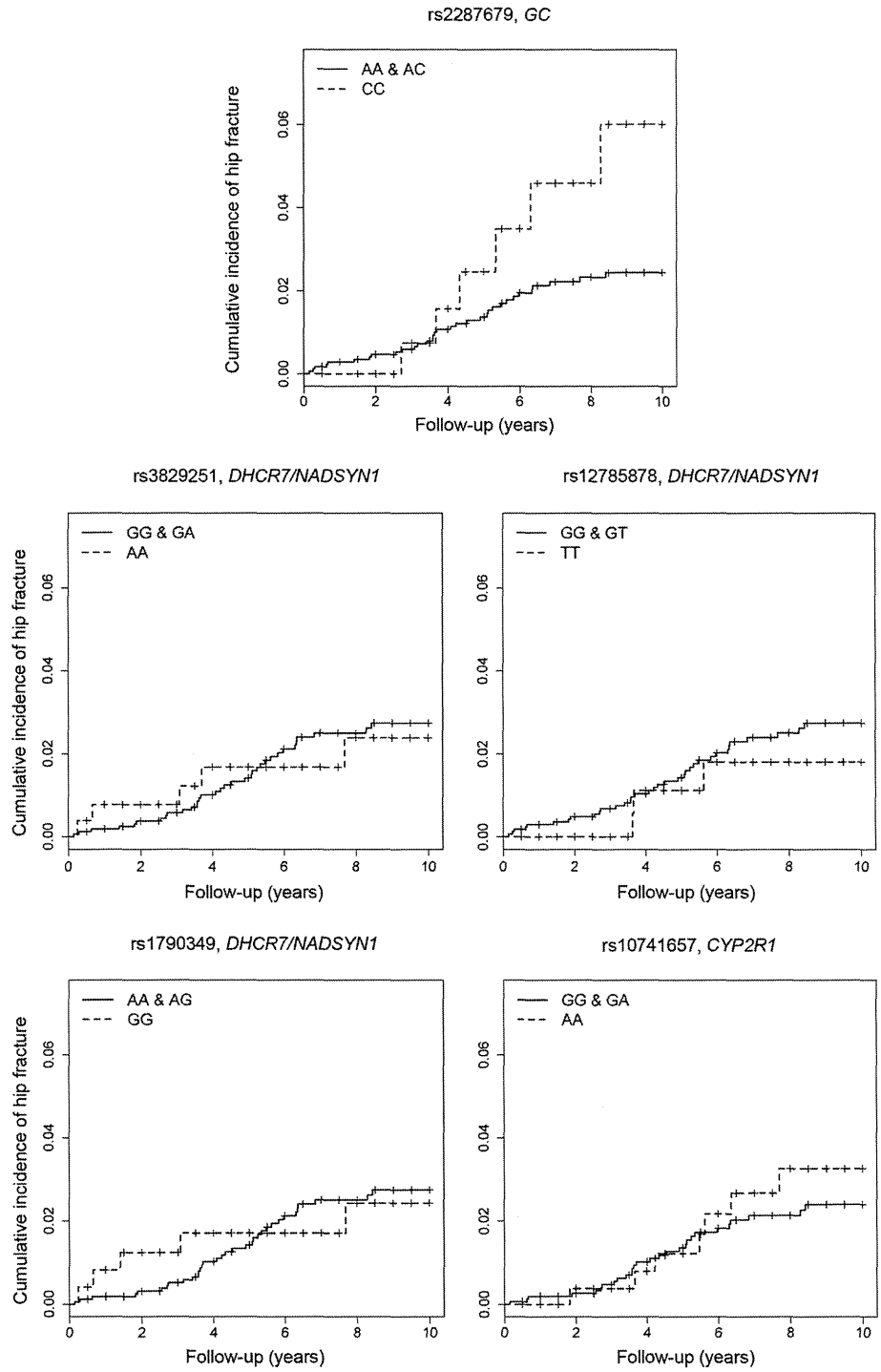


Figure 2 Cumulative incidence of hip fracture for patients who were homozygous or heterozygous for the non-risk allele and patients homozygous for the risk allele of each single nucleotide polymorphism (analyzed by the Kaplan-Meier method). Homozygous for the risk allele of rs2282679 (C) in the *GC* locus, a serum 25(OH)D-linked genetic variant, was significantly associated with the occurrence of hip fracture.

Abbreviations

25(OH)D: 25-hydroxyvitamin D; ALP: Alkaline phosphatase; BMD: bone mineral density; BMI: body mass index; CYP2R1: Cytochrome P450, family 2, subfamily R, polypeptide 1; DAS28: disease activity score in 28 joints; DBP: Vitamin D binding protein; DHCR7/NADSYN1: 7-dehydrocholesterol reductase/nicotinamide-adenine dinucleotide synthetase 1; DMARD: Disease modifying antirheumatic drug; GC: Group-specific component; GWAS: Genome-wide association studies; IORRA: Institute of Rheumatology, Rheumatoid Arthritis; IQR: inter-quartile range; J-HAQ: Japanese version of the Health Assessment Questionnaire; MAF: minor allele frequency; NSAID: non-steroidal anti-inflammatory drug; RA: rheumatoid arthritis; RF: rheumatoid factor; SE: standard error; SNP: single nucleotide polymorphism; TKR: total knee replacement.

Competing interests

The authors declared that they have no competing interests.

Authors' contributions

KI designed the study. KI, TF, YT, AT, HY and SM collected DNA samples and the data on fracture. SY and KI performed genotyping. SY and KI contributed to the statistical analyses. SY and KI wrote most of the manuscript. All authors contributed to writing and correcting the manuscript and have approved the final version.

Acknowledgements

We appreciate all DNA donors for making this study possible. We thank all members of the Institute of Rheumatology, Tokyo Women's Medical University for their successful management of the IORRA cohort. We are also grateful to Ms Kaori Arai and Ms Akiko Sato for their technical assistance and to Dr Eisuke Inoue for his guidance and suggestions in statistical analyses. This work was supported by grants provided by the Japanese Ministry of Education, Culture, Sports, Science and Technology Grant-in-Aid for Scientific Research (KI, SM), the Japanese Ministry of Health, Labour and Welfare (SM, HY) and the Japan Science and Technology Agency (HY).

Author details

[1]Institute of Rheumatology, Tokyo Women's Medical University, 10-22 Kawada, Shinjuku, Tokyo 162-0054, Japan. [2]Department of Orthopaedic Surgery, Keio University School of Medicine, 35 Shinano, Shinjuku, Tokyo 160-8582, Japan.

