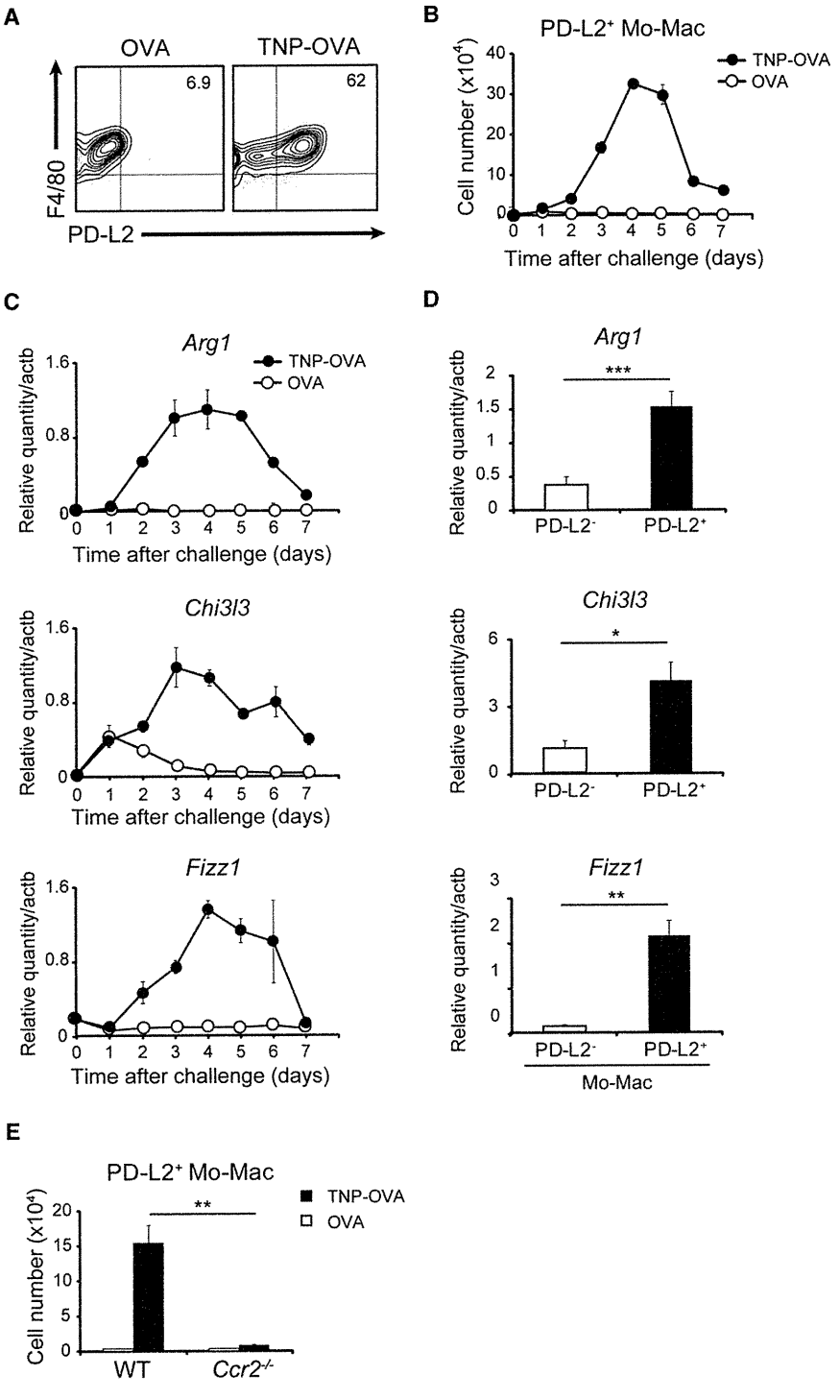

Figure 4. Monocytes-Macrophages Accumulating in the IgE-CAI Skin Lesions Display a Phenotype of M2-type Macrophages

(A–D) C57BL/6 mice were treated as in Figure 1 to induce IgE-CAI.

(A) The expression of PD-L2 on F4/80$^+$CD11b$^+$ SSC^{lo} monocytes-macrophages in the skin lesions of mice challenged with TNP-OVA or control OVA was examined on day 4 post-challenge.

(B and C) Time course of the PD-L2$^+$ monocytes-macrophage number (B, mean ± SEM, n = 3 each) and the expression of indicated mRNAs (C, mean ± SEM, n = 3 each) in the skin lesions of mice challenged with TNP-OVA (closed circles) or control OVA (open circles).

(D) The expression of indicated mRNAs in PD-L2$^-$ and PD-L2$^+$ monocytes-macrophages that were isolated on day 3 postchallenge from the ear skin of mice challenged with TNP-OVA (mean ± SEM, n = 6 each).

(E) Wild-type and *Ccr2*$^{-/-}$ BALB/c mice were treated as in Figure 3 to induce IgE-CAI. Data show the numbers of PD-L2$^+$ monocytes-macrophages that were isolated on day 4 postchallenge from the ear skin of mice challenged with TNP-OVA or control OVA (mean ± SEM, n = 4–5 each). Data shown are representative of at least three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figures S1–S5.

of inflammatory monocytes in *Ccr2*$^{-/-}$ mice resulted in the exacerbation rather than alleviation of allergic inflammation, and adoptive transfer of CCR2$^+$ inflammatory monocytes normalized it. Thus, M2-like monocytes-macrophages derived from inflammatory monocytes appear to negatively regulate allergic inflammation in IgE-CAI.

