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The 116th Japan Pediatric Society Scientific Research Award Winner

Primary immunodeficiency in Japan; epidemiology, diagnosis, and pathogenesis

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Abstract Primary immunodeficiency (PID) constitutes a large group of diseases, including almost 180 hereditary disorders. The patients show susceptibility to various infections due to congenital defects of the immune system. It is also known that PID patients suffer from non-infectious complications, including autoimmune diseases and malignant disorders. During the last 20 years the number of known PID has increased considerably. New PID conferring a specific predisposition to infections with one or a few pathogens have been described. Disorders of innate immunity and various autoinflammatory disorders were included in new categories. In contrast, the incidence, clinical manifestations, and genetic factors of PID seem to be different among countries or races. The clinical manifestations can differ depending on the hygiene conditions, health-care environment, and vaccination policy, and so on. A nationwide survey on PID patients in Japan provided a lot of information regarding these issues, and it uncovered a previously unknown complication of PID, endocrine disorders. In this review, the data concerning epidemiology and clinical characteristics of PID in Japan obtained in the nationwide questionnaire survey, and the results of studies on the clinical and genetic characteristics of Japanese patients with Mendelian susceptibility to mycobacterial disease and interleukin-1 receptor-associated kinase 4 deficiency are presented in the light of their pathogenesis and pathophysiology.

Key words: innate immunity, Mendelian susceptibility to mycobacterial diseases, primary immunodeficiency.

Patients with primary immunodeficiency (PID) show susceptibility to infections due to congenital immune system defects. International Union of Immunological Societies Expert Committee on PID classified primary immunodeficiency into eight categories: (i) combined immunodeficiencies; (ii) predominantly antibody deficiencies; (iii) well-defined syndromes with immunodeficiency; (iv) diseases of immune dysregulation; (v) congenital defects of phagocyte number, function, or both; (vi) defects in innate immunity; (vii) autoinflammatory disorders; and (viii) complement deficiencies.[1,2] Presented here are data concerning PID patients in Japan regarding incidence and clinical characteristics according to the PID classification.

Incidence of PID (from nationwide analysis)

To determine the prevalence and clinical characteristics of patients with PID in Japan, a nationwide survey of PID was conducted according to the nationwide epidemiological survey manual of patients with intractable disease (2nd edition 2006, Ministry of Health, Labour, and Welfare of Japan).[3] Questionnaires were distributed to 1224 pediatric departments and 1670 internal medicine departments of hospitals in Japan. It was found that the estimated number of patients with PID in Japan was 2900 (95% confidence interval: 2300–3500), and the prevalence was 2.3 per 100 000 inhabitants,[4] which was equivalent to that reported from Singapore (2.7/100 000)[5] and Taiwan (0.77–2.17/100 000),[6] although lower than that reported in Middle Eastern countries such as Kuwait (11.98/100 000)[7,8] or in European countries such as France (4.4/100 000).[9,10] The high rate of consanguinity may be a cause of the high prevalence rate of PID reported in Middle Eastern countries.[7,8] There might be some sample selection bias in this study because many asymptomatic cases (selective IgA deficiency [SIgAD] etc.), clinically recovered cases (transient hypogammaglobulinemia of infancy etc.) and deceased cases were not registered.

Clinical characteristics of PID (from nationwide analysis)

The most common form of PID was predominantly antibody deficiencies (40%), followed by congenital defects of phagocyte number, function, or both (19%) and other well-defined immunodeficiency syndromes (16%).[4] The most common PID was Bruton's tyrosine kinase (BTK) deficiency (14.7%), followed by chronic granulomatous disease (CDG; 11.9%). The prevalences of the two disorders were higher than those in a previous report from Europe (5.87% and 4.33%, respectively).[11] BTK deficiency appears to be more common in Japan,[12] although this may be partially because more patients, including those with atypical clinical manifestations, were diagnosed accurately by the

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recently established genetic diagnostic network.[13] The reason for the low number of registered CGD patients in Europe in a recent report (1/620 000)[11] is unknown; the prevalence of CGD was 1 in 250 000 in a previous European survey,[14] which was similar to the nationwide survey results (1 in 380 000). Common variable immunodeficiency disease (CVID) and SIgAD were observed only in 11.0% and 4.0%, respectively. CVID was the most common PID (20.7%) in Europe.[11,15] A lower number of registered CVID patients in the nationwide survey may be due to the low number of adult patients registered, although it is still possible that CVID is not as common in Japan as in Europe. There was no significant difference in the distribution rate of SIgAD between Japanese and European subjects according to questionnaire studies, although it has been reported that SIgAD is rare in Japanese people (1/18 500) compared with Caucasian people (1/330–2200) according to seroepidemiologic studies.[16] This may be because most SIgAD patients lack clinical manifestations.

Malignant disorders were observed in 2.7% of PID patients. Lymphoma, in particular Epstein–Barr virus-related, and leukemia were predominant. CVID, Wiskott–Aldrich syndrome, and ataxia telangiectasia were more frequently associated with malignant disease. Immune-related disease was observed in 8.5% of PID patients. Autoimmune lymphoproliferative syndrome, immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome, and nuclear factor kappa B (NF-κB) essential modulator (NEMO) deficiency were associated with immune-related diseases at a very high incidence.

Recently, the interaction of the immune and endocrine systems has been getting increasing attention, but there have been no reports on endocrine complications associated with PID in a large-scale survey. Many endocrine disorders in PID patients are thought to be based on the autoimmunity, which is closely related to the pathophysiology of PID. In contrast, it is not known how the immunological and molecular defects in individual PID contribute to the development of various autoimmune endocrine disorders. In addition, genetic defects in some PID can lead to these complications directly or indirectly via non-immunological mechanisms. Therefore, the endocrine complications of PID were analyzed. This was the first large-scale survey focusing on the endocrine complications in PID.[17] Among the 923 PID patients, 49 patients (5.3%) had endocrine diseases. The prevalence of endocrine diseases was much higher in the PID patients than in the general population (Table 1), even excluding the patients with immune dysregulation. Endocrine disorders are important complications that should not be overlooked in PID patients.

