ng/mL). Total RNA was extracted from the cells for quantitative real-time RT-PCR. The transcript levels of the cytokine-coding genes in each sample were normalized to *SP1* transcripts. Relative changes in the cytokine mRNA production were presented by dividing the normalized transcript levels from *rs2280964G>A*-CXCR3-transfected Jurkat T cells by those from vector (pEM7)-transfected cells. The data shown in Fig 4, C, are representative of 2 independent

experiments, each done with 5 replicates. For more information on primer sequences, see this article's Methods section available in the Online Repository.

Electrophoretic mobility shift assay

To examine whether the *rs2280964*-carrying intronic sequences would exhibit any protein-binding activity, we conducted electrophoretic mobility shift assay (EMSA) by incubating radiolabeled DNA probes with nuclear extract from either Jurkat or primary T (CD3⁺) cells. Stimulation of Jurkat T cells was carried out by incubating them with PMA (100 ng/mL) plus PHA (5 μ g/mL) for 24 hours. The TFIID probe was used as a positive control. Self- and cross-competition assays were performed by incubating the nuclear extract with 25- or 100-fold molar excess of unlabeled probe DNA for 10 minutes before adding the labeled probes (25-fold excess of competitor DNA was added to the reactions shown in Fig 5, C). The results shown in Figs 2, C, and 5, C, are representative of several independent experiments. See this article's Methods section available in the Online Repository for more detailed experimental conditions, including nuclear protein preparation from lymphocytes.

RESULTS

The presence of the CXCR3 intronic SNP *rs2280964G>A* correlates with altered gene expression levels

It is possible that an intronic SNP can alter splicing of primary transcripts or gene expression. Interestingly, CXCR3 has recently been shown to express 3 distinctive splice variants: CXCR3-A, CXCR3-B, and CXCR3-alt (very low expression).^{11,14,17} Therefore the aim of our study was to provide functional evidence for the association of *rs2280964* with asthma and determine whether the intronic SNP would lead to any phenotypic consequences resulting from alterations in molecular processes. To these ends, we first investigated the effects of *rs2280964* on alternative splicing by quantitatively comparing the transcript levels of CXCR3-A and CXCR3-B in EBV-transformed LCLs from male subjects carrying the *rs2280964G* or A alleles (*G* carriers, *n* = 18; *A* carriers, *n* = 19). The LCL donors were selected from the unaffected control group in the previous case-control study (Table I) because we wanted to examine allelic expression patterns independent of disease status. As previously described, LCLs have been widely considered a useful tool to quantify *in vivo* expression phenotypes for individuals.¹⁸ Quantitative real-time RT-PCR analyses with PCR primers specific for each transcript showed that CXCR3-A levels were 6 to 7 times higher than CXCR3-B levels, independent of LCL genotype, which is in accordance with a previous report by Lasagni et al.¹¹ This result clearly indicates a lack of allele-dependent differences in the ratio of CXCR3-A to CXCR3-B (Fig 1, B; CXCR3-alt was not tested because of its low expression¹⁷). On the other hand, we noticed that both CXCR3-A and CXCR3-B transcript levels were higher in *rs2280964G* carriers than in *rs2280964A* carriers (a 2-fold increase with respect to mean values; see Fig E1, A and B, in this article's Online Repository at www.jacionline.org). Quantitative real-time RT-PCR with primers for the total CXCR3 transcripts demonstrated *rs2280964*-dependent discrepancies in gene expression (Fig 1, C); that is, the *rs2280964A*-carrying cells had significantly lower total CXCR3 transcript levels (0.14 vs 0.08 with respect to mean values, *P* = .003).

Regulatory effect of the intronic SNP on CXCR3 gene expression

To further examine the role of *rs2280964* in the regulation of CXCR3 gene expression, we transiently transfected HEK293 or human CD4⁺ T cell-derived Jurkat T cells with equal molar concentrations of each of a series of β -lactamase reporter constructs.