Previous studies have shown two distinct modes of M2 generation. One is based on the observation during infection with *Listeria monocytogenes*. Ly6C$^{lo/-}$ resident monocytes recruited to the infection site turn on the expression of typical M2 marker genes, including *Arg1* and *Fizz1*, indicating their differentiation into M2 macrophages that probably contribute to tissue repair (Auffray et al., 2007). In contrast, Ly6C$^+$ inflammatory monocytes differentiate into dendritic cells that produce inflammatory mediators (Kurihara et al., 1997; Serbina et al., 2003). The other mode of M2 generation has been demonstrated in infection with helminth *Litomosoides sigmodontis*, in that M2 macrophages are generated through proliferation and alternative activation of tissue-resident macrophages rather than the recruitment of circulating monocytes (Jenkins et al., 2011). Our study on IgE-CAI has identified the third mode of M2 generation, in that Ly6C$^+$ inflammatory monocytes give rise to M2-type macrophages. The differentiation of inflammatory monocytes to M2 macrophages may not be restricted to allergic responses. A similar differentiation was suggested in experimental autoimmune encephalomyelitis, even though no direct evidence for this in vivo was provided (Denney et al., 2012).

The in vivo function of M2 macrophages has been less well characterized, compared to that of M1 macrophages, but has

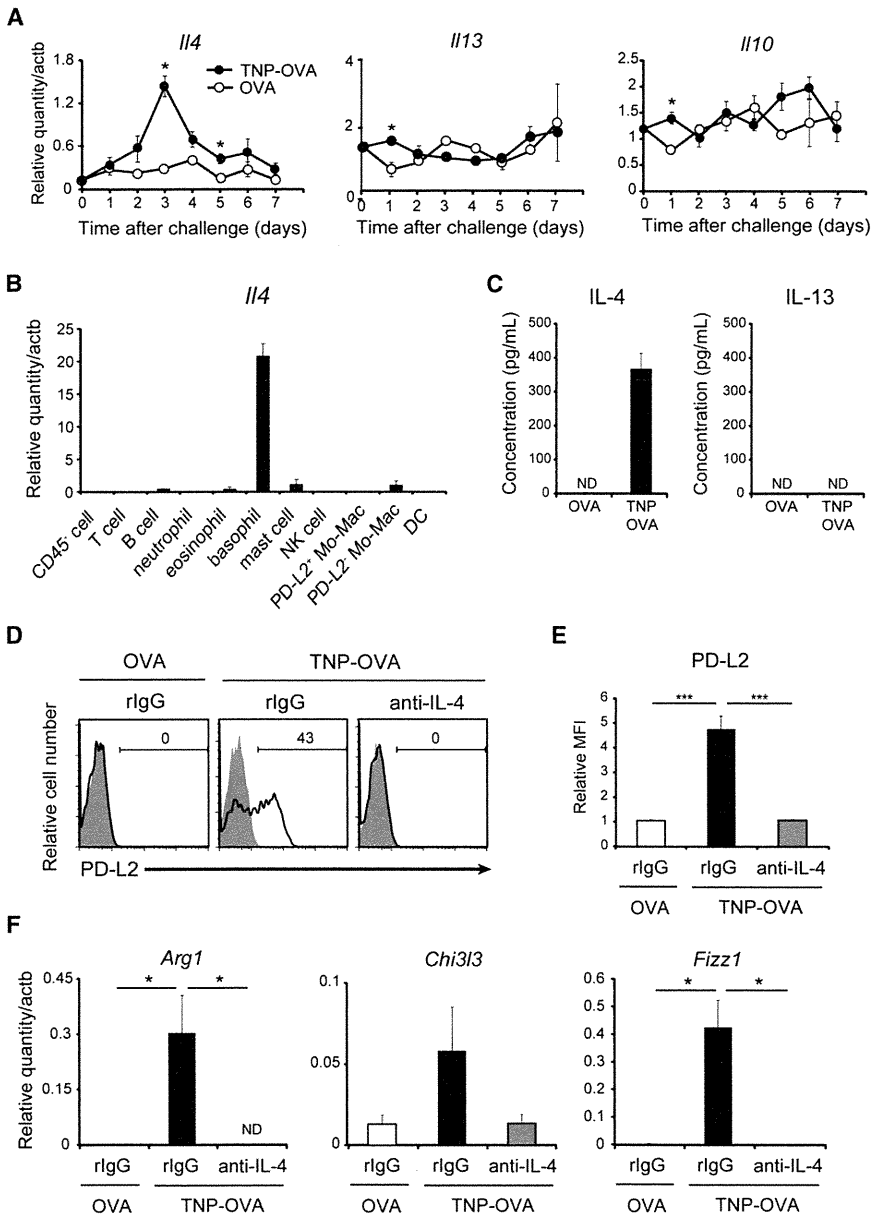

Figure 5. Basophil-Derived IL-4 Confers an M2-type Phenotype on Ly6C$^+$ Inflammatory Monocytes Ex Vivo

(A and B) C57BL/6 mice were treated as in Figure 1 to induce IgE-CAI. Time course of the expression of indicated mRNAs in the skin lesions of mice challenged with TNP-OVA (closed circles) or control OVA (open circles) is shown in (A) (mean ± SEM, n = 3 each). *p < 0.05. In (B), the indicated cell lineages were isolated on day 3 postchallenge from the skin lesions of mice challenged with TNP-OVA and subjected to quantitative RT-PCR for the analysis of *Il4* expression (mean ± SEM, n = 3 each).

(C) Basophils (2 × 10^5 cells/ml) enriched from bone marrow cells were sensitized ex vivo with anti-TNP IgE and then stimulated with TNP-OVA or control OVA at 37°C for 10 hr, and the concentration of IL-4 and IL-13 in their culture supernatants was determined by ELISA (mean ± SEM, n = 5 each). ND, not detectable.