Clinical characteristics of Mendelian susceptibility to mycobacterial disease

Mendelian susceptibility to mycobacterial diseases (MSMD; MIM 209950) is a rare primary immunodeficiency syndrome characterized by a predisposition to intracellular bacterial infection. The patients are susceptible to infections even by weakly virulent mycobacteria, such as *Mycobacterium bovis* bacille Calmette–Guerin (BCG), environmental non-tuberculous mycobacteria (NTM), *Salmonella* species, *Listeria monocytogenes*, and so on. Inborn errors of interleukin (IL)-12/23- and interferon (IFN)-γ-mediated immunity is the major cause of this disorder.[18,19] Hoshina *et al.* investigated the clinical characteristics and genetic background of MSMD in Japan, which are associated with a high prevalence of tuberculosis.[20] A total of 46 patients (30 male, 16 female) were studied, who were diagnosed as having recurrent, blood-borne (such as osteomyelitis/arthritis), or multiple (at different anatomic sites) infections, by intracellular bacteria including BCG, NTM, *Salmonella* species, *L. monocytogenes*, or *M. tuberculosis* in 34 hospitals in Japan from 1999 to 2009. Median patient age was 8 years (range, 6 months–41 years) and the median age at onset of infection was 1 year 4 months (range, 4 months–6 years). The male : female ratio was 1.9: 1. Only one patient had not received BCG vaccination. There were 59 episodes of disseminated mycobacterial infections in 46 patients. Nine (19%) of 46 patients had two or more episodes of these infections. In all episodes, BCG was the most common pathogen (82.6%). *M. avium* complex was isolated in eight episodes, and *M. tuberculosis* was also confirmed in two episodes of these infections. Severe *Salmonella* species, *L. monocytogenes*, or viral infections were not observed.

The common clinical manifestations were osteomyelitis/arthritis, lymphadenitis, subcutaneous or pulmonary abscess, and dermatitis. Among the BCG infections, the median interval between BCG vaccination and the development of primary BCG infection was 2 months (range, 1–6 months), 4 months (range, 2–36 months), 6 months (range, 3–10 months) and 11 months (range, 5–46 months) in dermatitis, lymphadenitis, subcutaneous abscess, and osteomyelitis/arthritis, respectively.

Table 1 Prevalence of endocrine disorders in PID vs the general population (<20 years old)

Endocrine disorders	Diabetes mellitus		Hypothyroidism		GH deficiency	Hypogonadism	Hypoparathyroidism	Isolated ACTH deficiency
	T1D	T2D	Hashimoto's thyroiditis	Non-autoimmune				
Estimated prevalence in PID patients	93	16	47	109	93	47	233	16
Prevalence in the general Japanese population	1.19	0.461†	ND	13.5‡	1.47	ND	0.072§	0.035

†Incidence instead of prevalence is given because of the lack of appropriate reports concerning prevalence in Japan. ‡Prevalence in USA is given because of the lack of appropriate data from Japan. §Prevalence for all age groups is given because of the lack of data for this age group. ACTH, adrenocorticotropic hormone; GH, growth hormone; PID, primary immunodeficiency; T1D, type 1 diabetes mellitus; T2D, type 2 diabetes mellitus.

Genetic analysis for *IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, *STAT1*, and *NEMO* genes was done for these patients. Six patients (five families) and one patient had mutations in *IFNGR1* and *NEMO* genes, respectively. All the IFN-γR1-deficient patients were heterozygotes, and the mutation was in the transmembrane domain in one patient (774del4, patient 5) and in the intracellular domain in five patients (811del4, patient 1; 818del4, patients 2–4; and 832G>T, E278X, patient 6), which are important hotspots in the patients diagnosed as having dominant partial IFNγ-R1 deficiency. IFN-γR1 expression level was significantly increased in all six patients with IFN-γR1 deficiency, as reported previously.[20,21] Patient 7 had a missense mutation in *NEMO* (943G>C, E315Q). The mutation was located in exon VIII within the leucine zipper domain of the *NEMO* gene. A previous study reported that the mutation within that domain disrupted a common salt bridge in the leucine zipper domain and impaired T-cell-dependent IL-12 production.[22] The CD14-positive cells from the patient produced a lower level of tumor necrosis factor-α (TNF-α) in response to lipopolysaccharide (LPS) stimulation, which was consistent with the defect of NF-κB signaling.

Fatal mycobacterial infections were not observed in that study.[20] Unlike complete IFNγ-R1 and IFNγ-R2 deficiencies which often cause fatal mycobacterial infection, the patients with dominant partial IFNγ-R1 and NEMO deficiencies have been reported to have a relatively mild disease and a better prognosis.[22,23] These factors might have contributed to the good outcome of the patients in the Hoshina *et al.* study. In addition, the low virulence of BCG might contribute to the characteristics of BCG infection in Japan because BCG Tokyo 172 strain that is used in Japan for vaccination is the least virulent BCG substrain.

It has been reported that *IL12RB1* mutation is the most common cause of MSMD.[18] None of the patients in the Hoshina *et al.* study, however, was diagnosed with IL-12 receptor β1 deficiency. One of the reasons for a low incidence of *Salmonella* infections might be the lack of IL-12 receptor β1 subunit-deficient patients, because *Salmonella* infection is observed often in IL-12 receptor β subunit deficiency.[18,19]

Clinical characteristics of interleukin-1 receptor-associated kinase 4 deficiency in Japan

It was reported that interleukin-1 receptor-associated kinase 4 (IRAK4) is indispensable for IL-1 and Toll-like receptor (TLR) signaling after analyzing IRAK4 knockout mice in 2002.[24] Human IRAK4 deficiency was first reported in 2003. The patients had severe extracellular pyogenic bacterial infections, predominantly by Gram-positive *Streptococcus pneumonia* and *Staphylococcus aureus*, recurrently early in life, but less frequently with age. Some IRAK4-deficient patients had lethal pneumococcal meningitis.[25] A family with IRAK4 deficiency was identified, and a rapid screening method using flow cytometry for IRAK4 deficiency was established (Fig. 1).[26] In addition, delayed separation of umbilical cord was noted in these patients.[26] On analysis of monocytic intracellular TNF-α production after LPS stimulation, it was found that in IRAK4-deficient patients, the number of TNF-α-producing monocytes was markedly reduced. In 2008, human myeloid differentiation factor (MyD) 88 deficiency was reported.[27] MyD88 is a key cytosolic adapter molecule, providing a bridge from TLR and IL-1R to the IRAK complex. IRAK4 and MyD88 deficiencies are considered phenocopies with respect to their immunologic phenotype.[27] An international survey of IRAK4 and MyD88 deficiencies identified clinical and genetic characteristics of these disorders.[28,29] Among the 48 IRAK4 deficiency and 12 MyD88 deficiency patients, the leading threat was invasive pneumococcal disease, documented in 41 patients (68%). Invasive infections caused by *Pseudomonas aeruginosa* and *S. aureus* were documented in 25% and in 25% of patients, respectively. The first invasive infection occurred before the age of 2 years in 88.3% and in the neonatal period in 32.7%. Clinical outcome was poor,