Received: 9 December 2013 Accepted: 13 March 2014
Published: 20 March 2014

References

1. DeLuca HF: **Overview of general physiologic features and functions of vitamin D.** *Am J Clin Nutr* 2004, **80:**1689S–1696S.
2. Anderson PH, Turner AG, Morris HA: **Vitamin D actions to regulate calcium and skeletal homeostasis.** *Clin Biochem* 2012, **45:**880–886.
3. Pludowski P, Holick MF, Pilz S, Wagner CL, Hollis BW, Grant WB, Shoenfeld Y, Lerchbaum E, Llewellyn DJ, Kienreich K, Soni M: **Vitamin D effects on musculoskeletal health, immunity, autoimmunity, cardiovascular disease, cancer, fertility, pregnancy, dementia and mortality-a review of recent evidence.** *Autoimmun Rev* 2013, **12:**976–989.
4. Shui IM, Mucci LA, Kraft P, Tamimi RM, Lindstrom S, Penney KL, Nimptsch K, Hollis BW, Dupre N, Platz EA, Stampfer MJ, Giovannucci E: **Vitamin D-related genetic variation, plasma vitamin D, and risk of lethal prostate cancer: a prospective nested case–control study.** *J Natl Cancer Inst* 2012, **104:**690–699.
5. Ritterhouse LL, Crowe SR, Niewold TB, Kamen DL, Macwana SR, Roberts VC, Dedeke AB, Harley JB, Scofield RH, Guthridge JM, James JA: **Vitamin D deficiency is associated with an increased autoimmune response in healthy individuals and in patients with systemic lupus erythematosus.** *Ann Rheum Dis* 2011, **70:**1569–1574.
6. Gennari C: **Calcium and vitamin D nutrition and bone disease of the elderly.** *Public Health Nutr* 2001, **4:**547–559.
7. Bhan A, Rao AD, Rao DS: **Osteomalacia as a result of vitamin D deficiency.** *Endocrinol Metab Clin North Am* 2010, **39:**321–331.
8. Ceglia L, Harris SS: **Vitamin D and its role in skeletal muscle.** *Calcif Tissue Int* 2013, **92:**151–162.
9. Orstavik RE, Haugeberg G, Uhlig T, Mowinckel P, Falch JA, Halse JI, Kvien TK: **Self reported non-vertebral fractures in rheumatoid arthritis and population based controls: incidence and relationship with bone mineral density and clinical variables.** *Ann Rheum Dis* 2004, **63:**177–182.
10. van Staa TP, Geusens P, Bijlsma JW, Leufkens HG, Cooper C: **Clinical assessment of the long-term risk of fracture in patients with rheumatoid arthritis.** *Arthritis Rheum* 2006, **54:**3104–3112.
11. Armstrong C, Swarbrick CM, Pye SR, O'Neill TW: **Occurrence and risk factors for falls in rheumatoid arthritis.** *Ann Rheum Dis* 2005, **64:**1602–1604.
12. Furuya T, Hosoi T, Tanaka E, Nakajima A, Taniguchi A, Momohara S, Yamanaka H: **Prevalence of and factors associated with vitamin D deficiency in 4,793 Japanese patients with rheumatoid arthritis.** *Clin Rheumatol* 2013, **32:**1081–1087.
13. Bischoff-Ferrari HA, Giovannucci E, Willett WC, Dietrich T, Dawson-Hughes B: **Estimation of optimal serum concentrations of 25-hydroxyvitamin D for multiple health outcomes.** *Am J Clin Nutr* 2006, **84:**18–28.
14. Rossini M, Maddali Bongi S, La Montagna G, Minisola G, Malavolta N, Bernini L, Cacace E, Sinigaglia L, Di Munno O, Adami S: **Vitamin D deficiency in rheumatoid arthritis: prevalence, determinants and associations with disease activity and disability.** *Arthritis Res Ther* 2010, **12:**R216.
15. Kim SY, Schneeweiss S, Liu J, Daniel GW, Chang CL, Garneau K, Solomon DH: **Risk of osteoporotic fracture in a large population-based cohort of patients with rheumatoid arthritis.** *Arthritis Res Ther* 2010, **12:**R154.
16. Furuya T, Inoue E, Hosoi T, Taniguchi A, Momohara S, Yamanaka H: **Risk factors associated with the occurrence of hip fracture in Japanese patients with rheumatoid arthritis: a prospective observational cohort study.** *Osteoporos Int* 2013, **24:**1257–1265.
17. Shea MK, Benjamin EJ, Dupuis J, Massaro JM, Jacques PF, D'Agostino RB Sr, Ordovas JM, O'Donnell CJ, Dawson-Hughes B, Vasan RS, Booth SL: **Genetic and non-genetic correlates of vitamins K and D.** *Eur J Clin Nutr* 2009, **63:**458–464.
18. Hunter D, De Lange M, Snieder H, MacGregor AJ, Swaminathan R, Thakker RV, Spector TD: **Genetic contribution to bone metabolism, calcium excretion, and vitamin D and parathyroid hormone regulation.** *J Bone Miner Res* 2001, **16:**371–378.
19. Wang TJ, Zhang F, Richards JB, Kestenbaum B, van Meurs JB, Berry D, Kiel DP, Streeten EA, Ohlsson C, Koller DL, Peltonen L, Cooper JD, O'Reilly PF, Houston DK, Glazer NL, Vandenput L, Peacock M, Shi J, Rivadeneira F, McCarthy MI, Anneli P, de Boer IH, Mangino M, Kato B, Smyth DJ, Booth SL, Jacques PF, Burke GL, Goodarzi M, Cheung CL, *et al*: **Common genetic determinants of vitamin D insufficiency: a genome-wide association study.** *Lancet* 2010, **376:**180–188.
20. Ahn J, Yu K, Stolzenberg-Solomon R, Simon KC, McCullough ML, Gallicchio L, Jacobs EJ, Ascherio A, Helzlsouer K, Jacobs KB, Li Q, Weinstein SJ, Purdue M, Virtamo J, Horst R, Wheeler W, Chanock S, Hunter DJ, Hayes RB, Kraft P, Albanes D: **Genome-wide association study of circulating vitamin D levels.** *Hum Mol Genet* 2010, **19:**2739–2745.
21. Okada Y, Terao C, Ikari K, Kochi Y, Ohmura K, Suzuki A, Kawaguchi T, Stahl EA, Kurreeman FA, Nishida N, Ohmiya H, Myouzen K, Takahashi M, Sawada T, Nishioka Y, Yukioka M, Matsubara T, Wakitani S, Teshima R, Tohma S, Takasugi K, Shimada K, Murasawa A, Honjo S, Matsuo K, Tanaka H, Tajima K, Suzuki T, Iwamoto T, Kawamura Y, *et al*: **Meta-analysis identifies nine new loci associated with rheumatoid arthritis in the Japanese population.** *Nat Genet* 2012, **44:**511–516.
22. Kobayashi S, Ikari K, Kaneko H, Kochi Y, Yamamoto K, Shimane K, Nakamura Y, Toyama Y, Mochizuki T, Tsukahara S, Kawaguchi Y, Terai C, Hara M, Tomatsu T, Yamanaka H, Horiuchi T, Tao K, Yasutomo K, Hamada D, Yasui N, Inoue H, Itakura M, Okamoto H, Kamatani N, Momohara S: **Association of STAT4 with susceptibility to rheumatoid arthritis and systemic lupus erythematosus in the Japanese population.** *Arthritis Rheum* 2008, **58:**1940–1946.
23. Kasahara AK, Singh RJ, Noymer A: **Vitamin D (25OHD) Serum seasonality in the United States.** *PLoS One* 2013, **8:**e65785.
24. **R: A language and environment for statistical computing.** 2010 [http://www.r-project.org/]
25. Speeckaert M, Huang G, Delanghe JR, Taes YE: **Biological and clinical aspects of the vitamin D binding protein (Gc-globulin) and its polymorphism.** *Clin Chim Acta* 2006, **372:**33–42.
26. Ahn J, Albanes D, Berndt SI, Peters U, Chatterjee N, Freedman ND, Abnet CC, Huang WY, Kibel AS, Crawford ED, Weinstein SJ, Chanock SJ, Schatzkin A, Hayes RB: **Vitamin D-related genes, serum vitamin D concentrations and prostate cancer risk.** *Carcinogenesis* 2009, **30:**769–776.

27. Jones G: **Pharmacokinetics of vitamin D toxicity.** *Am J Clin Nutr* 2008, **88**:582S–586S.
28. de Koning L, Henne D, Hemmelgarn BR, Woods P, Naugler C: **Non-linear relationship between serum 25-hydroxyvitamin D concentration and subsequent hip fracture.** *Osteoporos Int* 2013, **24**:2061–2065.
29. Bischoff-Ferrari HA, Willett WC, Wong JB, Giovannucci E, Dietrich T, Dawson-Hughes B: **Fracture prevention with vitamin D supplementation: a meta-analysis of randomized controlled trials.** *JAMA* 2005, **293**:2257–2264.
30. Bischoff-Ferrari HA, Dawson-Hughes B, Staehelin HB, Orav JE, Stuck AE, Theiler R, Wong JB, Egli A, Kiel DP, Henschkowski J: **Fall prevention with supplemental and active forms of vitamin D: a meta-analysis of randomised controlled trials.** *BMJ* 2009, **339**:b3692.
31. Estrada K, Styrkarsdottir U, Evangelou E, Hsu YH, Duncan EL, Ntzani EE, Oei L, Albagha OM, Amin N, Kemp JP, Koller DL, Li G, Liu CT, Minster RL, Moayyeri A, Vandenput L, Willner D, Xiao SM, Yerges-Armstrong LM, Zheng HF, Alonso N, Eriksson J, Kammerer CM, Kaptoge SK, Leo PJ, Thorleifsson G, Wilson SG, Wilson JF, Aalto V, Alen M, *et al*: **Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture.** *Nat Genet* 2012, **44**:491–501.
32. Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB, Ingvarsson T, Jonsdottir T, Saemundsdottir J, Center JR, Nguyen TV, Bagger Y, Gulcher JR, Eisman JA, Christiansen C, Sigurdsson G, Kong A, Thorsteinsdottir U, Stefansson K: **Multiple genetic loci for bone mineral density and fractures.** *N Engl J Med* 2008, **358**:2355–2365.
33. Levin GP, Robinson-Cohen C, de Boer IH, Houston DK, Lohman K, Liu Y, Kritchevsky SB, Cauley JA, Tanaka T, Ferrucci L, Bandinelli S, Patel KV, Hagstrom E, Michaelsson K, Melhus H, Wang T, Wolf M, Psaty BM, Siscovick D, Kestenbaum B: **Genetic variants and associations of 25-hydroxyvitamin D concentrations with major clinical outcomes.** *JAMA* 2012, **308**:1898–1905.
34. Kerr GS, Sabahi I, Richards JS, Caplan L, Cannon GW, Reimold A, Thiele GM, Johnson D, Mikuls TR: **Prevalence of vitamin D insufficiency/deficiency in rheumatoid arthritis and associations with disease severity and activity.** *J Rheumatol* 2011, **38**:53–59.

doi:10.1186/ar4516
Cite this article as: Yoshida *et al.*: A *GC* polymorphism associated with serum 25-hydroxyvitamin D level is a risk factor for hip fracture in Japanese patients with rheumatoid arthritis: 10-year follow-up of the Institute of Rheumatology, Rheumatoid Arthritis cohort study. *Arthritis Research & Therapy* 2014 **16**:R75.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

BioMed Central

OPEN ACCESS Freely available online

PLOS | ONE

Neutrophils Are Essential as a Source of IL-17 in the Effector Phase of Arthritis

Masaki Katayama[1], Koichiro Ohmura[1]*, Naoichiro Yukawa[1], Chikashi Terao[1,2], Motomu Hashimoto[3], Hajime Yoshifuji[1], Daisuke Kawabata[1], Takao Fujii[3], Yoichiro Iwakura[4], Tsuneyo Mimori[1]

1 Department of Rheumatology and Clinical Immunology, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **2** Center for Genetic Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **3** Department of the Control for Rheumatic Diseases, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **4** Center for Experimental Medicine and Systems Biology, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Abstract

Objective: Th17 has been shown to have a pivotal role in the development of arthritis. However, the role of IL-17 in the T cell-independent effector phase has not fully been examined. We investigated whether IL-17 is involved in the effector phase of arthritis by using K/BxN serum-induced arthritis model.

Methods: K/BxN serum was transferred into IL-17 knockout (KO) mice, SCID mice and their control mice, and arthritis was evaluated over time. In order to clarify the source of IL-17 in the effector phase, neutrophils or CD4+ T cells collected from IL-17 KO or control mice were injected into IL-17 KO recipient mice together with K/BxN serum. To examine if neutrophils secrete IL-17 upon stimulation, neutrophils were stimulated with immune complex in vitro and IL-17 in the supernatant was measured by ELISA.

Results: K/BxN serum-induced arthritis was much less severe in IL-17 KO mice than in WT mice. Since K/BxN serum-transferred SCID mice developed severe arthritis with high serum IL-17 concentration, we speculated neutrophils are the responsible player as an IL-17 source. When wild type (WT) but not IL-17 KO neutrophils were co-injected with K/BxN serum into IL-17 KO mice, arthritis was exacerbated, whereas co-injection of WT CD4+ T cells had no effect. In vitro, stimulation of neutrophils with immune complexcaused IL-17 secretion.

Conclusions: Neutrophils are essential as a source of IL-17 in the effector phase of arthritis. The trigger of secreting IL-17 from neutrophils may be immune complex.

Citation: Katayama M, Ohmura K, Yukawa N, Terao C, Hashimoto M, et al. (2013) Neutrophils Are Essential as a Source of IL-17 in the Effector Phase of Arthritis. PLoS ONE 8(5): e62231. doi:10.1371/journal.pone.0062231

Editor: Philip J. Norris, Blood Systems Research Institute, United States of America

Received December 23, 2012; **Accepted** March 19, 2013; **Published** May 6, 2013

Copyright: © 2013 Katayama et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was supported by grants from the Japan Society for Promotion of Science and the Ministry of Health and Welfare of Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: ohmurako@kuhp.kyoto-u.ac.jp

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovitis leading to destruction of articular cartilage and bone. The pathophysiology of RA is still unclear, but recently the important role of IL-17-producing T cells (Th17) has been highlighted in arthritis development in human and several mouse models. IL-17 (especially IL-17A) is a proinflammatory cytokine that is thought to contribute to the inflammation, cartilage destruction and bone erosion in RA. IL-17 is upregulated in the synovium and the synovial fluids of RA patients [1]. IL-17 induces fibroblasts, endothelial cells or macrophages to secrete IL-6, TNFα and IL-1 [2,3,4]. IL-17 can synergize with IL-1 and TNFα, although it may also have direct pathological effects. In experimental arthritis models, the critical role of IL-17 has been clearly demonstrated. Spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist was completely abrogated in the absence of IL-17 [5]. Collagen-induced arthritis [3] was also apparently suppressed in IL-17 knock out (KO) mice and was prevented by anti-IL-17 antibody treatment [6].