(D–F) Ly6C$^+$ inflammatory monocytes were purified from C57BL/6 bone marrow cells and incubated at 37°C for 24 hr in the presence of anti-IL-4 or control rat IgG (rIgG), with the culture supernatants of basophils that had been stimulated as in (C).

(D and E) The cultured monocytes were subjected to flow cytometric analysis for PD-L2 expression. Representative staining profiles are shown in (D). Shaded histograms show control staining with isotype-matched antibody. All the data are summarized in (E) (mean ± SEM, n = 5–7 each), in that the relative mean fluorescence intensity (MFI) was calculated as MFI (PD-L2 staining)/MFI (control staining).

(F) The cultured monocytes were subjected to quantitative RT-PCR analysis for expression of indicated mRNAs (mean ± SEM, n = 5 each).

Data shown are representative of at least three independent experiments. *p < 0.05, ***p < 0.001.

been implicated in a variety of processes, including protection against parasitic infection, promoting Th2 cell-type immune responses, wound healing, tissue fibrosis, metabolic regulation, angiogenesis, and tumorigenesis (Kreider et al., 2007; Martinez et al., 2009; Murray and Wynn, 2011). The role of M2 macrophages in allergy and asthma has not been well understood, in contrast to the extensive study on its role in parasitic infections. Of note, in mice infected with helminth *Schistosoma mansoni*, M2 macrophages and their products have been shown to suppress rather than promote Th2 cell-type inflammation (Nair et al., 2009; Pesce et al., 2009). No such anti-inflammatory property of M2 macrophages was definitely demonstrated in allergic responses, as far as we aware. Instead, in mouse models of airway allergic inflammation, M2 macrophages reportedly contribute to the pathogenesis of disease, including promotion of inflammation (Ford et al., 2012; Kim et al., 2008; Kurowska-Stolarska et al., 2009; Melgert et al., 2010; Moreira et al., 2010; Nagarkar et al., 2010) and angiogenesis (Sun et al., 2008). Intriguingly, *Ccr2*$^{-/-}$ mice were reported to display enhanced airway allergic inflammation, but the underlying mechanism remains to be determined (Kim et al., 2001).

In the present study, we clearly demonstrated that M2-like monocytes-macrophages derived from inflammatory monocytes exert an anti-inflammatory function in IgE-CAI. Their absence or the failure in their conversion to M2 type resulted in the exacerbation of allergic inflammation. Thus, M2-like monocytes-macrophages appear to dampen excessive inflammation in IgE-CAI. It remains to be definitely demonstrated how they exert an anti-inflammatory function. Treatment of mice with an inhibitor of arginase-1 showed no apparent effect on IgE-CAI (data not show), although arginase-1 has been shown to suppress Th2 cell-type inflammation in helminth

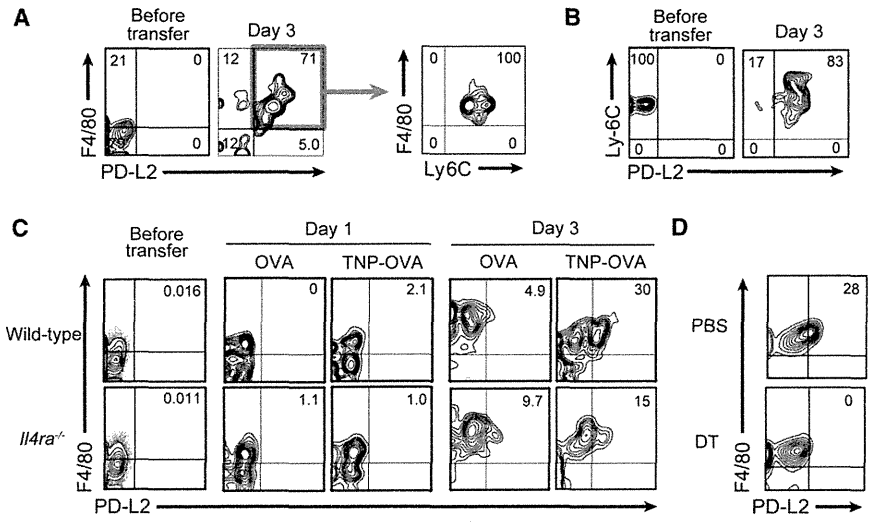

Figure 6. CD115$^+$ Monocytes Acquire PD-L2 Expression after Their Infiltration into the Skin Lesions, in a Manner Dependent on IL-4R and Basophils

(A and B) CD115$^+$ bone marrow cells isolated from BALB/c mice (A) or Ly6C$^+$Ly6G$^-$ inflammatory monocytes (purity > 99%) sorted from CD115$^+$ bone marrow cells (B) were labeled with CFSE and intravenously transferred (2 × 10^6 cells/mouse) into BALB/c mice that had been sensitized with anti-TNP IgE 1 day earlier. Recipient mice were challenged with intradermal administration of TNP-OVA immediately after the cell transfer. Flow cytometric analysis was performed for the surface expression of F4/80, PD-L2, and Ly6C in CFSE-labeled cells before the transfer and in those isolated from the TNP-OVA-injected skin on day 3 posttransfer.

(C) CD115$^+$ bone marrow cells were prepared from wild-type or *Il4ra*$^{-/-}$ mice, labeled with CFSE, and intravenously transferred (3 × 10^6 cells/mouse) into IgE-sensitized BALB/c mice, followed by the antigen challenge (TNP-OVA or control OVA) as in (A) and (B). Flow cytometric analysis was performed for the surface expression of F4/80 and PD-L2 in CFSE-labeled cells before the transfer (left) and in those isolated from the ear skin on day 1 and day 3 posttransfer (middle and right, respectively).