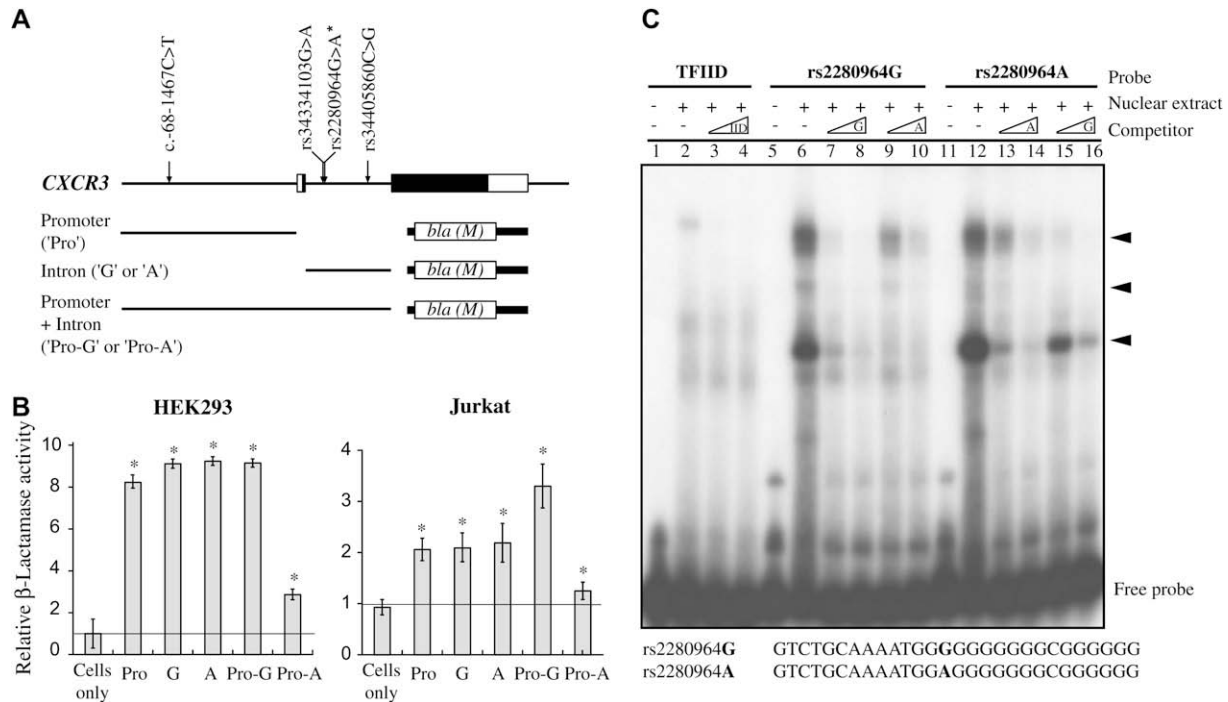


FIG 2. Test for *rs2280964* regulation of gene expression. **A**, Schematic presentation of reporter constructs carrying only the promoter region (*Pro*), intron (*G* or *A*), or both (*Pro-G* or *Pro-A*) upstream of the β -lactamase-coding *bla* (*M*) gene. **B**, Reporter activity in transiently transfected cells. Relative activity is indicated with respect to no-DNA mock-transfected control cells (*Cells only*). Mean values $\pm$ SDs are shown. Statistical significance between the negative control and each construct was evaluated by using the Wilcoxon rank sum test (* $P < .0005$). **C**, Test for protein binding of the *rs2280964* intronic region by means of EMSA. Specific DNA-protein complexes are indicated with closed arrowheads.

In the constructs reporter gene expression was driven by the *CXCR3* promoter, the intron, or both the promoter and the intron with either the *rs2280964G* or *A* allele (Fig 2, A). We observed that the reporter gene controlled by the combined promoter and *rs2280964A*-containing intron sequence exhibited the lowest expression levels (see "Pro-A" in Fig 2, B), even when compared with the promoter-only construct (relative to "Pro," $P < .0001$ for HEK293 cells and $P = .0009$ for Jurkat T cells). This result strongly suggests that *rs2280964A* can inhibit *CXCR3* promoter activity, which is consistent with the allele-dependent expression pattern in LCLs (Fig 1, B).

