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ORIGINAL ARTICLE

Association Study of 71 European Crohn's Disease Susceptibility Loci in a Japanese Population

Atsushi Hirano, MD,† Keiko Yamazaki, DVM, PhD,* Junji Umeno, MD, PhD,*† Kyota Ashikawa, MS,* Masayuki Aoki, MD, PhD,* Takayuki Matsumoto, MD, PhD,† Shotaro Nakamura, MD, PhD,† Toshiharu Ninomiya, MD, PhD,‡ Toshiyuki Matsui, MD, PhD,§ Fumihito Hirai, MD, PhD,§ Takaaki Kawaguchi, MD,‖ Masakazu Takazoe, MD,‖ Hiroki Tanaka, MD, PhD,¶ Satoshi Motoya, MD, PhD,¶ Yutaka Kiyohara, MD, PhD,‡ Takanari Kitazono, MD, PhD,† Yusuke Nakamura, MD, PhD,** Naoyuki Kamatani, MD, PhD,* and Michiaki Kubo, MD, PhD**

Background: A large-scale meta-analysis of a series of European genome-wide association studies revealed 71 susceptibility loci for Crohn's disease (CD). However, it is not clear whether these susceptibility loci are also shared with Japanese populations.

Methods: We genotyped 71 single-nucleotide polymorphisms (SNPs) comprising 1311 CD cases and 6585 controls of Japanese descent, and their associations with CD were evaluated using the Cochran–Armitage trend test. In addition, genotype–phenotype analyses were conducted on the SNPs showing associations with Japanese CD based on the Montreal classification.

Results: Twenty-seven SNPs showed at least nominal association ($P < 0.05$) and 11 of them remained significant even after Bonferroni correction ($P < 0.0007$). Despite high statistical power, we could not find any association in 17 loci. Moreover, SNPs in 9 loci were rare or absent in the Japanese population. Genetic variations involved in the innate immune system (*NOD2*, *ATG16L1*, and *IRGM*) showed no association with CD susceptibility in the Japanese population. Genotype–phenotype analyses showed that rs3810936, a marker of *TNFSF15*, correlated with severe CD phenotypes.

Conclusions: Our study suggests that there is a differential genetic background of CD susceptibility between Japanese and European populations.

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Key Words: Crohn's disease, Japanese, innate immune system, *TNFSF15*

Crohn's disease (CD) is one of the major subtypes of inflammatory bowel disease. It is hypothesized that CD is a multifactorial disease caused by multiple environmental and genetic factors. To date, several genome-wide association (GWA) studies of CD have been conducted, and recently a large-scale meta-analysis of GWA studies has identified 71 CD susceptibility loci in European populations.[1] This knowledge has brought several insights into the pathogenesis of CD. The importance of the innate and adaptive immune responses was particularly highlighted. In the innate arm of the immune system, it has been shown that *NOD2*, which encodes an intracellular sensor of bacterial muramyl dipeptide, and 2 autophagy-related genes (*ATG16L1* and *IRGM*) are strongly associated with CD.[1–4] In the adaptive arm of the immune system, GWA studies have identified genes related to the interleukin (IL)-23–T_H17 pathway, including *IL23R*, *IL12B*, *JAK2*, *STAT3*, *CCR6*, and *TNFSF15*.[1,2,5,6]

So far, the majority of GWA studies on CD have been performed in European populations. Although it has been reported that several CD susceptibility loci identified in European GWA studies are not associated with CD in Japanese populations,[7–11] it has not been thoroughly investigated whether the remaining European CD susceptibility loci are shared with other ethnicities. To the best of our knowledge, only a few genetic studies have been performed in Asian populations,[6–13] and it is unclear which genomic regions contribute to the genetic susceptibility of the Asians to CD. Therefore, we conducted a case–control association study to investigate the association of 71 European CD susceptibility loci in a Japanese population.

In addition, a number of genotype–phenotype analyses have been conducted on the CD susceptibility loci in European

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From the *Laboratory for Genotyping Development, Center for Genomic Medicine, RIKEN Yokohama Institute, Yokohama, Japan; †Department of Medicine and Department of Clinical Science, ‡Department of Environmental Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; §Department of Gastroenterology, Fukuoka University Chikushi Hospital, Fukuoka, Japan; ‖Department of Medicine, Division of Gastroenterology, Social Insurance Chuo General Hospital, Tokyo, Japan; ¶Department of Gastroenterology, Sapporo-Kosei General Hospital, Sapporo, Japan; and **Human Genome Center, Institute of Medical Science, the University of Tokyo, Tokyo, Japan.

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Reprints: Michiaki Kubo, MD, PhD, Laboratory for Genotyping Development, Center for Genomic Medicine, RIKEN Yokohama Institute, 1-7-22, Suehiro-cho, Tsurumi, Yokohama, Kanagawa 230-0045, Japan (e-mail: mkubo@src.riken.jp).

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CD populations, and *NOD2*, the strongest CD susceptibility gene in Europeans, is shown to be associated with CD severity such as early onset[14,15] and complicated behavior.[15–17] However, in the Japanese CD population, there is little information about the relationship between CD susceptibility loci and disease severity. We thus performed genotype–phenotype analyses on the associated loci in the Japanese CD population.

MATERIALS AND METHODS

Patients and Controls

A total of 1311 CD cases and 6585 controls were enrolled in this study. CD cases were collected at the Kyushu University Hospital (n = 160), its 16 affiliated hospitals (n = 447), Social Insurance Central General Hospital (n = 526), and Sapporo Kosei General Hospital (n = 178). All CD cases were diagnosed by clinical, radiological, endoscopic, and histological findings according to the Lennard–Jones criteria.[18] Clinical information of patients with CD was assessed at the time of sample collection, and phenotypes were categorized using Montreal classification.[19] Control subjects consisted of 3196 participants of the Hisayama study between 2002 and 2003[20] and 3389 samples selected from the BioBank Japan Project. This project (http://biobankjp.org) was started in 2003 to collect genomic DNA, serum, and clinical information of approximately 200,000 patients diagnosed with any of the 47 diseases by a collaborative network of 66 hospitals in Japan.[21] All participants were of Japanese descent.

Informed consent was obtained for all cases and controls before the study participation. This study was approved by the ethics committees of both RIKEN Yokohama Institute and Kyushu University.

Single-Nucleotide Polymorphism Selection for Case–Control Analysis and Genotyping

We selected 71 single-nucleotide polymorphisms (SNPs) that were reported to tag CD susceptibility loci in European populations. The SNPs were those imputed from data taken from 6 European GWA studies using HapMap3 reference data, and that were found to be significantly associated with CD in the meta-analysis of these GWAS followed by a replication study.[1] In this study, all SNPs were genotyped using the Invader assay (Third Wave Technologies, Madison, WI).[22] The overall call rate was 99.0%.