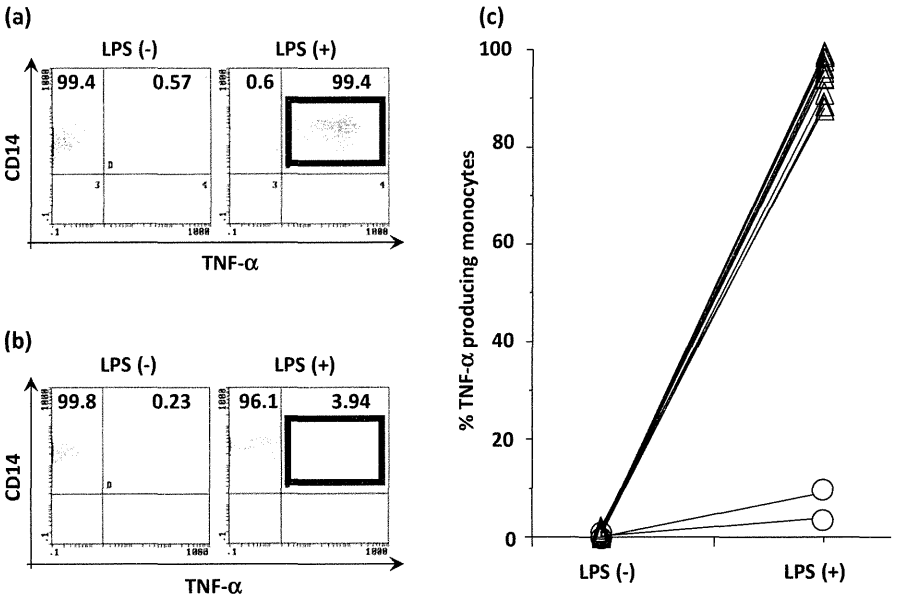

Fig. 1 (a,b) Intracellular staining for tumor necrosis factor (TNF)-α and its expression in monocytes with and without lipopolysaccharide (LPS) stimulation in (a) healthy controls and (b) interleukin-1 receptor-associated kinase 4 (IRAK4)-deficient patients. (c) Percentage of TNF-α producing cells in monocytes with and without LPS stimulation in (△) healthy controls and (○) IRAK4-deficient patients.

with 24 deaths, in 16 cases of invasive pneumococcal disease, but no deaths or invasive infectious disease were reported in patients after the age of 8 years and 14 years, respectively. Separation of the umbilical cord later than 28 days after birth was observed in 10 IRAK4-deficient patients.

Screening to detect IRAK4-deficient patients in Japan is continuing, and eight IRAK4-deficient patients from five families have been identified. Genetic analysis was performed for these patients, and the usefulness of this screening method was confirmed. Five patients died of pneumococcal meningitis in early infancy. Seven patients had delayed separation of the umbilical cord. The high frequency of delayed separation of umbilical cord in Japanese patients may indicate that this is an important clinical sign to lead to the early diagnosis of IRAK4 deficiency.

Conclusion

Analysis of the host defense system in humans and clinical observation of PID contribute to the fundamental knowledge on immune system biology and its perturbation in disease. This is of considerable clinical benefit to patients and family members. Establishment and improvement of the procedures for diagnosis and effective therapies will further promote the wellbeing of PID patients.

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nature genetics

High-density genotyping study identifies four new susceptibility loci for atopic dermatitis

Atopic dermatitis is a common inflammatory skin disease with a strong heritable component. Pathogenetic models consider keratinocyte differentiation defects and immune alterations as scaffolds[1], and recent data indicate a role for autoreactivity in at least a subgroup of patients[2]. *FLG* (encoding filaggrin) has been identified as a major locus causing skin barrier deficiency[3]. To better define risk variants and identify additional susceptibility loci, we densely genotyped 2,425 German individuals with atopic dermatitis (cases) and 5,449 controls using the Immunochip array followed by replication in 7,196 cases and 15,480 controls from Germany, Ireland, Japan and China. We identified four new susceptibility loci for atopic dermatitis and replicated previous associations. This brings the number of atopic dermatitis risk loci reported in individuals of European ancestry to 11. We estimate that these susceptibility loci together account for 14.4% of the heritability for atopic dermatitis.

Genome-wide association studies (GWAS) have shown remarkable overlap across immune-mediated diseases[4]. Two European GWAS on atopic dermatitis established four susceptibility loci (*C11orf30*, *OVOL1*, *ACTL9* and *RAD50-IL13-KIF3A*) in addition to *FLG*[5,6]. At *C11orf30*, the same allele also confers risk to asthma[7] and Crohn's disease[8]. For *RAD50-IL13*, locus agonistic effects were observed for asthma[9], and locus antagonistic effects were observed for psoriasis[10]. Two further loci were reported in a Chinese GWAS (*TNFRSF6B-ZGPAT* and *TMEM232-SLC25A46*)[11]. All loci were confirmed in a recent Japanese GWAS, which additionally reported eight new loci (*IL1RL1-IL18R1-IL18RAP*, *MHC*, *OR10A3-NLRP10*, *GLB1*, *CCDC80*, *CARD11*, *ZNF365* and *CYP24A1-PFDN4*)[12]. However, the causal variants at all loci except *FLG* are unknown. To better define susceptibility variants and evaluate loci implicated in other immune-mediated diseases, we genotyped 2,425 German cases with atopic dermatitis and 5,449 German population controls (**Supplementary Table 1a**) using the Immunochip array[13] followed by replication in four independent collections (**Supplementary Table 1b–d**).

After quality control, 128,830 SNPs with a minor allele frequency >1% were available for analysis (Online Methods). The initial comparison of the case-control frequencies yielded 131 and 663 SNPs within 5 and 33 genomic loci with $P_{\text{Immunochip}} < 5 \times 10^{-8}$ and $P_{\text{Immunochip}} < 10^{-4}$, respectively (**Fig. 1**). Of the five atopic dermatitis loci previously reported in European ancestry populations, three reached conservative genome-wide significance (GWS, defined as $P < 5 \times 10^{-8}$; $P_{1q21.3} = 4.51 \times 10^{-20}$, $P_{5q31.1} = 1.99 \times 10^{-14}$ and $P_{11q13.5} = 5.22 \times 10^{-11}$) (**Table 1**). For all three of these loci, we observed stronger association signals as compared to those of previously reported SNPs[5,6] (**Supplementary Table 2**), for which Immunochip data were used to refine the 5q31.1 locus[6]. Variant rs72702813 at 1q21.3 is located 5,275 bases upstream of *LCE3A* (encoding late cornified envelope gene 3A), a member of the *LCE3* group, which contains a psoriasis risk-associated deletion (*LCE3C_LCE3B*-del)[14] and encodes proteins that are involved in barrier repair with differential expression in atopic dermatitis[15].