It has been reported that gene expression is modulated by regulatory factor-binding sites in intronic regions.¹⁹ To investigate the molecular mechanism that underlies the inhibition of *CXCR3* by *rs2280964A*, we determined whether the intronic sequence would serve as a protein-binding site. Indeed, the EMSAs revealed strong *in vitro* protein binding in the intronic sequences surrounding *rs2280964* (Fig 2, C; compare lanes 6 and 12) by Jurkat nuclear extracts. Stimulation of cells with PHA and PMA made no difference in the binding pattern (data not shown). It was also noted that the presence of *rs2280964G* or *A* led to a difference in DNA-protein affinity, as shown in a competitive binding experiment with unlabeled self- or cross-competitor oligo-nucleotide DNA (compare lanes 7 to 8 and 9 to 10 and lanes 13 to 14 and 15 to 16). Quantification of band intensity is shown in Fig E2 (available in the Online Repository at www.jacionline.org). Interestingly, the *rs2280964G*- or *A*-containing sequences were able to completely abrogate *in vitro* transcription driven by a strong

Cauliflower mosaic virus (CMV) promoter when incubated with HeLa nuclear extracts, without an apparent allele-dependent discrepancy (see Fig E3 in the Online Repository at www.jacionline.org). These data, together with the LCL expression patterns, suggest that the *A* allele-containing region can regulate *CXCR3* gene expression at least in part by modulating the binding of a regulator protein that could ultimately inhibit overall gene expression.

The presence of *rs2280964* correlates with phenotypic polymorphisms in immune responsiveness of *CXCR3*-transfected Jurkat T cells

Given the physiologic roles of the chemokine receptor and its predominant expression in T_H cells, we hypothesized that *rs2280964*-dependent variation in *CXCR3* expression might influence T-cell immune responsiveness. To directly address this possibility, we established Jurkat T-cell lines stably expressing exogenous *CXCR3* with either the *rs2280964G* or *rs2280964A* allele types. The entire genomic region, including upstream and downstream sequences, all exons, and the intron, was inserted into a pEM7 vector for a low copy number (about 5 kb; Fig 1, A). The recombinant constructs were expressed in Jurkat T cells originally derived from a male subject bearing the *rs2280964G* allele, as determined by our sequencing. Using subclones of the established cell lines, we first examined *CXCR3* expression levels. We found that *rs2280964A*-*CXCR3*-transfected Jurkat T cells showed significantly less *CXCR3* at both the transcript and surface receptor levels compared with *rs2280964G*-transfected cells

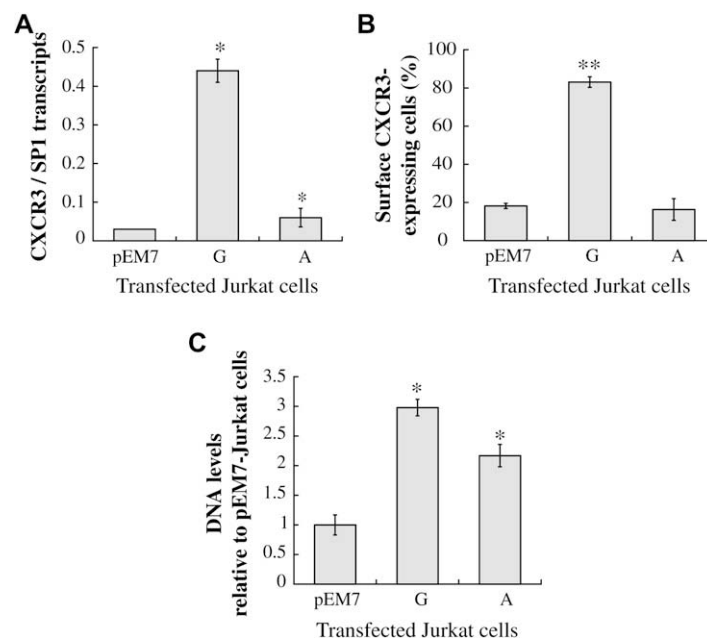


FIG 3. Phenotypic assessment of *rs2280964G>A*-*CXCR3*-transfected Jurkat T cells. **A** and **B**, *CXCR3* expression levels. Two clones were selected from each transfection and used to assess the transcript and surface protein levels by using real-time RT-PCR (**Fig 3, A**) and flow cytometric analysis (**Fig 3, B**), respectively. Mean values $\pm$ SD are shown. The significance of differences was evaluated by using the Wilcoxon rank sum test (** $P = .007$, * $P < .05$). **C**, Comparison of *CXCR3* gene copy numbers. Relative DNA levels indicate the ratio of DNA in *rs2280964G>A*-*CXCR3*-transfected Jurkat cells compared with that in vector-carrying cells. Mean values $\pm$ SDs are shown. The significance between the vector cells and each *CXCR3*-transfected cell type was estimated by using the Wilcoxon rank sum test (* $P < .05$).