K/BxN mouse is a KRN T cell receptor transgenic mouse crossed with NOD mouse, and develops severe arthritis similar to RA [7]. KRN T cells recognize glycolytic enzyme glucose-6-phosphatase isomerase (GPI), and the autoantibodies against GPI cause arthritis. Transfer of K/BxN arthritic serum or purified anti-GPI antibodies into normal mice induces arthritis similar to K/BxN mice [8]. In the K/BxN model, T cells and B cells are required for inducing arthritis, but once the anti-GPI antibody is generated, arthritis can develop without lymphocytes [9]. K/BxN serum-transfer arthritis is thus a useful model to analyze the effector phase of arthritis. The innate immune system including neutrophils [10], mast cells [11], Fcγ receptor and C5a [12] has been shown to be essential in this arthritis development. Although IL-1 and TNFα, but not IL-6, were shown to be important in this arthritis model [13], it has not been clear whether IL-17 works in the effector phase of arthritis, regardless of the findings that non-T cells produce IL-17. In the present study, we tested whether IL-17

is involved in K/BxN serum-induced arthritis using IL-17 KO mice and we found that IL-17 derived from neutrophils affects arthritis severity in the effector phase.

Materials and Methods

Mice

C57BL/6JJcl (B6), FOX CHASE SCID C.B-17/Icr-scid/scidJcl (SCID), FOX CHASE SCID C.B-17/Icr-+/+Jcl (SCID WT), NOD/SciJcl (NOD) mice were purchased from Japan Clea Inc. (Tokyo, Japan). KRN TCR transgenic (B6 background: K/B) and Cα (TCRα chain) KO mice were kindly provided by Drs. D. Mathis and C. Benoist, Harvard Medical School, Boston, MA. Fcγ receptor KO (FcR KO) mice were kindly provided by Dr. T. Takai, Tohoku University, Sendai. All mice were maintained in our animal facility under specific pathogen–free conditions. K/BxN arthritic mice were obtained by crossing K/B with NOD mice, and the sera were pooled at eight weeks old. Arthritic adult K/BxN mice were bled, and the sera collected from eight-week-old K/BxN mice were pooled. All animal procedures were approved by the Ethics Committee of Kyoto University.

Induction of K/BxN Serum-induced Arthritis and Arthritis Scoring

Recipient mice were usually i.p. injected with 200 μl of K/BxN sera at days 0 and 2. In the experiments of neutrophil or $CD4^+$ T cell transfer, recipient mice were i.v. injected with 200 μl of K/BxN sera at days 0 and 2. Arthritis was evaluated visually, and the swelling of each paw was scored on a scale of 0–3, where 0 = no evidence of inflammation, 1 = subtle inflammation or localized edema, 2 = easily identified swelling localized to either the dorsal or ventral surface of the paw, and 3 = swelling of all aspects of the paw. Clinical indices for all four paws were added as a composite score. Ankle thickness was measured with a caliper.

Histological Examination

Dissected ankles were fixed in 4% neutral buffered paraformaldehyde, demineralized and stained with hematoxylin and eosin (H&E).

Isolation of Murine Neutrophils from Bone Marrow (BM) and $CD4^+$ T Cells from Spleen

BM neutrophils were isolated using a mouse Anti-Ly-6G MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of neutrophils was >95% as determined by May-Giemsa staining. Splenic $CD4^+$ T cells were isolated using a mouse Pan T Cell Isolation Kit II (Miltenyi Biotec).

Neutrophil Stimulation in Vitro

Soluble murine peroxidase-anti-peroxidase (mPAP) ICs consisting primarily of two horseradish peroxidase (HRP) molecules bound to three anti-peroxidase IgGs were obtained from Jackson ImmunoResearch Laboratories (Bar Harbor, HE, USA). Immunomagnetically purified neutrophils from mouse BM were incubated at a concentration of 8×10^6/ml in RPMI 1640 medium containing 10%FCS, penicillin, or streptomycin for 3 hours, followed by culture with mPAP IC (200, 20, and 2 μg/ml), HRP (Sigma Aldrich, Saint Luis, MO, USA: 200 μg/ml), anti-HRP antibody (Thermo Fisher Scientific, Waltham, MA, USA: 200 μg/ml) or PBS for 1 hour before collecting the culture supernatant.

IL-17 Measurements by ELISA

Mouse IL-17 Quantikine EILSA kit was purchased from R&D systems (Minneapolis, MN, USA). Cell culture supernatant and sera from arthritic mice were assayed in accordance with the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using the Student's *t*-test. Data are expressed as means ± SEM unless otherwise stated.

Results

IL-17 Exacerbates K/BxN Serum-induced Arthritis

To clarify whether IL-17 has any roles in the effector phase of arthritis, we induced K/BxN serum-induced arthritis in IL-17 KO and WT B6 mice. We assessed the clinical index and ankle thickness over time. Interestingly, the severity of arthritis in IL-17 KO mice was much milder than that in WT mice (Fig. 1). Histological examination of ankle joints also revealed that the inflammation, bone and cartilage destruction of serum induced arthritis were much less in IL-17 KO mice than in WT mice (Fig. 2). These results clearly indicate that IL-17 has pivotal roles in aggravating arthritis in the effector phase.

CD4+T Cells are not the Source of IL-17 in Arthritis Effector Phase

We hypothesized that T cells were not the source of IL-17 in arthritis effector phase since it was reported that T cells are not essential in this phase. To confirm this, we injected K/BxN arthritic sera or BxN control sera into SCID and control mice (C.B-17/Icr-+/+). We assessed their clinical index and ankle thickness over time, and measured their serum concentration of IL-17 at days 7 and 21. The arthritis induced in SCID mice was as severe as that in control mice (Fig. 3A), and the concentrations of serum IL-17 in the SCID mice were similar to those in the control mice (Fig. 3B). In contrast, in both SCID and control mice, the concentrations of serum IL-17 in BxN serum-injected non-arthritic mice were significantly lower than those in K/BxN serum-induced arthritic mice (Fig. 3B). Since the concentration of IL-17 in the injected K/BxN serum was ~50 pg/ml (data not shown) and that of K/BxN serum-induced SCID mice was ~140 pg/ml (Fig. 3B), it was evident that the serum IL-17 in the K/BxN serum-induced SCID mice was not derived from K/BxN serum itself but from non-T non-B inflammatory cells.

Neutrophils Exacerbate Arthritis via IL-17 in the Effector Phase

We were curious what types of cells secrete IL-17 in the effector phase of arthritis. We focused on neutrophils, because it was reported that they could secrete IL-17 [14] and that they are essential for the development of K/BxN serum transfer arthritis [10]. We injected neutrophils (2×10^6/body) collected from BM of IL-17 KO or WT mice into IL-17 KO mice together with K/BxN serum at days 0 and 2, and the disease parameters were followed over time. As we expected, arthritis became significantly more severe when IL-17 sufficient neutrophils were injected compared with when IL-17 KO neutrophils were injected (Fig. 4A). The results were consistent in the three independent experiments. In order to exclude IL-17 producing-T cells are contaminated, we injected neutrophils (2×10^6/body) collected from BM of IL-17 KO or αβT cell deficient (Cα KO) mice into IL-17 KO mice together with K/BxN serum at days 0 and 2. As shown in Fig. 4B, Cα KO neutrophils (which can produce IL-17) exacerbated

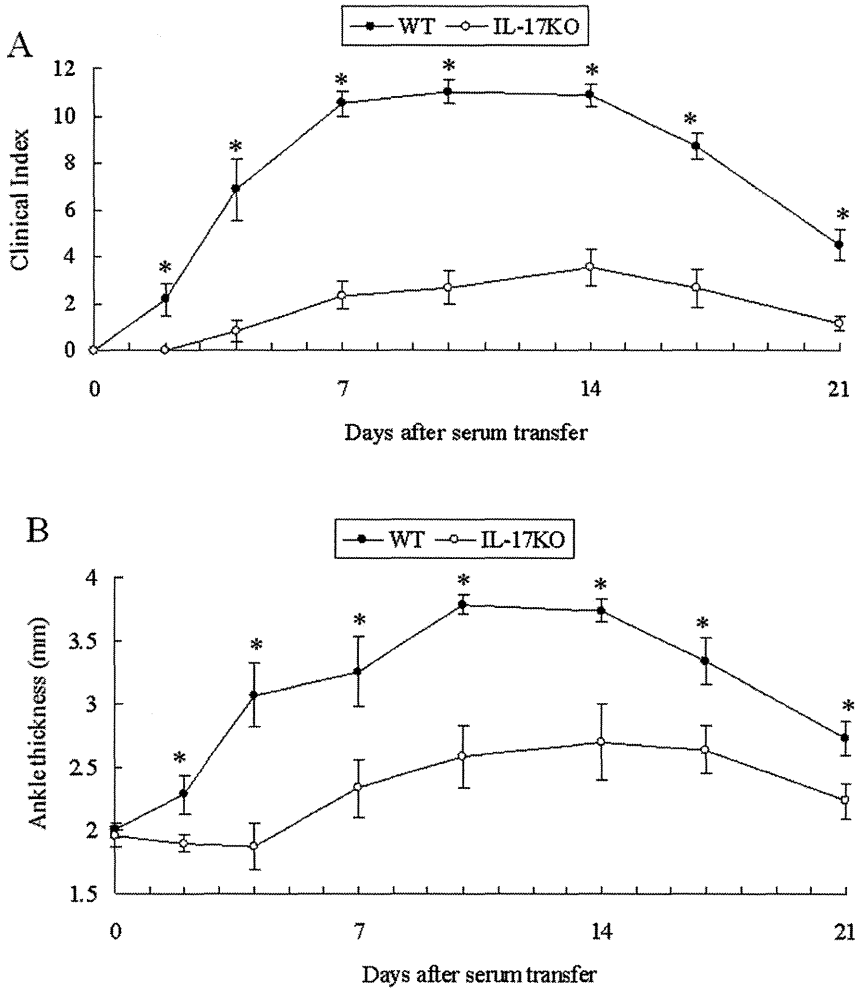


Figure 1. K/BxN serum-induced arthritis in IL-17 knockout mice. K/BxN sera (200 μl/body) were i.p. injected at days 0 and 2 into IL-17 KO (○) and WT mice (●) (n = 6 each). Clinical index (A) and ankle thickness (B) were monitored for 21 days. The results were from two independent experiments, both of which showed similar results. *$p<0.05$.
doi:10.1371/journal.pone.0062231.g001

arthritis of recipient mice more severely than the IL-17 KO neutrophils.