Statistical Analysis

Case–control association was analyzed using the Cochran–Armitage trend test, since χ^2 statistics from the 2×2 allelic table is inappropriate if the Hardy–Weinberg equilibrium does not hold in the combined case–control population.[23] Assessments of departure from the Hardy–Weinberg equilibrium in control samples were performed using χ^2 test. We considered a $P < 0.05$ as nominal association and a $P < 0.0007$ (0.05/71) as significant association in this study. To facilitate determination of risk direction, we calculated the odds ratios (ORs) and 95% confidence intervals of each SNP according to the risk alleles, as described in the original study.[1] Statistical power was estimated by the Genetic Power Calculator[24] using both ORs described in the original article[1] and allele frequencies from HapMap JPT data. Linkage disequilibrium coefficients (r^2) were calculated with the Haploview v4.2 [25] using HapMap JPT and CEU data. Genotype–phenotype analyses were conducted on those SNPs that showed significant association with CD in the present study. Primarily, CD cases were stratified on the basis of the Montreal classification for age at diagnosis, disease location, and disease behavior. In addition, CD cases were further sorted into 2 groups with respect to each category as follows: A1 (16 years or younger) versus A2 + A3 (17 years or older) for age at diagnosis, L1 (±L4) + L2 (±L4) (mainly localized in either ileum or colon) versus L3 (±L4) (spread in both ileum and colon) for disease location, and B1 (nonstricturing and nonpenetrating behavior) versus B2 + B3 (stricturing or penetrating behavior) for disease behavior. In these 3 categories, we performed case–case comparisons using the Cochran–Armitage trend test. Phenotypic groups of A1 (diagnosed at 16 years or younger), L3 (±L4) (spread in both ileum and colon), and B2 + B3 (stricturing or penetrating behavior) were regarded as severe phenotypes, whereas complementary phenotypic groups were regarded as reference phenotypes. For general statistical analysis, we used the R statistical environment (Version 2.13.0).

RESULTS

Clinical Characteristics

A total of 1311 CD cases and 6585 controls were examined. The clinical characteristics of the study population are summarized in Table 1. The proportion of males was higher in CD cases (70.2%) than in the controls (49.4%), and the mean age at sampling was lower in CD cases (36.0 years) than in the controls (55.0 years). The phenotype information was not available in almost 14% of the cases, and the remaining cases were stratified on the basis of the Montreal classification.

Case Control Analysis for 71 SNPs in a Japanese Population

No marker showed significant deviation from the Hardy–Weinberg equilibrium after Bonferroni correction. Twenty-seven SNPs showed at least nominal association with CD ($P < 0.05$), and 11 of them showed significant association even after Bonferroni correction ($P < 0.05/71 = 0.0007$) (Table 2). These significant loci included genes encoding *TNFSF15*, *NKX2-3*, *ICOSLG*, *HLA*, *IL12B*, *REL*, *VAMP3*, and *PLCL1*. We inferred that these loci might be common CD susceptibility loci for both Japanese and European populations.

On the other hand, 17 SNPs did not show any association with CD despite the high statistical power of our study (Table 3). Moreover, we found that the minor alleles of an additional 9 SNPs were rare (<1%) or absent in both cases and controls (Table 4), which contrasts with that observed in the GWA meta-analysis in European populations.[1] With regard to the remaining 18 SNPs, no conclusion could be drawn because the statistical power of this

TABLE 1. Clinical Characteristics of Patients with CD and Controls

	Cases (N = 1311)	Controls (N = 6585)
Sex		
Male/female	920 (70.2%)/ 390 (29.8%)	3227 (49.4%)/ 3302 (50.6%)
Not available	1	56
Age at sampling (yr)		
Mean (±SD)	36.0 (±11.6)	55.0 (±15.2)
Age at diagnosis[b]		
16 years or younger (A1)	160 (14.1%)	
Between 17 and 40 yr (A2)	884 (78.0%)	
Over 40 yr (A3)	89 (7.9%)	
Not available	178	
Disease location[b]		
Terminal ileum (L1) ± L4[a]	445 (39.3%)	
Colon (L2) ± L4[a]	172 (15.2%)	
Ileocolon (L3) ± L4[a]	514 (45.5%)	
Not available	180	
Disease behavior[bc]		
Nonstricturing, nonpenetrating (B1)	297 (26.3%)	
Stricturing (B2)	487 (43.1%)	
Penetrating (B3)	345 (30.6%)	
Not available	182	

[a]L4:upper gastrointestine.
[b]Phenotypes were categorized using Montreal classification.
[c]Perianal disease was not evaluated because clinical information was inadequate.

study was too low (see Table, Supplemental Digital Content 1, http://links.lww.com/IBD/A74).

To highlight the genetic differences in the CD susceptibility loci between Japanese and European populations, we compared the result of our data with the top 10 CD-associated SNPs in the European meta-analysis.[1] In these 10 SNPs, only a marker of *ZNF365* (rs10761659) was associated with a similar genetic risk in both populations. Out of the other 9 SNPs, the 5 markers of *MUC19/LRRK2* (rs11564258), *IRGM* (rs7714584), *ATG16L1* (rs3792109), *PTGER4* (rs11742570), and *PTPN2* (rs1893217) had no significant genetic effect in the Japanese population. Moreover, minor alleles of 4 markers of *IL23R* (rs11209026), *NOD2* (rs2076756), *PTPN22* (rs2476601), and *SLC22A4/SLC22A5/IRF1/IL3* (rs12521868) loci were very rare or absent in the Japanese population. These results reflect the genetic diversity at the single SNP level and suggest that there might be differences in the genetic background of CD between Europeans and Asians.

Association Between Genotypes and CD Severity

We conducted genotype–phenotype analyses on the 11 markers that showed significant association in this study. The marker SNP of *TNFSF15* (rs3810936), which was strongly associated with CD in the Japanese population, was shown to be associated with all 3 phenotype categories (Table 5). The CD risk allele C of rs3810936 was associated with the diagnosis at the age of 16 or younger (OR = 1.42), ileocolonic location (OR = 1.34), and stricturing or penetrating behavior (OR = 1.34). These phenotypes represent early onset, extensive lesion, and complicated behavior, respectively. Therefore, the result suggests that the variation in *TNFSF15* is associated with CD severity. The other 10 markers did not show any subphenotype association. Our study had sufficient power (≥80%) to detect the subphenotype association in almost all analyses, assuming an OR of 1.7 or more (data not shown).

