RASFs in vivo (5). The high expression of NOD1 which we found in gout points to a pathophysiological role of NOD1 also in this disease. Similar to the NOD-like receptor protein 3 (NLRP3) inflammasome, which is well known to play an important role in gout, NOD1 has been shown to bind to caspase 1 and promote IL-1 secretion (20).

Stimulation of NOD1 led to the production of a wide range of pro-inflammatory mediators and MMPs in RASFs. In addition there was a synergistic effect in the production of IL-6 by stimulation of NOD1 and TLRs. Surprisingly, MDMs reacted much less to stimulation with Tri-DAP than synovial fibroblasts. Together these data underline the important role of synovial fibroblasts as cells of the innate immune system that rapidly integrate and elicit an innate immune response. Of special interest seems the fact that stimulation of NOD1 selectively induced increased expression of other peptidoglycan sensing PRRs, namely TLR2 and NOD2, but not TLR4 and TLR3. This points to a directed chain reaction for proper immune defense rather than to a general increase in PRRs after sensing of invading pathogens. Exogenous ligands for PRRs such as the NOD2 ligand MDP or bacterial peptidoglycans were described to be present in joints of RA patients (19, 21). Also more and more endogenous ligands for PRRs are found and it has become clear that in addition to their role in immune defence, PRRs are also important sensors of tissue damage. No endogenous ligands for the NLRs NOD1 and NOD2 have been found up to now. In fact, it has to be mentioned that also direct interactions of NOD1 and NOD2 with their respective ligands were not demonstrated yet. Thereby indirect mechanisms by which these NLRs respond to their ligands cannot be excluded. The current study suggests that the presence of NOD1 is essential for the phosphorylation of IRAK1 after recruitment to MyD88. This is particularly interesting since NLRP12, a member of the pyrin-domain containing NLR subfamily, has been shown to bind IRAK1 and to block its phosphorylation (22). In general, evidence accumulates that NLRs tend to associate with other proteins to form large complexes and that the composition of this complexes determines the biological function of the various NLRs (23).

In summary, we show increased expression of NOD1 in synovial tissues of RA patients and a strong pro-inflammatory response of RASFs after stimulation of NOD1 with its ligand Tri-DAP. Downregulation of NOD1 led to reduced levels of IL-6 after TLR2 as well as IL-1β stimulation and blocked phosphorylation of IRAK1. Therefore, our data indicate that NOD1 alone and in interaction with other inflammatory activators plays an important role in the chronic and destructive joint inflammation in RA.

Acknowledgements: We thank Peter Künzler, Ferenc Pataky and Maria Comazzi for invaluable technical advice and assistance.

REFERENCES

1. Zhang X, Glogauer M, Zhu F, Kim TH, Chiu B, Inman RD. Innate immunity and arthritis: neutrophil Rac and toll-like receptor 4 expression define outcomes in infection-triggered arthritis. Arthritis Rheum. 2005;52(4):1297-304.
2. Deng GM, Nilsson IM, Verdrengh M, Collins LV, Tarkowski A. Intra-articularly localized bacterial DNA containing CpG motifs induces arthritis. Nat Med. 1999;5(6):702-5.
3. Strober W, Murray PJ, Kitani A, Watanabe T. Signalling pathways and molecular interactions of NOD1 and NOD2. Nat Rev Immunol. 2006;6(1):9-20.
4. Franchi L, Warner N, Viani K, Nunez G. Function of Nod-like receptors in microbial recognition and host defense. Immunol Rev. 2009;227(1):106-28.
5. Brentano F, Schorr O, Gay RE, Gay S, Kyburz D. RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via Toll-like receptor 3. Arthritis Rheum. 2005;52(9):2656-65.
6. Ospelt C, Brentano F, Rengel Y, Stanczyk J, Kolling C, Tak PP, et al. Overexpression of toll-like receptors 3 and 4 in synovial tissue from patients with early rheumatoid arthritis: toll-like receptor expression in early and longstanding arthritis. Arthritis Rheum. 2008;58(12):3684-92.
7. Ospelt C, Brentano F, Jungel A, Rengel Y, Kolling C, Michel BA, et al. Expression, regulation, and signaling of the pattern-recognition receptor nucleotide-binding oligomerization domain 2 in rheumatoid arthritis synovial fibroblasts. Arthritis Rheum. 2009;60(2):355-63.
8. Hisamatsu T, Suzuki M, Podolsky DK. Interferon-gamma augments CARD4/NOD1 gene and protein expression through interferon regulatory factor-1 in intestinal epithelial cells. J Biol Chem. 2003;278(35):32962-8.
9. Fritz JH, Girardin SE, Fitting C, Werts C, Mengin-Lecreulx D, Caroff M, et al. Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and NOD1- and NOD2-activating agonists. Eur J Immunol. 2005;35(8):2459-70.
10. Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L, et al. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nat Immunol. 2003;4(7):702-7.
11. Viala J, Chaput C, Boneca IG, Cardona A, Girardin SE, Moran AP, et al. Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. Nat Immunol. 2004;5(11):1166-74.
12. Welter-Stahl L, Ojcius DM, Viala J, Girardin S, Liu W, Delarbre C, et al. Stimulation of the cytosolic receptor for peptidoglycan, Nod1, by infection with Chlamydia trachomatis or Chlamydia muridarum. Cell Microbiol. 2006;8(6):1047-57.
13. Opitz B, Puschel A, Beermann W, Hocke AC, Forster S, Schmeck B, et al. Listeria monocytogenes activated p38 MAPK and induced IL-8 secretion in a nucleotide-binding oligomerization domain 1-dependent manner in endothelial cells. J Immunol. 2006;176(1):484-90.
14. van Heel DA, Ghosh S, Butler M, Hunt K, Foxwell BM, Mengin-Lecreulx D, et al. Synergistic enhancement of Toll-like receptor responses by NOD1 activation. Eur J Immunol. 2005;35(8):2471-6.
15. McGovern DP, Hysi P, Ahmad T, van Heel DA, Moffatt MF, Carey A, et al. Association between a complex insertion/deletion polymorphism in NOD1 (CARD4) and susceptibility to inflammatory bowel disease. Hum Mol Genet. 2005;14(10):1245-50.
16. Hysi P, Kabesch M, Moffatt MF, Schedel M, Carr D, Zhang Y, et al. NOD1 variation, immunoglobulin E and asthma. Hum Mol Genet. 2005;14(7):935-41.

17. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 1988;31(3):315-24.
18. Ospelt C, Brentano F, Jungel A, Rengel Y, Kolling C, Michel BA, et al. Expression, Regulation, and Signaling of the Pattern-Recognition Receptor Nucleotide-Binding Oligomerization Domain 2 in Rheumatoid Arthritis Synovial Fibroblasts. Arthritis and Rheumatism. 2009;60(2):355-63.
19. Joosten LA, Heinhuis B, Abdollahi-Roodsaz S, Ferwerda G, Lebourhis L, Philpott DJ, et al. Differential function of the NACHT-LRR (NLR) members Nod1 and Nod2 in arthritis. Proc Natl Acad Sci U S A. 2008;105(26):9017-22.
20. Yoo NJ, Park WS, Kim SY, Reed JC, Son SG, Lee JY, et al. Nod1, a CARD protein, enhances pro-interleukin-1beta processing through the interaction with pro-caspase-1. Biochem Biophys Res Commun. 2002;299(4):652-8.
21. van der Heijden IM, Wilbrink B, Tchetverikov I, Schrijver IA, Schouls LM, Hazenberg MP, et al. Presence of bacterial DNA and bacterial peptidoglycans in joints of patients with rheumatoid arthritis and other arthritides. Arthritis Rheum. 2000;43(3):593-8.
22. Williams KL, Lich JD, Duncan JA, Reed W, Rallabhandi P, Moore C, et al. The CATERPILLER protein monarch-1 is an antagonist of toll-like receptor-, tumor necrosis factor alpha-, and Mycobacterium tuberculosis-induced pro-inflammatory signals. J Biol Chem. 2005;280(48):39914-24.
23. Ting JP, Duncan JA, Lei Y. How the noninflammasome NLRs function in the innate immune system. Science. 2010;327(5963):286-90.

FIGURE LEGENDS

Figure 1. A) Representative pictures of NOD1 expression in RA and OA synovial tissues are shown. As negative controls, NOD1-blocking peptide (inset in upper panel) or isotype controls (inset in lower panel) were used. Nuclei were counterstained with hematoxylin. Positive staining appears in red. Stronger staining intensity of lining and sublining areas in RA patients was found after scoring (Mann-Whitney test. * = $p < 0.05$). B) RA synvovial tissues were double stained for NOD1 (green) and CD68 (red) for macrophages. C) RA synvovial tissues were double stained for NOD1 (green) and vimentin (red) for mesenchymal cells. Nuclei appear in blue (DAPI). Arrows point at double stained cells which are magnified in the insets. D) Strong staining for NOD1 was seen in synovial tissues from patients with gout, but not in patients with psoriasis arthritis (PA).

Figure 2. Expression of NOD1 mRNA and protein in RASFs, OASFs, healthy controls (HC) PBMCs and MDMs. A) Similar expression levels of NOD1 mRNA (n=4-5) and of NOD1 protein (n=5-6) were found in the various cell types tested. Delta Ct = cycle of threshold NOD1-cycle of threshold 18S; MFI = mean fluorescence intensity. **B)** After stimulation of RASFs (n=6) with Tri-DAP, Pam3, PIC, LPS, IL-1β or TNF-α for 24h, levels of NOD1 mRNA only significantly changed after PIC stimulation as calculated by Wilcoxon matched pairs test. Also, NOD1 protein was more abundant after 24h stimulation of RASFs with PIC (n=6; non-parametric Friedman test followed by Dunn's multiple comparison test). *=$p < 0.05$. **C)** NOD1 mRNA or protein expression did not change in MDMs (n=3) after incubation with TLR ligands for 24h.

Figure 3. Tri-DAP induces the production of proinflammatory cytokines, chemokines, matrix degrading enzymes and peptidoglycan-sensing PRRs in RASFs. Incubation of RASFs (n=6) with 10 ng/ml Tri-DAP for 8h and 24h significantly induced mRNA levels of

A) IL-6 and CCL5, and **B)** MMP-1, MMP-3, and MMP-13 but not MMP-9. From the measured pattern-recognition receptors only TLR2 (n=8) **(C)** and NOD2 (n=3-8) were significantly changed **(D).** Non-parametric Friedman test followed by Dunn's multiple comparison test was used. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Figure 4. Tri-DAP specificity and NOD1 silencing. A) RASFs (n=2-6) were stimulated with Tri-DAP alone or Tri-DAP with polymyxin B for 24h and IL-6 levels in the cell supernatants were measured by ELISA. **B)** RASFs (n=3-4) were transfected with control siRNA (sc) or NOD1 targeting siRNA (siNOD1) and NOD1 mRNA and protein expression was measured 24h and 48h after transfection. **C)** 24h after transfection of RASFs (n=6) with NOD1 siRNA or scrambled control siRNA (sc), cell were stimulated for 24h with Tri-DAP and IL-6 was measured in the supernatants. Non-parametric Friedman test followed by Dunn's multiple comparison test was used. * = $p < 0.05$.