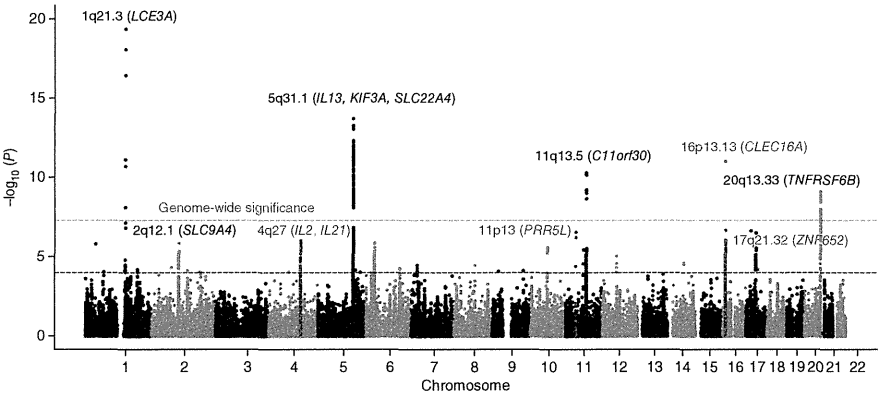

Figure 1 Manhattan plot of the Immunochip association statistics highlighting atopic dermatitis susceptibility loci. The red horizontal line indicates a genome-wide significance threshold of $P = 5 \times 10^{-8}$, and the black horizontal line indicates the threshold for follow-up genotyping of the most strongly associated SNPs ($n = 34$) with $P_{\text{Immunochip}} < 10^{-4}$ from each associated locus in an independent case-control collection (**Supplementary Table 1b**). SNPs within five known and four newly associated loci (depicted in blue) (**Table 1**) reached the GWS threshold for association with atopic dermatitis in the combined analysis of the Immunochip discovery and replication stages (**Supplementary Table 1a–c**).

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Table 1 Susceptibility loci associated with atopic dermatitis in Europeans at GWS

Chr.	Association boundaries (kb)	dbSNP ID	A1	A2	AF_{cases}	$AF_{controls}$	Key genes (*n* additional genes in the locus)	Discovery Immunochip (2,425/5,449)[a]		Replication (1,951/4,599)[a]		Immunochip + replication (4,376/10,048)[a]		Associations to other traits
								P	OR (95% CI)	*P*	OR (95% CI)	$P_{combined}$	OR (95% CI)	
Previously reported atopic dermatitis susceptibility loci[b] meeting genome-wide significance														
1q21.3	150803–151051	rs72702813	T	G	0.08147	0.04056	*LCE3A* (15)	4.51×10^{-20}	1.91 (1.66–2.19)	2.22×10^{-16}	2.46 (1.98–3.04)	1.49×10^{-33}	2.06 (1.83–2.31)	PS
2q12.1	102225–102619	rs759382	C	A	0.2866	0.2454	*SLC9A4* (5)	1.40×10^{-6}	1.21 (1.12–1.30)	9.69×10^{-6}	1.23 (1.12–1.35)	6.01×10^{-11}	1.22 (1.15–1.29)	AS, CD, CeliD, EC, WBCT
5q31.1	131812–132167	rs848	T	G	0.2679	0.2067	*IL13* (8)	1.99×10^{-14}	1.36 (1.25–1.47)	2.33×10^{-15}	1.47 (1.34–1.62)	8.22×10^{-28}	1.40 (1.32–1.49)	AS[c], CD, IgE[c], PS[c], HL[c], PIC, FIB, EC, CRP
11q13.5	75724–76017	rs7110818	A	G	0.4992	0.4392	*C11orf30* (1)	5.22×10^{-11}	1.25 (1.17–1.34)	7.20×10^{-7}	1.35 (1.20–1.53)	3.33×10^{-16}	1.28 (1.21–1.36)	AS, CD, ALRH, IgEgs, UC
20q13.33	61678–61872	rs909341	A	G	0.1809	0.226	*TNFRSF6B* (8)	7.73×10^{-10}	0.76 (0.70–0.83)	1.90×10^{-7}	0.77 (0.70–0.85)	7.77×10^{-16}	0.77 (0.72–0.82)	CD, U[c], polBD, GL
New atopic dermatitis susceptibility loci meeting genome-wide significance														
4q27	123204–123784	rs17389644	A	G	0.2774	0.2396	*IL2-IL21* (2)	1.16×10^{-6}	1.21 (1.12–1.30)	0.0026	1.16 (1.05–1.28)	1.39×10^{-8}	1.19 (1.12–1.26)	RA, CeliD, UC, T1D, T1DA, IgEgs, PSNP, AA
11p13	36355–36438	rs12295535	A	G	0.04045	0.02376	*PRR5L*	2.71×10^{-7}	1.63 (1.35–1.96)	4.32×10^{-7}	1.75 (1.41–2.17)	7.96×10^{-13}	1.68 (1.46–1.93)	–
16p13.13	10930–11218	rs2041733	A	G	0.4903	0.4528	*CLEC16A-DEXI*	1.00×10^{-11}	1.26 (1.18–1.35)	3.07×10^{-5}	1.18 (1.09–1.28)	3.44×10^{-15}	1.23 (1.17–1.29)	MS, T1D, T1DA, PBC, IgA, AA, ALRH
17q21.32	44641–44875	rs16948048	G	A	0.4252	0.3848	*ZNF652* (5)	6.46×10^{-5}	1.15 (1.07–1.23)	8.45×10^{-6}	1.20 (1.11–1.30)	2.92×10^{-9}	1.17 (1.11–1.23)	H

[a]Number of cases/number of controls. [b]We replicated the association at the 20q13.33 (*TNFRSF6B*) and 2q12.1 (*SLC4A1*) loci, previously reported in Chinese and Japanese populations, respectively, with GWS in Europeans. Two other known atopic dermatitis loci in Europeans from previous GWAS (*OVOL1* and *ACTL9*) are sparsely covered on the Immunochip (see the main text and **Supplementary Fig. 1**). [c]A known risk SNP for the listed trait is in high LD ($r^2 > 0.9$) with the atopic dermatitis hit SNP (Online Methods). The *P* values and ORs were calculated with respect to the minor allele. Chr., chromosome of the marker; genomic positions were retrieved from NCBI dbSNP build v130 (genome build hg18); A1, minor allele (corresponds to the risk allele, except for rs909341, which is protective); A2, major allele; AF, allele frequency of A1 estimated from Immunochip and replication; key gene(s), candidate gene(s) in the region; AA, alopecia areata; ALRH, allergic rhinitis; AS, asthma; CD, Crohn's disease; CeliD, celiac disease; CRP, C-reactive protein levels; EC, eosinophil counts; FIB, fibrinogen; GL, glioma; H, height; HL, Hodgkin's lymphoma; IgA, immunoglobulin A; IgE, total IgE levels; IgEgs, IgE grass sensitization; MS, multiple sclerosis; PBC, primary biliary cirrhosis; PIC, platelet counts; polBD, pediatric-onset inflammatory bowel disease; PS, psoriasis; PSNP, progressive supranuclear palsy; RA, rheumatoid arthritis; T1D, type 1 diabetes; T1DA, type 1 diabetes autoantibodies; UC, ulcerative colitis; WBCT, white blood cell types.

