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GRAIL (Gene Related to Anergy in Lymphocytes) Regulates Cytoskeletal Reorganization through Ubiquitination and Degradation of Arp2/3 Subunit 5 and Coronin 1A*[S]

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Anergy is an important mechanism for the maintenance of peripheral tolerance and avoidance of autoimmunity. The up-regulation of E3 ubiqitin ligases, including GRAIL (gene related to anergy in lymphocytes), is a key event in the induction and preservation of anergy in T cells. However, the mechanisms of GRAIL-mediated anergy induction are still not completely understood. We examined which proteins serve as substrates for GRAIL in anergic T cells. Arp2/3-5 (actin-related protein 2/3 subunit 5) and coronin 1A were polyubiquitinated by GRAIL via Lys-48 and Lys-63 linkages. In anergic T cells and GRAIL-overexpressed T cells, the expression of Arp2/3-5 and coronin 1A was reduced. Furthermore, we demonstrated that GRAIL impaired lamellipodium formation and reduced the accumulation of F-actin at the immunological synapse. GRAIL functions via the ubiquitination and degradation of actin cytoskeleton-associated proteins, in particular Arp2/3-5 and coronin 1A. These data reveal that GRAIL regulates proteins involved in the actin cytoskeletal organization, thereby maintaining the unresponsive state of anergic T cells.

The regulation of T cell activation ensures efficient elimination of pathogens, as well as the maintenance of tolerance to self. Peripheral tolerance prevents the expansion of self-reactive T cells that escaped thymic selection, thus avoiding autoimmunity. T cell anergy is one form of peripheral tolerance that results in nonresponsiveness to antigen rechallenge following an initial partial activation; partial initial activation may result from the stimulation of T cell receptor (TCR)[2] in the absence of co-stimulation or the stimulation of T cells with the calcium ionophore ionomycin (1, 2). The induction of T cell anergy is inhibited by the addition of cyclohexamide, suggesting that anergy induction requires new protein synthesis (3). Recent reports have demonstrated that the induction of E3 ubiquitin ligases, including CBL-b, Itch, Deltex-1, and GRAIL (gene related to anergy in lymphocytes), is required to induce and maintain T cell anergy (4–8). In particular, it is well known that Cbl and Cbl-b act as negative regulators of TCR or CD28 signal transduction cascade through their ability to ubiquitinate tyrosine kinases including Src family kinases such as Fyn and Lck; Syk family kinases such as ZAP-70, Syk, PKC-θ, phospholipase C-γ, and p85; and the regulatory subunit of PI3K (4, 5, 9–15).

GRAIL is a type I transmembrane E3 ligase identified as an early gene that promotes T cell anergy (8). The up-regulation of GRAIL was observed in anergic CD4 T cells after treatment with ionomycin *in vitro* (4). Overexpression of GRAIL in T cell hybridomas or in primary cells reduces IL-2 production as well as proliferation upon antigen stimulation. Naive T cells from *GRAIL*-deficient mice exhibit increased proliferation and cytokine expression upon activation compared with those from control mice and do not depend on co-stimulation for effector generation (16, 17). Moreover, *GRAIL*-deficient mice exhibit lymphocyte infiltration into the lung and kidney and exacerbation of experimental autoimmune encephalomyelitis, indicating an important role for GRAIL in preventing lymphoproliferative and autoimmune responses (17). Although several candidates for GRAIL targets have been reported, including membrane proteins such as CD40 ligand and cytosolic proteins such as Rho GDIs, the mechanisms of GRAIL-mediated anergy induction are still not completely understood (18–21).

T cell activation and function require a structured engagement of antigen-presenting cells. These cell contacts are characterized by prolonged contacts from stable junctions called immunological synapses (IS). Reorganization of the actin cytoskeleton plays an important role in IS formation and signaling. Treatment of T cells with the actin-destabilizing agent cytochalasin D inhibits TCR-mediated IL-2 gene transcription (22). The Arp2/3 (actin-related protein 2/3) complex has been reported to be essential for TCR-mediated cytoskeletal reorganization (23, 24), and Arp2/3 complex-mediated actin nucleation is required for the formation of an F-actin-rich lamellipod (22, 25, 26). Coronin 1A is preferentially expressed in hematopoietic cells and co-localizes with F-actin-rich membranes in activated T cells (27). Coronin 1A has been shown to bind the Arp2/3 complex and inhibit F-actin nucleation by freezing the Arp2/3 complex in its inactive conformation (28). *Coronin 1A*-deficient T cells exhibit reduced cytokine production, including of IL-2 and IFN-γ, and altered F-actin reorganization (29). Moreover, a nonsense mutation in coronin 1A was identified as a gene alteration associated with the Lmb3 locus, which

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental text, Table S1, and Figs. S1 and S2.

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[2] The abbreviations used are: TCR, T cell receptor; IS, immunological synapse(s); OVA, ovalbumin; Ab, antibody; Ub, ubiquitin.

plays a major role in modulating autoimmunity in Fas^{lpr} mice (30).

In the present study, we demonstrate that both Arp2/3 subunit 5 (Arp2/3-5), a component of the Arp2/3 complex, and coronin 1A serve as substrates for GRAIL. The expression of Arp2/3-5 and coronin 1A is reduced in anergic T cells and in T cells in which GRAIL is overexpressed. Retroviral-driven expression of Arp2/3-5 or coronin 1A in anergic ovalbumin (OVA)-specific T cells restores their proliferation upon antigen activation. The accumulation of F-actin, Arp2/3-5, and coronin 1A at the IS is decreased in anergic T cells as well as in T cells overexpressing GRAIL. Thus, our findings demonstrate that GRAIL maintains the anergic states of T cells by regulating IS formation via degradation of the actin cytoskeleton-associated proteins Arp2/3-5 and coronin 1A.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—We obtained ionomycin, polybrene, and OVA from Sigma-Aldrich, OVA peptide ($OVA_{323-339}$) from TORAY Laboratory (Tokyo), lactacystin from Boston Biochem Inc., and recombinant IL-2 from Pepro Tech. We purchased antibodies (Abs) against Arp2/3-5 (C3), c-Myc (9E10), HA (F7), and GAPDH (6C5) from Santa Cruz Biotechnology, anti-Arp2/3-5 from Epitomics Inc. (Burlingame, CA), anti-coronin 1A from Everest Biotech Ltd. (Oxfordshire, UK), anti-CD28 Ab from BD Bioscience (San Jose, CA), and peroxidase-conjugated anti-rabbit IgG, anti-goat IgG, and anti-mouse IgG from DAKO-Japan (Tokyo). We obtained the pcDNA4-V5/His vector, pcDNA4-Myc/His vector, and SNARF-1 from Invitrogen and the pAcGFP1-N1 vector from Clontech Laboratories, Inc. HA-conjugated wild-type or mutated ubiquitin constructs were kind gifts from Dr. C. Akazawa at Tokyo Medical and Dental University. pAlter-MAX HA-Cbl-b was a kind gift from Dr. H. Band (University of Nebraska Medical Center).

Mice—DO11.10, OVA-specific TCR-transgenic mice were purchased from Jackson Laboratories. Seven-week-old female C57BL/6J mice were purchased from CLEA Laboratory Animal Corporation (Tokyo, Japan). The animals were maintained in specific pathogen-free conditions, and all care and use procedures were in accordance with institutional guidelines.

Cell Culture and Proliferation—DO11.10 splenocytes were cultured in complete DMEM (Invitrogen) supplemented with 0.05 mM 2-mercaptoethanol, 100 units/ml penicillin/streptomycin, and 10% FBS. Proliferative responses after 2 days of stimulation with plate-bound anti-CD3 (0.5 μg/ml) and anti-CD28 (1 μg/ml) Abs were determined by [^{3}H]thymidine incorporation using a β-1205 counter (Pharmacia). To induce anergy *in vitro*, DO11.10 splenocytes incubated with 1 mg/ml OVA for 3 days were rested for 7–10 days and were then stimulated for 18 h with ionomycin (1 μg/ml) (3).

Constructs—GRAIL, Arp2/3-5, coronin 1A, RhoGDIα, RhoGDIβ, Lasp1, and RGS10 cDNAs from DO11.10 T cells in which anergy had been induced by ionomycin were amplified with following the specific PCR primers: GRAIL, 5′-CAGTGAATTCATGGGGCCGCCGCCCGGGATC-3′ and 5′-CAGTCTCGAGAGATTTAATCTCCCGAACAGCAGC-3′; Arp2/3-5, 5′-CATGGAATTCTCCGGGATGTCGAAGAACACGGTGTC-3′ and 5′-GATCGCGGCCGCCACGGTTTTCCTTGCAGTCA-3′; coronin1A, 5′-GATCGCGGCCGCCTACTTGGCCTGAACAGTCT-3′ and 5′-CAGTCTCGAGCTTGGCCTGAACAGTCTCCTC-3′; RhoGDIα, 5′-CATGGAATTCGTAAGCATGGCAGAACAGGAACCCAC-3′ and 5′-GATCGCGGCCGCGTCCTTCCACTCCTTTTTGA-3′; RhoGDIβ, 5′-CATGGGATCCATCAAGATGACGGAGAAGGATGCACA-3′ and 5′-GATCGCGGCCGTTCTGTCCAATCCTTCTTAA-3′; and RGS10, 5′-CAGTGGATCCATGTTCACCCGCGCCGTG-3′ and 5′-CAGTCTCGAGTGTGTTGTAAATTCTGGAGGCTCG-3′. SOD1 cDNA from brain was amplified with the following PCR primers: 5′-CAGTGAATTCATGGCGATGAAAGCGGTGTGC-3′ and 5′-CAGTCTCGAGCTGCGCAATCCCAATCACTCC-3′. PCR products were cloned into a pcDNA4 V5/His vector or pcDNA4 Myc/His vector. The H297N and H300N mutations in the RING domain of murine GRAIL were generated using a PCR site-directed mutagenesis kit (Stratagene, Santa Clara, CA). Deletion of the RING domain in murine GRAIL was generated using the following PCR primers: for the 5′-PCR product, CAGTGAATTCATGGGGCCGCCGCCCGGGATC and CAGTTTCGAATCTCCATCAGGGCCAATTTC; and for the 3′-PCR product, CAGTTTCGAAGTGTGACATTCTCAAAGCT and CAGTCTCGAGAGATTTAATCTCCCGAACAGCAGC. After these reactions, the DNAs were digested with BamHI and HpaI, and the fragments, which were WT-GRAIL-V5/His, H2N2-GRAIL-V5/His, ΔRF-GRAIL-V5/His, Arp2/3-5-Myc/His, coronin 1A-Myc/His, RhoGDIα-Myc/His or RhoGDIβ-Myc/His, were subcloned into a pMIG vector. After pcDNA4 WT-GRAIL-V5/His was digested with NheI and XhoI, the fragment was subcloned into pAcGFP N1 vector.

Retroviral Transductions and Proliferation of Transfected T Cells—HEK293T cells were transfected with a pMIG plasmid and pCLEco helper plasmid by calcium phosphate precipitation. Supernatants were collected 48 and 72 h later and filtered through 0.45-μm syringe filters (Millipore, MA). Activated DO11.10 $CD4^+$T cells were resuspended in the collected supernatant (1×10^6 cells/ml) with recombinant IL-2 (50 units/ml) and polybrene (8 μg/ml) and were centrifuged at 2,500 rpm for 90 min. Transfected cells were expanded in complete DMEM with recombinant IL-2 for 48 h and were rested without IL-2. After treatment with ionomycin (0.3 μg/ml) for 18 h, the cells were stained with SNARF-1 (5 μM) for 15 min and were stimulated with plate-bound anti-CD3 and anti-CD28 Abs. Two days later, proliferation was analyzed using a FACSCalibur and the CELLQuest program (BD Biosciences).

Western Blot Analysis—The cells were washed with PBS and lysed in 1% Nonidet P-40 lysis buffer (137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 20 mM Tris, pH 7.5). After incubation for 10 min on ice, lysates were centrifuged at 13,200 rpm for 15 min at 4 °C, and supernatants were collected. After adjustment of protein concentrations using the Dc protein assay (Bio-Rad), the lysates were mixed with Laemmli's buffer (1.33% SDS, 10% glycerol, 2% 2-mercaptoethanol, 0.002% bromphenol blue, 83 mM Tris, pH 6.8) and were boiled for 5 min. Lysates (10–30 μg) were subjected to 10 or 12% SDS-PAGE and immobilized on nitrocellulose membranes. The membranes were blocked with 5% milk, PBS, 0.05% Tween for 1 h at room temperature. Proteins were detected with various Abs (mostly diluted at 1:1000)

and horseradish peroxidase-coupled anti-rabbit, anti-mouse, or anti-goat IgG Abs (1:1000). The proteins were visualized using an enhanced chemiluminescence Western blot detection system (Amersham Biosciences).