Figure 5. Silencing of NOD1 downregulates TLR2 and IL-1β induced IL-6 production. A) TwoWay ANOVA analysis showed synergistic interaction of Tri-DAP stimulation with Pam3 and LPS in the production of IL-6 in RASFs (n=6). **B)** Knock-down of NOD1 in RASFs (n=6) led to decreased levels of IL-6 after Pam3 stimulation when compared to control transfected RASFs by Wilcoxon matched pairs test (left panel). Knock-down of NOD2 did not induce any change in IL-6 levels after stimulation with Pam3 (n=4) (right panel). **C**) Stimulation with heat inactivated *Staphylococcus aureus* (SA) or *Listeria monocytogenes* (LM) resulted in similar levels of IL-6 in NOD1 siRNA and control transfected cells (n=6) (left panel). Also, mRNA levels of TLR2 did not differ between NOD1 silenced and control RASFs, but were induced by stimulation with Pam3 (n=3) (right panel). **(D)** IL-6 levels were significantly lower in siNOD1 transfected cells than in control transfected cells after stimulation with IL-1β (n=6). Wilcoxon matched pairs test was used for

calculations. * = $p < 0.05$; ** = $p < 0.01$.

Figure 6. NOD1 influences IRAK1 phosphorylation. IRAK1 phosphorylation significantly increased after stimulation of control transfected RASFs with Pam3 for 20min, but not in NOD1 siRNA transfected cells (n=15). Wilcoxon matched pairs test was used for calculations. ** = $p < 0.01$

Supplemental Figure 1. Increased production of CCL5, MMPs, TLR2 and NOD2 protein after Tri-DAP stimulation. RASFs were incubated with 10 ng/ml Tri-DAP and levels of **A)** CCL5 (n=8) and **B)** MMP-1 (n=8) and MMP-3 (n=6) were measured in the supernatants by ELISA after 24h. Wilcoxon matched pairs test was used for calculations. * = $p < 0.05$; ** = $p < 0.01$. **C)** TLR2 protein was measured in RASFs 24h after stimulation with 10 ng/ml Tri-DAP by flow cytometry. **D)** NOD2 protein levels were determined by Western blotting 48h after stimulation with 10 ng/ml Tri-DAP or 10 ng/ml TNF-α (n=2).

Supplemental Figure 2. Time course of the induction of IL-6, CCL5, MMPs, TLR2 and NOD2 after Tri-DAP stimulation. RASFs (n=3) were incubated with 10 μg/ml Tri-DAP for the indicated time points and transcripts of **A)** IL-6, **B)** CCL5, **C)** MMP-1, MMP-3, MMP-9, MMP-13, and **D)** TLR2 and NOD2 were determined by real-time PCR. One representative time course experiment out of 3 is shown.

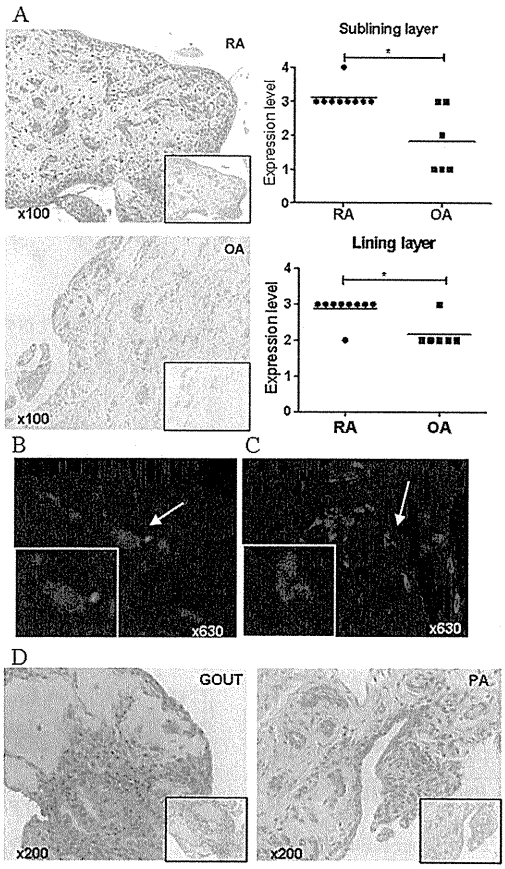

Figure 1. A) Representative pictures of NOD1 expression in RA and OA synovial tissues are shown. As negative controls, NOD1-blocking peptide (inset in upper panel) or isotype controls (inset in lower panel) were used. Nuclei were counterstained with hematoxylin. Positive staining appears in red. Stronger staining intensity of lining and sublining areas in RA patients was found after scoring (Mann-Whitney test. * = $p < 0.05$). **B)** RA synvovial tissues were double stained for NOD1 (green) and CD68 (red) for macrophages. **C)** RA synvovial tissues were double stained for NOD1 (green) and vimentin (red) for mesenchymal cells. Nuclei appear in blue (DAPI). Arrows point at double stained cells which are magnified in the insets. **D)** Strong staining for NOD1 was seen in synovial tissues from patients with gout, but not in patients with psoriasis arthritis (PA).

200x480mm (300 x 300 DPI)