DISCUSSION

We examined 71 European CD susceptibility loci in a Japanese population and found that at least 11 susceptibility loci are common to both European and Japanese populations. Among these 11 loci, 3 loci (*TNFSF15*, *NKX2-3*, and *IL12B*) were already reported to be associated with Japanese CD,[6,12] but, to the best of our knowledge, this study is the first to show the significant association of the additional 8 loci in the Japanese population. On the other hand, our study revealed that 17 loci did not show any association with CD in the Japanese population, despite high statistical power.

In interpreting our results, it is important to note that we tested the SNPs reported in the European GWA study.[1] Although some of these SNPs were the same as functional variants, the remaining SNPs were markers tagging functional variants in European populations. To evaluate the association of CD susceptibility loci properly, we should consider whether the tested SNPs tagged the functional variants in the Japanese subjects. In this study, tested SNPs in *TNFSF15* locus and *NKX2-3* locus are highly linked to the functional variants,[26,27] and these SNPs showed significant association in the Japanese population (Table 2). Although the tested SNPs in *ATG16L1* locus, *IRGM* locus, *PTPN2* locus, and *MST1* locus are also highly linked to the functional variants[28–32] in both European and Japanese populations, these SNPs showed no association in the Japanese population despite the high statistical power. This result indicates that these functional variants are indeed unlikely important in the Japanese population (Table 3).

Although it is not clear why the SNPs in *ATG16L1* and *IRGM* loci tagging functional variants did not show any association with CD, there may be several reasons for this. First, the differences in the environmental factors among populations may alter the effect of these variations. For instance, a previous study of twins indicated that differences in microbial compositions are correlated with the disease phenotype in monozygotic twins with identical genetic backgrounds.[33] If such differences in microbial composition exist at the population level, they may modulate the effect of the variants in the *ATG16L1* and *IRGM* loci in Japanese versus European

TABLE 2. SNPs Showing At Least Nominal Association with CD

Tested SNPs	Functional Variants[a]	r^2[b] JPT	r^2[b] CEU	Risk Allele[c]	RAF Cases	RAF Controls	*P*	OR (95% CI)[d]	Candidate Genes[e]
rs3810936	rs6478109	0.77	0.72	C	0.75	0.61	2.67×10^{-38} [g]	1.87 (1.70–2.06)	*TNFSF15, TNFSF8*
rs4409764	rs11190140	0.97	0.96	T	0.48	0.41	1.43×10^{-9} [g]	1.29 (1.19–1.41)	*NKX2-3*
rs2838519	—	—	—	G	0.66	0.61	2.51×10^{-7} [g]	1.26 (1.16–1.38)	*ICOSLG*
rs1799964	—	—	—	C	0.20	0.17	2.23×10^{-6} [g]	1.29 (1.16–1.43)	*LTA, HLA-DQA2, TNF, LST1, LTB*
rs6556412	—	—	—	A	0.52	0.47	9.01×10^{-6} [g]	1.21 (1.11–1.32)	*IL12B*
rs1736020	—	—	—	C	0.86	0.83	6.10×10^{-5} [g]	1.28 (1.14–1.44)	
rs10181042	—	—	—	T	0.06	0.05	6.63×10^{-5} [g]	1.44 (1.20–1.71)	*C2orf74, REL*
rs2797685	—	—	—	A	0.50	0.46	1.48×10^{-4} [g]	1.18 (1.08–1.28)	*VAMP3*
rs3764147	—	—	—	G	0.39	0.35	2.01×10^{-4} [g]	1.18 (1.08–1.28)	*C13orf31*
rs6738825	—	—	—	A	0.75	0.72	5.73×10^{-4} [g]	1.18 (1.08–1.30)	*PLCL1*
rs7702331	—	—	—	A	0.84	0.81	6.79×10^{-4} [g]	1.21 (1.08–1.36)	
rs780093	—	—	—	T	0.59	0.55	7.57×10^{-4} [f]	1.16 (1.06–1.26)	*GCKR*
rs151181	—	—	—	G	0.15	0.13	1.58×10^{-3} [f]	1.21 (1.07–1.36)	*IL27, SH2B1, EIF3C, LAT, CD19*
rs8005161	—	—	—	T	0.20	0.18	1.79×10^{-3} [f]	1.18 (1.06–1.31)	*GALC, GPR65*
rs10761659	rs7076156	0.05	0.21	G	0.73	0.70	1.84×10^{-3} [f]	1.16 (1.06–1.28)	*ZNF365*
rs694739	—	—	—	A	0.81	0.79	6.97×10^{-3} [f]	1.16 (1.04–1.29)	*PRDX5, ESRRA*
rs1250550	—	—	—	G	0.56	0.53	7.70×10^{-3} [f]	1.12 (1.03–1.22)	*ZMIZ1*
rs4809330	—	—	—	G	0.41	0.38	8.29×10^{-3} [f]	1.12 (1.03–1.22)	*RTEL1, TNFRSF6B, SLC2A4RG*
rs12242110	—	—	—	G	0.27	0.25	0.0108 [f]	1.13 (1.03–1.24)	*CREM*
rs713875	—	—	—	C	0.26	0.24	0.0155 [f]	1.13 (1.02–1.24)	*MTMR3*
rs11871801	—	—	—	A	0.87	0.85	0.0157[f]	1.16 (1.03–1.32)	*MLX, STAT3*
rs4656940	—	—	—	A	0.62	0.60	0.0174 [f]	1.11 (1.02–1.21)	*CD244, ITLN1*
rs181359	—	—	—	T	0.50	0.48	0.0180 [f]	1.11 (1.02–1.20)	*YDJC*
rs1819658	—	—	—	C	0.61	0.58	0.0238 [f]	1.10 (1.01–1.20)	*UBE2D1*
rs415890	—	—	—	C	0.52	0.49	0.0247 [f]	1.10 (1.01–1.20)	*CCR6*
rs17309827	—	—	—	T	0.57	0.55	0.0343 [f]	1.10 (1.01–1.19)	
rs7517810	—	—	—	T	0.92	0.91	0.0366 [f]	1.18 (1.01–1.38)	*TNFSF18, TNFSF4, FASLG*

[a]These variants have been reported as functional variants in the previous studies.
[b]r^2 between a tested SNP and a functional variant was calculated with Haploview using HapMap JPT and CEU database.
[c]Risk alleles are concordant with those of the original paper.[1]
[d]All associations are in the same direction, as described in the original paper.[1]
[e]Candidate genes are those presented in the original paper.[1]
[f]SNPs showed nominal association with CD ($0.0007 \leq P < 0.05$).
[g]SNPs showed significant association with CD even after Bonferroni correction ($P < 0.0007$).
RAF, risk allele frequency; CI, confidence interval.