We next collected splenic CD4+ T cells from IL-17KO or WT mice and injected them (1×10^7/body) into IL-17 KO mice at day0, while K/BxN serum (200 μl/body) was injected at days 0 and 2. As shown in Fig. 5, there were no statistical differences in arthritis severity between the recipients of IL-17KO and WT CD4+ T cells. All these results clearly indicate that neutrophils but not $CD4^+$ T cells are the major source of IL-17 in the effector phase and affect the severity of arthritis. The results were consistent in the two independent experiments.

Neutrophils can Secrete IL-17

Wipke et al. showed that mouse peroxidase-anti-peroxidase (mPAP) immune complex (IC) activates neutrophils through Fcγ receptor to get autoantibodies into joints in this model [15]. We hypothesized that ICs trigger neutrophils to secrete IL-17. We collected neutrophils from BM of B6 mice and incubated them for 3 hours, followed by culture with mPAP-IC for additional 1 hour. We measured the IL-17 concentration in the culture supernatant by ELISA. IL-17 was detected in the supernatant of cultures in a dose-dependent manner of IC (Fig. S1).

To clarify whether ICs stimulate neutrophils through Fcγ receptor for IL-17 secretion, we used FcR KO neutrophils to stimulate with mPAP-IC. We found that they secrete significantly

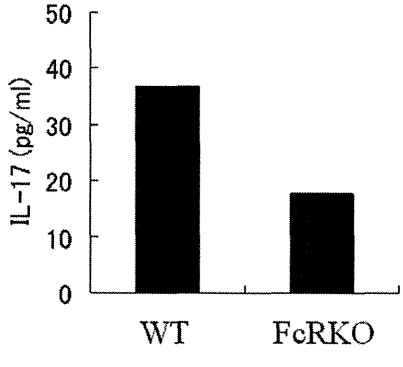


Figure 2. Histopathology of ankle joints from arthritic IL-17KO and WT mice. Arthritis was induced as described in the legend for figure 1 and the mice were sacrificed at days 7 and 21 after initial serum transfer. H&E stainings of ankle joints are shown (scale bar represents 100 μm).
doi:10.1371/journal.pone.0062231.g002

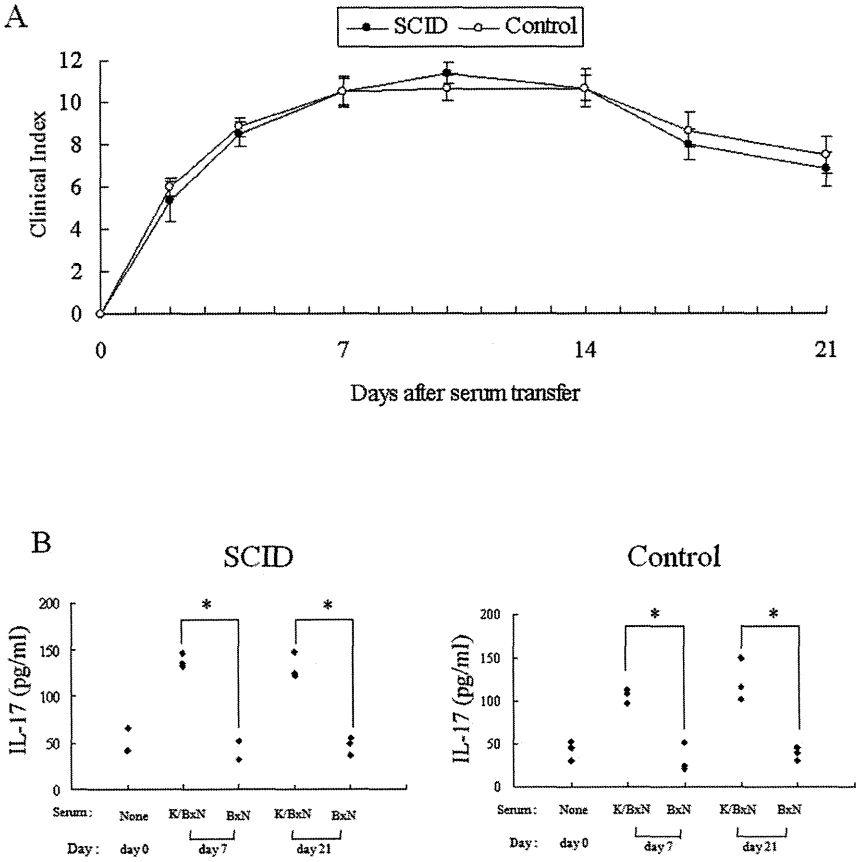


Figure 3. Arthritis severity and IL-17 in the sera of SCID or control mice which received K/BxN or BxN sera. K/BxN arthritic sera or BxN control sera were injected into SCID (C.B-17/Icr-scid/scid) and corresponding control mice (C.B-17/Icr-+/+) at days 0 and 2. (A) Clinical Index was monitored for 21 days. (B) Blood was sampled at days 0, 7 and 21 and IL-17 concentrations in the sera of each mouse were measured by EILSA. *$p<0.05$.
doi:10.1371/journal.pone.0062231.g003

lower amount of IL-17 than the WT neutrophils (Fig. S2). This result clearly shows that ICs stimulate neutrophils through FcR, although there may be other pathways as well.

Furthermore, we stimulated B6 neutrophils with HRP only, mouse anti-HRP antibody only, or PBS. IL-17 was detected in the supernatant of cultures stimulated by anti-HRP antibody and mPAP-IC, but not HRP or PBS (Fig. 6). On the other hand, IL-17 mRNA was not detected in any groups (data not shown). These results indicate that IL-17 is secreted from prestored pool in neutrophils by the stimulation of IC or anti-HRP antibodies themselves.

Discussion

In this report, we clearly showed that IL-17 operates as a proinflammatory cytokine in the effector phase of the K/BxN arthritis model. In this phase, neutrophils are the important source of IL-17 production.

It was rather surprising that IL-17 KO mice showed hyporesponsiveness to K/BxN serum transfer, since this means that IL-17 from cells other than $CD4^+$ T cells are important for arthritis development. Although the distinct $CD4^+$ helper T cell subset is famous for IL-17 producing cells (known as Th17), several innate immune cells have recently been reported to secrete IL-17 in inflammatory or autoimmune diseases. TCRγδ T cells secrete IL-17 in Mycobacterium Tuberculosis infection [16], in experimental antoimmune encephalomyelitis model mice [17], and in collagen-induced arthritis [18]. Invariant natural killer T cells also produce IL-17 [19]. Neutrophils are reported to secrete IL-17 in several mouse models of asthma [20], ANCA-associated vasculitis [21] and kidney ischemia-reperfusion injury [22]. Since IL-17 was detected in the sera of SCID mice which had been treated with K/BxN sera (Fig. 3), the source of IL-17 in the effector phase should not be TCRαβ, TCRγδ, or NKT cells. From the neutrophil transfer experiments (Fig. 4*A*), it was evident that neutrophils are the major source of IL-17 in this K/BxN serum-induced arthritis model. However, K/BxN serum-induced arthritis in IL-17KO mice which had been reconstituted with WT neutrophils was not as severe as that in the WT mice (Figs. 1A & 4A). Even when we injected 5 times more number (1×10^7/body) of WT neutrophils, the arthritis severity did not increase (data not shown). These results suggest that some innate immune cells other than neutrophils are also sources of IL-17 in K/BxN serum transfer arthritis. Mast cells are a possible source of IL-17 [23].

Jacobs et al. reported that IL-17-producing KRN T cells amplified the inflammatory process in the K/BxN serum-induced arthritis model [24]. In contrast, T cells did not augment the arthritis in our study (Fig. 5). The differences between these results are that Jacobs et al. transferred KRN T cells into recipients in which APC express $I\text{-}A^{g7}$ and lack B cells, whereas we transferred normal B6 T cells into IL-17 KO mice. Therefore, their T cells were activated but ours were not. If activated T cells are present, such T cells may augment the effector phase of arthritis. Jacobs

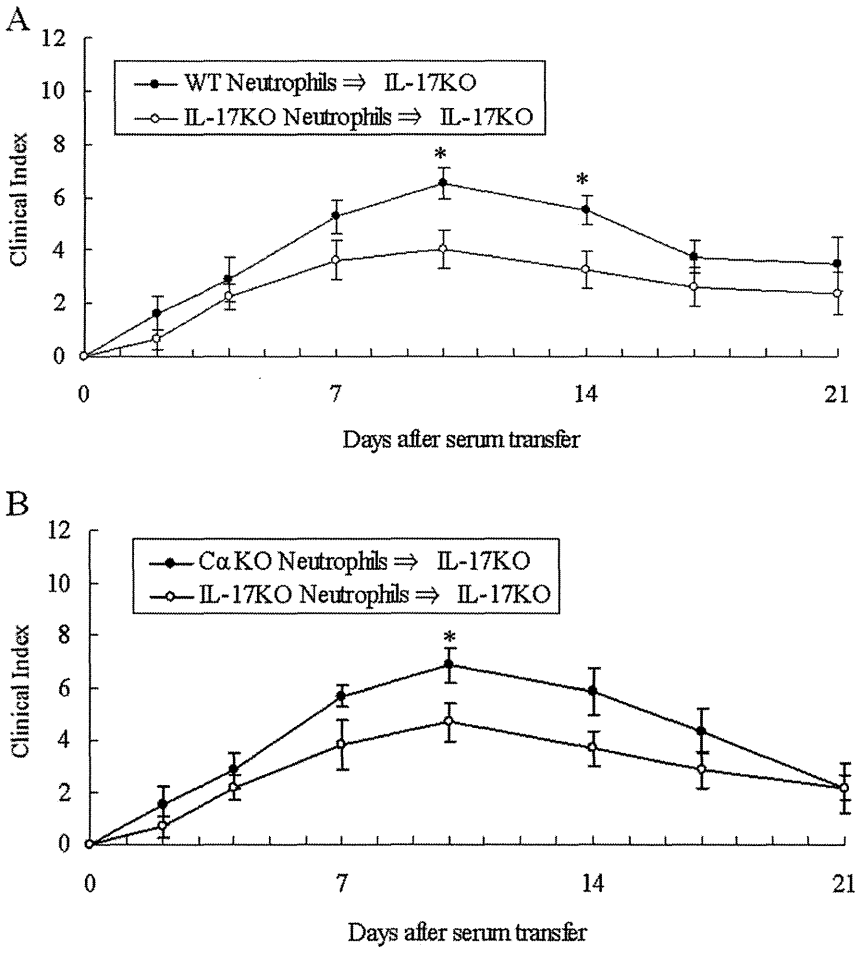


Figure 4. IL-17 from neutrophils aggravates K/BxN serum-induced arthritis. (A) Neutrophils (2×10^6/body) collected from bone marrow of IL-17 KO (○) or WT (●) mice were injected into IL-17 KO mice together with K/BxN sera (200 μl/body) at days 0 and 2. Clinical index of arthritis is shown. The results were from three independent experiments, both of which showed similar results. n = 8 mice in each group. (B) Neutrophils (2×10^6/body) collected from bone marrow of IL-17 KO (○) or Cα KO (●) mice were injected into IL-17 KO mice together with K/BxN sera (200 μl/body) at days 0 and 2. Clinical index of arthritis is shown. n = 6 mice in each group.*$p<0.05$.
doi:10.1371/journal.pone.0062231.g004