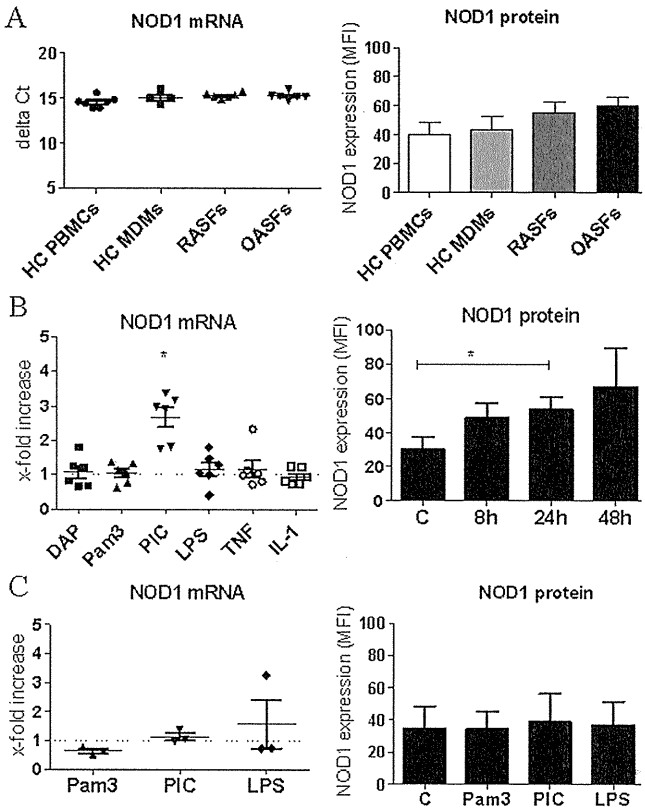

Figure 2. Expression of NOD1 mRNA and protein in RASFs, OASFs, healthy controls (HC) PBMCs and MDMs. A) Similar expression levels of NOD1 mRNA (n=4-5) and of NOD1 protein (n=5-6) were found in the various cell types tested. Delta Ct = cycle of threshold NOD1-cycle of threshold 18S; MFI = mean fluorescence intensity. **B)** After stimulation of RASFs (n=6) with Tri-DAP, Pam3, PIC, LPS, IL-1b or TNF-a for 24h, levels of NOD1 mRNA only significantly changed after PIC stimulation as calculated by Wilcoxon matched pairs test. Also, NOD1 protein was more abundant after 24h stimulation of RASFs with PIC (n=6; non-parametric Friedman test followed by Dunn's multiple comparison test). *=$p < 0.05$. **C)** NOD1 mRNA or protein expression did not change in MDMs (n=3) after incubation with TLR ligands for 24h.

83x158mm (300 x 300 DPI)

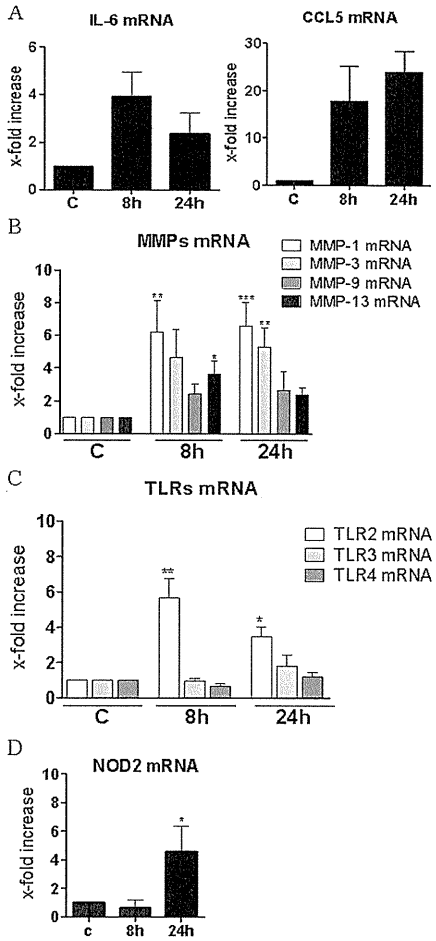

Figure 3. Tri-DAP induces the production of proinflammatory cytokines, chemokines, matrix degrading enzymes and peptidoglycan-sensing PRRs in RASFs. Incubation of RASFs (n=6) with 10 ng/ml Tri-DAP for 8h and 24h significantly induced mRNA levels of A) IL-6 and CCL5, and B) MMP-1, MMP-3, and MMP-13 but not MMP-9. From the measured pattern-recognition receptors only TLR2 (n=8) (C) and NOD2 (n=3-8) were significantly changed (D). Non-parametric Friedman test followed by Dunn's multiple comparison test was used. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

76x214mm (300 x 300 DPI)

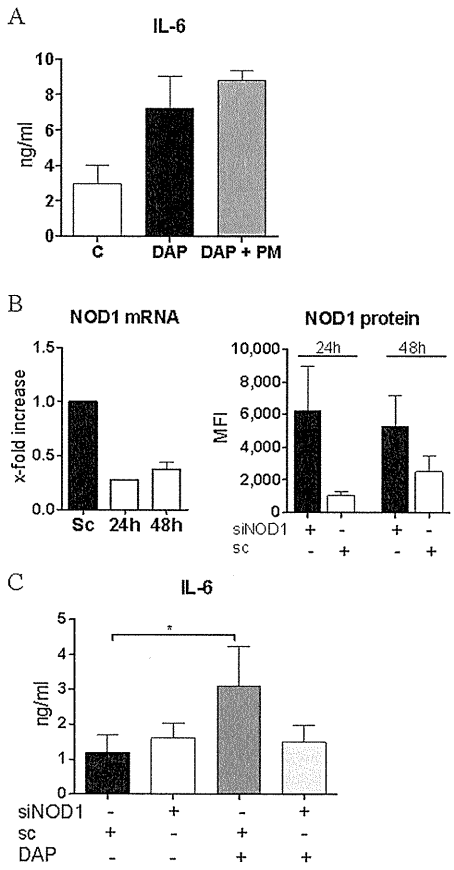

Figure 4. Tri-DAP specificity and NOD1 silencing. **A)** RASFs (n=2-6) were stimulated with Tri-DAP alone or Tri-DAP with polymyxin B for 24h and IL-6 levels in the cell supernatants were measured by ELISA. **B)** RASFs (n=3-4) were transfected with control siRNA (sc) or NOD1 targeting siRNA (siNOD1) and NOD1 mRNA and protein expression was measured 24h and 48h after transfection. **C)** 24h after transfection of RASFs (n=6) with NOD1 siRNA or scrambled control siRNA (sc), cell were stimulated for 24h with Tri-DAP and IL-6 was measured in the supernatants. Non-parametric Friedman test followed by Dunn's multiple comparison test was used. * = $p < 0.05$.