Ubiquitination Assay—HEK293T cells were co-transfected with V5/His-tagged GRAIL, HA-tagged ubiquitin, and Myc/His-tagged substrate-containing expression vectors. Twenty-four hours later, the cells were incubated with 0.3 μM lactacystin for 12 h. The cells were lysed using 1% Nonidet P-40 lysis buffer containing protease inhibitors (Complete protease inhibitor mixture; Roche Applied Science) and were subjected to immunoprecipitation with anti-Myc Ab. Ubiquitination of substrates was analyzed by SDS-PAGE after blotting with anti-HA Ab.

Immunofluorescence Microscopy—To investigate co-localization of GRAIL and its substrates, HEK293T cells were co-transfected by calcium phosphate precipitation with the pAcGFP1-N1 vector containing GRAIL and pcDNA4-DsRed vector containing the substrate. Twenty-four hours later, the cells were incubated with lactacystin (0.3 μM) for 12 h and were fixed with MeOH for 15 min at 4 °C. To analyze T cell-B cell conjugation, A20 cells pulsed with 1 μg/ml $OVA_{323-339}$ for 2 h at 37 °C were incubated at a ratio of 1:1 with transfected GFP^+DO11.10 $CD4^+$T cells sorted on a FACS Aria cell sorter (BD Biosciences) at 37 °C for 10 min. The cells were then plated on poly-L-lysine-coated slides for 15 min. To analyze lamellipodium formation, T cells overexpressing the control or indicated constructs were settled onto anti-CD3-coated coverslips for 5 min as described previously (26). The cells were fixed with 4% paraformaldehyde for 15 min at 4 °C and washed with PBS, 0.01% Tween 20. After blocking with PBS, 1% BSA for 1 h at room temperature, the cells were incubated with either anti-Arp2/3-5 (C3) or anti-coronin 1A Ab for 18 h at 4 °C. After washing, the cells were labeled with Cy5-conjugated anti-mouse IgG or anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at room temperature. The slides were mounted with ProLong Gold antifade reagent (Invitrogen) with or without DAPI. Confocal images were acquired using FV1000-D (Olympus, Tokyo, Japan).

Statistical Analysis—Statistical differences between control and treatment groups were assessed with the Student's *t* test.

Additional Procedures—Information on semiquantitative RT-PCR and generation of shRNA is available in the supplemental materials.

RESULTS

Reduced Expression of Arp2/3-5 and Coronin 1A—E3 ubiquitin ligases including GRAIL are up-regulated in anergized T cells and play an important role in the induction of anergy (4, 8). To determine which proteins serve as substrates for GRAIL, we used two-dimensional difference gel electrophoresis to analyze proteins that were down-regulated in T cells in which anergy had been induced by ionomycin. Down-regulated proteins were identified by MALDI-TOF-MS and the nonredundant NCBI (NCBInr) database using MASCOT software (supplemental Table S1). Proteins related to cytoskeletal reorganization were the most frequently down-regulated proteins in anergic T cells. We decided to focus on actin-related proteins Arp2/3-5 and coronin 1A. We first confirmed that the expression levels of these proteins were reduced in T cells in ionomycin-induced anergy. We stimulated splenocytes of DO11.10 mice with OVA protein for 3 days and then rested them for 7 days. Anergy was induced by the addition of ionomycin for 18 h and the proliferative response upon the addition of anti-CD3 and anti-CD28 Abs detected by the incorporation of [^{3}H]thymidine. The proliferative response was significantly suppressed in ionomycin-treated cells, confirming that anergy was properly induced (Fig. 1*A*). In these anergized cells, the protein expression of Arp2/3-5 and coronin 1A was reduced (Fig. 1*B*, *lanes 2* and *4*). To address the functional involvement of Arp2/3-5 and coronin 1A in T cell anergy, we examined whether overexpression of these proteins in DO11.10 $CD4^+$ T cells enhanced their proliferative response upon stimulation. DO11.10 $CD4^+$ T cells were transfected with Arp2/3-5 or coronin 1A. To analyze proliferation of transfected T cells by flow cytometry, the cells were treated with ionomycin and labeled with SNARF-1, which can monitor proliferating cells through dye dilution in a similar fashion to CFSE dilution assay. The number of proliferating cells upon stimulation (GFP^+ $SNARF\text{-}1^-$ cells) was increased in Arp2/3-5 or coronin 1A-overexpressing cells compared with that of control cells (Fig. 1*C*). We also analyzed whether an anergy-like state was displayed by knockdown of Arp2/3-5 or coronin 1A. The percentage of proliferation increase upon the restimulation with anti-CD3/anti-CD28 was decreased in Arp2/3-5 shRNA-expressing T cells (8%) and in coronin 1A shRNA-expressing T cells (3%) compared with that in control shRNA-expressing cells (13%). These results indicate that the expression of Arp2/3-5 and coronin 1A is correlated with T cell responses and is reduced in anergic T cells.

GRAIL Polyubiquitinates Arp2/3-5 and Coronin 1A—We next examined whether Arp2/3-5 and coronin 1A serve as substrates for GRAIL. Myc-tagged Arp2/3-5, coronin 1A or other candidate substrate proteins were transiently co-expressed with V5-tagged GRAIL and HA-tagged ubiquitin (Ub) in HEK293T cells. Twenty-four hours after transfection, the cells were treated with the proteasome inhibitor lactacystin for 12 h, and then lysates were prepared and immunoprecipitated with an anti-Myc Ab. SDS-PAGE followed by immunoblotting with anti-HA revealed a polyubiquitinated laddering pattern of Arp2/3-5 and coronin 1A in the presence of GRAIL (Fig. 2*A*, *lanes 6* and *10*). As Rho GDP dissociation inhibitors (RhoGDI) α and β were previously reported as substrates of GRAIL, we confirmed that these two proteins were polyubiquitinated as well (Fig. 2*A*, *lanes 8* and *4*). On the other hand, Lasp1 (LIM and SH3 protein 1), RGS10 (regulator of G-protein signaling 10), and SOD1 (superoxide dismutase 1), which were identified as proteins with reduced expression in anergized T cells by the two-dimensional difference gel electrophoresis analysis, were not ubiquitinated in the presence of GRAIL (Fig. 2*A*, *lanes 2*, *12*, and *14*). These results indicate that Arp2/3-5 and coronin 1A are selectively polyubiquitinated by GRAIL. Histidine to asparagine substitution in the RING finger domain (H2N2) or deletion of the RING finger domain (ΔRF) of GRAIL (Fig. 2*B*) reportedly inactivates GRAIL. These mutant forms of GRAIL abrogated the ability of GRAIL to ubiquitinate Arp2/3-5 and coronin 1A as well as RhoGDIα and β (Fig. 2*C*). Recent evi-

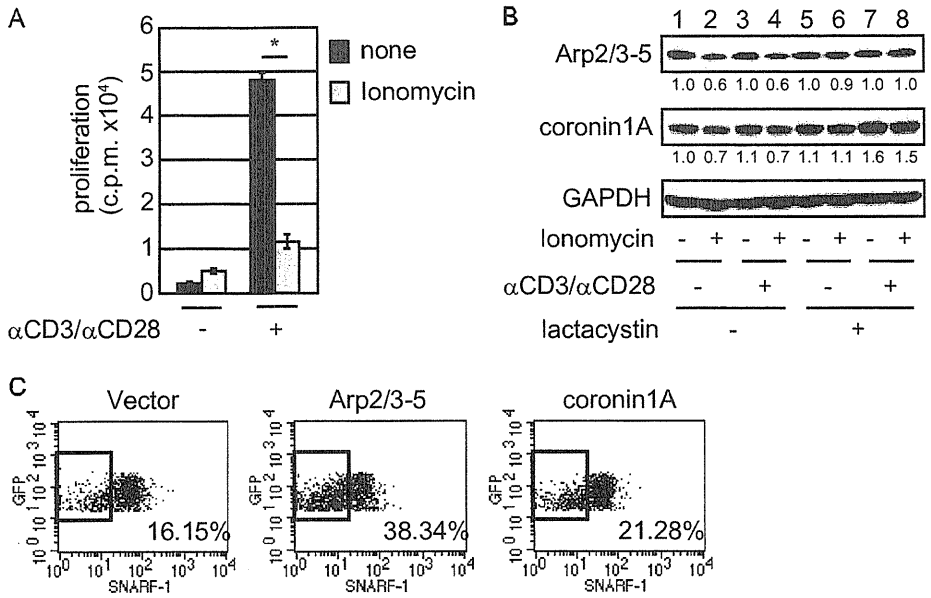

FIGURE 1. **Arp2/3-5 and coronin 1A are down-regulated in T cells anergized by ionomycin.** *A* and *B*, splenocytes derived from DO11.10 mice were stimulated with OVA for 3 days and rested for 7–10 days. The rested T cells were then treated with or without ionomycin for 18 h and restimulated with plate-bound anti-CD3 and soluble anti-CD28. *A*, proliferation was assessed by [^{3}H]thymidine uptake for 48 h. The mean c.p.m. of triplicate wells ± S.E. is shown (n = 9). *, p = 0.0000033 *versus* control. *B*, cells were lysed and analyzed by immunoblotting after 1-hour activation with plate-bound anti-CD3 and soluble anti-CD28. Each protein level analyzed by ImageJ software was normalized to the corresponding GAPDH level and is expressed as relative quantity to that of untreated control. *C*, DO11.10 CD4$^+$ T cells were transfected with vector control (GFP alone), Arp2/3-5, or coronin 1A. Forty-eight hours later, the transfected cells were treated with ionomycin for 18 h and were labeled with SNARF-1. The cells were restimulated with plate-bound anti-CD3 and soluble anti-CD28. Forty-eight hours later, proliferation was analyzed by FACS.

dence suggests that Cbl-b, which is E3 ligase as well as GRAIL, is important for induction of T cell anergy. We also analyzed whether Arp2/3-5 and coronin 1A are substrates of Cbl-b. However, Arp2/3-5 and coronin 1A are not ubiquitinated by Cbl-b (supplemental Fig. S1). These data indicate that GRAIL but not Cbl-b E3 ligase selectively ubiquitinates Arp2/3-5 and coronin 1A.

GRAIL Co-localizes with Arp2/3-5 and Coronin 1A—To address the interaction of Arp2/3-5 and coronin 1A with GRAIL, we examined the co-localization of these proteins. We transiently expressed GFP-tagged GRAIL together with HA-tagged ubiquitin and DsRed-tagged Arp2/3-5, coronin 1A, or RhoDGIα/β. After treatment with lactacystin, the localization of GRAIL and its substrates was analyzed by confocal microscopy. Indeed, Arp2/3-5 (Fig. 3*A*), coronin 1A (Fig. 3*B*), and RhoDGIα and β (Fig. 3, *C* and *D*) all co-localized with GRAIL. The substrates were localized together with GRAIL in contrast to the diffuse localization of GFP and substrate proteins in the cells transfected with GFP control vector and substrate proteins, indicating the co-localization of GRAIL and Arp2/3-5 or coronin 1A. These findings suggest that Arp2/3-5 and coronin 1A interact with GRAIL.

GRAIL Ubiquitinates Arp2/3-5 and Coronin 1A via Lys-63 and Lys-48—GRAIL has been reported to form polyubiquitin chains through lysine 63, resulting in proteolysis-independent functional modulation of Rho GDIs. However, when CD151 is the substrate, polyubiquitin chains are formed through lysine 48, which leads to protein degradation (18). We therefore assessed whether GRAIL ubiquitinates Arp2/3-5 and coronin 1A through Lys-63 and Lys-48. A similar polyubiquitinated ladder pattern of Arp2/3-5 was observed in the presence of WT Ub or Ub containing a lysine to arginine substitution at residue 29 (K29R) (Fig. 4*A*, *lanes 4* and *6*). In contrast, Ub conjugation of Arp2/3-5 was barely detected in the presence of Ub containing a lysine to arginine substitution at residue 48 (K48R) or at residue 63 (K63R) (Fig. 4*A*, *lanes 8* and *10*). Similarly, Ub conjugation of coronin 1A was observed in the presence of WT or K29R Ub (Fig. 4*B*, *lanes 4* and *6*) but was much lower when K48R or K63R Ub was used (Fig. 4*B*, *lanes 8* and *10*). These data reveal that Arp2/3-5 and coronin 1A were modified by Lys-48 and Lys-63 mixed linkage ubiquitin chains. To address the effect of GRAIL on the protein levels of Arp2/3-5 and coronin 1A, we overexpressed GRAIL and its enzymatically inactive mutant, H2N2-GRAIL or ΔRF-GRAIL, in DO11.10 CD4$^+$ T cells and determined Arp2/3-5 and coronin 1A expression by immunoblotting with specific Abs. Both Arp2/3-5 and coronin 1A were reduced when GRAIL, but not the enzymatically inactive forms of GRAIL, was overexpressed (Fig. 4*C*). These results indicate that GRAIL polyubiquitinates Arp2/3-5 and coronin 1A through Lys-48 and Lys-63 and eventually leads them to be degraded.