Analysis of linkage disequilibrium (LD) patterns showed that rs72702813 does not tag the psoriasis deletion ($D' = 1.0$, $r^2 = 0.09$ with proxy SNP rs4112788 (ref. 14)) but is in moderate LD ($D' = 0.63$, $r^2 = 0.38$) with the known *FLG* mutation c.2282del4 (**Supplementary Table 3**). After conditioning on *FLG* mutations (p.Arg501X, c.2282del4, p.Arg2447X and p.Ser3247X), rs72702813 no longer showed association ($P_{cond} = 0.94$, odds ratio (OR) = 0.99, 95% confidence interval (CI) 0.77–1.28).

Another locus at *CLEC16A* (16p13.13), which was not previously known to be associated with atopic dermatitis, attained GWS ($P_{rs2041733} = 1.00 \times 10^{-11}$, OR = 1.26, 95% CI 1.18–1.35) (**Table 1**). For the two remaining established loci, we observed a significant signal for *OVOL1* (11q13.1) ($P_{rs11820062} = 3.60 \times 10^{-6}$) but not *ACTL9* (19p13.2) ($P_{rs2967682} = 0.18$) (**Supplementary Fig. 1**), which is sparsely covered on the Immunochip ($r^2 = 0.15$ between rs2967682 and the lead SNP rs2164983 from a previous GWAS[6]). Furthermore, we replicated the association at the *TNFRSF6B* (20q13.33) locus ($P_{rs909341} = 7.73 \times 10^{-10}$, OR = 0.76, 95% CI 0.70–0.83), which was previously reported in a Chinese population, with GWS in Europeans. This gene encodes a soluble decoy receptor (DcR3) that acts as an immunomodulator (for example, in support of T helper type 2 (T_H2) cell polarization, which is a hallmark feature of atopic dermatitis)[16]. DcR3 is overexpressed in inflamed epithelia, and increased serum concentrations of this protein have been reported in autoimmune and inflammatory diseases[17,18]. In line with this, we observed a slight overexpression in serum from patients with atopic dermatitis ($P_{Fisher} = 0.00049$; **Supplementary Fig. 2**). However, immunohistochemistry showed strong epidermal staining with no clear differences between atopic dermatitis lesional and healthy skin (**Supplementary Fig. 3**). No proxy SNPs ($r^2 > 0.5$) were available for the 5q22.1 locus identified in the Chinese population (*TMEM232-SLC25A46*). For the recently reported loci in a Japanese population, we observed significant associations at 2q12.1 (*IL1RL1-IL18R1-IL18RAP*, $P_{rs13015714} = 2.81 \times 10^{-5}$, OR = 1.18, 95% CI 1.09–1.27), 6p21.3 (*GPSM3*, $P_{rs176095} = 2.53 \times 10^{-5}$, OR = 0.83, 95% CI 0.76–0.90) and 7p22 (*CARD11*, $P_{rs6978200} = 2.34 \times 10^{-3}$, OR = 1.12, 95% CI 1.04–1.20, $r^2 = 0.58$ with the reported SNP rs4722404). We found no association for 3p21.33 (*GLB1*, $P_{rs35480293} = 0.80$, $r^2 = 0.92$ with the reported SNP rs6780220) or 10q21.2 (*ZNF365*, $P_{rs10995251} = 0.08$). The 3q13.2 (*CCDC80*) and 20q13 (*CYP24A1-PFDN4*) loci have more limited coverage on the Immunochip array.

To identify additional susceptibility loci, we analyzed the most strongly associated SNPs ($n = 34$) with $P_{Immunochip} < 10^{-4}$ after the clumping procedure from each associated locus in an independent set of 794 German cases and 3,338 controls (**Supplementary Table 1b**). We further genotyped SNPs replicated at the 0.05 significance level ($n = 15$; **Supplementary Table 4**) in 1,157 Irish childhood cases and 1,261 controls (**Supplementary Table 1c**). In a meta-analysis ($P_{Immunochip+Repl}$) of the discovery ($P_{Immunochip}$) and replication (P_{Repl}) stages (**Supplementary Table 1a–c**), SNPs within six distinct regions met the GWS threshold (**Table 1**). Again, we observed association at 16p13.13 for SNP rs2041733 in *CLEC16A* ($P_{Immunochip+Repl} = 3.44 \times 10^{-15}$, $OR_{Immunochip+Repl} = 1.23$, 95% CI 1.17–1.29). *CLEC16A* encodes a sugar-binding, C-type lectin expressed on B lymphocytes, natural killer cells and dendritic cells that is functionally active through an immunoreceptor tyrosine-based activation motif (ITAM)[19]. Several SNPs in *CLEC16A* have been associated with immune-mediated diseases such as multiple sclerosis, type 1 diabetes[20,21] and alopecia areata[22], a frequent comorbidity of atopic dermatitis.

We found a significant association at 11p13 for rs12295535 in *PRR5L* ($P_{Immunochip+Repl} = 7.96 \times 10^{-13}$, $OR_{Immunochip+Repl} = 1.68$, 95% CI 1.46–1.93), which encodes a protein that promotes apoptosis[23].

Table 2 Susceptibility loci associated with atopic dermatitis in Japanese and Chinese replication case-control studies