81x214mm (300 x 300 DPI)

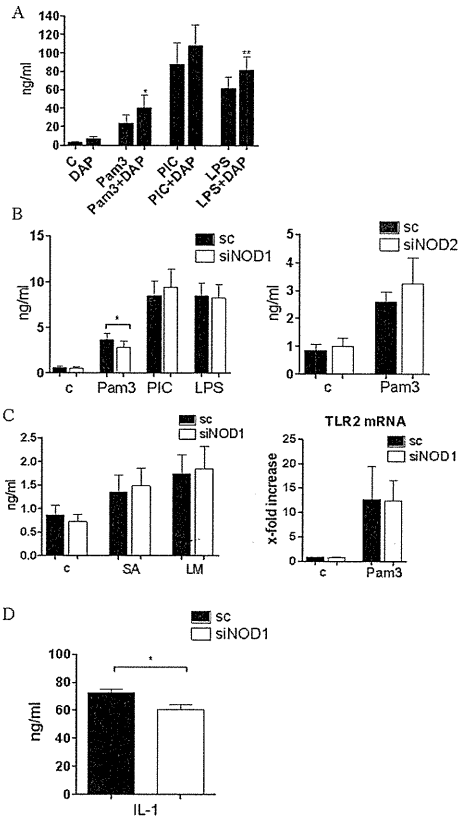

Figure 5. Silencing of NOD1 downregulates TLR2 and IL-1 induced IL-6 production. A) TwoWay ANOVA analysis showed synergistic interaction of Tri-DAP stimulation with Pam3 and LPS in the production of IL-6 in RASFs (n=6). **B)** Knock-down of NOD1 in RASFs (n=6) led to decreased levels of IL-6 after Pam3 stimulation when compared to control transfected RASFs by Wilcoxon matched pairs test (left panel). Knock-down of NOD2 did not induce any change in IL-6 levels after stimulation with Pam3 (n=4) (right panel). **C)** Stimulation with heat inactivated Staphylococcus aureus (SA) or Listeria monocytogenes (LM) resulted in similar levels of IL-6 in NOD1 siRNA and control transfected cells (n=6) (left panel). Also, mRNA levels of TLR2 did not differ between NOD1 silenced and control RASFs, but were induced by stimulation with Pam3 (n=3) (right panel). **D)** IL-6 levels were significantly lower in siNOD1 transfected cells than in control transfected cells after stimulation with IL-1 (n=6). Wilcoxon matched pairs test was used for calculations. * = $p < 0.05$; ** = $p < 0.01$.

83x214mm (300 x 300 DPI)

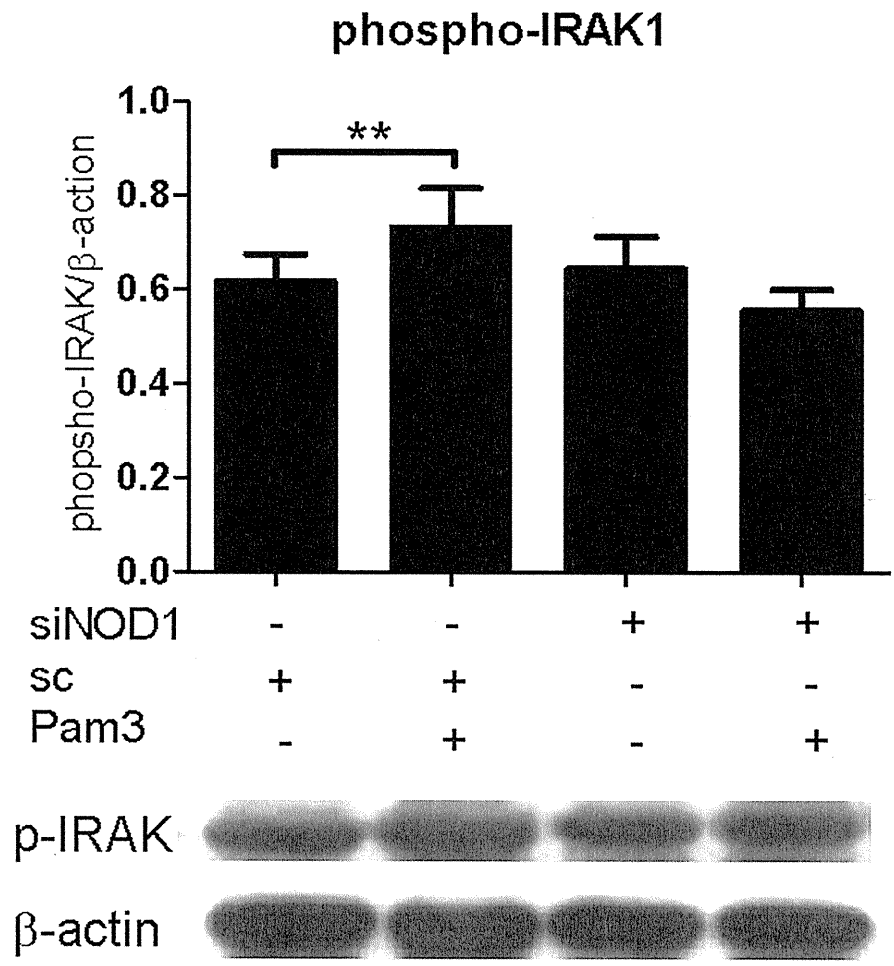

Figure 6. NOD1 influences IRAK1 phosphorylation. IRAK1 phosphorylation significantly increased after stimulation of control transfected RASFs with Pam3 for 20min, but not in NOD1 siRNA transfected cells (n=15). Wilcoxon matched pairs test was used for calculations. ** = $p < 0.01$