Less Arp2/3-5 and Coronin 1A Localize at the IS in Anergy—To investigate the role of Arp2/3-5 and coronin 1A in anergic T cells, we next examined the accumulation of F-actin, Arp2/3-5, and coronin 1A at the IS using confocal microscopy. As described previously, F-actin and Arp2/3-5 were recruited to the IS formed between DO11.10 CD4$^+$ T cells and $OVA_{323–339}$ peptide-pulsed A20 B cells (Fig. 5, *A* and *B*, *top panels*). In contrast, the accumulation of F-actin and the recruitment of Arp2/3-5 to the IS were reduced in ionomycin-treated DO11.10 CD4$^+$ T cells compared with those in control cells (Fig. 5*A*, *bottom panel*). Similarly, the recruitment of coronin 1A to the

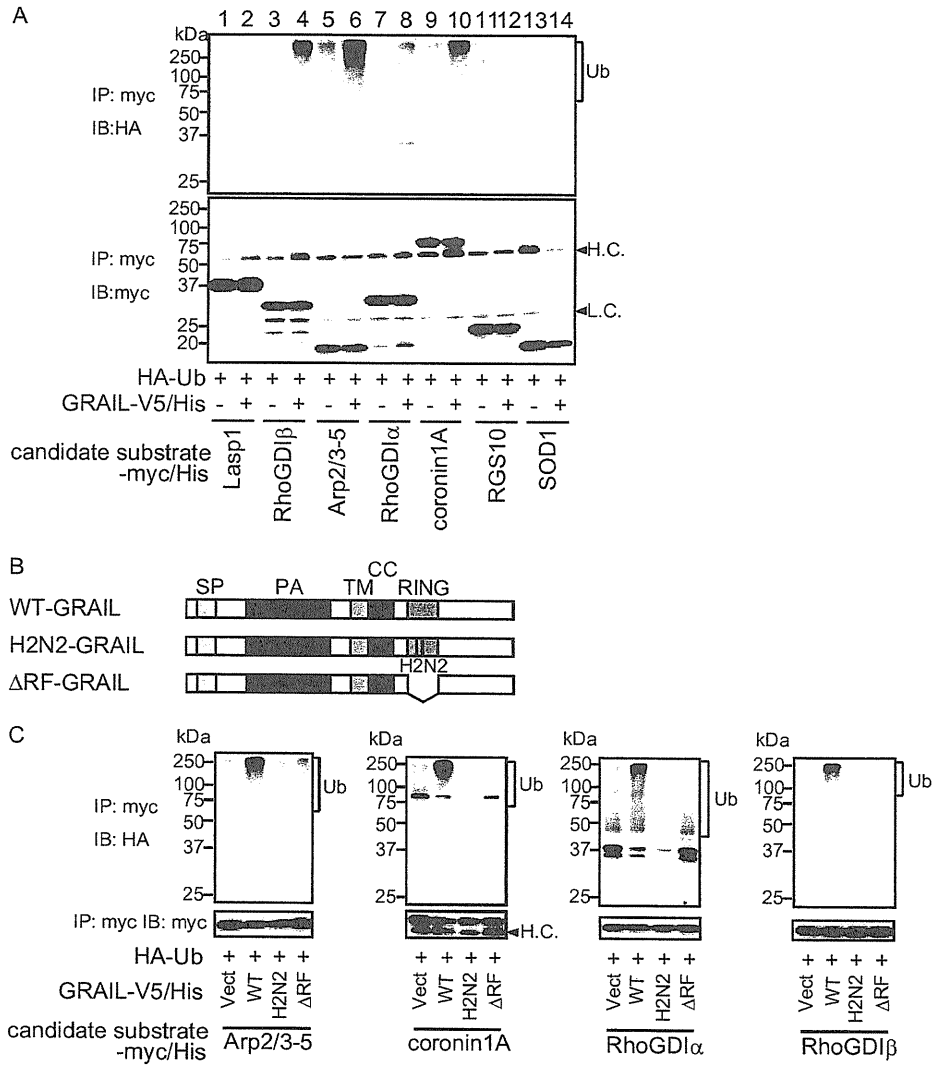

FIGURE 2. **Arp2/3-5 and coronin 1A are ubiquitinated by GRAIL.** *A* and *C*, HEK293T cells were transiently transfected with the indicated constructs and were treated with lactacystin for 12 h before lysis. Ubiquitination of the indicated proteins was detected by immunoprecipitation (*IP*) with anti-Myc Ab, followed by anti-HA immunoblotting (*IB*). The membrane was stripped and reprobed with anti-Myc Ab. *B*, schematic structures of the WT-, H2N2-, and ΔRF-GRAIL proteins.

IS in ionomycin-treated DO11.10 CD4$^+$ T cells was reduced compared with that in nontreated DO11.10 CD4$^+$ T cells (Fig. 5*B*, *bottom panel*). These data demonstrate that the accumulation of Arp2/3-5 and coronin 1A together with F-actin at the IS is impaired in anergic T cells.

GRAIL Inhibits Arp2/3 and Coronin 1A Accumulation at the IS—To address the contribution of GRAIL to IS formation, we overexpressed GRAIL, ΔRF-GRAIL, or a control vector in DO11.10 CD4$^+$ T cells and analyzed the accumulation of Arp2/3-5, coronin 1A, and F-actin at the IS. First, the expression of Arp2/3-5 and coronin 1A was reduced in T cells (GFP-positive cells) in which GRAIL was overexpressed compared with expression levels in control cells (Fig. 6, *A* and *B*, compare *top* and *middle panels*). The accumulation of both Arp2/3-5 and coronin 1A together with F-actin was reduced in DO11.10 CD4$^+$ T cells overexpressing GRAIL compared with that in control vector-transfected T cells (Fig. 6, *A* and *B*, compare *top* and *middle panels*). On the other hand, the accumulation of Arp2/3-5, coronin 1A, and F-actin at the IS in DO11.10 CD4$^+$ T cells overexpressing ΔRF-GRAIL was similar to that in controls (Fig. 6, *A* and *B*, *bottom panels*). We also examined whether the formation of IS occurred in ionomycin-treated T cells in which GRAIL was down-regulated by GRAIL shRNA-encoding retroviral infection. Coincident with the results for GRAIL-overexpressing experiments, both Arp2/3–5 and coronin 1A together with F-actin fully accumulated at the IS in ionomycin-treated GRAIL knockdown DO11.10 CD4$^+$ T cells compared with that in ionomycin-treated control T cells (anergic T cells) (supplemental Fig. S2). These results indicated that GRAIL regulates the recruitment of Arp2/3-5 and coronin 1A into the IS and the subsequent accumulation of F-actin at the site of the IS.

GRAIL Inhibits Lamellipodium Formation—Because Arp2/3 has been reported to be essential for the formation of lamellipodia at the IS, we next examined the effect of GRAIL on lamellipodium formation. Because the spreading of T cells on anti-TCR-coated coverslips requires the formation of stable actin structures and the generation of lamellipodia, we first analyzed whether T cells could spread onto anti-CD3-coated coverslips

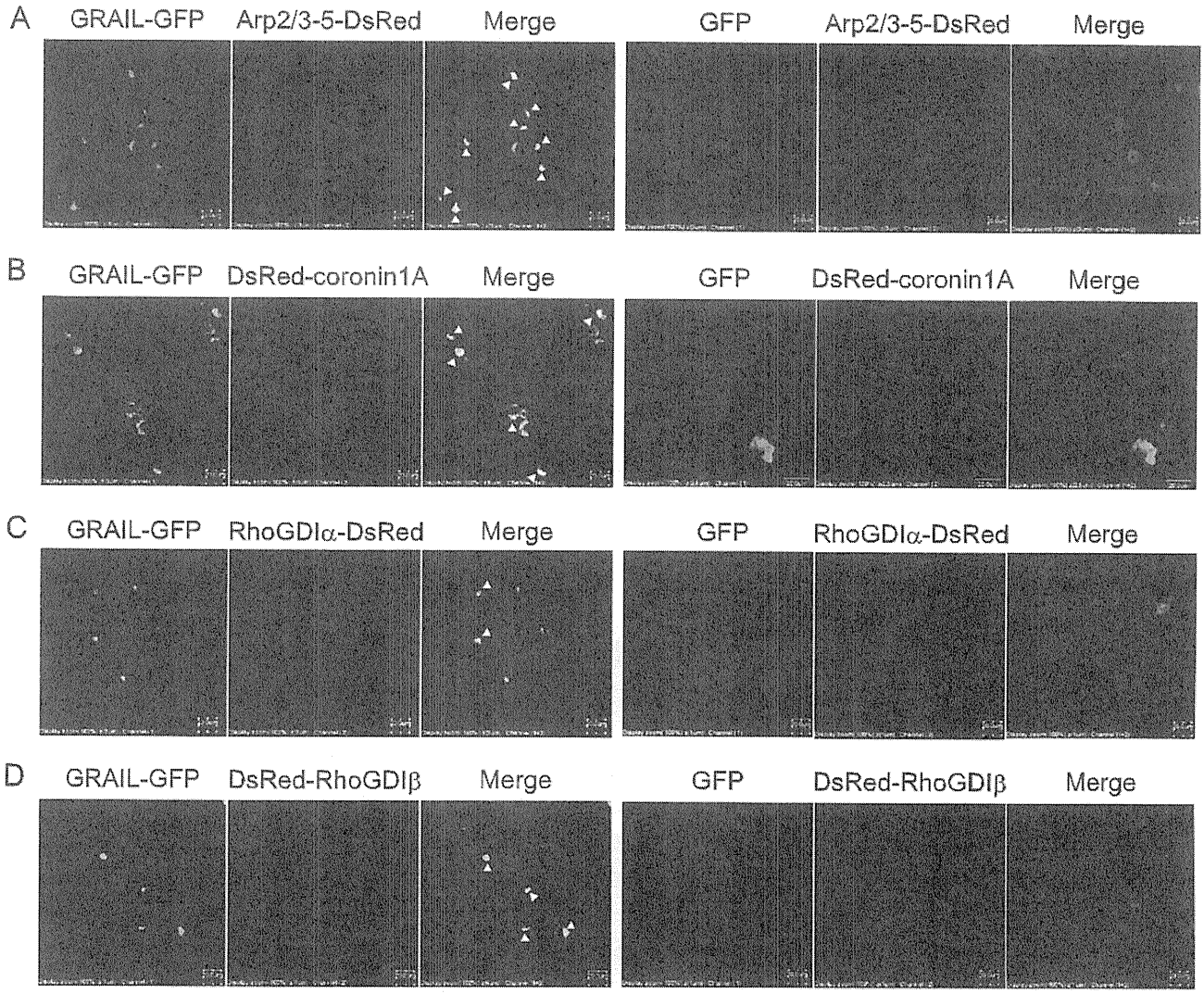

FIGURE 3. **GRAIL co-localizes with Arp2/3-5 and coronin 1A.** HEK293T cells were transiently transfected with constructs expressing GFP-tagged GRAIL, DsRed-tagged substrates (Arp2/3-5, *A*; coronin1A, *B*; RhoGDIα, *C*, and RhoGDIβ, *D*), and HA-ubiquitin and were treated with lactacystin for 12 h before being fixed. Co-localization with GFP-GRAIL was analyzed by confocal microscopy.

under anergic conditions. Control DO11.10 $CD4^+$ T cells spread onto anti-TCR-coated coverslips and formed round lamellipodial interfaces containing F-actin-rich structures (Fig. 7*A*). In contrast, DO11.10 $CD4^+$ T cells in which anergy had been induced by ionomycin barely formed lamellipodia (Fig. 7*A*, *bottom panels*). We next analyzed the lamellipodium formation in $CD4^+$ T cells overexpressing GRAIL. Lamellipodia were not efficiently formed on anti-CD3-coated coverslips when GRAIL was overexpressed in DO11.10 $CD4^+$ T cells (Fig. 7*B*, *middle panels*). In contrast, lamellipodia were efficiently formed at the IS when a catalytically inactive mutant GRAIL (ΔRF) was overexpressed in DO11.10 $CD4^+$ T cells (Fig. 7*B*, *bottom panels*). These data demonstrate that GRAIL inhibits lamellipodium formation at the IS.