Chr.	Association boundaries (kb)	dbSNP ID	A1	A2	Key genes (*n* additional genes in the locus)	Immunochip + Replication Europeans (4,376/10,048)[a]		Replication Japan (2,397/7,937)[a]		Replication China (2,848/2,944)[a]	
						P	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)
Previously reported atopic dermatitis susceptibility loci[b]											
1q21.3	150803–151051	rs72702813	T	G	*LCE3A* (15)	1.49×10^{-33}	2.06 (1.83–2.31)	–	–	0.3261	1 (0–∞)
2q12.1	102225–102619	rs759382	C	A	*SLC9A4* (5)	6.01×10^{-11}	1.22 (1.15–1.29)	1.36×10^{-9}	1.22 (1.15–1.30)	0.4540	0.97 (0.90–1.05)
5q31.1	131812–132167	rs848	T	G	*IL13* (8)	8.22×10^{-28}	1.40 (1.32–1.49)	5.14×10^{-10}	1.24 (1.16–1.33)	1.28×10^{-6}	1.21 (1.12–1.31)
11q13.5	75724–76017	rs7110818	A	G	*C11orf30* (1)	3.33×10^{-16}	1.28 (1.21–1.36)	6.34×10^{-6}	1.16 (1.09–1.24)	0.0320	1.08 (1.01–1.17)
20q13.33	61678–61872	rs909341	A	G	*TNFRSF6B* (8)	7.77×10^{-16}	0.77 (0.72–0.82)	7.74×10^{-4}	0.83 (0.84–0.95)	1.52×10^{-7}	0.82 (0.76–0.88)
New atopic dermatitis susceptibility loci											
4q27	123204–123784	rs17389644	A	G	*IL2-IL21 (2)*	1.39×10^{-8}	1.19 (1.12–1.26)	0.2492	1.06 (0.96–1.18)	0.1599	1.08 (0.97–1.21)
11p13	36355–36438	rs12295535	A	G	*PRR5L*	7.96×10^{-13}	1.68 (1.46–1.93)	0.0074	1.31 (1.08–1.60)	0.1588	1.13 (0.95–1.34)
16p13.13	10930–11218	rs2041733	A	G	*CLEC16A-DEXI*	3.44×10^{-15}	1.23 (1.17–1.29)	0.0063	1.09 (1.03–1.18)	1.23×10^{-4}	1.18 (1.08–1.28)
17q21.32	44641–44875	rs16948048	G	A	*ZNF652* (5)	2.92×10^{-9}	1.17 (1.11–1.23)	1.87×10^{-5}	1.22 (1.12–1.34)	0.04224	1.10 (1.00–1.20)

[a]Number of cases/number of controls. [b]We replicated the association at the 20q13.33 (*TNFRSF6B*) and 2q12.1 (*SLC4A4*) loci, previously reported in Chinese and Japanese populations, respectively, with GWS in Europeans. Two other known atopic dermatitis loci in Europeans from previous GWAS (*OVOL1* and *ACTL9*) are sparsely covered on the Immunochip (see the main text and **Supplementary Fig. 1**). Chr., chromosome of the marker; genomic positions were retrieved from NCBI dbSNP build v130 (genome build hg18); A1, minor allele; A2, major allele; key gene(s), candidate gene(s) in the region. *P* values and ORs were calculated with respect to the minor allele. rs72702813 failed replication genotyping in the Japanese study due to technical reasons.

At 2q12.1, the associated SNP (rs759382, $P_{\text{Immunochip+Repl}} = 6.01 \times 10^{-11}$, $\text{OR}_{\text{Immunochip+Repl}} = 1.22$, 95% CI 1.15–1.29) maps to a 400-kb LD block encompassing four genes (*IL1RL1*, *IL18R1*, *IL18RAP* and *SLC9A4*). *IL1RL1* encodes a receptor for IL-33, which promotes T_H2 cell responses[24], and the products of *IL18RAP* and *IL18R1* form the receptor for IL-18, which has multiple immunologic functions, including the induction of T_H1 cell responses. Various SNPs in *IL1RL1*, *IL18R1* and *IL18RAP* are associated with asthma and related traits, and the effect has been attributed to *IL1RL1* (refs. 9,25,26). In addition, variants in *IL18R1* and *IL18RAP* have been associated with Crohn's disease[27] and celiac disease[28]. Stepwise conditional regression identified evidence for three independent signals (rs759382 in *SLC9A4*; rs3771180 in *IL1RL1*, which was previously implicated in asthma[29]; and rs10185897 in *IL1RL1-IL18R1*) with $P < 5 \times 10^{-4}$ and showed that the recently reported variant rs13015714 (ref. 12) tags rs759382 (**Supplementary Table 5**).

We found additional significant associations for rs16948048 at 17q21.32 (*ZNF652*, $P_{\text{Immunochip+Repl}} = 2.92 \times 10^{-9}$, $\text{OR}_{\text{Immunochip+Repl}} = 1.17$, 95% CI 1.11–1.23) and rs17389644 at 4q27 (*IL2-IL21*, $P_{\text{Immunochip+Repl}} = 1.39 \times 10^{-8}$, $\text{OR}_{\text{Immunochip+Repl}} = 1.19$, 95% CI 1.12–1.26). *ZNF652* encodes a transcriptional repressor that is implicated in epithelial cancers[30]. IL-2 has pleiotropic immunoregulatory functions, in particular control of the proliferation and survival of regulatory T cells[31]. The *IL2* locus is tightly linked with *IL21*, and variants in *IL2*, its high-affinity receptor *IL2RA* and *IL21* have been associated with multiple immune-mediated diseases. None of the SNPs chosen from the major histocompatibility complex replicated. Regional association plots of the nine atopic dermatitis susceptibility loci with GWS in Europeans are shown in **Supplementary Figure 4**. The four newly associated loci collectively increase the explained heritability from 9% to 14.4% (**Supplementary Table 6**).

To further determine the impact of the new susceptibility loci identified in Europeans on atopic dermatitis risk in diverse populations, we tested them for association in 2,397 adult Japanese cases and 7,937 controls from a recent GWAS[12] and 2,848 adult Chinese cases and 2,944 controls (**Supplementary Table 1d**). In the Japanese study population, all new loci except *IL2-IL21* passed the Bonferroni-corrected significance threshold ($P < 0.05/6 = 0.008$) for replication, and in the Chinese study population, two loci (*CLEC16A* and *TNFRSF6B*) passed this threshold (**Table 2** and **Supplementary Table 4**). Thus, *CLEC16A* and *TNFRSF6B* seem to be relevant to atopic dermatitis in both these European and Asian populations, whereas results for the other loci might reflect phenotypic and ancestry differences between the studies.

Because atopic dermatitis is often coexpressed with asthma, to enhance the interpretation of our findings, we analyzed Immunochip data from an independent set of 733 German cases with asthma[32] and 2,503 controls for atopic dermatitis, asthma, atopic dermatitis without asthma and asthma without atopic dermatitis (Online Methods). We found that all of the newly identified susceptibility loci associated primarily with atopic dermatitis (**Supplementary Table 7**).

For the nine loci associated at GWS (**Table 1**), we identified seven coding SNPs highly correlated ($r^2 > 0.9$ in 1000 Genomes Project European samples) with the lead SNPs (**Supplementary Table 8**). However, these nonsynonymous SNPs are predicted *in silico* to have a nondamaging effect on protein products.

Analysis of whole-blood samples from 740 German control individuals identified evidence for correlation between the expression of *IL1RL1*, *ARAP3*, *MAP3K11* and *STMN3* and SNP alleles in high LD ($r^2 > 0.95$) with the most strongly associated SNPs listed in **Table 1** (**Supplementary Table 9**). Examination of expression levels in skin biopsies from 64 healthy controls run on HU133 Plus 2.0 arrays[33] yielded no evidence for *cis*-regulatory effects (**Supplementary Table 10**). However, a regulatory effect in another physiological state (for example, atopic dermatitis) cannot be ruled out.