TABLE 3. SNPs Showing No Association with CD Despite High Statistical Power

Tested SNPs	Functional Variants[a]	r^{2b}		Risk Allele[c]	RAF		P	OR (95% CI)	Candidate Genes[d]	Statistical Power[e] (%)
		JPT	CEU		Cases	Controls				
rs3180018	—	—	—	A	0.75	0.74	0.191	1.07 (0.97–1.17)	SCAMP3, MUC1	68.1
rs3197999	rs3197999	1	1	A	0.05	0.04	0.232	1.13 (0.92–1.38)	MST1, GPX1, BSN	78.4
rs7927997	—	—	—	T	0.14	0.14	0.636	1.03 (0.91–1.16)	C11orf30	79.7
rs2872507	—	—	—	A	0.26	0.26	0.669	1.02 (0.93–1.12)	*GSMDL, ZPBP2, ORMDL3, IKZF3*	82.6
rs1456896	—	—	—	T	0.52	0.51	0.243	1.05 (0.97–1.14)	*IKZF1, ZPBP, FIGNL1*	85.5
rs6908425	—	—	—	C	0.80	0.79	0.426	1.04 (0.94–1.16)	*CDKAL1*	86.1
rs1893217	rs1893217	1	1	G	0.12	0.12	0.520	0.96 (0.84–1.09)	*PTPN2*	91.8
rs740495	—	—	—	G	0.43	0.44	0.775	0.99 (0.91–1.08)	*GPX4, SBNO2*	93.3
rs4077515	—	—	—	T	0.32	0.33	0.197	0.94 (0.86–1.03)	*CARD9, SNAPC4*	94.4
rs4871611	—	—	—	A	0.38	0.38	0.958	1.00 (0.92–1.09)		95.5
rs10758669	—	—	—	C	0.37	0.36	0.201	1.06 (0.97–1.15)	*JAK2*	96.9
rs2058660	—	—	—	G	0.46	0.45	0.117	1.07 (0.98–1.16)	*IL18RAP, IL12RL2, IL18R1, IL1RL1*	98.2
rs11564258	rs3761863	0.01	0.04	A	0.03	0.03	0.898	1.02 (0.81–1.28)	*MUC19, LRRK2*	98.7
rs3091315	—	—	—	A	0.36	0.36	0.624	0.98 (0.90–1.07)	*CCL2, CCL7*	98.7
rs11742570	—	—	—	C	0.16	0.16	0.312	1.06 (0.95–1.19)	*PTGER4*	99.9
rs7714584	20 kb deletion[f]	1	1	G	0.40	0.38	0.060	1.09 (1.00–1.18)	*IRGM*	100.0
	rs10065172	1	1							
rs3792109	rs2241880	1	0.97	A	0.24	0.23	0.418	1.04 (0.94–1.15)	*ATG16L1*	100.0

[a]These variants have been reported as functional variants in the previous studies.
[b]r^2 between a tested SNP and a functional variant was calculated with Haploview using HapMap JPT and CEU database.
[c]Risk alleles are concordant with those of the original paper.[1]
[d]Candidate genes are those presented in the original paper.[1]
[e]Statistical power was estimated using both ORs described in the original paper[1] and allele frequencies from HapMap database.
[f]This variant is 20 kb deletion that maps 1.6 kb upstream of *IRGM*.
RAF, risk allele frequency; CI, confidence interval.

TABLE 4. SNPs Whose Minor Alleles Were Very Rare (<1%) or Absent in a Japanese Population

Tested SNPs	Functional Variants[a]	r^{2b}		Risk Allele[c]	RAF		P	OR (95% CI)	Candidate Genes[d]
		JPT	CEU		Cases	Controls			
rs10495903	—	—	—	T	0.0053	0.0060	0.722	0.90 (0.51–1.59)	*THADA*
rs2476601	rs2476601	1	1	G	0.9996	0.9999	0.204	0.20 (0.01–3.18)	*PTPN22*
rs2076756	rs2066844	NA	NA	G	0	0.0014	0.052	NA	*NOD2*
	rs2066845	NA	NA						
	rs2066847	NA	NA						
rs6568421	—	—	—	G	0	0.0003	0.372	NA	*PRDM1*
rs12521868	rs1050152	—[e]	0.93	T	0.0008	0.0004	0.395	2.01 (0.39–10.37)	*SLC22A4*, *SLC22A5*, *IRF1*, *IL3*
	rs2631367	—[e]	NA						
rs281379	rs601338	—[e]	0.75	A	0	0.0002	0.439	NA	*FUT2*, *RASIP1*
rs11209026	rs11209026	1	1	G	1	1	NA	NA	*IL23R*
rs12720356	—	—	—	G	0	0	NA	NA	*TYK2*, *ICAM1*, *ICAM3*
rs7423615	—	—	—	T	0	0	NA	NA	*SP140*

[a]Reported as functional variants in the previous studies.
[b]r^2 between a tested SNP and a functional variant was calculated with Haploview using HapMap JPT and CEU database.
[c]Risk alleles are concordant with those of the original paper.[1]
[d]Candidate genes are those presented in the original paper.[1]
[e]Rs12521868, rs1050152, rs2631367, and rs281379 are monomorphic in HapMap JPT database.
RAF, risk allele frequency; CI, confidence interval; NA, not available.

populations. Second, although functional interactions between *ATG16L1* and *NOD2* in the intracellular antibacterial response have been reported,[34,35] 3 coding variants of *NOD2*, which were strongly associated with CD in European populations, were not polymorphic in Asian populations.[7,8,13] If *NOD2* variants have an impact on the contribution of the *ATG16L1* variant to pathogenesis, the absence of *NOD2* variants may weaken the effect of the *ATG16L1* variant in the Japanese population. Third, it was revealed that a synonymous variant in *IRGM* (rs10065172) alters a microRNA-binding site and causes deregulation of IRGM-dependent xenophagy.[30] If the microRNA profile of Japanese individuals is different from that of Europeans, the effect of the microRNA-binding site variant may be less penetrant in the Japanese population.

In addition to *ATG16L1* and *IRGM*, *NOD2* is involved in the innate immune system. A tested SNP of *NOD2* locus, which is

TABLE 5. Genotype–Phenotype Association in *TNFSF15* (rs3810936)

Phenotype[a]	Genotype			RAF	P^b	OR (95% CI)[c]
	CC	CT	TT			
Age at diagnosis						
A1 (16 yr or younger)	104	47	8	0.80	0.0180	1.42 (1.06–1.91)
A2 + A3 (17 yr or older)	528	381	62	0.74		
Disease location						
L3 (±L4) (ileocolon)	311	178	24	0.78	0.0025	1.34 (1.11–1.63)
L1 (±L4) + L2 (±L4) (ileum or colon)	319	249	44	0.72		
Disease behavior						
B2 + B3 (stricturing or penetrating)	479	310	42	0.76	0.0060	1.34 (1.09–1.65)
B1 (nonstricturing and nonpenetrating)	150	118	28	0.71		

[a]Phenotypes were categorized using Montreal classification.
[b]P values were calculated using the Cochran–Armitage trend test.
[c]ORs were calculated using phenotypic subgroups described in lower rows as references.
RAF, risk allele frequency; CI, confidence interval.

a marker tagging functional variants in the European populations, did not show any association in this study because the minor allele of the tested SNP was very rare in the Japanese subjects. Furthermore, it has been reported that 3 known functional variants of *NOD2* locus are absent in Japanese populations.[7,8] These results suggest that genetic variations involved in the innate immune system may not have crucial roles in CD susceptibility in the Japanese population. Meanwhile, our study showed that several genes involved in the IL-23–T_H17 pathway (*TNFSF15*, *IL12B*, *STAT3*, and *CCR6*) are associated with CD susceptibility in the Japanese population. These findings underscore the importance of the adaptive immune system in CD pathogenesis across various populations.