DISCUSSION

In this study, we demonstrate that Arp2/3-5 and coronin 1A are down-regulated in anergic T cells as well as in T cells that overexpress GRAIL. Arp2/3-5 and coronin 1A co-localize with GRAIL and are ubiquitinated by GRAIL but not by Cbl-b via Lys-48 and Lys-63 linkage. Furthermore, the accumulation of Arp2/3-5 and coronin 1A together with F-actin is reduced at the IS in anergic T cells or in T cells that overexpress GRAIL. Coincident with the results for GRAIL-overexpressing experiments, IS formation in ionomycin-treated anergic T cells occurred by knockdown of GRAIL. Finally, we showed that overexpression of GRAIL suppresses lamellipodium formation at the IS.

CD40 ligand, CD151, CD83, and RhoGDI have been reported to be candidate substrates of GRAIL; however, the mechanism of GRAIL-mediated anergy induction is not yet fully understood (18–21). In fact, the expression of CD40 ligand was not up-regulated, and the down-regulation of CD3 was impaired in GRAIL-deficient mice. Because GRAIL is the only membrane protein among E3 ligases up-regulated in anergic T cells, it is reasonable that membrane proteins such as CD151 or CD83 are regulated by GRAIL. In this study, we confirmed that cytosolic proteins such as RhoGDIs serve as substrates for GRAIL. Fur-

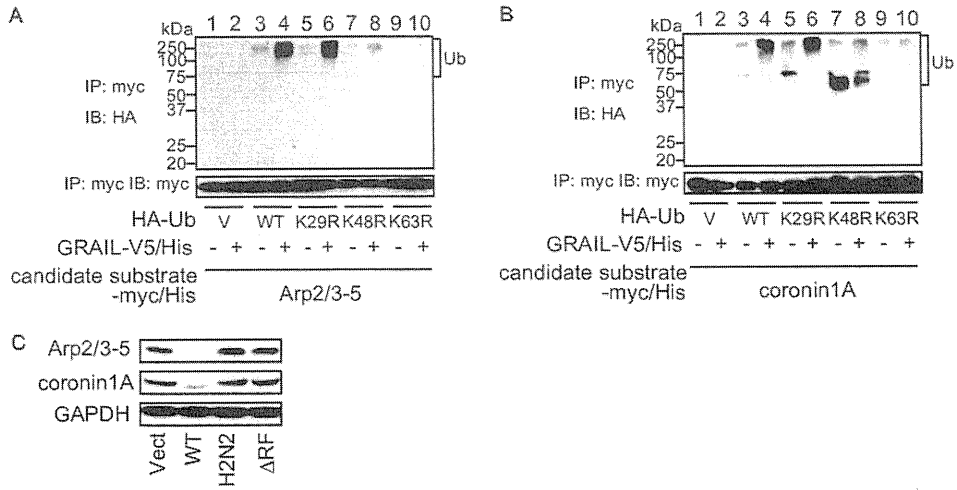

FIGURE 4. **Arp2/3-5 and coronin 1A are polyubiquitinated though Lys-48 and/or Lys-63 ubiquitin linkages and are down-regulated by catalytically active GRAIL.** *A* and *B*, HEK293T cells were transiently transfected with the indicated vectors and were treated with lactacystin for 12 h before lysis. Arp2/3-5 (*A*) and coronin 1A (*B*) were immunoprecipitated (*IP*) with anti-Myc Ab followed by immunoblotting (*IB*) with anti-HA Ab. The membrane was stripped and reprobed with anti-Myc Ab. *C*, $CD4^+$ T cells were transfected with vector control (GFP alone) or WT-, H2N2-, or ΔRF-GRAIL expression constructs. Forty-eight hours later, the transfected cells (GFP^+ cells) were sorted using a FACS Aria cell sorter. Sorted cells were rested for 2 days and were subjected to immunoblot analysis with anti-coronin 1A or Arp2/3-5 Ab.

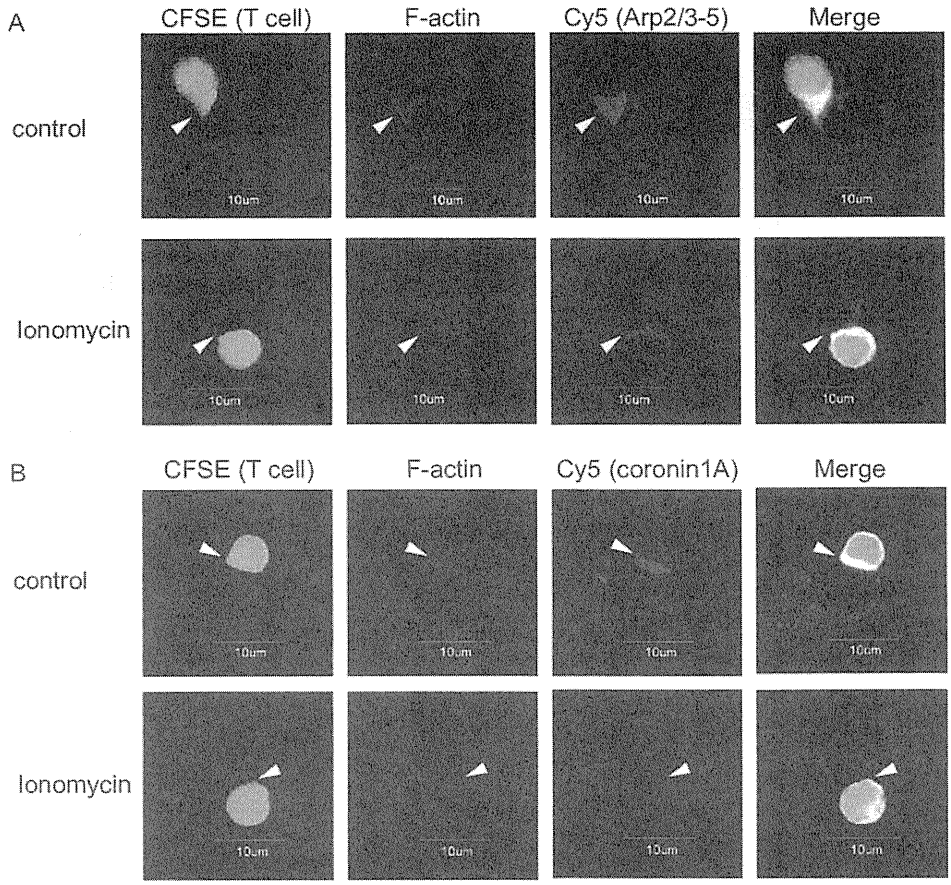

FIGURE 5. **The accumulation of Arp2/3-5, coronin 1A, and F-actin at the IS is reduced in anergic T cells.** *A* and *B*, OVA-stimulated DO11.10 splenocytes were rested for 7–10 days. Rested T cells were stained with CFSE, treated with or without ionomycin for 18 h, incubated with $OVA_{323-339}$-pulsed A20 cells, and co-stained with rhodamine-phalloidin (*red*) to visualize F-actin and either anti-Arp2/3-5 Ab (*A*) or anti-coronin 1A Ab (*purple*) (*B*). The *arrowheads* indicate IS.

thermore, we identified Arp2/3-5 and coronin 1A as novel substrates for GRAIL. Interestingly, these proteins as well as RhoGDIs are reportedly involved in the regulation of cytoskeletal organization. Although ubiquitination of target proteins was almost completely lost when either K63R or K48R mutant ubiquitin was used, it remains unclear whether Arp2/3-5 and coronin 1A are ubiquitinated via Lys-48, Lys-63, or both sites. To address this issue, characterization of ubiquitin chain using

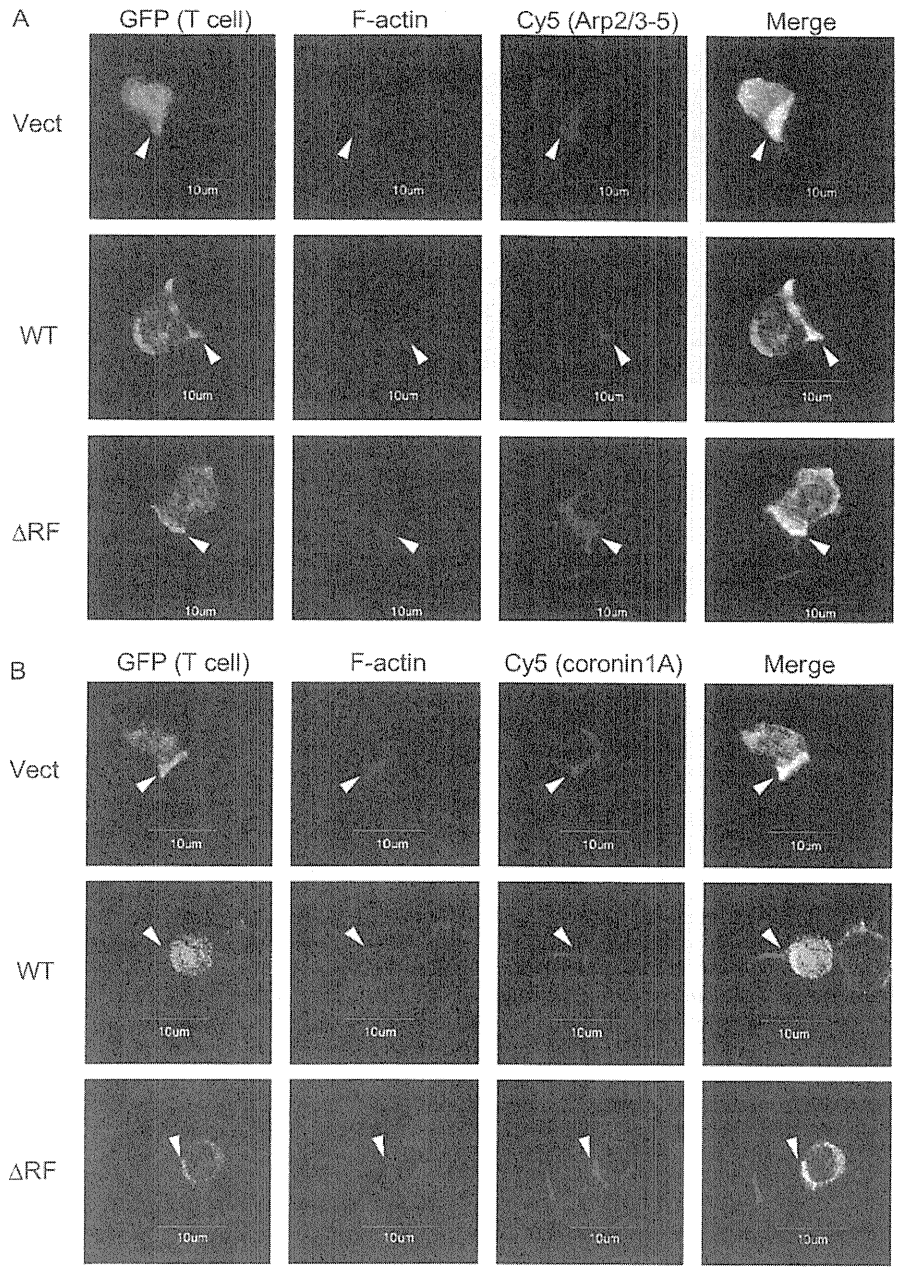

FIGURE 6. **GRAIL inhibits the accumulation of Arp2/3-5, coronin 1A, and F-actin at the IS.** *A* and *B*, DO11.10 CD4$^+$ T cells were transfected with vector control (GFP alone) or WT- or ΔRF-GRAIL expression constructs (*green*). Each population was incubated with $OVA_{323-339}$-pulsed A20 cells and co-stained with rhodamine-phalloidin (*red*) and either anti-Arp2/3-5 (*A*) or anti-coronin 1A (*purple*) (*B*). The *arrowheads* indicate IS.