We next looked for statistical interactions (allelic-by-allelic epistasis) between lead SNPs of each locus shown in **Table 1** (**Supplementary Table 11**). One SNP pair (rs848 (*IL13*) and rs2041733 (*CLEC16A*)) showed evidence for interaction ($P = 5.41 \times 10^{-4}$) after Bonferroni correction ($P < 0.05/36 = 1.39 \times 10^{-3}$). rs848 is in tight LD with the functional *IL13* variant rs20541 ($r^2 = 0.979$), which affects the activation of the signal transducer and activator of transcription 6 (STAT6) signaling pathway[34]. CLEC16A is thought to act through its ITAM, the ligation of which modulates JAK-STAT signaling[35]. Thus, the observed interactions reflect potential functional links, which need further investigation.

In summary, our dense genotyping approach using the Immunochip array identified four new atopic dermatitis risk loci in Europeans (**Table 1**), adding 5.4% to the estimate of explained atopic dermatitis heritability and bringing the total to 14.4% heritability explained by currently reported susceptibility loci. Our results expand the catalog of genetic loci implicated in atopic dermatitis and provide evidence for a substantial contribution of loci shared with other immune-mediated diseases.

URL. PopGen biobank, http://www.popgen.de.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.W., A.F., Y.-A.L. and D.E. designed the experiments. E.R., J.E.-G., A.M., I.M., N.H., H.S., U.M.-H., M.H., M. Kubo, W.H.I.M. and N.N. performed wet lab experiments. D.E., H.B. and S.M. analyzed the data. S.W., Y.-A.L., N.N., L.M., R.F.-H. and T.W. provided German case samples. H.S. helped providing case samples. M.T., A.T., Y.N., T.H. and M. Kubo provided Japanese replication data. A.D.I., S. Brown, M.A.M. and C.M.F. provided Irish replication data. L.S., X. Zuo, S.Y. and X. Zhang provided Chinese replication data. B.O.B. provided German control samples from the Echinococcus Multilocularis and Internal Diseases in Leutkirch (EMIL) study. P.H. and M.M.N. provided German control samples. S. Brand, J.G. and C.B. provided German control samples, which were genotyped at the University of Pittsburgh Genomics and Proteomics Core Laboratories (R.H.D., principal investigator). J.W. and T.I. provided German control samples. M. Kabesch provided Immunochip data from the cases with asthma from the Multicenter Asthma Genetics in Childhood (MAGIC) and International Study of Asthma and Allergies in Childhood (ISAAC) studies. H.P., K.H., T.I., C.H., L.C.T., P.S. and J.T.E. contributed and analyzed expression data. S.W., A.F., S.S., N.H. and Y.-A.L. supervised the experiments. D.E., H.B., S.W. and A.F. wrote the paper. All authors reviewed, edited and approved the final manuscript.

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The authors declare no competing financial interests.

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ONLINE METHODS

Study subjects.

Immunochip data. All German cases with atopic dermatitis (**Supplementary Table 1a**) were recruited from tertiary dermatology and pediatrics clinics based at four centers (Technische Universität Munich, as part of the Gene-Environment Association (GENEVA) study, the University of Kiel, the University of Bonn and University Children's Hospital, Charité Universitätsmedizin Berlin, as part of the Genetic Studies in Nuclear Families with Atopic Dermatitis (GENUFAD) study). Atopic dermatitis was diagnosed on the basis of a skin examination performed by experienced dermatologists and pediatricians according to standard criteria, which included the presence of chronic or chronically relapsing pruritic dermatitis with the typical morphology and distribution[36]. A total of 2,461 German controls were obtained from the PopGen biorepository[37]. A total of 1,545 cases with atopic dermatitis and all 2,461 controls from PopGen were genotyped at the Institute of Clinical Molecular Biology, Christian-Albrechts-University of Kiel, and 880 cases with atopic dermatitis were genotyped at the Max-Delbrück-Centrum (MDC) for Molecular Medicine (Berlin-Buch, Germany). Nine-hundred seventy-nine German controls were selected as part of an independent population-based sample from the general population living in the region of Augsburg (Cooperative Heath Research in the Region of Augsburg (KORA)), southern Germany[38], and were genotyped at the Helmholtz Center in Munich. Three hundred and two control individuals were of south German ancestry and were part of the control population from Munich recruited from the Bavarian Red Cross; 208 control individuals were recruited from the Charité-Universitätsmedizin Berlin. These samples were genotyped at the University of Pittsburgh Genomics and Proteomics Core Laboratories (R.H.D., principal investigator). The Bonn controls (n = 1,499) were recruited from the population-based epidemiological Heinz Nixdorf Recall study[39] and genotyped at the Life and Brain Center at the University Clinic in Bonn. Of the cases with atopic dermatitis used for the screen with available phenotype information, 33.1% and 31.0% (**Supplementary Table 1a**) suffered from comorbid asthma.

For *in silico* analysis of the selected SNPs for asthma, Immunochip data from 733 German cases with asthma from the MAGICS and German ISAAC studies[9], as well as 2,503 controls from PopGen, were used.

Replication data. For follow-up genotyping (**Supplementary Table 1b**), we used 794 cases recruited at tertiary dermatology clinics in Munich, Bonn, Kiel and Hannover (Technische Universität Munich, as part of the GENEVA study, the University of Bonn, the University of Kiel and Medizinische Hochschule of Hannover). A total of 2,412 German control individuals were selected as part of the EMIL study, an independent population-based sample from the general population living in Leukirch, southern Germany[40]. A total of 926 German control individuals were selected as part of an independent population-based sample from the general population living in the region of Augsburg (KORA), southern Germany[38], and were genotyped at the Helmholtz Center in Munich. The Irish case-control collection consisted of 1,157 unrelated children of self-reported Irish ancestry with moderate-to-severe atopic dermatitis recruited from the tertiary referral pediatric dermatology clinic based at Our Lady's Children's Hospital, Dublin (**Supplementary Table 1c**). A total of 1,261 unselected control samples were obtained from the population-based Trinity Biobank Control samples.