Although it has been reported that *NOD2* is correlated with CD severity such as early onset[14,15] and complicated behavior[15–17] in European CD populations, no loci correlated with CD severity has been identified in the Japanese CD population. To elucidate the loci correlated with CD severity, we conducted genotype–phenotype analyses on 11 common susceptibility loci for European and Japanese populations and identified that a marker of *TNFSF15* (rs3810936) might be related to severe phenotypes such as early age of diagnosis, ileocolonic location, and complicated behavior. However, previous genotype–phenotype analyses of the *TNFSF15* locus did not find any significant association in either European[36,37] or Korean population.[38] One reason for this conflicting result might be the smaller sample sizes in the previous studies.[36–38] The association between variations in *TNFSF15* and CD phenotypes should be confirmed by additional studies in the future.

There are some limitations in this study. We used ORs derived from European populations to calculate statistical power in this study. However, this approach might be imperfect because ORs are highly correlated with allele frequencies within a particular population. When we calculated the statistical power in this study, the lowest effect sizes detected with 80% power were 1.64, 1.29, 1.21, and 1.16 for the risk allele frequencies of 0.01, 0.05, 0.1, and 0.2, respectively. Therefore, further studies with larger sample size will be required to clarify the associations of established CD susceptibility loci with low allele frequency in Japanese populations.

In conclusion, we assessed whether 71 CD susceptibility loci identified in the European GWA studies were associated with Japanese CD. Our study identified at least 11 susceptibility loci that are common to both European and Japanese populations. We also revealed distinct ethnic differences in the genetic background of CD susceptibility, especially in the innate immune system–related loci. Further studies will be needed to clarify the shared or differential genetic background of CD susceptibility between European and Asian populations.

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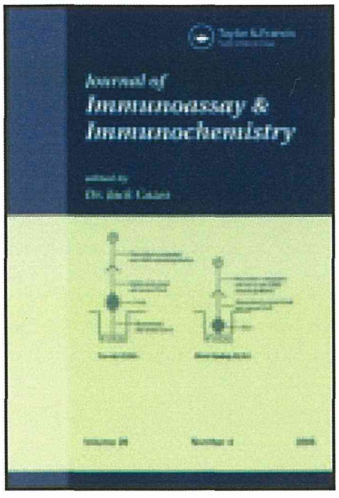

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COMPREHENSIVE ANALYSIS OF CHEMOKINES AND CYTOKINES SECRETED IN THE PERITONEAL CAVITY DURING LAPAROTOMY

Rei Kawashima [a b], Yuki I. Kawamura [a], Tomoyuki Oshio [a], Noriko Mizutani [a], Toshihiko Okada [a], Yutaka J. Kawamura [c], Fumio Konishi [c] & Taeko Dohi [a]

[a] Department of Gastroenterology, Research Center for Hepatitis and Immunology, Research Institute, National Center for Global Health and Medicine, Tokyo, Japan

[b] Department of Biochemistry, School of Allied Health Sciences, Kitasato University, Kanagawa, Japan

[c] Department of Surgery, Jichi Medical University, Saitama Medical Center, Saitama, Japan

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[1]*Department of Gastroenterology, Research Center for Hepatitis and Immunology, Research Institute, National Center for Global Health and Medicine, Tokyo, Japan*
[2]*Department of Biochemistry, School of Allied Health Sciences, Kitasato University, Kanagawa, Japan*
[3]*Department of Surgery, Jichi Medical University, Saitama Medical Center, Saitama, Japan*

□ *We recently found that chemokine-driven peritoneal cell aggregation is the primary mechanism of postoperative adhesion in a mouse model. To investigate this in humans, paired samples of peritoneal lavage fluid were obtained from seven patients immediately after incision (preoperative) and before closure (postoperative), and were assayed for the presence of 27 cytokines and chemokines using multiplex beads assay. As a result, IL-6 and CCL5 showed the most striking increase during operation. Recombinant CCL5 or lavage fluid induced chemotaxis of human peripheral blood mononuclear cells. We propose that CCL5 is possibly involved in the mechanism of postoperative adhesion in humans.*

Keywords CCL5, chemokine, cytokine, laparotomy, multiplex beads assay, peritoneal fluid

INTRODUCTION

Postoperative adhesions occur in the majority of patients following laparotomy and laparoscopy.[1,2] Treatment of adhesions can be expensive, and in some cases, life-threatening. In spite of the large number of surgical operations performed daily, the mechanism of peritoneal adhesion is not well understood. Previous reports have shown that peritoneal injury is triggered by leakage of plasma proteins, followed by formation of fibrinous

Address correspondence to Taeko Dohi, Department of Gastroenterology, Research Center for Hepatitis and Immunology, Research Institute, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan. E-mail: dohi@ri.ncgm.go.jp

deposits and proliferation of fibroblasts.[3] Rapid and transient influx of neutrophils into the peritoneal cavity also occurs, followed by the accumulation of mononuclear cells, usually macrophages.[4,5] $CD4^+$ T cells and the T cell-derived proinflammatory cytokine interleukin (IL)-17 also play significant roles in peritoneal adhesion.[6] Active roles of these cells in adhesion have been shown.[7,8] We previously found that peritoneal macrophages (PMF) form a large aggregated mass that packs the serosal side of the perforating ulcer with adhesion of visceral tissues in a colitis model in mice.[9] Transcripts encoding chemokine C-C motif receptor 8 (CCR8) were upregulated in the aggregating cells, while other chemokine receptors were downregulated compared with naïve PMF. Of note, chemokine C-C motif ligand 1 (CCL1), the ligand for CCR8, was also produced by macrophages and mesothelial cells in response to inflammatory stimuli. The presence of CCL1-induced formation of cell aggregates by PMF and mesothelial cells *in vitro*, and neutralization of CCL1 prevented formation of peritoneal membranous adhesion *in vivo*.[9] Thus, autoactivation of peritoneal macrophages via chemokines was the initial trigger for peritoneal adhesion. These results from the mouse system prompted us to investigate similar chemokine-/cytokine-driven mechanisms of peritoneal adhesion in humans. In this study, we collected peritoneal lavage fluid at the beginning and the end of laparotomy, and quantified the presence of 27 cytokines and chemokines in an effort to identify potential molecules that could be targeted to prevent adhesion formation in humans.