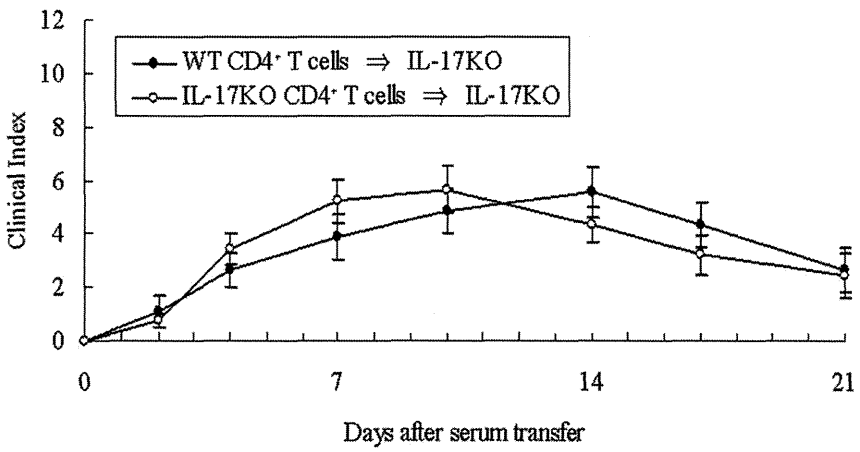


Figure 5. CD4+ T cells do not aggravate K/BxN serum-induced arthritis. $CD4^+$ T cells (1×10^7/body) collected from spleen of IL-17 KO (○) or WT (●) mice were injected into IL-17 KO mice at day 0. K/BxN sera (200 μl/body) were injected at days 0 and 2. Clinical index of arthritis is shown. The results were from two independent experiments, both of which showed similar results. n = 9 mice in each group.
doi:10.1371/journal.pone.0062231.g005

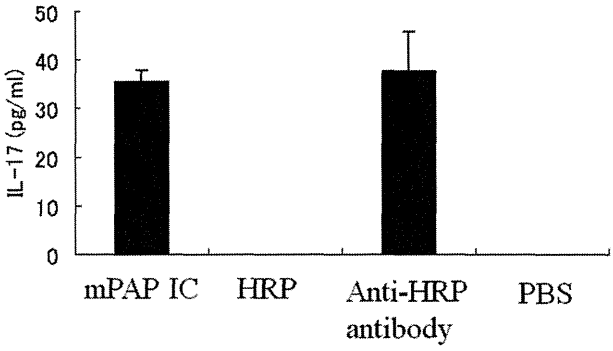


Figure 6. Neutrophils secrete IL-17 after stimulation in vitro. Neutrophils (8×10^6 cells/ml) collected from the bone marrow cells of B6 mice were cultured for three hours, followed by incubation with mPAP: murine peroxidase (HRP) -anti-peroxidase immune complex (IC), HRP only, anti-HRP antibody only or PBS for additional one hour. The supernatants were collected and the concentration of IL-17 was measured by ELISA. The results were reproducible in four independent experiments and the representative results are shown.
doi:10.1371/journal.pone.0062231.g006

et al. also showed that anti-IL-17 antibodies offer no protection against K/BxN serum-induced arthritis. Such results may be due to the shortage of antibody amount or incomplete blocking of IL-17 in the intimate interaction between effector cells.

Actually it was recently reported that the severity of arthritis in IL-17 receptor (IL-17R) deficient mice is milder than that of IL-17R WT mice using K/BxN serum-induced arthritis model [25]. Their results are consistent with our results, but they did not refer to the source of IL-17. Since the severity of K/BxN serum-induced arthritis in their IL-17R deficient mice is similar to that in our IL-17A KO mice, IL-17A may have the dominant role in the IL-17 family members for arthritis induction, although possible important role of IL-17F cannot be excluded.

The trigger causing neutrophils to secrete IL-17 is not yet clear. Wipke et al. demonstrated that the immune complex can stimulate neutrophils through FcR to increase local vascular permeability as the initiation of arthritis [15]. We found similar results, in that the same immune complex (HRP-anti-HRP antibodies) can trigger neutrophils to secrete IL-17 in vitro (Fig. 5). However, we found that not only HRP-anti-HRP antibody immune complex, but also anti-HRP antibody itself can stimulate neutrophils, which was not tested in Wipke's paper. There still remains a possibility that anti-HRP antibody may directly stimulate neutrophils. Another possibility is that anti-HRP Ab crossreacts with a certain molecule to form ICs that stimulate neutrophils. In order to test whether GPI-anti-GPI Ab IC can stimulate neutrophils, we cultured neutrophils in RPMI medium containing 10% K/BxN arthritic serum with or without GPI protein (200 μg/ml), but we could not detect IL-17 in the culture supernatant (data not shown). This implies that neither GPI-anti-GPI antibody immune complex nor soluble factors in K/BxN serum can trigger neutrophils to secrete IL-17. The stimulating factor of neutrophils to secrete IL-17 remains to be determined.

Pathological functions of IL-17 in human arthritides such as RA have not completely clarified, but IL-17 is thought to be working on accumulation of neutrophils in synovial space, activation of synovial cells and osteoclasts, which lead to joint inflammation, synovial cell proliferation, cartilage and bone destruction. In this paper, we focused on the effector phase of arthritis and did not analyze the initiation phase in which antigen recognition and T-B cell interaction occur. Since IL-17 is produced from various types of cells and works on multiple cell types, it is difficult to dissect the pathological mechanisms in arthritis. By dividing the phase of arthritis, we were able to find the major player of the arthritis effector phase, IL-17-producing neutrophils. However, we have to be careful when we apply our findings to human diseases, which sometimes behave differently from mouse model. Human studies are also needed to warrant our mouse results.

In summary, this is the first report to clearly show that IL-17 is critical in the effector phase of arthritis and that neutrophils are the major source of IL-17, at least in the effector phase. These results demonstrate a new pathogenic role of neutrophils in the arthritis development.

Supporting Information

Figure S1 Immune complex stimulate neutrophils to secrete IL-17 in a dose depend manner. We collected neutrophils from the bone marrow cells of B6 mice and cultured them (8×10^6 cells/well) for 3 hours, followed by incubation with 200, 20 and 2 μg/ml of mPAP: murine peroxidase (HRP) -anti-peroxidase immune complex (IC) for additional 1 hour. The supernatants were collected and the concentration of IL-17 was measured by ELISA.
(JPG)

Figure S2 Immune complex stimulate neutrophils through Fcγ receptor. We collected neutrophils from the bone marrow cells of Fcγ receptor (FcR) knockout (KO) mice or wild type (WT) B6 mice and cultured them (8×10^6 cells/well) for 3 hours, followed by incubation with 200 μg/ml of mPAP-IC for additional 1 hour. The supernatants were collected and the concentration of IL-17 was measured by ELISA.
(JPG)

Acknowledgments

We would like to thank Drs. Diane Mathis and Christophe Benoist for providing the KRN and CαKO mice, and Dr. Toshiyuki Takai for the FcR KO mice.

Author Contributions

Conceived and designed the experiments: MK KO NY. Performed the experiments: MK KO. Analyzed the data: MK KO NY CT MH HY DK TF TM. Contributed reagents/materials/analysis tools: MK KO CT YI. Wrote the paper: MK KO.

References

1. Chabaud M, Durand JM, Buchs N, Fossiez F, Page G, et al. (1999) Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. Arthritis Rheum 42: 963–970.
2. Yao Z, Fanslow WC, Seldin MF, Rousseau AM, Painter SL, et al. (1995) Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. Immunity 3: 811–821.
3. Fossiez F, Djossou O, Chomarat P, Flores-Romo L, Ait-Yahia S, et al. (1996) T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. J Exp Med 183: 2593–2603.
4. Jovanovic DV, Di Battista JA, Martel-Pelletier J, Jolicoeur FC, He Y, et al. (1998) IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. J Immunol 160: 3513–3521.
5. Nakae S, Saijo S, Horai R, Sudo K, Mori S, et al. (2003) IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. Proc Natl Acad Sci U S A 100: 5986–5990.