MALDI-TOF-MS or mutants in which Lys-48 or Lys-63 is the only lysine residue that can mediate the ubiquitin chain formation will be needed for future studies

The immunological synapse is important in sustained signaling and delivery of a subset of effector cytokines by CD4$^+$ T cells (25, 29, 31, 32). Although the precise contribution of actin cytoskeletal remodeling to T cell signaling and biologic function is not completely understood, both anergic T cells and T cells overexpressing GRAIL have been reported to form unstable immunologic synapses (4, 38). Actin nucleation in T cells is induced by the WAVE2 complex (33) and the actin-nucleation-promoting factor WASPs, which are required to promote and stabilize interactions between T cells and APC *in vitro* and TCR clustering on artificial surfaces. WASPs bind to actin monomers, whereas the acidic stretch associates with the Arp2/3-5 complex (23, 34), a seven-subunit complex that has intrinsic actin-nucleating activity and is essential for polarization of F-actin at the IS (25, 35). In addition, co-localization of WASPs and the Arp2/3-5 complex at the interface between anti-CD3-coated beads and Jurkat T cells suggests that these cytoskeletal components are essential for the dynamics of the actin cytoskeleton and for T cell function (24). Arp2/3-5 is essential for the formation of a stable synapse by creating lamellipodia (25). Consistent with these findings, overexpression of GRAIL reduced the protein expression of Arp2/3-5 and impaired lamellipodium formation. These results suggest that proteins

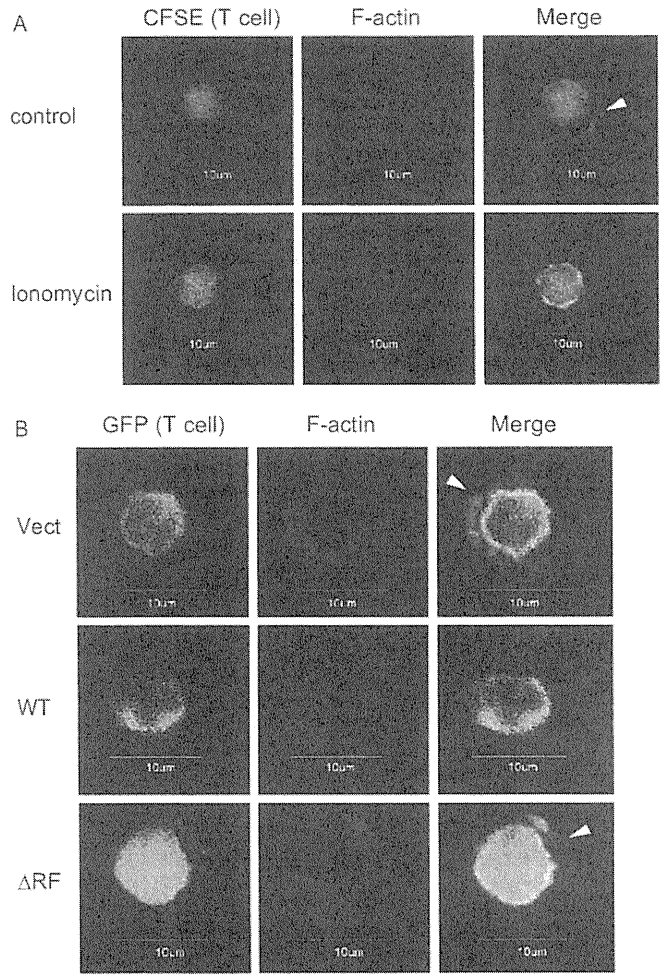

FIGURE 7. **GRAIL inhibits lamellipodium formation during TCR stimulation.** *A*, OVA-stimulated DO11.10 splenocytes were rested for 7–10 days and stained with CFSE. The cells were treated with or without ionomycin for 18 h. The cells were stimulated with plate-bound anti-CD3 mAb and stained with rhodamine-phalloidin (*red*) to visualize F-actin. *B*, DO11.10 $CD4^+$ T cells were transfected with a control vector (GFP alone) or WT- or ΔRF-GRAIL expression vectors. The cells were stimulated with coated anti-CD3 mAb and stained with rhodamine-phalloidin (*red*). The *arrowheads* indicate lamellipodium formation.

related to cytoskeletal reorganization at the IS are cytosolic targets for GRAIL.

An earlier study of coronin 1A knock-out mice reported that coronin 1A has an Arp2/3-5-dependent inhibitory effect on F-actin formation and concluded that coronin 1A is indispensable for TCR signaling (27, 29). In the present study, overexpression of coronin 1A restored the proliferative response. These findings suggest that coronin 1A participates in modulating T cell signaling and thereby contributes to the maintenance of anergy. In anergic T cells and in T cells overexpressing GRAIL, F-actin accumulation at the IS was decreased, although the expression of coronin 1A was reduced in contrast to previous studies. This may be because GRAIL regulates not only coronin 1A but also the Arp2/3-5 complex as well as RhoGDIs, which are important in the regulation of the accumulation of F-actin.

Anergic T cells have been reported to exhibit initial interaction, but implementation of T cell anergy results in reduced binding of LFA-1 to its ligand ICAM-1 (4). This process is mediated through degradation of PKC-θ and phospholipase C-γ by Cbl-b. A recent report demonstrated that overexpression of GRAIL impairs LFA-1 polarization at the IS (37). Stimulation through the TCR was shown to result in WAVE2-Arp2/3-5-dependent F-actin nucleation and the formation of a complex containing WAVE2, Arp2/3-5, vinculin, and talin (33). Moreover, TCR stimulation induces integrin clustering through the recruitment of vinculin and talin (33). Therefore, our study might link the unstable immunological synapse formation and impaired LFA-1 polarization at the IS in anergic T cells. Thus, whereas Cbl-b leads to unstable immunological synapse through degradation of tyrosine kinase, GRAIL leads to the phenotype of synapse disorganization via degradation of proteins involved in the actin cytoskeletal organization. In summary, we provide evidence that GRAIL regulates cytoskeletal reorganization to keep cells unresponsive to further antigen stimulation through the ubiquitination and down-regulation of the Arp2/3-5 complex and coronin 1A.

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Concise report

PADI4 polymorphism predisposes male smokers to rheumatoid arthritis

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ABSTRACT

Objective To elucidate the differential role of peptidyl arginine deiminase 4 (*PADI4*) polymorphism in rheumatoid arthritis (RA) between Asian and European populations, possible gene–environmental interactions among the *PADI4* polymorphism, sex and smoking status were analysed.

Methods Three independent sets of case–control samples were genotyped for single-nucleotide polymorphisms in *PADI4*; Japanese samples (first set, 1019 RA patients, 907 controls; second set, 999 RA patients, 1128 controls) using TaqMan assays and Dutch samples (635 RA patients, 391 controls) using Sequenom MassARRAY platform. The association of *PADI4* with RA susceptibility was evaluated by smoking status and sex in contingency tables and logistic regression models.

Results In the first set of Japanese samples, *PADI4* polymorphism (rs1748033) showed a greater risk in men (OR_{allele} 1.39; 95% CI 1.10 to 1.76; $p_{trend}=0.0054$) than in women and in ever-smokers (OR_{allele} 1.25; 95% CI 1.02 to 1.53; $p_{trend}=0.032$) than in never-smokers. Moreover, the highest risk was seen in male ever-smokers (OR_{allele} 1.46; 95% CI 1.12 to 1.90; $p_{trend}=0.0047$). Similar trends were observed in the second set of Japanese samples as well as in Dutch samples.

Conclusion *PADI4* polymorphism highly predisposes male smokers to RA, and the genetic heterogeneity observed between Asian and European populations may be partly explained by differences in smoking prevalence among men.

Rheumatoid arthritis (RA) is a multigenic disease caused by interactions between genetic predispositions and environmental factors that result in abnormal immune response and joint destruction. The *HLA-DRB1* region is considered to be the major genetic determinant of RA susceptibility, but recent genetic studies have revealed multiple non-human leucocyte antigen susceptibility genes for RA.[1] Among these, the peptidyl arginine deiminase 4 (*PADI4*) gene, which encodes a post-translational modification enzyme that converts arginine to citrulline residues in proteins, is thought to have significant relevance in RA pathogenesis as anti-citrullinated protein antibodies (ACPA) are specifically observed in the sera of patients.[2 3]

The association of the *PADI4* polymorphism with RA susceptibility was first reported in a Japanese population[2] and has been replicated in several Asian populations.[4 5] Conversely, inconsistent results have been observed in populations of European ancestry.[6–8] A meta-analysis confirmed the association in Asian populations, but not in European populations.[6 7] The genetic heterogeneity observed between different populations could be partly explained by the difference of disease severity between the study populations, as the *PADI4* polymorphism was reported to influence erosive joint status.[9] However, it could also be explained by unknown gene–gene or gene–environmental interactions with *PADI4*, and the higher magnitude of risk with *PADI4* in Asian populations suggests the presence of these interacting factors.

Smoking is one of the well-established environmental factors in RA,[10] and several studies have described associations with the appearance of ACPA in RA patients.[3] Klareskog *et al*[11] first reported that citrullinated proteins were detected in bronchoalveolar lavage cells from smokers but not in those from non-smokers. A later study by Makrygiannakis *et al*[12] showed that a significantly increased *PADI2* expression and a higher trend of *PADI4* expression were observed in bronchoalveolar lavage cells from smokers compared with non-smokers. These lines of evidence suggest that the upregulated expression of PADI enzymes provoked by smoking may promote the citrullination of proteins in the lung, leading to citrulline autoimmunity in RA.[3]

The present study examined possible interactions between *PADI4* polymorphism, sex and smoking status, and discusses the resulting influence on the genetic heterogeneity in *PADI4* observed between Asian and European populations.

METHODS

Subjects

Japanese RA patients (first set n=1019, second set n=999) were provided by the Leading Project for Personalized Medicine in the Ministry of Education, Culture, Sports, Science and Technology, Japan (BioBank Japan).[13] Unrelated Japanese controls (first set n=907, second set n=1128) were recruited through Midousuji Rotary club and several medical institutes in Japan. These Japanese case–control sets were independent from that used in the previous study.[2] Dutch cohorts and RA patients were previously described.[14] RA patients (n=635) were part of the Leiden Early Arthritis Clinic, which comprises an inception cohort of patients with recent-onset arthritis (duration of symptoms <2 years). Those patients were diagnosed with RA within the first year after their initial visit. All individuals with RA met the 1987 revised criteria of the American College of Rheumatology for RA.[10] The characteristics of the cohorts are described in detail in supplementary table 1 (available online only). All

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subjects entered into this study provided informed consent prior to participation in the study, and all study protocols were preapproved by the ethics committees of each institute.

Smoking status

Smoking status was determined for each individual on the basis of self-reported information. An ever-smoker was defined as a person who had smoked tobacco, cigarettes or pipes at any stage in their life, whereas a never-smoker was defined as someone who had never smoked any of these. Smoking status was available for all the samples in the first Japanese case–control set, cases in the second Japanese set and a part of the Dutch RA patients (52.9%), but not for the control subjects in the second Japanese and Dutch sets.

SNP genotyping

The four exonic single-nucleotide polymorphisms (SNP) comprising two major transcripts of *PADI4* (rs11203366=padi4_89, rs11203367=padi4_90, rs874881=padi4_92 and rs1748033=padi4_104) were genotyped.[2] Two of these SNP (rs11203367 and rs1748033) tag the three haplotypes (two common haplotypes and one rare haplotype, see supplementary table 2, available online only) and provide full information for *PADI4*. These were also tested in the Dutch population. In the Japanese population SNP were genotyped using predesigned TaqMan SNP genotyping assays (Applied Biosystems, Carlsbad, California, USA). Fluorescence was detected using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). In the Dutch population SNP were genotyped using time-of-flight mass spectrometry-based Sequenom MassARRAY Platform (Sequenom, San Diego, California, USA). Genotyping assessment was made for over 95% of samples, for all of the polymorphisms genotyped. All SNP were in Hardy–Weinberg equilibrium in control subjects according to χ^2 statistics ($p>0.01$).