(D) *Mcpt8*^{DTR} C57BL/6 mice were sensitized with anti-TNP IgE and challenged with TNP-OVA as in Figure 1A to induce IgE-CAI. On day 2 postchallenge, the mice were treated with intravenous injection of CFSE-labeled CD115$^+$ bone marrow cells (1 × 10^7 cells/mouse) derived from wild-type mice, in conjunction with DT or control PBS. On day 4 postchallenge, the expression of F4/80 and PD-L2 on CFSE-labeled cells isolated from the ear skin was examined.

Data shown are representative of three independent experiments.

infection (Pesce et al., 2009). Notably, M2-like monocytes-macrophages infiltrating skin lesions of IgE-CAI express PD-L2, a ligand for the inhibitory receptor PD-1 (Loke and Allison, 2003). Blockade of PD-L2 with a specific antibody enhanced a Th2 cell-type response in helminth infection (Huber et al., 2010). However, our preliminary experiments with PD-L2 antibody suggested no apparent contribution of PD-L2 to the damping of allergic inflammation in IgE-CAI. In skeletal muscle injury, Ly6C$^+$ inflammatory monocytes are recruited and converted into "anti-inflammatory" macrophages that express IL-10 and transforming growth factor-β (TGF-β) (Arnold et al., 2007). In IgE-CAI, however, neither IL-10 nor TGF-β seems to be involved in damping inflammation in IgE-CAI (not all data shown). Of note, PD-L2$^+$ monocytes-macrophages accumulating in the IgE-CAI skin lesions expressed high amounts of a mannose receptor CD206 (Figure S6A), suggesting that they might have enhanced endocytic activity (Montaner et al., 1999). Indeed, PD-L2$^+$ monocytes-macrophages in the skin lesions showed a much higher extent of antigen uptake compared to PD-L2$^-$ monocytes-macrophages or other cell lineages including eosinophils and neutrophils (Figure S6B). This suggests that the failure in the generation of M2-like monocytes-macrophages may lead to the insufficient clearance of antigens in the skin lesions. Considering the fact that the extent and duration of the IgE-CAI reaction correlate well with the dose of antigens (Sato et al., 2003), the anti-inflammatory property of M2-like monocytes-macrophages could be attributed, at least in part, to their efficient uptake and clearance of antigens, making antigens unavailable for basophil activation.

Alternatively activated M2-type macrophages are typically generated by stimulation with the Th2 cell cytokines, IL-4 and IL-13, that can be produced by Th2 cells, natural killer T (NKT) cells, mast cells, eosinophils, basophils, and innate-type lymphoid cells (Paul and Zhu, 2010). Memory Th2 cells are the major source of Th2 cell cytokines for M2 generation in helminth infection (Anthony et al., 2006), whereas NKT cell-derived IL-4 is important for M2 generation in experimental autoimmune encephalomyelitis (Denney et al., 2012). In adipose tissues, eosinophil-derived IL-4 and IL-13 are crucial for M2 generation to maintain glucose homeostasis (Wu et al., 2011). In the present study, we demonstrated that basophil-derived IL-4 can act on inflammatory monocytes and convert them to anti-inflammatory M2-like monocytes-macrophages. Thus, basophils can contribute to the activation and differentiation of monocytes and macrophages, in addition to those of T and B cells as reported previously (Perrigoue et al., 2009; Sokol et al., 2008, 2009; Yoshimoto et al., 2009; Chen et al., 2009; Denzel et al., 2008). Of note, when stimulated ex vivo with IL-4, human CD14$^+$ monocytes display a phenotype characteristic for human M2-type macrophages, including upregulated expression of PD-L2 on their surface (Semnani et al., 2011). Given the fact that human basophils produce large quantities of Th2 cell cytokines as do murine basophils (Piccinni et al., 1991), it is plausible that basophils contribute to the generation of M2-type monocytes-macrophages in humans as observed in mice.

In conclusion, the present study demonstrated a previously unappreciated mode of monocyte-to-macrophage transition, that is, a conversion from inflammatory monocytes to anti-inflammatory M2-type monocytes-macrophages in an allergic response. In repeated infections with parasites, host animals often raise IgE against parasite antigens, and hence basophils can be stimulated with IgE plus antigens as seen in IgE-CAI (Karasuyama et al., 2011b; Voehringer, 2009). Moreover, basophils can be directly activated in an IgE-independent manner,

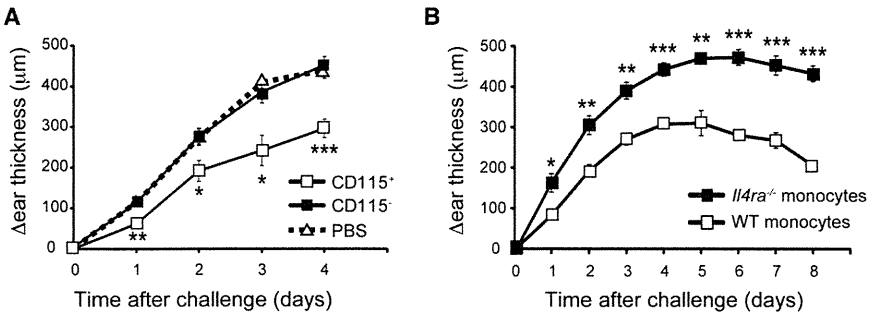

Figure 7. Adoptive Transfer of CCR2$^+$Ly6C$^+$ Inflammatory Monocytes from Wild-Type but Not *Il4ra*$^{-/-}$ Mice Normalizes the Exacerbated IgE-CAI in *Ccr2*$^{-/-}$ Mice

Ccr2$^{-/-}$ mice were treated as in Figure 3A to induce IgE-CAI.