(>4-fold decrease, as shown in **Fig 3, A** and **B**). These data agree with the allelic expression pattern in LCLs and the reporter gene expression profiles (**Figs 1, C**, and **2, B**). No difference in the ratio between the *CXCR3*-A and *CXCR3*-B splice variant levels was observed in transfected cells, confirming the LCL results (**Fig E1, C**). It is also noteworthy that differences in the expression between the *rs2280964G* and A cells (5-fold) substantially exceeded the variation in the *CXCR3* gene copy numbers (1.37-fold: 2.98- and 2.17-fold copy number increases, respectively, compared with pEM7 vector-transfected cells; **Fig 3, C**). This observation suggests that the allele-dependent discrepancy in gene expression is likely not due to copy number variation in the *CXCR3*-transfected cells.

Differential gene expression can lead to noticeable phenotypic polymorphisms. Thus we compared the allele-dependent immune responsiveness of *CXCR3*-transfected Jurkat T cells by analyzing their capacity to respond to activating signals. In general, immune responsiveness can be measured by assessing changes in Ca^{2+} mobilization, chemotactic activity, and cytokine production. It is well known that the interaction of G protein-coupled cell-surface receptor with its respective chemokines results in both an increase in intracellular $[\text{Ca}^{2+}]$ levels and in the chemotaxis of lymphocytes. We observed that intracellular $[\text{Ca}^{2+}]$ levels increased in transfected Jurkat T cells on chemokine treatment (**Fig 4, A**; data for CXCL4, CXCL9, and CXCL10 were not presented). Pretreating the cells with either IL-4 ($\text{T}_\text{H}2$ specific) or IFN- γ ($\text{T}_\text{H}1$ specific) substantially enhanced the Ca^{2+} in flux. Notably, *rs2280964A* Jurkat T cells demonstrated significantly lower Ca^{2+} flux than *rs2280964G* cells on stimulation with IFN- γ . In addition, we found that the combining ligand and cytokines more strongly induced chemotaxis in the *rs2280964G* cells than

in the *rs2280964A* cells, as measured by migration toward chemo-attractants (**Fig 4, B**). The *rs2280964G*-transfected cells more specifically responded to $\text{T}_\text{H}1$ -like cytokines than to $\text{T}_\text{H}2$ -like cytokines in both the Ca^{2+} flux and chemotaxis assays. Moreover, quantitative RT-PCR analyses demonstrated that the *rs2280964G* cells induced significantly higher levels of the cytokine-coding gene transcripts on $\text{T}_\text{H}1$ - or $\text{T}_\text{H}2$ -specific stimulating signals, along with much higher induction of *CXCR3* transcription (**Fig 4, C**). Thus *rs2280964G* cells might be more immune responsive than *rs2280964A* cells with regard to both $\text{T}_\text{H}1$ and $\text{T}_\text{H}2$ cytokine induction in response to a specific ligand-cytokine milieu. Notably, the weak responses to stimuli seen in *rs2280964A* cells were comparable with those observed in the vector-only cells, despite the fact that *rs2280964A* cells have more than twice the copy number of *CXCR3*.

Effects of *rs2280964* polymorphisms on phenotypes of primary cells from donors

Although our biochemical and phenotypic assay data indicate *rs2280964* differential gene expression and immune responsiveness to stimuli, we could not exclude the possibility that the *rs2280964A* allele-associated downregulation of *CXCR3* expression could have been caused by artificial effects from using LCLs and transfected Jurkat T cells. To address this issue, we examined the phenotypes of primary $\text{CD}3^+$ cells that were extracted from PBMCs of affected or unaffected subjects with appropriate genotypes (referred to as G and A in **Fig 5**). Only the individuals with homozygous genotypes with respect to *rs2280964* were selected in the case of female subjects. We observed statistically