6. Nakae S, Nambu A, Sudo K, Iwakura Y (2003) Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. J Immunol 171: 6173–6177.
7. Kouskoff V, Korganow AS, Duchatelle V, Degott C, Benoist C, et al. (1996) Organ-specific disease provoked by systemic autoimmunity. Cell 87: 811–822.
8. Korganow AS, Ji H, Mangialaio S, Duchatelle V, Pelanda R, et al. (1999) From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. Immunity 10: 451–461.
9. Matsumoto I, Staub A, Benoist C, Mathis D (1999) Arthritis provoked by linked T and B cell recognition of a glycolytic enzyme. Science 286: 1732–1735.
10. Wipke BT, Allen PM (2001) Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis. J Immunol 167: 1601–1608.
11. Lee DM, Friend DS, Gurish MF, Benoist C, Mathis D, et al. (2002) Mast cells: a cellular link between autoantibodies and inflammatory arthritis. Science 297: 1689–1692.
12. Ji H, Ohmura K, Mahmood U, Lee DM, Hofhuis FM, et al. (2002) Arthritis critically dependent on innate immune system players. Immunity 16: 157–168.
13. Ji H, Pettit A, Ohmura K, Ortiz-Lopez A, Duchatelle V, et al. (2002) Critical roles for interleukin 1 and tumor necrosis factor alpha in antibody-induced arthritis. J Exp Med 196: 77–85.
14. Lin AM, Rubin CJ, Khandpur R, Wang JY, Riblett M, et al. (2011) Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis. J Immunol 187: 490–500.
15. Wipke BT, Wang Z, Nagengast W, Reichert DE, Allen PM (2004) Staging the initiation of autoantibody-induced arthritis: a critical role for immune complexes. J Immunol 172: 7694–7702.
16. Lockhart E, Green AM, Flynn JL (2006) IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. J Immunol 177: 4662–4669.
17. Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC, et al. (2009) Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. Immunity 31: 331–341.
18. Ito Y, Usui T, Kobayashi S, Iguchi-Hashimoto M, Ito H, et al. (2009) Gamma/delta T cells are the predominant source of interleukin-17 in affected joints in collagen-induced arthritis, but not in rheumatoid arthritis. Arthritis Rheum 60: 2294–2303.
19. Michel ML, Mendes-da-Cruz D, Keller AC, Lochner M, Schneider E, et al. (2008) Critical role of ROR-gammat in a new thymic pathway leading to IL-17-producing invariant NKT cell differentiation. Proc Natl Acad Sci U S A 105: 19845–19850.
20. Ferretti S, Bonneau O, Dubois GR, Jones CE, Trifilieff A (2003) IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. J Immunol 170: 2106–2112.
21. Hoshino A, Nagao T, Nagi-Miura N, Ohno N, Yasuhara M, et al. (2008) MPO-ANCA induces IL-17 production by activated neutrophils in vitro via classical complement pathway-dependent manner. J Autoimmun 31: 79–89.
22. Li L, Huang L, Vergis AL, Ye H, Bajwa A, et al. (2010) IL-17 produced by neutrophils regulates IFN-gamma-mediated neutrophil migration in mouse kidney ischemia-reperfusion injury. J Clin Invest 120: 331–342.
23. Hueber AJ, Asquith DL, Miller AM, Reilly J, Kerr S, et al. (2010) Mast cells express IL-17A in rheumatoid arthritis synovium. J Immunol 184: 3336–3340.
24. Jacobs JP, Wu HJ, Benoist C, Mathis D (2009) IL-17-producing T cells can augment autoantibody-induced arthritis. Proc Natl Acad Sci U S A 106: 21789–21794.
25. Sadik CD, Kim ND, Alekseeva E, Luster AD (2011) IL-17RA signaling amplifies antibody-induced arthritis. PLoS One 6: e26342.

PLOS | GENETICS

Genome-Wide Association Study and Gene Expression Analysis Identifies *CD84* as a Predictor of Response to Etanercept Therapy in Rheumatoid Arthritis

Jing Cui[1☯], Eli A. Stahl[1,2,3☯¤a], Saedis Saevarsdottir[4,5], Corinne Miceli[6,7], Dorothee Diogo[1,2,3], Gosia Trynka[1,2,3], Towfique Raj[2,3,8], Maša Umićević Mirkov[9], Helena Canhao[1,10,11], Katsunori Ikari[12], Chikashi Terao[13,14], Yukinori Okada[1,2,3], Sara Wedrén[4,5], Johan Askling[4,15], Hisashi Yamanaka[12], Shigeki Momohara[12], Atsuo Taniguchi[12], Koichiro Ohmura[13], Fumihiko Matsuda[14], Tsuneyo Mimori[13], Namrata Gupta[3], Manik Kuchroo[3,8], Ann W. Morgan[16], John D. Isaacs[17], Anthony G. Wilson[18], Kimme L. Hyrich[19], Marieke Herenius[20], Marieke E. Doorenspleet[20], Paul-Peter Tak[20¤b], J. Bart A. Crusius[21], Irene E. van der Horst-Bruinsma[22], Gert Jan Wolbink[23,24,25], Piet L. C. M. van Riel[9], Mart van de Laar[26], Henk-Jan Guchelaar[27], Nancy A. Shadick[1], Cornelia F. Allaart[28], Tom W. J. Huizinga[28], Rene E. M. Toes[28], Robert P. Kimberly[29], S. Louis Bridges Jr.[29], Lindsey A. Criswell[30], Larry W. Moreland[31], João Eurico Fonseca[10,11], Niek de Vries[20], Barbara E. Stranger[2,3], Philip L. De Jager[2,3,7], Soumya Raychaudhuri[1,2,3,32], Michael E. Weinblatt[1], Peter K. Gregersen[33], Xavier Mariette[6,7], Anne Barton[34], Leonid Padyukov[5], Marieke J. H. Coenen[9], Elizabeth W. Karlson[1], Robert M. Plenge[1,2,3]*

1 Division of Rheumatology, Immunology, and Allergy, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, United States of America, **2** Division of Genetics, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, United States of America, **3** Medical and Population Genetics Program, Chemical Biology Program, Broad Institute, Cambridge, Massachusetts, United States of America, **4** Rheumatology Unit, Department of Medicine, Karolinska Institutet and Karolinska University Hospital Solna, Stockholm, Sweden, **5** Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden, **6** Université Paris-Sud, Orsay, France, **7** APHP–Hôpital Bicêtre, INSERM U1012, Le Kremlin Bicêtre, Paris, France, **8** Program in Translational NeuroPsychiatric Genomics, Institute for the Neurosciences, Department of Neurology, Brigham and Women's Hospital, Boston, Massachusetts, United States of America, **9** Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, **10** Rheumatology Research Unit, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisbon, Portugal, **11** Rheumatology Department, Santa Maria Hospital–CHLN, Lisbon, Portugal, **12** Institute of Rheumatology, Tokyo Women's Medical University, Tokyo, Japan, **13** Department of Rheumatology and Clinical Immunology, Kyoto University Graduate School of Medicine, Kyoto, Japan, **14** Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan, **15** Clinical Epidemiology Unit, Department of Medicine, Karolinska Institute/Karolinska University Hospital, Stockholm, Sweden, **16** NIHR–Leeds Musculoskeletal Biomedical Research Unit and Leeds Institute of Molecular Medicine, University of Leeds, Leeds, United Kingdom, **17** Musculoskeletal Research Group, Institute of Cellular Medicine, Newcastle Upon Tyne, United Kingdom, **18** Rheumatology Unit, Medical School, University of Sheffield, Sheffield, United Kingdom, **19** School of Translational Medicine, Arthritis Research UK Epidemiology Unit, University of Manchester, Manchester, United Kingdom, **20** Department of Clinical Immunology and Rheumatology, Academic Medical Center/University of Amsterdam, Amsterdam, The Netherlands, **21** Laboratory of Immunogenetics, Department of Pathology, Vrije Universiteit Medical Center, Amsterdam, The Netherlands, **22** Department of Rheumatology, Vrije Universiteit University Medical Center, Amsterdam, The Netherlands, **23** Sanquin Research Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands, **24** School of Medicine and Biomedical Sciences, Sheffield University, Sheffield, United Kingdom, **25** Jan van Breemen Institute, Amsterdam, The Netherlands, **26** Arthritis Center Twente, University Twente and Medisch Spectrum Twente, Enschede, The Netherlands, **27** Department of Clinical Pharmacy and Toxicology, Leiden University Medical Center, Leiden, The Netherlands, **28** Department of Rheumatology, Leiden University Medical Centre, Leiden, The Netherlands, **29** Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama, United States of America, **30** Rosalind Russell Medical Research Center for Arthritis, Division of Rheumatology, Department of Medicine, University of California San Francisco, San Francisco, California, United States of America, **31** Division of Rheumatology and Clinical Immunology, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America, **32** NIHR Manchester Musculoskeletal Biomedical Research Unit, Central Manchester NHS Foundation Trust, Manchester Academic Health Sciences Centre, Manchester, United Kingdom, **33** The Feinstein Institute for Medical Research, North Shore–Long Island Jewish Health System, Manhasset, New York, United States of America, **34** Arthritis Research UK Epidemiology Unit, Musculoskeletal Research Group, University of Manchester, Manchester Academic Health Sciences Centre, Manchester, United Kingdom

Abstract

Anti-tumor necrosis factor alpha (anti-TNF) biologic therapy is a widely used treatment for rheumatoid arthritis (RA). It is unknown why some RA patients fail to respond adequately to anti-TNF therapy, which limits the development of clinical biomarkers to predict response or new drugs to target refractory cases. To understand the biological basis of response to anti-TNF therapy, we conducted a genome-wide association study (GWAS) meta-analysis of more than 2 million common variants in 2,706 RA patients from 13 different collections. Patients were treated with one of three anti-TNF medications: etanercept (n = 733), infliximab (n = 894), or adalimumab (n = 1,071). We identified a SNP (rs6427528) at the *1q23* locus that was associated with change in disease activity score (ΔDAS) in the etanercept subset of patients ($P = 8 \times 10^{-8}$), but not in the infliximab or adalimumab subsets ($P > 0.05$). The SNP is predicted to disrupt transcription factor binding site motifs in the 3′ UTR of an immune-related gene, *CD84*, and the allele associated with better response to etanercept was associated with higher *CD84* gene expression in peripheral blood mononuclear cells ($P = 1 \times 10^{-11}$ in 228 non-RA patients and $P = 0.004$ in 132 RA patients). Consistent with the genetic findings, higher *CD84* gene expression correlated with lower cross-sectional DAS ($P = 0.02$, n = 210) and showed a non-significant trend for better ΔDAS in a subset of RA patients with gene expression data (n = 31, etanercept-treated). A small, multi-ethnic replication showed a non-significant trend towards an association among etanercept-treated RA

patients of Portuguese ancestry (n = 139, $P = 0.4$), but no association among patients of Japanese ancestry (n = 151, $P = 0.8$). Our study demonstrates that an allele associated with response to etanercept therapy is also associated with *CD84* gene expression, and further that *CD84* expression correlates with disease activity. These findings support a model in which *CD84* genotypes and/or expression may serve as a useful biomarker for response to etanercept treatment in RA patients of European ancestry.