Statistical analysis

The case–control association of each SNP was tested with the Cochran Armitage trend test and the χ^2 test. Genotype and

Table 1 Association of the *PADI4* polymorphism and RA stratified with sex and smoking status in a Japanese population*

		Sum		MAF			p Value for
	Set	Case	Contol	Case	Contol	Per allele OR	trend test
rs11203367							
All	1st	1019	907	0.43	0.40	1.14 (1.00 to 1.29)	0.045
	2nd	996	1124	0.42	0.40	1.09 (0.96 to 1.23)	0.16
Men	1st	190	672	0.48	0.39	1.44 (1.14 to 1.81)	0.0022
	2nd	185	448	0.44	0.40	1.18 (0.92 to 1.51)	0.19
Women	1st	829	235	0.42	0.41	1.02 (0.82 to 1.25)	0.84
	2nd	811	676	0.41	0.39	1.07 (0.92 to 1.24)	0.31
Ever-smoker	1st	337	488	0.47	0.39	1.35 (1.11 to 1.65)	0.0024
	2nd	302	1124	0.43	0.40	1.15 (0.95 to 1.38)	0.12
Never-smoker	1st	682	418	0.41	0.40	1.03 (0.86 to 1.23)	0.71
	2nd	694	1124	0.41	0.40	1.06 (0.92 to 1.21)	0.36
Male ever-smoker	1st	155	451	0.50	0.39	1.61 (1.24 to 2.09)	0.00031
	2nd	145	448	0.46	0.40	1.25 (0.96 to 1.63)	0.10
Male never-smoker	1st	35	221	0.39	0.40	0.92 (0.54 to 1.54)	0.77
	2nd	40	448	0.39	0.40	0.95 (0.59 to 1.52)	0.84
Female ever-smoker	1st	182	37	0.44	0.47	0.86 (0.52 to 1.42)	0.56
	2nd	157	676	0.41	0.39	1.05 (0.82 to 1.35)	0.64
Female never-smoker	1st	647	197	0.41	0.40	1.04 (0.83 to 1.32)	0.68
	2nd	654	676	0.41	0.39	1.08 (0.92 to 1.26)	0.31
rs1748033							
All	1st	1018	904	0.37	0.35	1.12 (0.98 to 1.27)	0.089
	2nd	996	1125	0.36	0.34	1.08 (0.95 to 1.22)	0.20
Men	1st	190	669	0.42	0.34	1.39 (1.10 to 1.76)	0.0054
	2nd	185	448	0.40	0.34	1.25 (0.97 to 1.60)	0.08
Women	1st	828	235	0.36	0.36	1.00 (0.81 to 1.24)	0.96
	2nd	811	677	0.35	0.34	1.05 (0.90 to 1.22)	0.50
Ever-smoker	1st	336	485	0.40	0.35	1.25 (1.02 to 1.53)	0.032
	2nd	302	1125	0.38	0.34	1.19 (0.99 to 1.43)	0.055
Never-smoker	1st	682	418	0.36	0.34	1.07 (0.89 to 1.28)	0.47
	2nd	694	1125	0.35	0.34	1.03 (0.90 to 1.19)	0.59
Male ever-smoker	1st	155	448	0.44	0.34	1.46 (1.12 to 1.90)	0.0047
	2nd	145	448	0.41	0.34	1.34 (1.02 to 1.75)	0.039
Male never-smoker	1st	35	221	0.36	0.34	1.09 (0.64 to 1.85)	0.75
	2nd	40	448	0.34	0.34	0.96 (0.59 to 1.56)	0.90
Female ever-smoker	1st	181	37	0.37	0.41	0.87 (0.52 to 1.45)	0.60
	2nd	157	677	0.36	0.34	1.06 (0.82 to 1.37)	0.60
Female never-smoker	1st	647	197	0.36	0.35	1.03 (0.81 to 1.30)	0.79
	2nd	654	677	0.35	0.34	1.04 (0.89 to 1.22)	0.55

*rs112033673 (T/C, T is the minor allele) and rs174803 (T/C, T is the minor allele) were genotyped for the test. Both case and control subjects were stratified with smoking status in the first set, whereas only case subjects were stratified with smoking status in the second set.
MAF, minor allele frequency; *PADI4*, peptidyl arginine deiminase 4; RA, rheumatoid arthritis.

allele frequencies for patients and controls were used to calculate the OR and the 95% CI using the method of Woolf.[15] Gene–environmental interactions were assessed by both 'case-only' analysis and logistic regression analysis.[16] All statistical analysis were performed using Plink software.[17]

RESULTS AND DISCUSSION

A significant association between the *PADI4* polymorphism and RA susceptibility was observed in the whole set of case–control subjects in the first Japanese set (rs11203367; per allele OR (OR_{allele}) 1.14; 95% CI 1.00 to 1.29; p value for a trend test (p_{trend})=0.045; table 1). In a stratified analysis with sex, the *PADI4* polymorphism was significantly associated only in men (OR_{allele} 1.44; 95% CI 1.14 to 1.81; p_{trend}=0.0022), but not in women (OR_{allele} 1.02; 95% CI 0.82 to 1.25; p_{trend}=0.84). Similarly, when subjects in both cases and controls were stratified for smoking status, the *PADI4* polymorphism had a greater effect in ever-smokers (OR_{allele} 1.35; 95% CI 1.11 to 1.65; p_{trend}=0.0024) compared with never-smokers (OR_{allele} 1.03; 95% CI 0.86 to 1.23; p_{trend}=0.71). Further stratification analysis with sex and smoking status revealed that the *PADI4* polymorphism had the highest risk in the subpopulation of male ever-smokers (OR_{allele} 1.61; 95% CI 1.24 to 2.09; p_{trend}=0.00031). Similar findings were also observed, when only ACPA-positive patients were analysed (supplementary table 3, available online only).

To support these observations, we also analysed other case–control sets in the Japanese population and Dutch population (unstratified controls for smoking status were used in both sets as no information was available). In the second Japanese set, the highest risk in the subpopulation of male ever-smokers was replicated in rs1748033 (OR_{allele} 1.34; 95% CI 1.02 to 1.75; p_{trend}=0.039; table 1). In the Dutch set, the association of the *PADI4* polymorphism (rs1748033) was statistically significant in a dominant model (OR_{dom} 1.32; 95% CI 1.02 to 1.72; p_{dom}=0.03; table 2), but not in a trend test, when evaluated in total (p_{trend}=0.14). When patients were stratified by sex or/and smoking status and compared with control subjects, OR in the dominant model was higher for men (OR 1.36; 95% CI 0.90 to 2.06; p=0.13) than for women and was higher for ever-smokers (OR 1.56; 95% CI 1.06 to 2.31; p=0.02) than for never-smokers. Furthermore, it was highest in male ever-smokers (OR 1.79; 95% CI 0.98 to 3.27; p=0.043).

Table 2 Association of *PADI4* polymorphism and RA stratified with sex and smoking status in a Dutch population*

	Sum		MAF		Genotype frequency test (dominant model)	
	Case	Contol	Case	Contol	OR	p Value
rs11203367						
All	646	385	0.44	0.42	1.19 (0.90 to 1.56)	0.2
Men	218	180	0.47	0.40	1.49 (0.96 to 2.33)	0.063
Women	398	188	0.42	0.43	1.06 (0.73 to 1.56)	0.7
Ever-smoker	174	385	0.43	0.42	1.14 (0.76 to 1.70)	0.5
Never-smoker	178	385	0.45	0.42	1.06 (0.72 to 1.57)	0.7
Male ever-smoker	76	180	0.45	0.40	1.46 (0.78 to 2.71)	0.2
Male never-smoker	40	180	0.53	0.40	1.38 (0.62 to 3.10)	0.4
Female ever-smoker	98	188	0.41	0.43	0.99 (0.58 to 1.72)	0.9
Female never-smoker	138	188	0.43	0.43	0.99 (0.61 to 1.61)	0.9
rs1748033						
All	635	391	0.34	0.30	1.32 (1.02 to 1.72)	0.03
Men	215	183	0.35	0.28	1.36 (0.90 to 2.06)	0.13
Women	389	191	0.32	0.31	1.33 (0.93 to 1.91)	0.11
Ever-smoker	158	391	0.36	0.30	1.56 (1.06 to 2.31)	0.02
Never-smoker	178	391	0.31	0.30	1.06 (0.73 to 1.53)	0.7
Male ever-smoker	70	183	0.38	0.28	1.79 (0.98 to 3.27)	0.043
Male never-smoker	41	183	0.35	0.28	1.35 (0.65 to 2.82)	0.4
Female ever-smoker	88	191	0.34	0.31	1.48 (0.86 to 2.55)	0.13
Female never-smoker	137	191	0.30	0.31	1.03 (0.65 to 1.64)	0.9

*rs112033673 (T/C, T is the minor allele) and rs174803 (T/C, T is the minor allele) were genotyped for the test. Only case subjects were stratified with smoking status.
MAF, minor allele frequency; *PADI4*, peptidyl arginine deiminase 4; RA, rheumatoid arthritis.

These stratified analyses suggested gene–environmental interactions between *PADI4* and sex, and/or between *PADI4* and smoking status. We performed case-only analysis to test these interactions statistically, by comparing the allele frequency of the *PADI4* polymorphism in the stratified subpopulation of patients (the first and second Japanese sets were combined). Allele frequency was significantly higher in men than in women (rs11203367; 0.48 vs 0.42; p_{trend}=0.0016) and in ever-smokers than in never-smokers (rs11203367; 0.47 vs 0.41; p_{trend}=0.00077), suggesting the presence of gene–environmental interactions for *PADI4*. Similar results were obtained for rs1748033. In addition to stratified analyses using the contingency tables, we analysed these gene–environmental interactions using logistic regression models. The first Japanese set was used for analysis because of the availability of smoking status. The *PADI4* polymorphism was associated with RA susceptibility in an additive model, adjusted by sex and smoking status (rs11203367; OR_{add} 1.18; 95% CI 1.01 to 1.38; p_{add}=0.035). When an interaction term between SNP genotype and sex (a product term of genotype×sex) was introduced into the regression model, the logistic coefficient for the term was significant (p=0.029). Similarly, when an interaction term between SNP genotype and smoking status (a product term of genotype×smoking status) was introduced into the model, the coefficient for the term was again significant (p=0.034). We also added the age of subjects into the model, because it could be a confounding factor considering that smoking prevalence has been decreasing in recent decades, especially in Japanese men (OECD Health Data, 2009).[18] The interaction term for SNP and smoking remained significant (p=0.038), whereas the significance level of the interaction term for SNP and sex became marginal (p=0.075).

Finally, we examined the association between the *PADI4* polymorphism and ACPA status in the patients of Japanese sets. The allele frequency of *PADI4* showed a higher trend in ACPA-positive patients compared with ACPA-negative patients (rs11203367; 0.43 vs 0.41; p_{trend}=0.54). When the genotype frequency was compared in a recessive model, the *PADI4* polymorphism was significantly associated with the ACPA status in ever-smokers (rs11203367; OR_{rec} 2.33; 95% CI 1.23 to 4.39; p_{rec}=0.0072; table 3), suggesting that the *PADI4* polymorphism may be involved in the appearance of ACPA in smokers.

Gene–environmental interactions in RA susceptibility have been well described between polymorphisms in *HLA-DRB1* and *PTPN22* genes and smoking habit in populations of European descent.[19 20] Our observations here indicate that the *PADI4* polymorphism is another genetic risk that would interact with smoking in RA susceptibility, although why this interaction is prominent in men remains to be solved. The status of sex hormones may influence the role of *PADI4*, as it is profoundly involved in the onset of RA.[21] Another possible explanation could be gender differences in smoking behaviour, which has also been argued in other smoking-related diseases.[22] Quantitative analysis of smoking history, such as pack-years smoked, may be needed to investigate further for the gender difference.