For further replication, a total of 2,397 Japanese cases and 7,937 Japanese controls were analyzed (**Supplementary Table 1d**). Cases were recruited from several medical institutes, including Fukujuji Hospital, Iizuka Hospital, Juntendo University, Hospital Iwate Medical University School of Medicine, National Hospital Organization Osaka National Hospital, Nihon University, Nippon Medical School, Shiga University of Medical Science, Cancer Institute Hospital of the Japanese Foundation for Cancer Research, Tokushukai Hospital and Tokyo Metropolitan Geriatric Hospital[41]. Controls included 6,018 cases with one of five diseases (cerebral aneurysm, esophageal cancer, endometrial cancer, chronic obstructive pulmonary disease or glaucoma) who did not have atopic dermatitis or bronchial asthma in BioBank Japan, 1,018 healthy volunteers from members of the Rotary Club of Osaka-Midosuji District 2660 Rotary International in Japan and 901 healthy subjects from the PharmaSNP Consortium. A total of 2,848 Chinese Han case samples with atopic dermatitis and 2,944 Chinese Han control samples (**Supplementary Table 1d**) were provided by S.Y. and X. Zhang.

Written, informed consent was obtained from all study participants, and the institutional ethical review committees of the participating centers approved all protocols.

Immunochip genotyping. DNA samples were genotyped using the Immunochip, which is an Illumina iSelect HD custom genotyping array. The Immunochip is a BeadChip developed for highly multiplexed SNP genotyping of complex DNA. Data were analyzed using Illumina's GenomeStudio Genotyping Module. The NCBI build 36 (hg18) map was used (Illumina manifest file Immuno_BeadChip_11419691_B.bpm), and normalized probe intensities were extracted for all samples that passed standard laboratory quality-control thresholds.

Immunochip genotype calling and quality control. Genotype calling was performed with the GenomeStudio GenTrain 2.0 algorithm (Illumina's GenomeStudio data analysis software) and the custom generated cluster file of Trynka *et al.* (based on an initial clustering of 2,000 UK samples and subsequent manual readjustment of cluster positions)[13].

SNPs that had >5% missing data, a minor allele frequency <1% or deviated from Hardy-Weinberg equilibrium (exact $P < 10^{-4}$ in controls) per sample study were excluded using PLINK software version 1.07 (ref. 42). Sample quality control measures included sample call rate, overall heterozygosity, relatedness testing and other metrics (**Supplementary Figs. 5–7**). The remaining 2,425 cases with atopic dermatitis and 5,449 controls were tested for population stratification using the principal components stratification method as implemented in EIGENSTRAT[43]. Principal component analysis revealed no population stratification in the remaining samples; no population outliers were detected. A total of 128,830 polymorphic SNPs were available for analysis. A quantile-quantile plot of the full association analysis showed a marked excess of significant associations in the tail of the distribution (**Supplementary Fig. 8a**), which was due primarily to hundreds of highly significant association signals from a few associated (fine-mapped) regions. A quantile-quantile plot using 2,714 'null' SNPs not associated with autoimmune disease (bipolar disease–associated SNPs)[13] is shown as negative control, and the inflation factor inferred from this showed only modest inflation (λ = 1.01; **Supplementary Fig. 8b**).

Replication genotyping. For replication genotyping, we selected the most strongly associated SNP (n = 34) with $P < 10^{-4}$ from each associated locus by means of PLINK's clumping procedure (using default settings: $P_1 < 0.0001$, $P_2 < 0.01$, $r^2 \geq 0.5$, kb = 250) representing 23 loci (see also **Supplementary Table 4**). Follow-up replication genotyping in the German study population was carried out using our Sequenom iPlex platform from Sequenom and TaqMan technology from Applied Biosystems. Replication typing in the Irish case-control collection was done using TaqMan technology from Applied Biosystems. Quality control was done for each country population separately. Individuals with >8% missing data were removed. SNPs that had >3% missing data or deviated from Hardy-Weinberg equilibrium (exact $P < 0.01$ in controls) per sample population were excluded. P values for allele-based tests of phenotypic association for each single replication population were calculated using R 2.14.2 (ref. 44). PLINK's meta-analysis function was used to obtain P values for the replication data set (P_{Repl}) (**Supplementary Table 1b,c**) and the combined discovery-replication data set ($P_{Immunochip+Repl}$) (**Supplementary Table 1a–c**). We used the commonly accepted threshold of $P = 5 \times 10^{-8}$ for joint P values to define statistical significance.

The Japanese replication set was typed using multiplex PCR–based Invader assay (Third Wave Technologies). Genotyping in the Chinese replication cohort was carried out using Sequenom technology.

Annotation of association boundaries. LD regions (association boundaries) around focal SNPs were defined by extending in both directions a distance of 0.1 centimorgans (cM) or until another SNP with $P < 10^{-5}$ was reached, in which case the process was repeated from this SNP. For each locus, candidate genes within regions are listed in columns labeled 'key gene(s)' in **Tables 1** and **2** and are listed in more detail in **Supplementary Table 4**.

Annotation of associations to other phenotypes. Overlaps with other phenotypes were annotated with the National Human Genome Research Institute

(NHGRI) GWAS catalog[45] (www.genome.gov/gwastudies, accessed December 19, 2012). All known associations with $P < 5 \times 10^{-8}$ to any disease or primary phenotype were included. For each atopic dermatitis susceptibility locus with association boundaries defined in **Table 1**, we annotated all phenotypes that had at least one associated SNP within the region. We also checked whether the hit SNP in the NHGRI GWAS catalog was the same as, or in high LD with ($r^2 > 0.9$), the atopic dermatitis hit SNP.

Stepwise conditional logistic regression and joint analysis. Multiple associated SNPs were selected through a stepwise selection procedure using GCTA (Genome-wide Complex Trait Analysis)[46] using SNP markers at 2q12.1 and a threshold P value of 5×10^{-4} to declare evidence for independently associated SNPs (–massoc-p 5e-4) (**Supplementary Table 5a**).

Expression quantitative trait loci look up. We analyzed gene expression data measured previously in whole blood (fasting conditions) and skin specimens. For analysis of whole blood, we used Illumina Human HT-12 v3 Expression BeadChip data from 740 adult individuals of the German population-based KORA (Cooperative Heath Research in the Region of Augsburg) F4 study performed in 2006–2008 (ref. 47) (**Supplementary Table 9**). For analysis of skin, we used Affymetrix HU133 Plus 2.0 arrays data from 57 healthy individuals[33] (**Supplementary Table 10**).

Statistical interaction analysis. To look for interactions between associated loci, we considered all distinct pairs ($n = 36$) of the nine lead SNPs listed in **Table 1** (see **Supplementary Table 11**).

Immunohistochemistry. Immunohistochemical staining of paraffin-embedded tissue of eight biopsies taken from lesions of patients with atopic dermatitis compared to eight healthy sex- and age-matched control persons was done by using monoclonal mouse anti-DcR3 (see **Supplementary Fig. 3**).

Serum measurements. Analysis of DcR3 serum concentrations was done using the DuoSet ELISA development system from R&D Systems (Wiesbaden, Germany) according to the manufacturer's instructions.

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