EXPERIMENTAL METHODS

Peritoneal Lavage Fluid and Exudate Collection

This study was performed with approval from the ethics committees of the National Center for Global Health and Medicine and Jichi Medical University. Seven patients (Table 1) who had laparotomy for colorectal resection of colorectal cancer were recruited in this study with informed

TABLE 1 Patient Demographics

Case	Age	Sex	Operation	Stage	Operation Time (h)
1	51	Male	Intersphincteric resection with loop ileostomy	I	5.00
2	74	Female	Sigmoid colectomy	II	2.25
3	57	Male	Low anterior resection with loop ileostomy	II	3.30
4	76	Male	Low anterior resection	IV	5.00
5	65	Male	Left hemicolectomy with extended left lobectomy of the liver	IV	8.75
6	62	Male	Anterior resection	III	3.17
7	40	Female	Low anterior resection	III	4.08

consent. These patients had not had a previous major laparotomy, and none of them suffered from inflammatory bowel disease. Immediately after incision at the beginning of the operation (preoperative) and before closure at the end (postoperative), 1 L of saline was poured into the peritoneal cavity, and as much fluid as possible was aspirated back into a bottle. The recovered fluid volume was approximately 777–934 mL [857 ± 76 mL, mean ± standard deviation (SD)]. After removal of visible fat debris, exudate cells were separated by centrifugation at 600 × *g*. Supernatant aliquots were maintained at −80°C until use, and were subjected to cytokine assays using the Bio-Plex Suspension Array system (Bio-Rad Japan, Tokyo, Japan). The assay tested for the presence of IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, basic fibroblast growth factor (FGF basic), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), platelet-derived growth factor (PDGF)-BB, vascular endothelial growth factor (VEGF), eotaxin (CCL11), IP-10 [chemokine C-X-C motif ligand 10 (CXCL10)], MCP-1 (CCL2), MIP1α (CCL3), MIP1β (CCL4), RANTES (CCL5), and IL-8 (CXCL8).

Cell Culture

Preoperative peritoneal exudate cells from five patients (Cases 1–3, 6, and 7) were cultured in a 24-well plate at 5.8×10^6 cells/mL per well, with or without 100 ng/mL lipopolysaccharide (LPS; from *Salmonella minnesota,* Sigma-Aldrich, St. Louis, MO, USA) for 20 h. Supernatant of duplicated cultures was harvested and subjected to the assay described above.

Chemotaxis Assay

Peripheral blood mononuclear cells (PBMCs) obtained from a healthy volunteer using Ficoll-Paque Plus (GE Healthcare Japan, Tokyo, Japan) were prestained for 30 min at 37°C with 3 μM 2′,7′-bis(2-carboxyethyl)–5-(and-6)-carboxyfluorescein acetomethyl ester (BCECF-AM, Molecular Probes, Eugene, OR, USA) and then suspended at 2×10^6 cells/mL in Hank's balanced saline solution containing 0.5% bovine serum albumin and 20 mM HEPES. Chemotaxis assay was performed using a Chemo Tx-96 Chemotaxis plate (NeuroProbe, Inc., Gaithersburg, MD, USA), as follows. After washing, 65 μL of cell suspension was loaded onto the membrane plate and placed onto a flat-bottomed microtiter plate with 96 wells containing 30 μL of recombinant CCL5 (R & D Systems, Minneapolis, MN, USA) solution in each well or peritoneal lavage fluid diluted with phosphate buffered saline (1:1). The plate was then incubated at 37°C

for 120 min, and cells that had undergone migration were collected. These collected cells were lysed with 0.1% triton X-100 and counted using a fluorescence microplate reader (FlexStation, Molecular Device Japan, Tokyo, Japan). Data were shown as the average of five scans. Anti-human CCL5 and anti-human CCL2 antibodies were purchased from R & D Systems.

Statistical Analysis

The results were statistically analyzed by the two-tailed paired t-test using Prism 4 software (GraphPad Software, Inc., La Jolla, CA, USA). When *P* values were less than 0.05, the results were considered as a significant difference.

RESULTS AND DISCUSSION

We measured the amounts of various cytokines and chemokines in the peritoneal fluid from patients prior to and following a colorectal operation in an effort to identify molecules that may be involved in the formation of adhesions in the gut. Many molecules were drastically increased in the peritoneal lavage fluid after the laparotomy procedure (Table 2). The concentration of IL-6 was increased 11-fold, while that of IL-1β, PDGF, and IL-1Ra showed more than a fivefold increase. Furthermore, IFN-γ, IL-13, IL-4, IL-10, and VEGF were increased more than twofold in postoperative samples. When the percent composition of each cytokine in the total sample was evaluated, we observed that FGF basic, GM-CSF, and IL-6 were dominant in the preoperative samples. However, only IL-6 increased to account for more than 50% of the measured cytokines in the postoperative samples (Figure 1A). For the chemokine response, we observed that CCL5, CCL3, CCL2, CXCL8, and CCL4 were increased after the operation. However, in terms of absolute amount of protein, CCL5, CCL2, CXCL8, and CCL4 were the major secreted chemokines. As for percent composition, increases in CCL5 were the most obvious, while the relative amount of other chemokines did not significantly change, except for CXCL10 (Figure 1B). One patient, case 5 in Table 1, who underwent lobectomy of the liver with relatively long operation time was included in our study. Since this case might have different conditions of the peritoneal cavity from other cases, we reviewed individual data. However, this patient did not show a particular secretion pattern of CCL5, IL-6, or other cytokines and chemokines when compared with other cases (Figure 1C). Since we had found that the CCL1/CCR8 system is critical in the formation of peritoneal adhesions in mice,[9] we attempted to measure the amount of CCL1 present in the samples, but the concentration in the original peritoneal lavage fluid was less than the

TABLE 2 Concentration of Cytokines and Chemokines (pg/mL) in Peritoneal Lavage Fluid