Citation: Cui J, Stahl EA, Saevarsdottir S, Miceli C, Diogo D, et al. (2013) Genome-Wide Association Study and Gene Expression Analysis Identifies *CD84* as a Predictor of Response to Etanercept Therapy in Rheumatoid Arthritis. PLoS Genet 9(3): e1003394. doi:10.1371/journal.pgen.1003394

Editor: Alison Motsinger-Reif, North Carolina State University, United States of America

Received August 13, 2012; **Accepted** January 13, 2013; **Published** March 28, 2013

Copyright: © 2013 Cui et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: JC is supported by grants from ACR REF HPNIA award and the NIH (R01-AR059073, P60-AR047782, U01-GM092691, R01-AR049880). RMP is supported by grants from the NIH (R01-AR057108, R01-AR056768, U01-GM092691, R01-AR059648) and holds a Career Award for Medical Scientists from the Burroughs Wellcome Fund. The American College of Rheumatology Research and Education Foundation provided funding to support this project. The eRA study was supported by R01 AI/AR47487. SS's work was supported by a clinical research fund from Stockholm County (ALF fund). LP's work was supported by the Swedish Rheumatism Association and the Swedish Medical Research Council. NdV and MED were sponsored by CTMM, the Center for Translational Molecular Medicine, and the Dutch Arthritis Foundation project TRACER (grant 04I-202). The Swedish studies (EIRA, Karolinska) were supported by grants from the Swedish Medical Research Council, the Stockholm County Council, the Swedish Council for Working Life and Social Research, King Gustaf V's 80-year foundation, the Swedish Rheumatism Association, the Swedish Foundation for Strategic Research, the Swedish COMBINE project, and the IMI funded BTCure project. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: rplenge@partners.org

¤a Current address: Division of Psychiatric Genomics, Mt. Sinai School of Medicine, New York, New York, United States of America
¤b Current address: GlaxoSmithKline, Stevenage, United Kingdom

These authors contributed equally to this work.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the synovial lining of the joint [1]. If left untreated, outcome varies from self-limited disease in a small proportion of RA patients to severe disease resulting in profound structural damage, excess morbidity and disability, and early mortality [2]. In the last twenty years, disease activity has been controlled in many patients by treatment with disease-modifying anti-rheumatic drugs (DMARDs), such as methotrexate, and the more recently developed biologic DMARDs that block inflammatory cytokines such as tumor necrosis factor-alpha (TNFa) [3]. Unfortunately, these medications are not effective in all RA patients, with up to one-third of patients failing to respond to any single DMARD [1–3]. Moreover, the biological mechanisms underlying treatment failure are unknown, which limits the development of clinical biomarkers to guide DMARD therapy or the development of new drugs to target refractory cases.

There are two classes of anti-TNF therapy: the TNF receptor fusion protein (etanercept), which acts as a soluble receptor to bind circulating cytokine and prevent TNF from binding to its cell surface receptor, and monoclonal antibodies that bind TNF (adalimumab, infliximab, certolizumab, and golimumab). There are undoubtedly shared mechanisms between the two drug classes (e.g., downstream signaling factors), as illustrated by similar effects on the change in inflammatory cytokines, complement activation, lymphocyte trafficking, and apoptosis [4,5,6]. Similarly, there are likely to be different biological factors that influence response: infliximab and adalimumab are approved for treatment of Crohn's disease; infliximab and adalimumab bind to transmembrane TNF on the surface of activated immune cells, whereas etanercept only binds soluble TNF [7]; and etanercept also binds a related molecule, lymphotoxin alpha (LTA), whereas infliximab/adalimumab do not [8].

Pharmacogenetics of response to anti-TNF therapy in RA remains in its early stages, with no single variant reaching an unambiguous level of statistical significance. Candidate gene studies suggest associations of TNFa or TNF receptor alleles, RA risk alleles or other SNPs with response to anti-TNF therapy [9,10,11]. Two GWAS in small sample sets (largest was 566 patients) have been performed, which identified loci with suggestive evidence for association [12,13]. Therefore, GWAS of large sample sizes may yet uncover genetic factors associated with response to anti-TNF therapy in RA, and larger cohorts enable separate analyses of the different types of anti-TNF drugs.

Here we report a GWAS of 2,706 samples with anti-TNF treatment response data collected from an international collaboration, including previously published GWAS data [12,13]. Our primary outcome measure was the change in disease activity score based on a joint count in 28 joints (DAS28) from baseline to 3–12 months after initiating anti-TNF therapy. Our secondary outcome measure was European League Against Rheumatism (EULAR) responder status [14,15], where patients are classified as EULAR good responders, moderate responders or non-responders based on follow up DAS28 after treatment and overall change in DAS28. We found a highly significant association for a variant that we also show is also a strong expression quantitative trait locus (eQTL) for the *CD84* gene. Our findings suggest that *CD84* genotype and/or expression may prove to be a biomarker for etanercept response in RA patients.

Results

Genome-wide association study

Clinical and GWAS data were compiled for 2,706 individuals of European ancestry from 13 collections as part of an international collaboration. Table 1 shows sample sizes, phenotypes and clinical variables for the four collections that were the units of analysis (additional details are shown in Table S1). Disease activity score based on a 28-joint count (DAS28) were collected at baseline and at one time point after anti-TNF therapy administration (mean 3.7 months, range 3–12 months). We defined our primary phenotype

Author Summary

There are no genetic predictors of response to one of the most widely used classes of drugs in the treatment of rheumatoid arthritis—biological modifiers of the inflammatory cytokine tumor necrosis factor-alpha (or anti-TNF therapy). To identify genetic predictors, we performed the largest genome-wide association study (GWAS) to date as part of an international collaboration. In our study, which included 2,706 RA patients treated with one of three anti-TNF drugs, the most significant finding was restricted to RA patients treated with etanercept ($P=8\times10^{-8}$), a drug that acts as a soluble receptor to bind circulating cytokine and prevents TNF from binding to its cell surface receptor. The associated variant influences expression of a nearby immune-related gene, *CD84*, whose expression is correlated with disease activity in RA patients. Together, our data support a model in which genomic factors related to *CD84* expression serve as a predictor of disease activity and response to etanercept therapy among RA patients of European ancestry, but not anti-TNF therapies that act through different biological mechanisms or potentially in RA patients of other genetic ancestries.

as a change in DAS28 (ΔDAS) from baseline (so that greater ΔDAS corresponded with better response to therapy; overall mean and standard deviation of 2.1 ± 1.3), adjusted for baseline DAS. A secondary phenotype was used based on European League Against Rheumatism (EULAR) response criteria. EULAR 'good response' was defined as ending DAS<3.2 and ΔDAS>1.2; 'non-response' was defined as ΔDAS <0.6 or ΔDAS≤1.2, and ending DAS >5.1; and 'moderate response' is in between [15]. We limited our secondary analysis to a dichotomous outcome, EULAR good responders (n = 998 for all patients treated with anti-TNF therapy) versus EULAR non-responders (n = 655), excluding the moderate category based on the hypothesis that a more extreme phenotype of response would yield improved discrimination.

Clinical variables were examined for association with phenotype, and therefore possible confounding in genetic association tests. In multivariate models (Table S2), only baseline DAS was strongly associated with the ΔDAS phenotype. As previously shown [11], age and gender showed univariate associations that were attenuated in the multivariate analysis. Accordingly, we used only baseline DAS as a clinical covariate, as this allowed us to maximize sample size given clinical variable missing data in some cohorts.

We performed quality control (QC) filtering and data processing of GWAS data for each of eleven genotyping batches. Genotyping array platforms are described in the Methods. HapMap2 imputation allowed us to test for association at >2 M SNPs with imputation quality scores >0.5. Genotype data were merged across several genotype batches to create four collections for genome-wide association testing. We performed linear regression association tests using baseline DAS and three principal components as covariates, and performed inverse-variance weighted meta-analysis to combine results across the four collections. Quantile-quantile plots with genomic control λ_{GC} values are shown in Figure S1. We found no evidence of systematic inflation of association test results, and no evidence of deflation for imputed versus genotyped SNPs. As a final filter, we excluded SNPs that

Table 1. Samples and clinical data.

Collection (analysis batch):	REF	BRAGGSS	DREAM	ReAct	Total
Sample sizes	959*	595	880*	272	2706
Drug subsets					
etanercept	365	259	109	0	733
infliximab	415	268	211	0	894
adalimumab	174	68	557	272	1071
EULAR Reponse categories					
Good responder	432**	161	313	92	998
Moderate responder	243	258	359	131	991
Non-responder	322	176	208	49	755
Genotype platform	mixed	Affy 500K	Illu550K +650K	Illumina OmniExpress	
Clinical variables					
Age, yr; mean (SD)	53.6 (12.7)	57.4 (10.9)	54.8 (12.9)	53.9 (10.8)	
Disease duration, yr; mean (SD)	6.7 (9.4)	14 (9.8)	9.6 (9.5)	12 (9.1)	
Gender, female %	75.6	77.3	68.3	77.9	
Seropositive, %	87	78	80	70	
MTX co-therapy, %	65.6	85.6	76.0	50.0	
Baseline DAS, mean (SD)	5.5 (1.2)	6.7 (0.9)	5.5 (1.2)	5.9 (1.0)	
ΔDAS, mean (SD)	1.9 (1.6)	2.5 (1.5)	1.9 (1.3)	2.2 (1.3)	
Mean treatment duration	4.6	5.6	3	3	
Study design	All***	Observational	Observational	Observational	

*8 patients had no TNF drug information.
**38 patients had only EULAR response (good, moderate or none) clinical data.
***ABCoN, GENRA are prospective cohorts, BeSt, eRA and TEAR are randomized controlled trial (RCT), and rest of REF group are observational cohorts.
doi:10.1371/journal.pgen.1003394.t001

showed strong evidence of heterogeneity across collections (Cochran's Q $P<0.001$).

We first analyzed all samples together (n = 2,706), regardless of drug type. We found no clear evidence of association with treatment response measured by ΔDAS (Figure 1A). Similar results were obtained using the binary phenotype of EULAR responder versus EULAR non-responder status (Figures S1 and S2).

We next separately analyzed patients treated with either etanercept (n = 733), infliximab (n = 894) or adalimumab (n = 1,071) (Figure 1B–1D), under the hypothesis that different genetic loci affect response to the different drugs based on their mechanism of action or other biochemical properties. GWAS results are publicly available for all SNPs tested at the Plenge laboratory and RICOPILI Web sites (see URLs). GWAS results for all SNPs achieving $P<10^{-6}$ from any analysis are detailed in the Table S3.