94x124mm (300 x 300 DPI)

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

Yoshihiro Yoshida, Mayuko Sakamoto, Kazuhiro Yokota, Kojiro Sato and Toshihide Mimura

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

(Intern Med 50: 1757-1760, 2011)
(DOI: 10.2169/internalmedicine.50.4771)

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-

Department of Rheumatology and Applied Immunology, Faculty of Medicine, Saitama Medical University, Japan
Received for publication November 1, 2010; Accepted for publication April 7, 2011
Correspondence to Dr. Kojiro Sato, satok@saitama-med.ac.jp

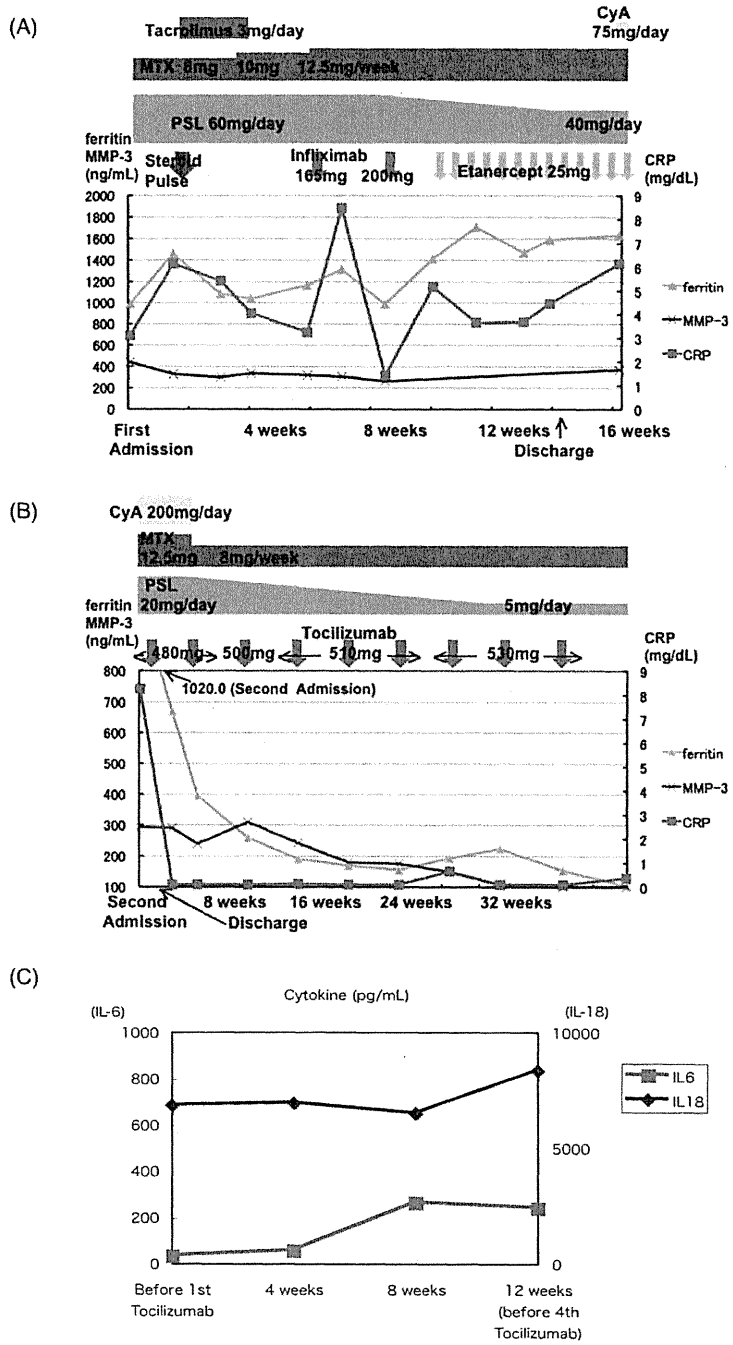

Figure 1. Clinical course of the patient before (A) and after (B) the treatment with tocilizumab. (C) The serum levels of IL-6 and IL-18.

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

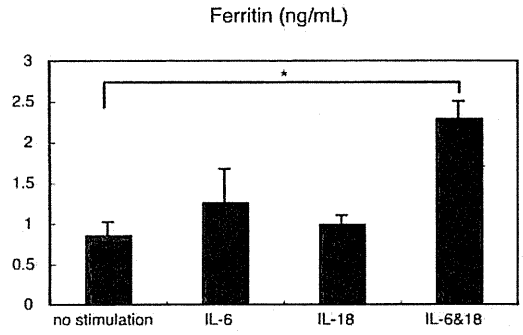

Figure 2. Monocytes were sorted by magnetic beads(MACS, Miltenyi Biotec, Auburn, CA) from peripheral blood mononuclear cells derived from healthy controls (Institutional Review Board ID No. 06-060-1). The cells were cultured with or without the presence of IL-6 and/or IL-18 (10 ng/mL each) for 15 hours, 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).
Toshihide Mimura, M.D.: Research funding, Chugai Pharmaceutical Co., Ltd.