	Pre-operation[a]	Post-operation[a]	*P* value	Fold Increase
Cytokines				
IL-6	1776 ± 2624	20074 ± 7240	0.001*	11.3
IL-1β	333 ± 155	2857 ± 4010	0.153	8.6
PDGF	185 ± 67	1084 ± 776	0.025*	5.8
G-CSF	435 ± 410	2098 ± 2119	0.100	4.8
IL-1Ra	220 ± 119	874 ± 709	0.058	4.0
IFN-γ	176 ± 30	567 ± 275	0.013*	3.2
IL-13	333 ± 158	763 ± 541	0.046*	2.3
IL-4	163 ± 69	328 ± 192	0.021*	2.0
IL-10	489 ± 149	982 ± 482	0.071	2.0
VEGF	822 ± 246	1609 ± 884	0.101	2.0
IL-12	217 ± 75	372 ± 221	0.121	1.7
TNF-α	742 ± 132	1222 ± 576	0.104	1.6
IL-15	874 ± 567	1352 ± 857	0.014*	1.5
IL-9	323 ± 61	495 ± 123	0.001*	1.5
IL-7	201 ± 75	273 ± 141	0.368	1.4
IL-2	223 ± 133	280 ± 105	0.157	1.3
GM-CSF	1218 ± 859	1012 ± 628	0.438	0.8
IL-5	90 ± 80	63 ± 10	0.405	0.7
FGFbasic	3882 ± 1886	2707 ± 3004	0.363	0.7
IL-17	669 ± 446	418 ± 333	0.122	0.6
Chemokines				
CCL5 (RANTES)	2241 ± 1921	24066 ± 2979	$P < 0.0001$*	10.7
CCL3 (MIP1α)	160 ± 69	1431 ± 2865	0.284	8.9
CCL2 (MCP-1)	2250 ± 1534	14511 ± 8692	0.008*	6.4
CXCL8 (IL-8)	1881 ± 2446	11647 ± 7836	0.024*	6.2
CCL4 (MIP1β)	1409 ± 1287	7216 ± 5660	0.033*	5.1
CCL11 (eotaxin)	318 ± 185	584 ± 253	0.031*	1.8
CXCL10 (IP-10)	980 ± 1267	827 ± 1448	0.109	0.8
Total protein mg/mL	1.593 ± 1.721	3.39 ± 2.99	0.269	2.1

[a]Data are shown as an average of seven cases with a standard deviation.
*Statistically significant difference ($P < 0.05$) between pre- and postoperative samples.

sensitivity of the assay. We detected 10 pg/mL CCL1 after removal of albumin, with a 10-fold greater concentration of the fluid in the preoperative sample from one individual, but this patient had severe peritoneal adhesion and was therefore excluded from this study.

The majority of cells harvested from the peritoneal lavage fluid prior to the operation were $CD33^{+}CD14^{+}$ macrophage-type cells (data not shown) and lymphocytes as reported previously.[10] In order to know how these cells respond to inflammatory stress that possibly occurs during colorectal operation, we tested if the treatment of these cells with LPS, a potent activator of macrophage type cells, would enhance the chemokine/cytokine response. As expected, secretion of inflammatory cytokines, which were upregulated during operation including TNF-α and IL-6, were significantly augmented in the presence of LPS (Figure 2). In spite of the striking

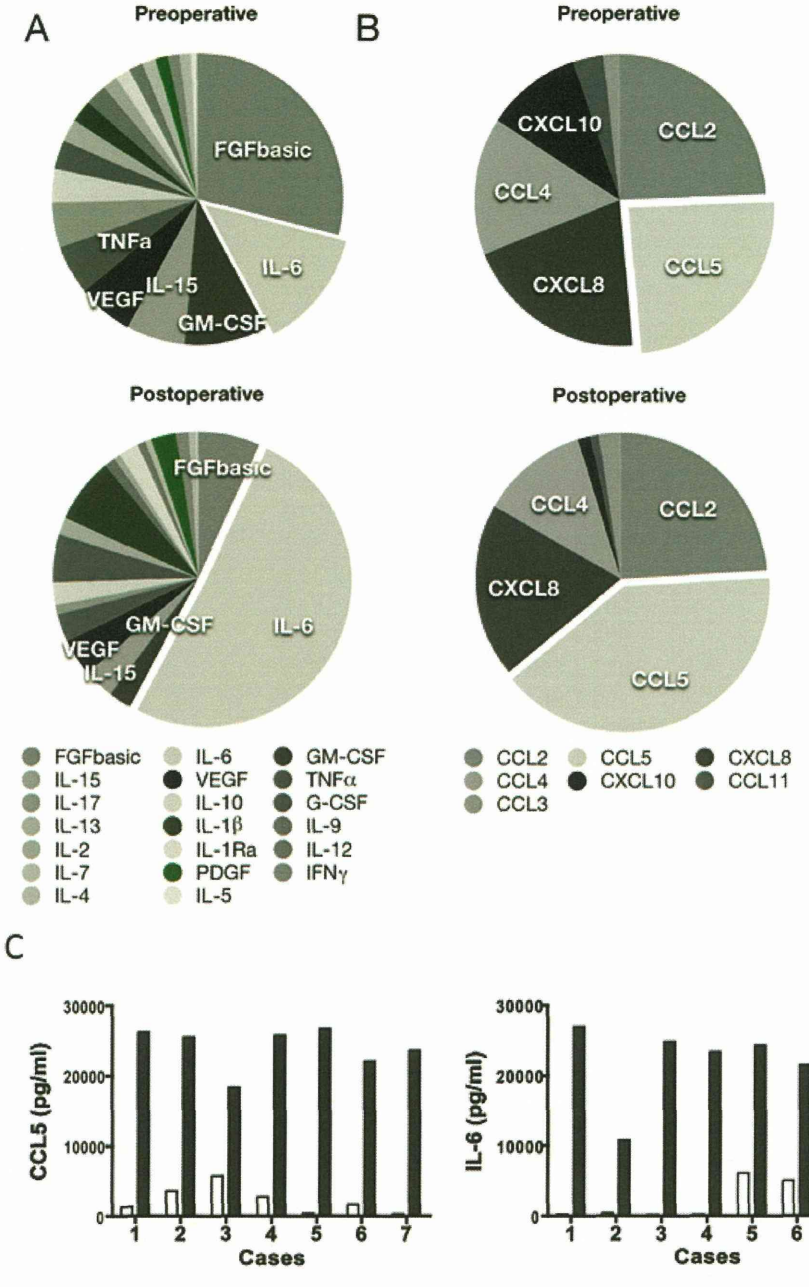


FIGURE 1 Relative amount of measured cytokines (A) and chemokines (B) listed in Table 1. Percentage was calculated by dividing the concentration of cytokines or chemokines (pg/mL) with sum of the concentrations (total concentration) of measured cytokines or chemokines (pg/mL). (C) Data from an individual case for CCL5 and IL-6 are shown. Blank bars, preoperative concentration; solid bars, postoperative concentration. The case number indicates the patient ID in Table 1 (color figure available online).

increase in CCL5 in the postoperative lavage samples, the amount secreted by cultured exudate cells was low and was not augmented by LPS. Similar results were obtained for CCL2 (data not shown). These results suggested that peritoneal exudate cells are not the origin of CCL5. Further, to support the inflammatory process that may be mediated by CCL5 in the