(A) CD115$^+$ (open squares) or CD115$^-$ (closed squares) bone marrow cells from BALB/c mice or control PBS (open triangles) were intravenously administered to the mice four times (1 × 10^6 cells/injection/mouse), on days 0, 1, 2, and 3 post-challenge. Time course of ear swelling (Δear thickness) is shown (mean ± SEM, n = 3–5 each).

(B) Ly6C$^+$Ly6G$^-$ inflammatory monocytes (purity > 99%) sorted from bone marrow cells of wild-type (open squares) or *Il4ra*$^{-/-}$ (closed squares) mice were intradermally administered once (1 × 10^6 cells/site) in the ear of *Ccr2*$^{-/-}$ mice, in conjunction with administration of TNP-OVA or control OVA. Time course of ear swelling (Δear thickness) is shown (mean ± SEM, n = 4 each).

Data shown are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. Note that error bars are displayed in all figures, but often are hidden behind symbols. See also Figures S6.

for example with certain proteases and pathogen products (Schroeder et al., 2001). Therefore, basophil-elicited M2 generation might be widely observed in various settings. Further studies will clarify their functional significance in each setting.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 and BALB/c mice were purchased from CLEA Japan. *Mcpt8*^{DTR} C57BL/6 (Wada et al., 2010), *Ccr2*$^{-/-}$ (Kuziel et al., 1997), and *Il4ra*$^{-/-}$ BALB/c (Noben-Trauth et al., 1997, 1999) mice were as described previously and maintained under specific-pathogen-free conditions in our animal facilities. All animal studies were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Induction of IgE-CAI

IgE-CAI was elicited as described previously (Mukai et al., 2005). In brief, mice were sensitized with intravenous injection of 300 μg of anti-TNP IgE, and on the following day challenged with an intradermal injection of 10 μg TNP_{12}-conjugated ovalbumin (OVA) and control OVA into the right and left ear, respectively. The value of Δear thickness, the differences in ear thickness (right − left) was calculated for the evaluation of inflammation.

Isolation of Bone Marrow Basophils and Monocytes

Basophils and monocytes were enriched from bone marrow cells via IMag system with biotinylated anti-CD49b and anti-CD115, respectively, followed by streptavidin-conjugated magnetic particles (BD PharMingen). Inflammatory monocytes were purified by sorting Ly6C^{hi}Siglec-F$^-$CD11c$^-$Ly6G$^-$ cells from the CD115$^+$ bone marrow cell population with FACSAria (BD Biosciences).

In Vitro Stimulation of Basophils and Monocytes

Basophils were stimulated for 10 hr with TNP_{12}-OVA or control OVA (300 ng/ml) after sensitization with TNP-specific IgE. The concentration of cytokines in culture supernatants was determined with Mouse IL-4 ELISA MAX Standard (Biolegend) for IL-4 and mouse Ready-Set-Go! ELISA kit (eBioscience) for IL-13. Monocytes were incubated for 24 hr with the culture supernatant of activated basophils in the presence or absence of anti-IL-4 or control rat IgG (20 μg/ml).

Ablation of Basophils

Mcpt8^{DTR} mice were treated once or twice with intravenous injection of diphtheria toxin (DT, Sigma-Aldrich, 500 ng/injection).

Statistical Analysis

Statistical analysis was performed with unpaired Student's t test. A p value <0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.11.014.

ACKNOWLEDGMENTS

We thank W.A. Kuziel (External Scientific Affairs, Daiichi Sankyo Group, Edison, NJ) for providing *Ccr2*$^{-/-}$ mice, D. Yamanaka and R. Matsunaga for technical support, and M. Miki for secretary assistance. This work was supported by a research grant from JST, CREST (to H.K.).

Received: August 4, 2012
Accepted: November 19, 2012
Published: February 21, 2013

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PEDIATRICS INTERNATIONAL Official Journal of the Japan Pediatric Society

Pediatrics International (2013) **55**, 671–674 doi: 10.1111/ped.12224

The 116th Japan Pediatric Society Scientific Research Award Winner

Primary immunodeficiency in Japan; epidemiology, diagnosis, and pathogenesis

Hidetoshi Takada

Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

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

Correspondence: Hidetoshi Takada, MD PhD, Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Email: takadah@pediatr.med.kyushu-u.ac.jp

Received 13 August 2013; revised 30 August 2013; accepted 10 September 2013.