References

1. Bywaters EG. Still's disease in the adult. Ann Rheum Dis **30**: 121-133, 1971.
2. Colebunders R, Stevens WJ, Vanagt E, Snoeck J. Adult Still's disease caused by *Yersinia enterocolitica* infection. Arch Intern Med **144**: 1880-1882, 1984.
3. Wouters JM, van der Veen J, van de Putte LB, de Rooij DJ. Adult onset Still's disease and viral infections. Ann Rheum Dis **47**: 764-767, 1988.
4. Masson C, Le Loet X, Liote F, et al. Adult Still's disease. Part II. Management, outcome, and prognostic factors. Rev Rhum Engl Ed **62**: 758-765, 1995.
5. Fautrel B, Borget C, Rozenberg S, et al. Corticosteroid sparing effect of low dose methotrexate treatment in adult Still's disease. J Rheumatol **26**: 373-378, 1999.
6. Marchesoni A, Ceravolo GP, Battafarano N, Rossetti A, Tosi S, Fantini F. Cyclosporin A in the treatment of adult onset Still's dis-

ease. J Rheumatol **24**: 1582-1587, 1997.
7. Kraetsch HG, Antoni C, Kalden JR, Manger B. Successful treatment of a small cohort of patients with adult onset of Still's disease with infliximab: first experiences. Ann Rheum Dis **60** (Suppl 3): iii55-iii57, 2001.
8. Kokkinos A, Iliopoulos A, Greka P, Efthymiou A, Katsilambros N, Sfikakis PP. Successful treatment of refractory adult-onset Still's disease with infliximab. A prospective, non-comparative series of four patients. Clin Rheumatol **23**: 45-49, 2004.
9. Fautrel B, Sibilia J, Mariette X, Combe B. Tumour necrosis factor alpha blocking agents in refractory adult Still's disease: an observational study of 20 cases. Ann Rheum Dis **64**: 262-266, 2005.
10. Husni ME, Maier AL, Mease PJ, et al. Etanercept in the treatment of adult patients with Still's disease. Arthritis Rheum **46**: 1171-1176, 2002.
11. Kotter I, Wacker A, Koch S, et al. Anakinra in patients with treatment-resistant adult-onset Still's disease: four case reports with serial cytokine measurements and a review of the literature. Semin Arthritis Rheum **37**: 189-197, 2007.
12. Nishimoto N, Sasai M, Shima Y, et al. Improvement in Castleman's disease by humanized anti-interleukin-6 receptor antibody therapy. Blood **95**: 56-61, 2000.
13. Yokota S, Miyamae T, Imagawa T, Katakura S, Kurosawa R, Mori M. Clinical study of tocilizumab in children with systemic-onset juvenile idiopathic arthritis. Clin Rev Allergy Immunol **28**: 231-238, 2005.
14. Nishimoto N, Terao K, Mima T, Nakahara H, Takagi N, Kakehi T. Mechanisms and pathologic significances in increase in serum interleukin-6 (IL-6) and soluble IL-6 receptor after administration of an anti-IL-6 receptor antibody, tocilizumab, in patients with rheumatoid arthritis and Castleman disease. Blood **112**: 3959-3964, 2008.
15. Holt I, Cooper RG, Hopkins SJ. Relationships between local inflammation, interleukin-6 concentration and the acute phase protein response in arthritis patients. Eur J Clin Invest **21**: 479-484, 1991.
16. Uchiyama Y, Yoshida H, Koike N, et al. Anti-IL-6 receptor antibody increases blood IL-6 level via the blockade of IL-6 clearance, but not via the induction of IL-6 production. Int Immunopharmacol **8**: 1595-1601, 2008.
17. Kishida D, Okuda Y, Onishi M, et al. Successful tocilizumab treatment in a patient with adult-onset Still's disease complicated by chronic active hepatitis B and amyloid A amyloidosis. Mod Rheumatol **21**: 215-218, 2011.
18. Kobayashi M, Takahashi Y, Yamashita H, Kaneko H, Mimori A. Benefit and a possible risk of tocilizumab therapy for adult-onset Still's disease accompanied by macrophage-activation syndrome. Mod Rheumatol **21**: 92-96, 2011.
19. Yoshimura M, Makiyama J, Koga T, et al. Successful treatment with tocilizumab in a patient with refractory adult-onset Still's disease (AOSD). Clin Exp Rheumatol **28**: 141-142, 2010.
20. Sumida K, Ubara Y, Hoshino J, et al. Etanercept-refractory adult-onset Still's disease with thrombotic thrombocytopenic purpura successfully treated with tocilizumab. Clin Rheumatol **29**: 1191-1194, 2010.
21. Matsumoto K, Nagashima T, Takatori S, et al. Glucocorticoid and cyclosporine refractory adult onset Still's disease successfully treated with tocilizumab. Clin Rheumatol **28**: 485-487, 2009.
22. Nakahara H, Mima T, Yoshio-Hoshino N, Matsushita M, Hashimoto J, Nishimoto N. A case report of a patient with refractory adult-onset Still's disease who was successfully treated with tocilizumab over 6 years. Mod Rheumatol **19**: 69-72, 2009.
23. Murakami K, Fujii T, Yukawa N, et al. Successful treatment of a patient with refractory adult Still's disease by tacrolimus. Mod Rheumatol **17**: 167-170, 2007.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 17, pp. 14963–14971, April 29, 2011

Marked Induction of c-Maf Protein during Th17 Cell Differentiation and Its Implication in Memory Th Cell Development*

Received for publication, January 6, 2011, and in revised form, February 23, 2011 Published, JBC Papers in Press, March 14, 2011, DOI 10.1074/jbc.M111.218867

Kojiro Sato[‡1,2], Fumihiko Miyoshi[‡1], Kazuhiro Yokota[‡], Yasuto Araki[‡], Yu Asanuma[‡], Yuji Akiyama[‡], Keigyou Yoh[§], Satoru Takahashi[¶], Hiroyuki Aburatani[‖], and Toshihide Mimura[‡]

From the [‡]Department of Rheumatology and Applied Immunology, Faculty of Medicine, Saitama Medical University, Saitama 350-0495, Japan, [§]Pathophysiology of Renal Diseases, Doctoral Program in Clinical Sciences, and [¶]Department of Anatomy and Embryology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki 305-8575, Japan, and the [‖]Department of Cancer Systems Biology, Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology, University of Tokyo, Tokyo 153-8904, Japan

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.