Smoking prevalence rates differed highly among the populations, and the attribution of smoking to the onset of RA may thus differ among populations. A recent epidemiological survey

Table 3 Association of *PADI4* polymorphism and ACPA status in a Japanese population*

	Sum		MAF		Genotype frequency test (recessive model)	
	ACPA+	ACPA−	ACPA+	ACPA−	OR	p Value
rs11203367						
All	1614	401	0.43	0.41	1.25 (0.93 to 1.68)	0.14
Men	295	80	0.46	0.45	1.22 (0.65 to 2.28)	0.52
Women	1319	321	0.42	0.40	1.27 (0.90 to 1.78)	0.17
Ever-smoker	523	116	0.46	0.41	2.33 (1.23 to 4.39)	0.0072
Never-smoker	1091	285	0.41	0.41	0.99 (0.70 to 1.39)	0.96
Male ever-smoker	245	55	0.49	0.44	1.90 (0.85 to 4.25)	0.11
Male never-smoker	50	25	0.34	0.48	0.28 (0.08 to 1.01)	0.045
Female ever-smoker	278	61	0.43	0.39	3.20 (1.11 to 9.22)	0.024
Female never-smoker	1041	260	0.41	0.41	1.09 (0.75 to 1.56)	1
rs1748033						
All	1614	400	0.37	0.37	1.39 (0.98 to 1.98)	0.063
Men	295	80	0.41	0.42	1.40 (0.69 to 2.83)	0.34
Women	1319	320	0.36	0.36	1.41 (0.93 to 2.12)	0.10
Ever-smoker	523	115	0.40	0.38	2.15 (1.08 to 4.28)	0.026
Never-smoker	1091	285	0.35	0.37	1.13 (0.75 to 1.72)	0.54
Male ever-smoker	245	55	0.43	0.41	2.09 (0.84 to 5.16)	0.10
Male never-smoker	50	25	0.30	0.44	0.34 (0.08 to 1.43)	0.13
Female ever-smoker	278	60	0.37	0.36	2.28 (0.78 to 6.65)	0.12
Female never-smoker	1041	260	0.35	0.36	1.27 (0.81 to 1.98)	0.29

Anti-citrullinated protein antibody (ACPA)+ and ACPA−, ACPA-positive and ACPA-negative rheumatoid arthritis (RA) patients, respectively.
*rs112033673 (T/C, T is the minor allele) and rs174803 (T/C, T is the minor allele) were genotyped for the test. Case subjects of Japanese sets (first and second) were combined for analysis.
MAF, minor allele frequency; *PADI4*, peptidyl arginine deiminase 4.

has shown that smoking prevalences are generally higher in men from Asian countries than in western European countries: Japan, 45.8%; Korea, 46.6%; UK, 25.0%; The Netherlands, 35.0%; Sweden, 13.9%; and USA, 19.1% in 2005.[16] Considering our observation that the *PADI4* polymorphism has the highest risk in male ever-smokers, the attribution of the *PADI4* polymorphism may be relatively high in populations with high smoking prevalences among men, such as Japan and Korea, corresponding to the positive results in association studies for *PADI4* polymorphisms in these countries.[2 4 5]

In conclusion, the *PADI4* polymorphism highly predisposes male smokers to RA, and the genetic heterogeneity observed in the *PADI4* polymorphism between populations of Asian and European countries may be partly explained by differences in smoking prevalences among men.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the ethics committee of RIKEN and Leiden University.

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INTERNAL MEDICINE

□ CASE REPORT □

Tocilizumab Improved both Clinical and Laboratory Manifestations Except for Interleukin-18 in a Case of Multiple Drug-Resistant Adult-Onset Still's Disease

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Abstract

A patient with adult-onset Still's disease (AOSD) resistant to multiple drugs was treated in our hospital. Even biologics that block tumor necrosis factor (TNF) were ineffective. However, this patient responded quite well to tocilizumab, an interleukin (IL)-6 receptor blocker, suggesting that it is among the promising candidate drugs for multiple-drug resistant AOSD. Although the serum levels of most inflammatory markers such as C-reactive protein (CRP) and ferritin were reduced promptly by tocilizumab, that of IL-18 remained high. Thus, IL-18 is considered to have a further upstream position than IL-6 or to be at the same level as IL-6 in the inflammatory cascade of AOSD. This finding casts light on the pathogenesis of AOSD, and drugs that target IL-18 may prove beneficial in the treatment of this inflammatory disease.

Key words: adult-onset Still's disease, IL-6, IL-18, tocilizumab

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Introduction

Adult-onset Still's disease (AOSD) is a systemic inflammatory disease characterized by a high spiking fever, arthritis, evanescent rash, and certain laboratory findings including abnormal liver function and elevated acute-phase proteins (1). AOSD is of unknown etiology although infectious triggers have been suggested (2, 3). The mainstay of treatment is glucocorticoids and/or non-steroidal anti-inflammatory drugs (NSAIDs) (4), but in addition, immunosuppressants, such as methotrexate (MTX) (5), cyclosporine A (CyA) (6), TNF blockers (7-10) or an IL-1 blocker (11), are sometimes necessary. Some patients are refractory even to the combination of the drugs mentioned above.

Recently, an IL-6 receptor blocker has been developed and made available in the clinical field. This agent, called tocilizumab, is a humanized anti-IL-6 receptor (IL-6R) antibody; it is used for Castleman's disease (12), juvenile idiopathic arthritis (JIA) (13), and rheumatoid arthritis (14). As IL-6 is a key player in the induction of various acute-phase proteins (15) and AOSD shares some characteristics with JIA, tocilizumab is promising as an agent for multiple-drug refractory AOSD. Indeed, we successfully treated such a patient with tocilizumab, for whom even treatment with the TNF blockers, infliximab and etanercept, had not proven effective. In the course of the treatment, the levels of both CRP and ferritin decreased rapidly, but interestingly, that of IL-18 did not. We discuss the implication of this phenomenon in light of certain reports in the literature.

Case Report

A 25-year-old man was admitted to a local hospital with fever and arthritis of the knees. As he exhibited marked leukocytosis (>20,000/μL) along with an elevated level of CRP (>20 mg/dL), infectious diseases were suspected at first. Treatment with various antibiotics did not ameliorate the symptoms, and no bacterium or virus was detected. Eventually, an evanescent salmon-colored rash appeared on his trunk. Rheumatoid factor, anti-nuclear antibody and anti-neutrophil cytoplasmic antibody (ANCA) were all negative. AOSD was suspected and the patient was treated with (i) prednisolone (PSL, 40 mg/day), (ii) intravenous methylpred-

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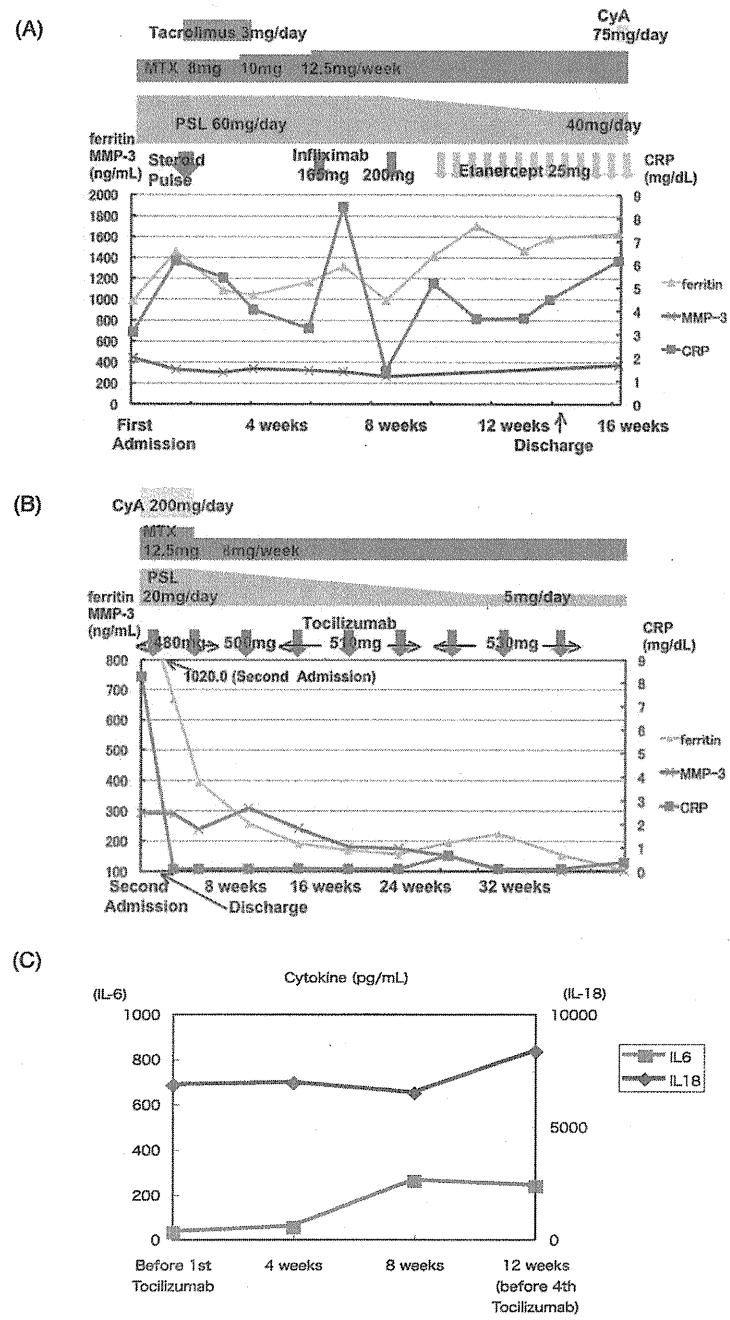

Figure 1. Clinical course of the patient before (A) and after (B) the treatment with tocilizumab. (C) The serum levels of IL-6 and IL-18.

nisolone 1 g/day for 3 days (steroid pulse) ×2 times, (iii) azathioprine (100 mg/day), and (iv) MTX (8 mg/week), but the levels of CRP, ferritin, and white blood cells (WBCs) did not normalize. He was referred and admitted to this hospital about 2 months after the onset of the symptoms.

On examination, the patient presented as obviously ill. His body temperature was 37.1℃, pulse 89 beats per minute, and blood pressure 125/62 mmHg. His weight was 60.0 kg and height 169 cm. Chest sounds were normal. The abdomen was flat and soft and bowel sounds were normal. The liver and spleen were not palpable. Skin rash was not observed. The leukocyte count was $25.37\times10^3/\mu L$ (92.9% neutrophil), hemoglobin level 13.8 g/dL, and platelet count $266\times10^3/\mu L$. The CRP level was 7.19 mg/dL, ferritin 1890 ng/mL, and matrix metalloproteinase-3 (MMP-3) 339.0 ng/mL. The aspartate aminotransferase level was not increased (16 IU/L, normal range 10.0-37.0) but lactate dehydrogenase (LDH) was slightly elevated (237 IU/L, normal range 107.0-220.0). No bone erosion was observed by X-ray. The patient was treated with PSL (60 mg/day) and MTX (8 mg/week), and one course of steroid pulse was performed again, followed by tacrolimus (3 mg/day). General fatigue persisted

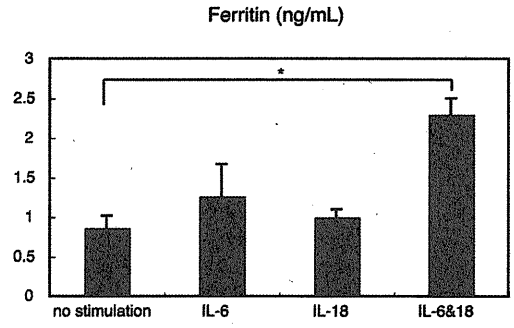

Figure 2. Monocytes were sorted by magnetic beads(MACS, Miltenyi Biotec, Auburn, CA) from peripheral blood mononuclear cells derived from healthy controls (Institutional Review Board ID No. 06-060-1). The cells were cultured with or without the presence of IL-6 and/or IL-18 (10 ng/mL each) for 15 hours, and the level of ferritin in the supernatant was quantified using ELISA. *: $p<0.05$ by Student's t-test. The data are representative of three independent experiments.

and the level of CRP remained high, as well as that of ferritin (Fig. 1A). The dose of MTX was increased to 10 mg/week and then to 12.5 mg/week with no evident improvement. As the blood levels of tacrolimus remained low, it was discontinued and infliximab (3 mg/kg, 165 mg in total) was begun with the result of a decrease in the CRP level. After the second infusion of infliximab (200 mg), however, the CRP level rebounded. Infliximab was switched to etanercept and the patient was discharged. However, the CRP level did not normalize in the outpatient clinic of this hospital and it was difficult to taper the dose of PSL. CyA (75 mg/day) was added and etanercept was discontinued. The dose of CyA was increased to 200 mg/day, without notable benefit. Finally, after discussion with the patient and his family, we decided to use tocilizumab. After the first treatment with tocilizumab (480 mg, 8 mg/kg), the level of CRP dropped to below 0.1 mg/dL. The patient also reported that his general fatigue disappeared. He was treated with tocilizumab every 4 weeks and the levels of ferritin and MMP-3 gradually and consecutively came into the normal range (Fig. 1B). As reported previously (16), the level of IL-6 elevated after the initiation of tocilizumab therapy, however, the level of IL-18 remained extremely high (more than 5,000 pg/mL, Fig. 1C). Almost 16 months have passed since the first treatment with tocilizumab. Now we use 560 mg (9 mg/kg) of the biologic every 5 weeks and no sign of relapse has been observed.