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,

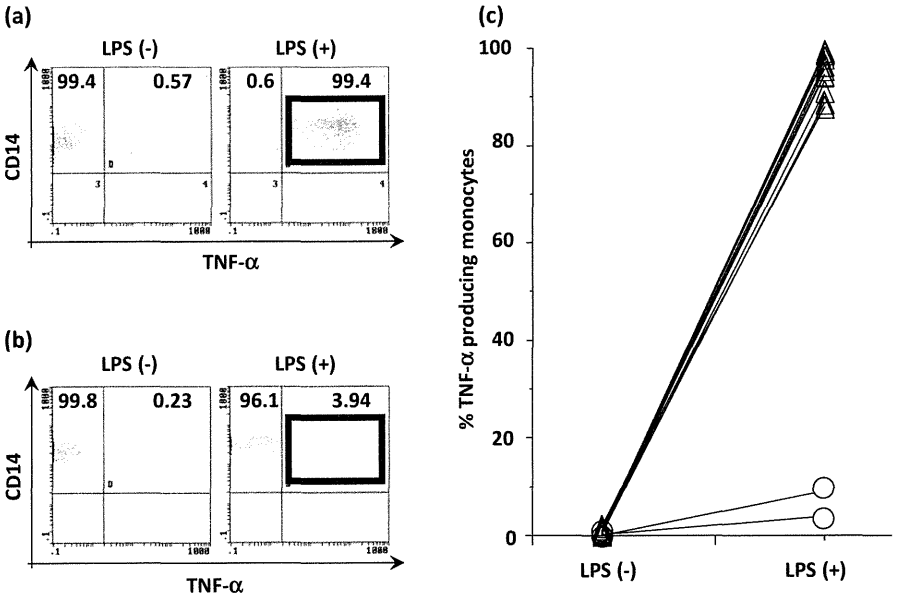

Fig. 1 (a,b) Intracellular staining for tumor necrosis factor (TNF)-α and its expression in monocytes with and without lipopolysaccharide (LPS) stimulation in (a) healthy controls and (b) interleukin-1 receptor-associated kinase 4 (IRAK4)-deficient patients. (c) Percentage of TNF-α producing cells in 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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Arthritis & Rheumatism
DOI 10.1002/art.38324

Full-length articles

Running head: ARID5A inhibits Th17 cell differentiation

Title: AT-rich interactive domain-containing protein 5a functions as a negative regulator of RORγt-induced Th17 cell differentiation

Yukari Saito, MSc*[1], Shin-ichiro Kagami, MD. PhD*[1,2], Yoshie Sanayama, MD[1], Kei Ikeda, MD. PhD[1], Akira Suto, MD. PhD[1], Daisuke Kashiwakuma, MD. PhD[2], Shunsuke Furuta, MD. PhD[1,2], Itsuo Iwamoto, MD. PhD[2], Ken Nonaka, PhD[3], Osamu Ohara, PhD[3], and Hiroshi Nakajima, MD. PhD[1]

[1]Department of Allergy and Clinical Immunology, Graduate School of Medicine, Chiba University, [2]Research Center for Allergy and Clinical Immunology, Asahi General Hospital, [3]Department of Human Genome Research, Kazusa DNA Research Institute, Chiba, Japan

*These authors equally contributed to this work.

Address correspondence to Dr. Hiroshi Nakajima (nakajimh@faculty.chiba-u.jp) or Dr. Shin-ichiro Kagami (kagami@hospital.asahi.chiba.jp), Department of Allergy and Clinical Immunology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chiba City, Chiba 260-8670, Japan. Tel: 81-43-226-2198; Fax: 81-43-226-2199.

Key words: ARID5A, RORγt, Th17, rheumatoid arthritis

The authors have no conflicting financial interests.

This work was supported in part by Grants-in-Aids for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government, Global COE Program (Global Center for Education and Research in Immune System Regulation and Treatment), MEXT, and Health and Labor Sciences Research Grant, Japan.

Received: Jun 11, 2013; Revised: Nov 14, 2013; Accepted: Dec 12, 2013

ABSTRACT

Objective: Pro-inflammatory cytokines TNF-α and IL-6 and a Th17 cytokine IL-17A are implicated in the pathogenesis of rheumatoid arthritis (RA), and the blockade of these cytokines by biologic agents provides clinical benefits for RA patients. This study aimed to clarify the mechanisms underlying the efficacy of IL-6 blockade for RA and to find a novel therapeutic target for RA.

Methods: We examined gene expression profiles of $CD4^+$ T cells by DNA microarray analysis before and after the treatment with an anti-IL-6 receptor antibody, Tocilizumab (TCZ), in RA patients who exhibited good clinical responses to the treatment. We then examined the roles of a newly identified molecule whose expression was significantly reduced in $CD4^+$ T cells by TCZ therapy in helper T cell differentiation by using murine $CD4^+$ T cells. We also examined the effect of the enforced expression of the molecule on RORγt-induced IL-17A production in $CD4^+$ T cells and on RORγt-induced IL-17A promoter activation.

Results: We identified AT-rich interactive domain-containing protein 5a (ARID5A) as a new molecule downregulated by IL-6 blockade by TCZ therapy. IL-6 induced the expression of ARID5A in $CD4^+$ T cells during Th17 cell differentiation by a Stat3-dependent mechanism, whereas IL-6-induced ARID5A expression was not affected by the absence of RORγt, a lineage-specifying transcription factor of Th17 cells. Furthermore, ARID5A physically associated with RORγt by its N-terminal region and inhibited RORγt-induced Th17 cell differentiation.

Conclusion: ARID5A is a lineage-specific attenuator of Th17 cell differentiation and may be involved in the pathogenesis of RA.

INTRODUCTION

Rheumatoid arthritis (RA) is characterized by the destruction of cartilage and bone with inflammation and cellular proliferation in the synovial joints. Accumulating evidence has shown that immune cells including T cells, B cells, dendritic cells, and macrophages play essential roles in the pathogenesis of RA (1). Pro-inflammatory cytokines such as TNF-α and IL-6 produced by these immune cells are involved not only in synovial inflammation but also in extraarticular manifestations in RA (2, 3). Clinical efficacy of biologic agents that block the effects of these pro-inflammatory cytokines has proved the roles of these cytokines in the pathogenesis of RA (4, 5).