* This work was supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and grants from the Mochida Memorial Foundation for Medical and Pharmaceutical Research, the Naito Foundation, the Kanae Foundation for the Promotion of Medical Science, Uehara Memorial Foundation, and the Kato Memorial Bioscience Foundation.

[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: Morohongo 38, Moroyama, Iruma-gun, Saitama 350-0495, Japan. Tel.: 81-49-276-1462; Fax: 81-49-295-4849; Email: satok@saitama-med.ac.jp.

[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*.

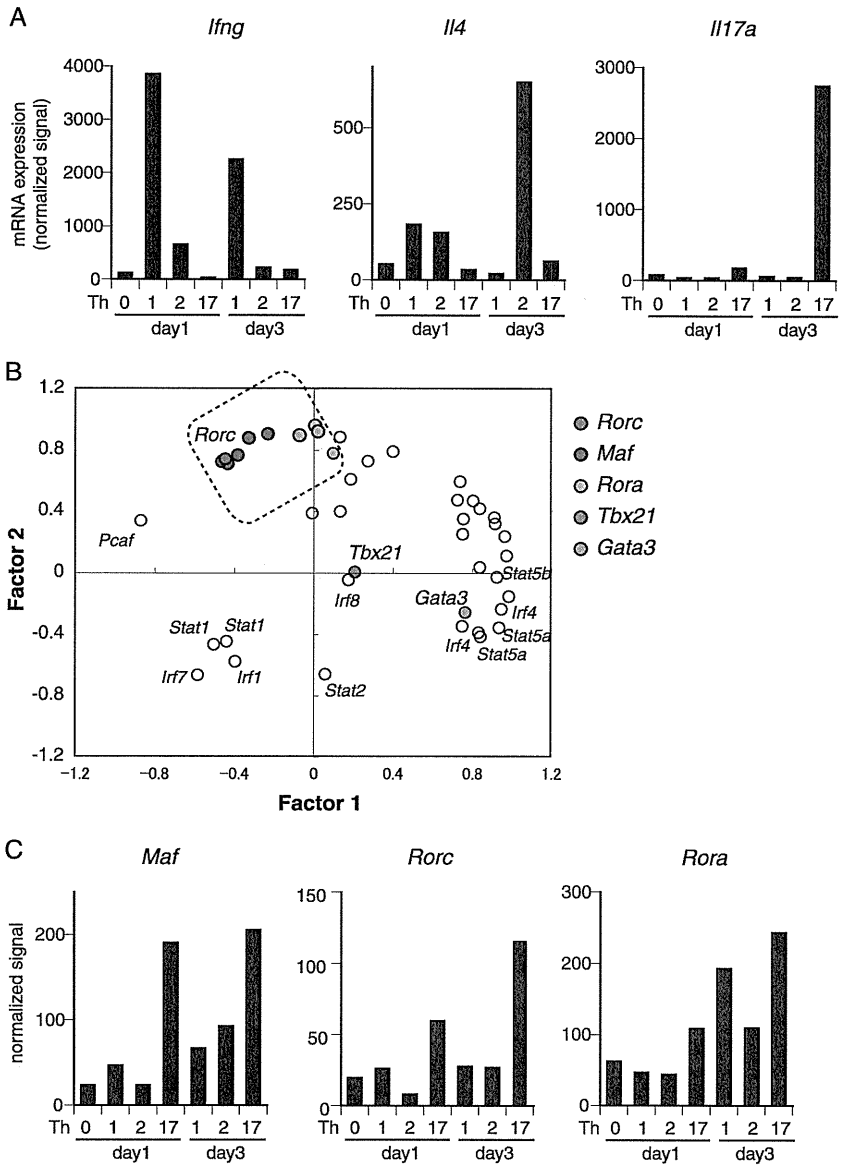

FIGURE 1. **Time series transcriptome analysis of genes representative of Th subsets at the mRNA level and factor analysis.** *A*, total RNA was obtained from Th cells cultured under the Th0, Th1, Th2, or Th17 conditions for 1 day (24 h) or 3 days (72 h). It was subjected to GeneChip analysis. As expected, IFN-γ, IL-4, and IL-17, the representative cytokines released from each Th subset, were induced in the relevant Th cells. *B*, distribution of transcription factors in a two-dimensional space defined by two factors extracted by factor analysis. Forty-three probes (including probes allotted to the same genes) were positioned in a plane defined by Factors 1 and 2. Note that all the probes representing RORγ, c-Maf, and RORα reside within a *box* with *dotted lines*. *C*, time series GeneChip data of *Maf* (data of one representative probe of five independent probes) and the known master regulator transcription factors for Th17 differentiation, namely, *Rorc* and *Rora* (data of one representative probe of four probes).

Expression of c-Maf but Not GATA-3 in Th17 Cells—The above data were rather unexpected in that c-Maf was reported to play important roles in Th2 differentiation (23). If c-Maf is highly expressed in Th17 cells, the question arises as to why they do not become Th2 cells. Indeed, similar levels of c-Maf protein expression were observed in the cells cultured under the Th2 and Th17 conditions (Fig. 2*A*, *left panel*). Interestingly, there was a large difference in GATA-3 expression level between the two subsets, whereas GATA-3 was barely detected in Th17 cells (Fig. 2*A*, *right panel*). This finding may explain why Th17 cells do not become Th2 cells. Indeed, IL-6 induces c-Maf by activating Stat3, which directly binds to the *Maf* promoter (24). GATA-3 is induced by IL-6, as well, but more indirect mechanisms are implicated (24). On the other hand, TGF-β, another important cytokine for the differentiation of mouse Th17 cells, is a potent inhibitor of Th2 development and has been shown to down-regulate GATA-3 (25). We added these cytokines to naive $CD4^+$ T cells separately or in combination and quantified the expression levels of two established markers of Th17 cells, IL-17 and IL-23R, in the screening for putative transcriptional targets of c-Maf. IL-6 alone induced *Il23r* but