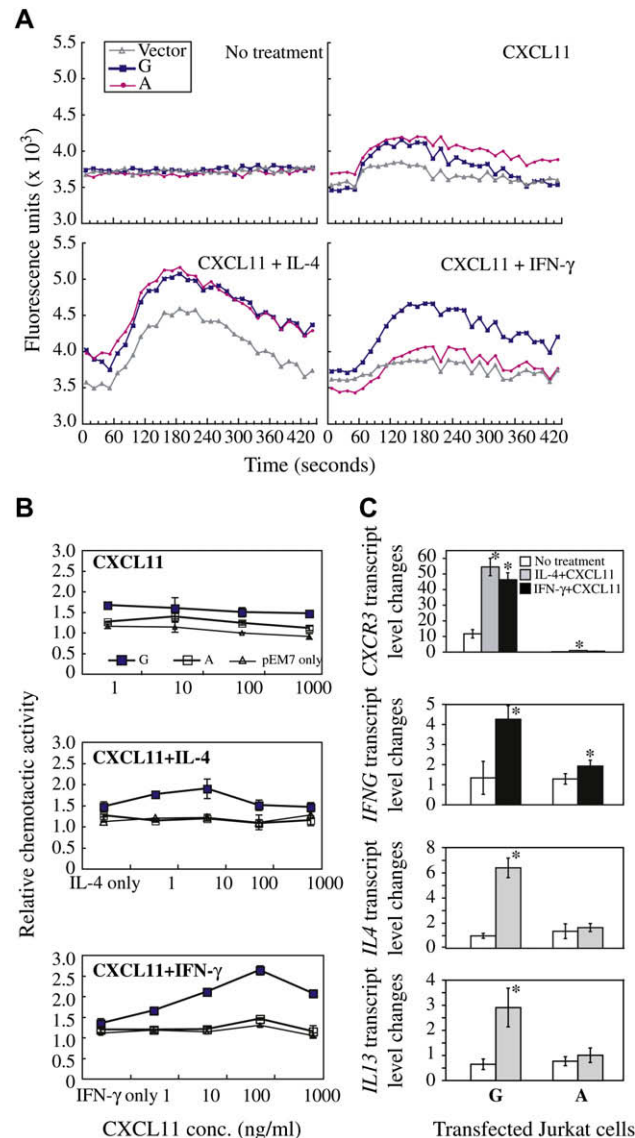


FIG 4. The *rs2280964* polymorphism induces differential immune responsiveness in Jurkat T cells *in vitro*. **A**, Ca^{2+} mobilization in *rs2280964G>A*-CXCR3 Jurkat T cells. The cells carrying pEM7 are shown as gray triangles, cells carrying *rs2280964G*-CXCR3 are shown as blue squares, and cell carrying *rs2280964A*-CXCR3 are shown as pink circles. **B**, Chemotactic activity. **C**, Cytokine mRNA expression assessed by using real-time RT-PCR. Mean values $\pm$ SDs are indicated. Statistical significance was estimated by using the Wilcoxon rank sum test (* $P < .005$).

significant allele-dependent discrepancies in both *CXCR3* transcript and chemotaxis levels, regardless of disease status of the subjects (Fig 5). These results clearly replicate our *in vitro* data, except for the surface CXCR3 protein levels, which did not show any significant differences in either stimulated or unstimulated primary cells, as was seen in the LCLs (data not shown). We presume that this could be attributed to internalization of the receptors and heterogeneity of the cell population. Similar *rs2280964A*-dependent downregulation patterns were obtained from the primary cells stimulated with either IL-4 or PHA plus PMA, although some results from unaffected group did not reach a level of statistical significance ($P < .05$; see Fig E4 in the Online Repository at www.jacionline.org). Notably, the distribution of chemotactic activities showed relatively small variance in relation

to that of the transcript profile in these datasets (Fig 5, B), and the asthmatic group exhibited a larger ratio of the mean chemotaxis between G and A carriers than the unaffected group (1.42 vs 1.16, $P = .0075$). In addition, there was no discrepancy found with respect to the ratio between CXCR3-A and CXCR3-B in either group (see Fig E5 in the Online Repository at www.jacionline.org), confirming that the intronic SNP would not affect the splicing, as was observed in the LCLs (Fig 1, B). Collectively, these findings demonstrate that the presence of the *rs2280964A* allele resulted in both downregulation of gene expression and decreased immune responses to chemokines and cytokines both *in vitro* and *ex vivo*.