In addition to TNF-α and IL-6, recent studies have demonstrated that Th17 cell-related cytokines such as IL-17A, IL-17F, and IL-22 participate in the pathogenesis of RA (6, 7). IL-17A levels have been shown to be elevated in synovium and synovial fluid in RA patients (8, 9). In addition, it has been shown that the number of $CD4^+$ T cells with Th17 cell phenotype ($CCR6^+$ IL-$17A^+$ TNF-α^+ memory T cells) is increased in untreated RA patients as compared with that in healthy controls (10). Intriguingly, when these Th17 cell-like $CD4^+$ T cells are co-cultured with synovial fibroblasts, they produce not only IL-17A but also pro-inflammatory cytokines and tissue-destructive enzymes (10). Furthermore, the induction of collagen-induced arthritis (CIA), a murine model of RA, is attenuated in mice lacking IL-17A (11) or IL-23 (12), the latter of which promotes the differentiation of pathogenic Th17 cells in conjunction with IL-6 and TGF-β13. These findings indicate that Th17 cells play a pivotal role in the pathogenesis of RA.

During the differentiation of Th17 cells, IL-6-Stat3 signaling along with TGF-β induces the expression of RORγt, which functions as a lineage-specifying transcription factor of Th17 cells (13). Consistently, T cells lacking Stat3 exhibit reduced expression of RORγt and impaired Th17 cell differentiation (14). These findings are in good

agreement with the clinical efficacy of Tocilizumab (TCZ), an anti-IL-6 receptor monoclonal antibody which blocks IL-6-Stat3 signaling, for RA (15). However, detailed mechanisms underlying the efficacy of IL-6 blockade for RA are not fully understood. In the present study, we examined gene expression profiles of $CD4^+$ T cells before and after TCZ therapy in RA patients who showed good clinical responses to the therapy to clarify the mechanisms underlying the efficacy of IL-6 blockade for RA.

MATERIALS and METHODS

Patients

Patients who fulfilled the 1987 revised criteria of American College of Rheumatology for RA were recruited to the study when they and their physicians intended to start TCZ therapy as a routine care for uncontrolled arthritis between June 2009 and October 2011 in the Department of Allergy and Clinical Immunology, Chiba University Hospital and the Research Center for Allergy and Clinical Immunology, Asahi General Hospital. As control groups, treatment-naïve RA patients, RA patients treated with TNF inhibitors or Abatacept, and age-matched healthy controls were also recruited. The disease status of patients was assessed at baseline and at 24-week of the therapy using Clinical Disease Activity Index (CDAI) score (16). Good clinical response to the therapy was defined as an improvement of >50% from the baseline CDAI. The entire study was approved by the Ethics Committees of Chiba University, Asahi General Hospital, and Kazusa DNA Research Institute and was performed in accordance with the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all subjects.

Isolation of human $CD4^+$ T cells

Mononuclear cells were isolated from peripheral blood by Ficoll-Paque density gradient centrifugation. $CD4^+$ T cells were purified from mononuclear cells by using $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec, Sunnyvale, CA) according to the manufacturer's instruction. The purity of $CD4^+$ T cells was routinely >98% by FACS analysis.

DNA microarray analysis

Total cellular RNA was extracted from human $CD4^+$ T cells with ISOGEN solution (Nippon GENE Co., LTD, Tokyo, Japan). DNA microarray analysis was performed using Quick Amp Labeling Kit (Agilent technologies Inc. Santa Clara, CA) and Whole

Human Genome DNA Microarray 4×44K (Agilent technologies Inc.) according to the manufacture's protocols. Microarray data were analyzed using the GeneSpring GX11.5.1 software (Agilent technologies Inc.)

Mice and reagents

C57BL/6 mice were purchased from Charles River Laboratories (Kanagawa, Japan). RORγt-deficient mice (13) were kind gifts from Dr. Y. Iwakura (Tokyo University of Science). All mice were housed in microisolator cages under specific pathogen-free conditions, and animal procedures used in this study were approved by Chiba University Animal Care and Use Committee.

Antibodies to murine CD3ε (145-2C11), CD28 (37.51), IL-4 (11B11) and IFN-γ (XMG1.2) were purchased from BD Biosciences (San Diego, CA). Murine IL-4 and IL-6 were purchased from Pepro Tech Inc. (Rocky Hill, NJ). Human TGF-β was purchased from R&D Systems (Minneapolis, MN). Stat3 inhibitor VI (S3I-201) was purchased from Santa Cruz biotechnology Inc. (Santa Cruz, CA).

Plasmids

cDNAs for murine ARID5A and RORγt were subcloned into pMX-IRES-GFP vector and MSCV-IRES-Thy1.1 vector, respectively. Truncated mutants of ARID5A were generated using KOD-Plus-Mutagenesis kit (TOYOBO Life Science, Tokyo, Japan) as described previously (17). Murine IL-17A promoter (18) was subcloned into pGL3-vector to generate -153 mIL17p-Luc or -94 mIL17p-Luc. All sequences were verified by DNA sequencing.

Cell culture

Murine naïve $CD4^+$ T cells ($CD62L^{high}$ $CD25^-$ $CD4^+$ T cells) were isolated from spleen and lymph nodes using $CD4^+$ T Cell Isolation Kit II, according to the manufacture's instructions

(Miltenyi Biotec). The purity of isolated cells was routinely >98% by FACS analysis. Naïve $CD4^+$ T cells (5 x 10^5 cells/ml) were stimulated with plate-bound anti-CD3ε mAb (1 μg/ml) plus anti-CD28 mAb (2 μg/ml) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μM β-mercaptoethanol, 2 mM L-glutamine, and antibiotics at 37°C. Where indicated, IL-6 (100 ng/ml), TGF-β (1 ng/ml), anti-IL-4 mAb (5 μg/ml), and anti-IFN-γ mAb (5 μg/ml) were added to induce Th17 cells (Th17-polarizing conditions). Anti-IL-4 mAb (5 μg/ml) and anti-IFN-γ mAb (5 μg/ml) were added to induce Th0 cells (Th0 conditions). IL-6 (100 ng/ml), IL-23 (100 ng/ml), anti-TGF-β mAb (5 μg/ml), anti-IL-4 mAb (5 μg/ml), and anti-IFN-γ mAb (5 μg/ml) were added to induce Th22 cells (Th22-polarizing conditions) (19). TGF-β (1 ng/ml) was added to induce regulatory T cells (Tregs) (Treg-polarizing conditions).