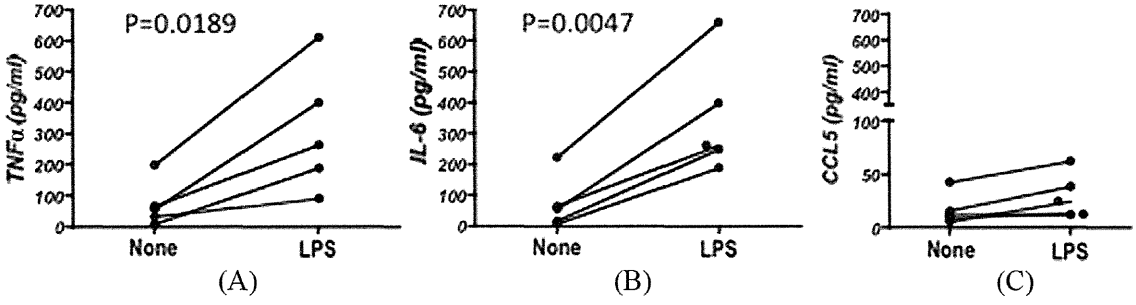
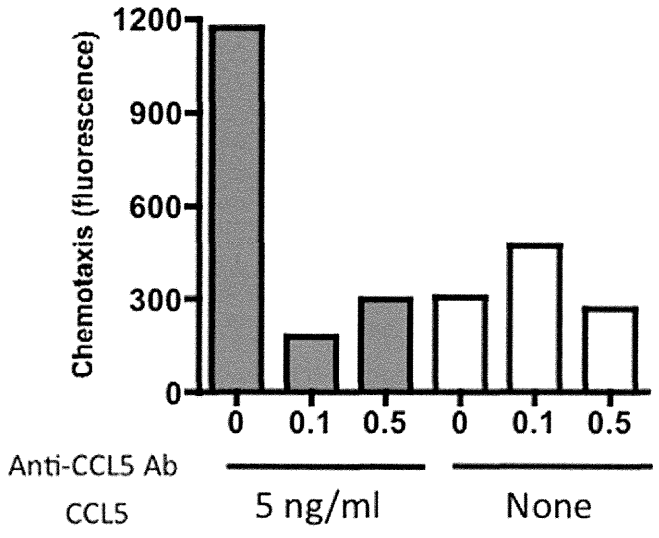


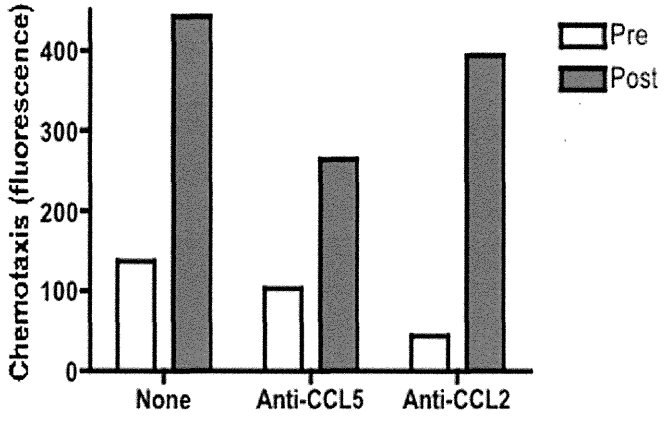
FIGURE 2 TNF-α, IL-6, and CCL5 production by peritoneal exudate cells. Peritoneal exudate cells harvested from preoperative lavage fluid were cultured with (LPS) or without LPS (none). Culture supernatant was subjected to the assay for TNF-α (A), IL-6 (B), and CCL5 (C).

peritoneal cavity, we tested PBMCs if they have chemotaxis potential in response to CCL5. As shown in Figure 3, recombinant CCL5 as well as peritoneal fluid was able to induce chemotaxis of PBMCs. Anti-CCL5 antibody but not anti-CCL2 antibody partially inhibited the chemotaxis induced by peritoneal lavage fluid.

This study reports the comprehensive analysis of the secretion of cytokines and chemokines in the peritoneal cavity during laparotomy. Prior studies have investigated individual cytokines in peritoneal fluid after operation,[11] but the total composition of cytokines present in the peritoneal cavity before and after operation has not been addressed until now. Importantly, assaying both the pre- and postoperative lavage fluid from the same individuals enabled us to quantify the cytokine levels under conditions close to the naïve status of each patient, as well as quantify the net secretion during the operation. Although the stage of the disease in the tested cases varied, the result was quite constant. For example, IL-1β, IL-6, IL-10, IL-12, IL-13, IL-15, and IL-17 were detected in the preoperative fluid. In combination with the high amount of GM-CSF, this array of cytokines may work to maintain the steady-state nature of the peritoneal cavity and its resident cells. After operation, increases in IL-6 were the most striking, though those of IL-13 and IL-15 were also statistically significant. A previous study reported that adhesion formation 48 h after laparoscopy was associated with a high concentration of IL-6.[12] Though most studies have not addressed IL-13 and IL-15 under these conditions, they are most likely involved in the response to surgical stress in the peritoneal cavity. Since IL-15 is known to activate T cells, which are involved in adhesion formation,[13,14] it is possible that it could trigger the reaction of these cells to induce further immune responses. IL-13 may induce alternative (M2 type) activation of macrophages,[15] and M2 type macrophages have reduced abilities to stimulate T cell proliferation and Th1 differentiation, with the production of large amounts of IL-10.[16] Therefore, the balance of IL-15 and IL-13 may be critical for avoiding excess immune reactions. In the



(A)



(B)

FIGURE 3 CCL5-driven chemotaxis of human PBMCs. (A) Chemotaxis of PBMCs induced by recombinant CCL5 was measured in the presence of indicated concentrations of anti-CCL5 antibody (mg/mL). (B) Pre- (blank) and postoperative (solid) peritoneal fluid from case 1 was tested for the chemotactic potential of human PBMCs in the presence of 0.1 mg/mL of anti-CCL5 antibody or anti-CCL2 antibody. Data are shown as an average of duplicated assay.

mouse, IL-17 is associated with adhesion formation[6]; however, we found that in humans, the amount of IL-17 either decreased or remained unchanged after the operation. It is possible that IL-17 may not play a role in this process until a later time. The amount of IFN-γ was not very high in the peritoneal fluid prior to the operation, but the fact that it increased by 3.2-fold following laparotomy may support the report that recommends IFN-γ as a target for prevention of adhesion in a mouse model.[17] Although differences were not statistically significant, elevation of IL-1Ra and IL-10 may be important, as both have antagonizing effects on the inflammatory response.