Moreover, we conducted EMSA with nuclear extract from primary T cells of healthy donors to verify the previous cell line

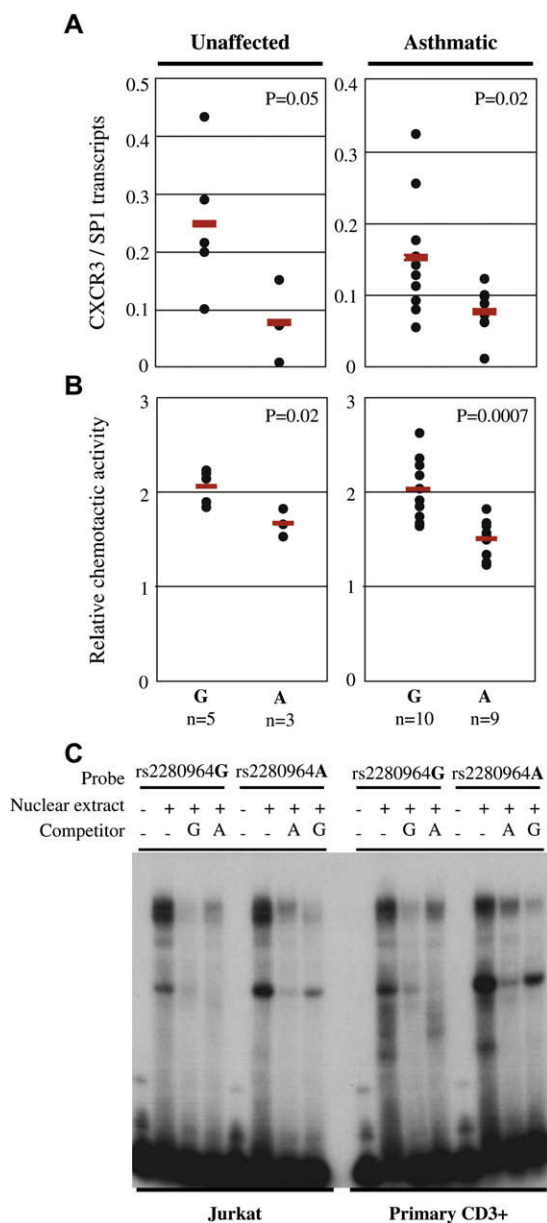


FIG 5. Phenotypic assessment of human primary T cells. **A**, *CXCR3* transcript levels in primary CD3⁺ cells isolated from unstimulated PBMCs. Each dot represents a mean value of 5 replicates for each subject. The mean value of each group is indicated with red bars. Statistical significance was calculated by using the Student *t* test. **B**, Chemotactic activities of CD3⁺ cells prestimulated with IFN- γ . Each dot indicates a mean value of a duplicate assay. **C**, Comparison of allele differential DNA-protein interaction between Jurkat and primary cell nuclear proteins.

data and found almost identical patterns of DNA-protein interaction generated by both Jurkat and primary CD3⁺ proteins (Fig 5, C, as performed with extracts from a male A carrier). No difference in DNA-binding profiles was observed among extracts prepared from different genotype carriers (female GG [n = 1] and GA [n = 1] and male A [n = 2] carriers, data not shown). This result suggests that cell types (Jurkat or primary cells) or the donor's genotypes appear not to influence the allele-differential protein binding activity of the SNP-containing region in our experimental conditions.

DISCUSSION

CXCR3 has been known as a marker for T cells that are associated with certain inflammatory reactions, particularly with T_H1-type responses. Accordingly, there have been reports linking *CXCR3* and the related chemokines to autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis.^{12,20} Conversely, our previous case-control association study showed that a *CXCR3* polymorphism might have an influence in modulating host susceptibility to T_H2-mediated asthma.¹⁵ Evidence for the critical roles of T_H2 cells and T_H2-specific cytokines in asthma pathogenesis has been provided by many studies both in animal model-based experimental asthma and by asthmatic pathophysiology in human subjects.^{21,22} Consequently, many investigators proposed that an enhanced T_H1 response might be effective to downregulate both T_H2 responses and asthma.⁴