Induction of human Th17 cells

Memory $CD4^+$ T cells were isolated from peripheral blood mononuclear cells of healthy controls or untreated RA patients by using Memory $CD4^+$ T Cell Isolation Kit Human (Miltenyi Biotec) and stimulated with plate-bound anti-CD3 antibody (1 μg/ml, eBioscience, San Diego, CA) plus anti-CD28 antibody (1 μg/ml, eBioscience) under Th0 conditions (IL-2 [10 ng/ml], anti-IL-4 antibody [5 μg/ml], and anti-IFN-γ antibody [5 μg/ml]) or Th17-polarizing conditions (IL-6 [10 ng/ml], IL-23 [10 ng/ml], IL-1β [10 ng/ml], IL-2 [10 ng/ml], anti-IL-4 antibody, and anti-IFN-γ antibody) for 14 days.

Real-time PCR analysis

Total cellular RNA was extracted from human and murine $CD4^+$ T cells with ISOGEN solution and reverse transcription was carried out using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The expression of ARID5A was measured by real-time quantitative PCR analysis (qPCR) by using a standard protocol on ABI PRISM 7300

instrument (Applied Biosystems, Foster City, CA). The levels of ARID5A were normalized to the levels of GAPDH.

Retrovirus-mediated gene induction

Retrovirus-mediated gene induction for murine naïve $CD4^+$ T cells was performed as described previously (20).

Intracellular staining

Cultured cells were harvested and restimulated with PMA (20 ng/ml) plus ionomycin (1 μg/ml) at 37°C for 5 h in the presence of monensin (2 μM, Sigma, St. Louis, MO). Intracellular staining for murine IL-17A, IL-17F, IL-22, and Foxp3 was performed as described previously (21).

Western blotting

Whole cell lysates were prepared and immunoblotting was performed as described previously (22). Anti-human ARID5A polyclonal antibody was purchased from Abcam (Cambridge, UK).

Immunoprecipitation assay

293T cells were transfected with either pcDNA3 Flag-ARID5A (wild-type) or the truncated mutants of ARID5A and/or MSCV-myc-RORγt-IRES-Thy1.1 by Lipofectamine (Invitrogen, Carlsbad, CA). Cells were lysed with lysis buffer and cell lysates were incubated with anti-Flag M2-Agarose Affinity Gel (Sigma) for 2 h at 4°C. After washing, samples were subjected to Western blotting with HRP-conjugated anti-Myc antibody (9E10, Santa Cruz biotechnology Inc.) or HRP-conjugated anti-Flag antibody (M2, Sigma).

Luciferase assay

EL4 cells (5 x10^5 cells) were transfected with the indicated plasmids using Neon Transfection System (Life Technologies, NY) according to the manufacture's instruction. Twenty-four hours later, luciferase reporter assay was performed with a dual luciferase assay system (Promega Biotech Inc., Madison, WI) according to the manufacturer's instruction. All values were obtained from experiments carried out in triplicate and repeated at least three times.

Data Analysis

Data are summarized as means ± SD. Statistical analysis was performed by means of ANOVA or unpaired t-test as appropriate. P values <0.05 were considered significant.

RESULTS

The expression of ARID5A is reduced in CD4$^+$ T cells from RA patients who exhibit good clinical responses to Tocilizumab therapy

Ten RA patients who received Tocilizumab (TCZ) therapy for uncontrolled arthritis were enrolled in this study. Eight out of 10 patients showed good clinical responses to TCZ therapy as evaluated by an improvement of >50% from the baseline Clinical Disease Activity Index (CDAI) (Table S1). To clarify the mechanisms underlying the efficacy of IL-6 blockade for RA, we examined gene expression profiles of CD4$^+$ T cells by DNA microarray analysis at baseline and at 12-week of TCZ therapy in the RA patients who showed good clinical responses to the therapy. Analysis of microarray data by a weighted average difference (WAD) method (23) identified several signaling molecules and transcription factors whose expression was significantly reduced in CD4$^+$ T cells by TCZ therapy (Table 1). The presence of several known IL-6-Stat3- or Th17 cell-related genes such as SOCS3 (24, 25), BCL3 (26), and BATF (27) in the list supported the reliability of this screening. We identified AT-rich interactive domain-containing protein 5a (ARID5A; also known as Mrf1) as one of new molecules downregulated by IL-6 blockade by TCZ therapy (Table 1).

First, we compared the expression of ARID5A in CD4$^+$ T cells between untreated RA patients (n = 17) and healthy controls (n = 10). The signals of ARID5A were significantly higher in untreated RA patients than those in healthy controls ($p<0.01$) (Fig. 1A). Consistent with the analysis by the WAD method, the signals of ARID5A in CD4$^+$ T cells were significantly decreased by the TCZ therapy in RA patients who showed good clinical responses to the therapy (Fig. 1B), whereas the signals of ARID5A were not decreased in patients who did not (data not shown). Importantly, the signals of ARID5A in CD4$^+$ T cells were not significantly decreased in RA patients who were treated with TNF inhibitors (TNFi) (n = 13) or Abatacept (ABT) (n = 12)