Discussion

In this case, multiple immunosuppressant drugs, including anti-rheumatic biologics that block TNF-α, were ineffective, but tocilizumab displayed a marked effect on both clinical symptoms and laboratory findings. Although there are reported cases in which TNF blockers were effective against AOSD, the response rate was not particularly robust. Recently, tocilizumab has been made available clinically and an increasing number of cases have been reported in which it was used successfully against the disease (17-22). Its response rate is, at present, unclear. Tacrolimus was reported to be useful against multiple-drug resistant AOSD (23), yet it was not beneficial in this case, probably because a sufficient blood concentration of the drug was not obtained.

One of the particularly interesting findings in this case is that the level of IL-18 remained high even though the levels of CRP, ferritin and MMP-3 were reduced markedly by tocilizumab (Fig. 1B and C). IL-18 was reported to be increased in AOSD patients, and it was quite high in this case. The fact that treatment with tocilizumab did not reduce the IL-18 level suggests that IL-18 is located either upstream of, or at the same level as, IL-6 in the pathogenesis of AOSD. Indeed, in the above-mentioned case in which tacrolimus was effective (23), the level of IL-18 was also reduced, suggesting that tacrolimus blocked the inflammatory cascade of AOSD at more upstream position than tocilizumab.

In order to evaluate if IL-6 and IL-18 have an additive or synergistic effect on the production of ferritin, we added IL-6 and/or IL-18 to monocytes derived from healthy controls *in vitro* and analyzed the level of ferritin in the supernatant of the culture medium (Fig. 2). Only the addition of both IL-6 and IL-18 (10 ng/mL each) led to a significant increase in the protein level of ferritin, indicating that IL-6 and IL-18 have a synergistic effect on the production of ferritin from macrophage-lineage cells. Although this does not directly prove the role IL-18 plays in AOSD, it appears to afford collateral evidence that IL-18 is involved in the pathogenesis of the disease.

This case reveals the potential of tocilizumab to be a quite effective drug for refractory AOSD. It is also possible, however, that tocilizumab may have to be continued as long as the level of serum IL-18 remains high. In such a case, drugs that target IL-18 may prove beneficial to the patients.

Author's disclosure of potential Conflicts of Interest (COI).
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Marked Induction of c-Maf Protein during Th17 Cell Differentiation and Its Implication in Memory Th Cell Development*

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Until recently, effector T helper (Th) cells have been classified into two subsets, Th1 and Th2 cells. Since the discovery of Th17 cells, which produce IL-17, much attention has been given to Th17 cells, mainly because they have been implicated in the pathogenesis of various inflammatory diseases. We have performed transcriptome analysis combined with factor analysis and revealed that the expression level of c-Maf, which is considered to be important for Th2 differentiation, increases significantly during the course of Th17 differentiation. The IL-23 receptor (IL-23R), which is important for Th17 cells, is among putative transcriptional targets of c-Maf. Interestingly, the analysis of c-Maf transgenic Th cells revealed that the overexpression of c-Maf did not lead to the acceleration of the early stage of Th17 differentiation but rather to the expansion of memory phenotype cells, particularly with Th1 and Th17 traits. Consistently, mouse wild-type memory Th cells expressed higher mRNA levels of c-Maf, IL-23R, IL-17, and IFN-γ than control cells; in contrast, *Maf*$^{-/-}$ memory Th cells expressed lower mRNA levels of those molecules. Thus, we propose that c-Maf is important for the development of memory Th cells, particularly memory Th17 cells and Th1 cells.

Acquired immune responses have been divided into two major categories according to the cytokine-production patterns of T helper (Th) cells. Th1 cells produce abundant IFN-γ and play important roles in cellular immune responses. On the other hand, Th2 cells produce various cytokines involved in humoral immunity, such as IL-4. It has been a predominant concept that a skewed balance of Th1/Th2 responses could lead to pathological conditions like autoimmune diseases. Recently, Th17 cells have been discovered as the third type of effector Th cells that produce large amounts of proinflammatory cytokine IL-17A (IL-17) but only minimal amounts of IFN-γ or IL-4 (1, 2). Th17 cells have been shown to play important roles in the pathogenesis of various inflammatory disease models previously considered to be Th1 diseases, such as collagen-induced arthritis and experimental autoimmune encephalomyelitis (3, 4). Thus, Th17 cells have been receiving considerable attention from the viewpoint of the pathological basis of human inflammatory diseases.

Initially, Th17 cells were believed to be differentiated in the presence of IL-23; however, it was thereafter reported that the differentiation factors for Th17 cells are actually TGF-β and IL-6 and that IL-23 is a proliferation factor in mice (5–7). On the other hand, the possibility has been raised that IL-23 is not a mere growth factor for Th17 cells but is important for the differentiation (8–10) and/or proper function (11) of these cells. Recently, TGF-β-independent but IL-23-, IL-6-, and IL-1β-dependent Th17 differentiation has been reported (12). These nonconventional Th17 cells may be more important than conventional TGF-dependent Th17 cells in inflammatory conditions such as experimental autoimmune encephalomyelitis.

In terms of the intracellular mechanisms of Th17 differentiation, Stat3 seems to play an essential role (13, 14). This is not surprising because Stat3 is activated by phosphorylation occurring downstream of IL-6 and IL-23 signaling. In 2006, RAR-related orphan receptor (ROR)[3]γt was reported to be a master regulator transcription factor for Th17 differentiation (15); it is a nuclear receptor the ligand of which is as yet unknown. Another nuclear receptor, RORα, was also implicated to function synergistically with RORγt in Th17 differentiation (16). The entire network of transcription factors in Th17 cells, however, remains to be elucidated. Thus, we first tried to shed light on the network and encountered the transcription factor c-Maf.

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[3] The abbreviations used are: ROR, RAR-related orphan receptor; qRT-PCR, quantitative RT-PCR; FC, fold change; cRNA, complementary RNA; IL-23R, IL-23 receptor; luc, luciferase; MARE, Maf recognition element; Tg, transgenic.

EXPERIMENTAL PROCEDURES

Mice—C57BL/6 mice were purchased from CLEA Japan, Inc., and T cell-specific c-Maf Tg mice (under the control of the human CD2 promoter and locus control region) were described previously (17). All the mice were maintained under specific pathogen-free conditions. All animal experiments were performed with the approval of the Animal Study Committee of Saitama Medical University and conformed to relevant guidelines and laws.

Th1/2/17 Cell Differentiation in Vitro—Mouse naive Th cells were purified from mouse spleens using a magnetic sorter and microbeads (AutoMACS system and $CD4^+CD62L^+$ T Cell Isolation Kit II, Miltenyi Biotec). They were cultured in RPMI 1640 medium containing 10% FCS (culture medium) and stimulated with plate-bound anti-CD3 and anti-CD28 mAbs (1 μg/ml each) for 3 days. Th1 cells were cultured with 10 ng/ml IL-12 and 10 μg/ml anti-IL-4 mAb; Th2 cells with 10 ng/ml IL-4 and 10 μg/ml anti-IFN-γ mAb; and Th17 cells with 10 ng/ml each of IL-6 and IL-23, 3 ng/ml TGF-β and 10 μg/ml each of anti-IFN-γ and anti-IL-4 mAbs. All of the antibodies were purchased from BD Biosciences, and the cytokines were purchased from R&D Systems.

GeneChip Analysis—Total RNA was used for cDNA synthesis by reverse transcription followed by the synthesis of biotinylated cRNA through *in vitro* transcription. After cRNA fragmentation, we performed hybridization with a mouse A430 GeneChip (Affymetrix). The raw data were analyzed using Affymetrix Microarray Suite (version 5.0) and normalized.

qRT-PCR and ELISA—We performed qRT-PCR analysis using an ABI PRISM 7000 Sequence Detection System with TaqMan Gene Expression Assay probes. The GAPDH expression level was used as the internal control. As to the analysis of cytokine production of Th cells, the cells were stimulated with phorbol 12-myristate 13-acetate (40 ng/ml) and ionomycin (1 μg/ml) for 5 h in the culture medium. Then, RNA was extracted from the cells, and the supernatant was subjected to ELISA. The mouse IL-17 ELISA kit was from R&D Systems.

Intracellular Staining of Transcription Factors and Cytokines—Mouse Th cells differentiated *in vitro* were preincubated with an anti-mouse CD16/CD32 (Fcγ receptor) mAb for 15 min on ice to block nonspecific staining. The cells were then fixed and permeabilized with BD Cytofix/Cytoperm (BD Bioscience) and stained with the primary Abs (anti-c-Maf Ab, M-153; anti-GATA-3 Ab, HG3-31, Santa Cruz Biotechnology). They were then stained with appropriate secondary Abs conjugated with Alexa Fluor 488 (Invitrogen). For intracellular cytokine staining, Th cells were cultured in the culture medium in the presence of phorbol 12-myristate 13-acetate (40 ng/ml) and ionomycin (1 μg/ml) for 5 h. In the last 1 h, monensin (Golgistop) was added. The cells were then fixed and permeabilized with BD Cytofix/Cytoperm and intracellularly stained with anti-IFN-γ-FITC plus anti-IL-17-PE, or anti-IFN-γ-FITC plus anti-IL-4-PE Abs (all reagents were from BD Biosciences). Stained cells were analyzed by FACScan or FACSCanto (BD Biosciences).

Factor Analysis—Transcription factors whose expression levels (normalized signals) were higher than 100 under at least one of the Th cell differentiation conditions were selected. Forty-three probes (including overlapping probes for the same genes) were subjected to factor analysis using SPSS software (version 15.0). By the unweighted least-squares method, two principal factors were extracted, and, after rotation by the varimax method with Kaiser normalization, each gene was positioned on a plane defined by Factors 1 and 2 according to its factor loadings.

Luciferase Assay—To construct the reporter plasmid pTA-*Il23r*-luc, the mouse *Il23r* promoter region (-1440 to +110) was ligated in the NheI and XhoI gap of the pTA-luc plasmid (Clontech). The following primers were used for PCR: 5′-GCT AGC TGG AGG CAT TTC CTC AGC TG-3′ (sense) and 5′-CTC GAG CTC AGG AAT TAG GGT CTC CT-3′ (antisense). A deletion mutant of pTA-*Il23r*-luc, which lacks a putative Maf binding site (MARE-like element) was constructed as described previously (18). DNA transfection and luciferase assays were performed as described previously (19, 20). Briefly, the reporter plasmid was transfected along with a c-Maf expression vector (21), a GATA-3 expression vector (22), and/or the control vector pcDNA3.1 into HEK293T cells using FuGENE 6 (Roche Applied Science). After 24 h, the cells were harvested, and luciferase activity was measured.

Statistical Analyses—*Error bars* indicate S.D. Student's *t* test was used for statistical analyses (*, $p < 0.05$ and **, $p < 0.01$).

RESULTS

Transcriptome Analysis of Th1/2/17 Cells during Course of Their Differentiation—First, we obtained the basic profiles of Th1/2/17 cell differentiation. Mouse naive Th cells were sorted and cultured under Th1, Th2, and Th17 cell differentiation conditions, and harvested on days 1 and 3. We also harvested Th0 cells on day 1. RNA extracted from the cells was subjected to transcriptome analysis using GeneChip. The expression levels of genes encoding IFN-γ, IL-4, and IL-17, which are "signature" cytokines produced by Th1, Th2, and Th17 cells, respectively, were up-regulated significantly in each subset, indicating that the differentiation conditions were appropriate (Fig. 1*A*).

Factor Analysis of Transcription Factors—To focus on transcription factors that are specifically up-regulated during the course of Th1/2/17 differentiation, we selected transcription factor genes whose expression levels (signals) were >100 in at least one of the Th conditions that we examined. Then, we performed factor analysis using the seven sets of data (days 1 and 3 for Th1/2/17 cells and day 1 for Th0 cells). Two principal "Factors" were extracted, and each gene is plotted in the two-dimensional space defined by Factors 1 and 2, according to its factor loading (Fig. 1*B*). As expected, dots representing RORγ (one dot) and RORα (four dots, because four different probes are attributed to RORα in the chip we used) were closely located in this diagram and both were high in Factor 2 but not in Factor 1. Interestingly, all the dots representing c-Maf were plotted nearest to the dot representing *Rorc*, suggesting that c-Maf is even more closely related to RORγ than RORα is to RORγ. Histograms of the GeneChip data of *Maf*, *Rorc*, and *Rora* are shown in Fig. 1*C*. Consistent with Fig. 1*B*, *Maf* and *Rorc* demonstrated a more Th17-specific expression pattern than *Rora*.