Nevertheless, there has been increasing evidence that emphasizes the importance of the interregulation and balance between T_H1- and T_H2-polarized lymphocytes in asthma pathogenesis. Some recent studies suggest that T_H1 and T_H2 responses are not mutually exclusive but could be controlled in a complex synergy to potentiate inflammatory reactions.²³ For example, Randolph et al²⁴ used a murine asthma model to show that both T_H1 and T_H2 cells were recruited to the airways of ovalbumin-challenged mice and that passively transferred antigen-specific T_H1 cells significantly increased mobilization of both T_H1 and T_H2 cells to the lungs or airways of the challenged animals. By using knockout model mice for *IFNG* or *IL4* and/or *IL13* genes, it was also shown that both T_H1 and T_H2 cells were required for both eosinophil- and neutrophil-associated airway inflammation.²⁵ Evidence for *CXCR3*'s functions in atopic responses of asthma has also been found. Thomas et al²⁶ suggested that *CXCR3* plays a role in inflammatory T-cell recruitment to the bronchoalveoli during the allergic inflammatory response by using an endobronchial segmental allergen challenge in human atopic asthmatic subjects. In addition to its predominant expression in T cells, *CXCR3* is also found in eosinophils.¹³ Results from studies by Jinquan et al¹³ revealed that the expression of *CXCR3* was important for the regulation of eosinophil chemotaxis by the *CXCR3*-recognizing chemokines CXCL9 and CXCL10. The authors proposed that *CXCR3*-mediated eosinophil activation might be important in initiating, progressing, and terminating allergic inflammation.¹³ Together these reports support the idea that T_H1 cells (cytokines) can have both downmodulating and upmodulating actions on T_H2 cell-driven inflammation.

Here we demonstrated that a common intronic *CXCR3* variant, *rs2280964G>A*, could lead to a marked difference in gene expression and significant differences in immune cell responsiveness to activating signals. Concomitant with higher *CXCR3* expression, the G allele-carrying cells exhibited a higher responsiveness to chemokines and cytokines, as shown by Ca²⁺ mobilization, chemotaxis, and T_H1 and T_H2 cytokine production in *CXCR3*-transfected Jurkat T cells and by chemotaxis profiles in primary CD3⁺ cells extracted from subjects. These data, we believe, strongly support idea of *rs2280964G*-dependent enhancement and *rs2280964A*-dependent attenuation of lymphocyte responsiveness to specific ligand/cytokine-mediated activating signals. Therefore we propose that *rs2280964G*-associated immune reactivity, along with the reported role of *CXCR3* in inflammatory responses,^{13,14,26} provides functional clues toward the previously reported association between *CXCR3* polymorphism and the risk for allergic asthma.¹⁵ We speculate that *rs2280964G*-

associated immune responsiveness increases the disease risk through T_H1 cell- or eosinophil-mediated upregulation of T_H2 -driven inflammation. However, we cannot exclude the possibility of *CXCR3* exerting its regulatory role through another mechanism. Given the minor allele frequency, we note that the contribution of *rs2280964* to the disease phenotype might be confounded by other risk factors, including variation in exposure level to inhaled allergens or an individual's pre-existing airway inflammation, both of which can affect the lung microenvironment. Such modulation of the genetic effects by environmental and other genetic factors might give rise to substantial polymorphism in individual phenotypic profiles. Interestingly, our *ex vivo* data showed that the discrepancy in chemotaxis between *G* and *A* carriers was greater in the asthmatic group than in the unaffected group. Further epidemiologic and functional studies are undeniably needed to elucidate the mechanisms underlying the role or roles of *rs2280964* in asthma pathogenesis and to define the magnitude of the genetic and other environmental effects (sex or hormonal effects) on disease susceptibility. The studies might also shed light on functional implications of the intronic SNP for both T_H1 and T_H2 cells, as well as memory and effector T-cell populations.

The EMSA results indicated allele differential protein bindings by the SNP-carrying sequence in the presence of either Jurkat or primary T-cell nuclear proteins. The intronic region was also shown to inhibit initiation of transcription *in vitro* driven by a strong CMV promoter. On the basis of these biochemical data, we propose that the differences in gene expression might at least in part result from a regulatory effect of the intronic SNP-carrying sequence by modifying DNA-protein interactions in the intron region. However, functional relevance of the DNA-protein interaction to the phenotypic outcomes is yet to be determined, and alternative models for the role of the intronic polymorphism should be taken into account. In particular, it will be of interest to design and conduct additional functional approaches to substantiate the binding of nuclear proteins in the intronic region and to identify regulatory factors or mechanisms that might be involved.

In summary, we have demonstrated that a common intronic nucleotide variant in *CXCR3*, a chemokine receptor gene, is functionally associated with the gene expression level and immune cell responses to stimuli. These findings implicate a functional link between a common intronic SNP, leading to a phenotypic polymorphism and a cause for a complex-trait disease. We believe that our findings lend significant insight into the role of *CXCR3* in allergic asthma and its potential usefulness as a disease marker.

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Clinical implications: These findings lend significant insight into the role of the chemokine receptor *CXCR3* in individual susceptibility to allergic asthma and its potential usefulness as a disease marker.

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