For etanercept-treated RA patients, a locus on chromosome *1q23* achieved near-genome-wide significance (rs6427528, $P_{META}=8\times10^{-8}$) (Figure 1B, Figure 2A, and Figure 3), but not in the infliximab or adalimumab subsets ($P>0.05$) (Figure S3). SNPs in linkage disequilibrium (LD) showed consistent association results (rs1503860, $P=1\times10^{-7}$, $r^2=1$ with rs6427528 in HapMap; three perfect-LD clusters of SNPs exemplified by rs3737792, rs10908787 and rs11265432 respectively; $P<5\times10^{-6}$; $r^2=0.83$, 0.63 and 0.59 with rs6427528, respectively). No single collection was responsible for the signal of association, as the effect size was consistent across all collections (Figure S4). The top SNP rs6427528 was genotyped in the ReAct dataset (Illumina Omni Express genotyping chip), and was well imputed across all other datasets (imputation quality score INFO ≥0.94, which is an estimate of genotype accuracy; the range of INFO scores is 0–1, where 1 indicates high confidence). All of these SNPs had minor

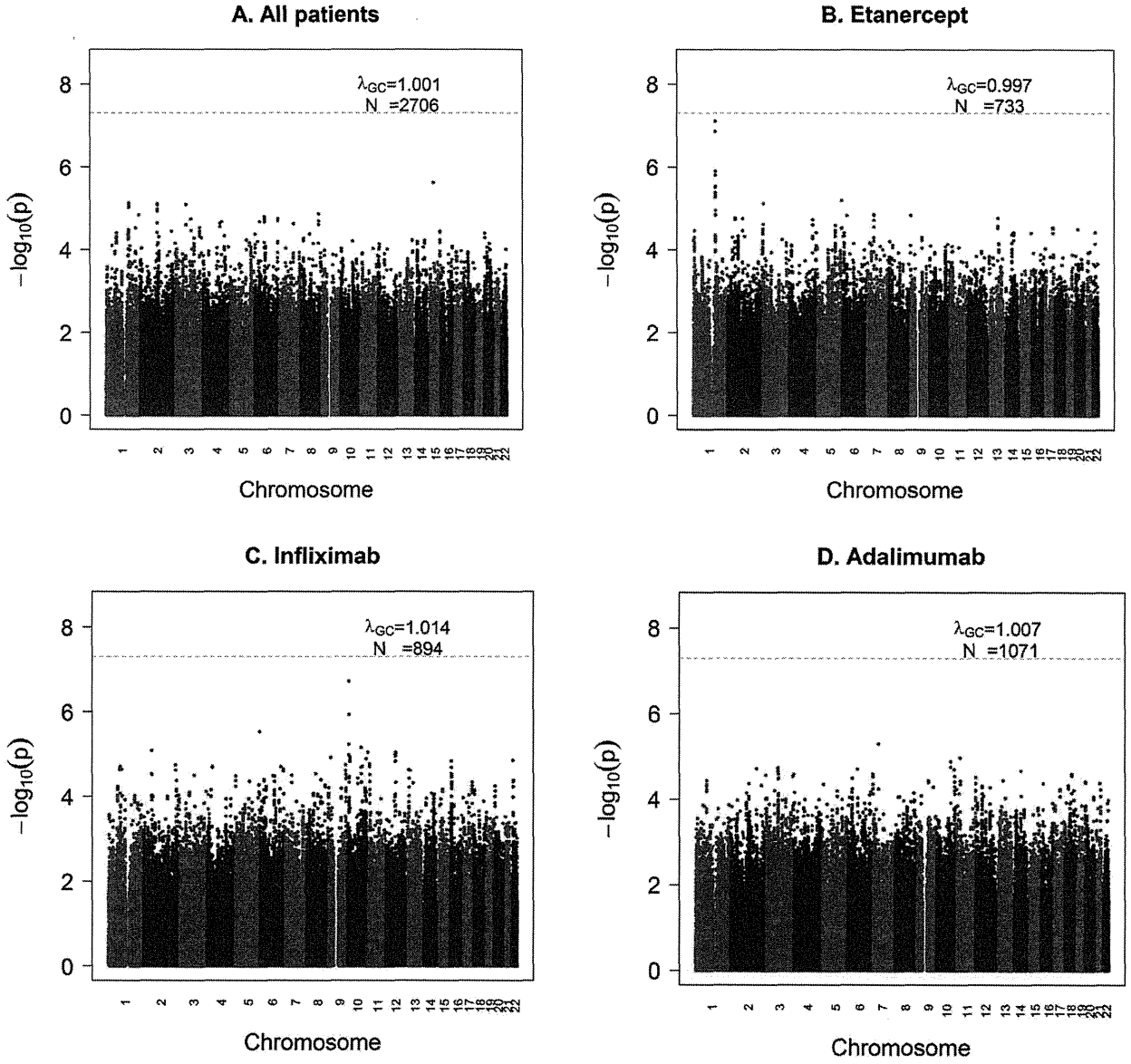
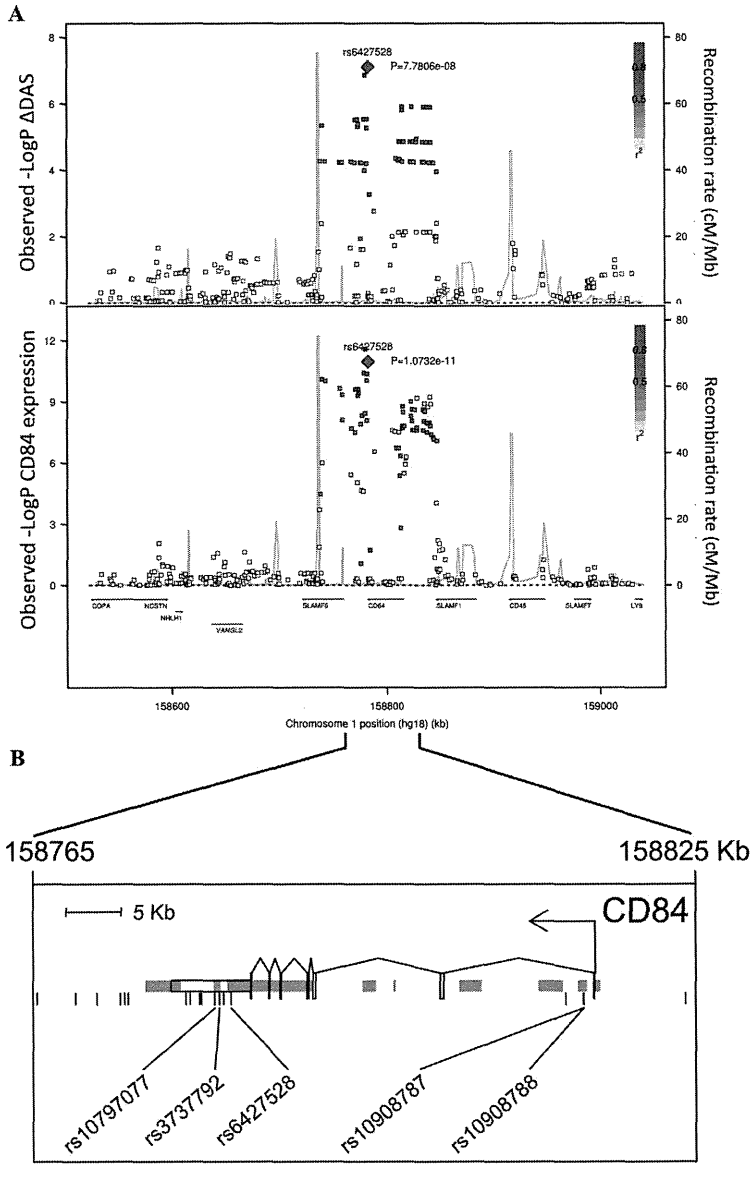


Figure 1. GWAS results for the ΔDAS phenotype. Shown are strengths of association (−Log10 P-value) for each SNP versus position along chromosomes 1 to 22. A) All samples (n = 2,706). B) Etanercept-treated patients (n = 733). C) Infliximab-treated patients (n = 894). D) Adalimumab-treated patients (n = 1,071).
doi:10.1371/journal.pgen.1003394.g001



C.

SNP (Major/ minor alleles)	Conserv-ation score[1]	DNase[2]	Transcription factor motifs altered		
			TF Motif	LOD(min) - LOD(maj)	Position weight matrix logo[3]
rs10797077 (G/A)	2.1	T-47D	AIRE_2	>6.6	
rs3737792 (G/A)	-1.3	-	-		
rs6427528 (A/G)	-2.8	-	KROX	>3	
			SREBP_4	-2.2	
rs10908787 (A/G)	-3.4	GM12878, Jurkat	-		
rs10908788 (A/G)	-7.8	GM12878	-		

Figure 2. Association results and SNP annotations in the *1q23 CD84* locus. A) Regional association plots with ΔDAS (top panel) and with *CD84* expression (bottom panel), showing strengths of association (−Log10 P-value) versus position (Kb) along chromosome 1. B) Schematic of *CD84* gene structure (RefSeq gene model, box exons connected by diagonal lines, arrow indicates direction of transcription) with strong enhancer chromatin states (orange rectangles) and SNPs in high LD (r2>0.8) with rs6427528 (vertical ticks). SNPs in enhancers are labeled below. C) Annotations of strong-enhancer rs6427528 proxy SNPs; listed are SNP rs-ID (major and minor alleles), conservation score, cell line with DNAse footprint if present, and transcription factor binding sites altered. 1- Genomic evolutionary rate profiling (GERP) conservation score, where a score >2 indicates conservation across mammals. 2- DNase footprint data are compiled from publicly available experiments by HaploReg. 3- Position weight matrix logos show transcription factor consensus binding sites with nucleotide bases proportional to binding importance. SNP position is boxed. Note that the rs10797077 AIRE_2 and the rs6427528 SREBP_4 motifs are on the minus strand (base complements correspond to SNP alleles), with the SREBP motif shown upside down to align with the rs6427528 KROX motif on the positive strand. Data are from HaploReg.
doi:10.1371/journal.pgen.1003394.g002

allele frequencies ranging from 7–10%. The SNP explains 2.6% variance in response to etanercept treatment.

For patients treated with infliximab, we observed a suggestive result on chromosome *10p14* (rs12570744, $P = 2\times10^{-7}$). No highly significant or suggestive results were observed for the ΔDAS phenotype in patients treated with adalimumab ($P_{META} > 10^{-5}$).

Qualitatively similar results were attained in the analysis of our secondary phenotype, EULAR good responder vs non-responder status (Figures S1 and S2). For SNPs at the *1q23* locus, the pattern of association with responder/non-responder status (etanercept-treated patients) was consistent with the results for ΔDAS ($P = 6\times10^{-3}$ for rs6427528 and rs1503860). We also identified potential novel associations, with suggestive results for infliximab (rs4336372, chromosome *5q35*, $P = 8\times10^{-7}$) and adalimumab (rs940928, chromosome *2q12*, $P = 2\times10^{-6}$).

eQTL and sequence analysis of the *CD84* gene

For each SNP with $P<10^{-6}$ identified by our GWAS (n = 6 independent SNPs), we searched for biological evidence to support a true positive association. We used genome-wide sequence data from the 1000 Genomes Project to search for putative functional variants in LD with the index SNP (defined as SNPs predicted to change protein-sequence or mRNA splicing). We also used genome-wide expression data to search for an expression quantitative trait locus (eQTL) in public databases and in peripheral blood mononuclear cells (PBMCs) in 228 non-RA patients and in 132 RA patients.

While we did not identify any variants disrupting protein-coding sequences or mRNA splicing, we did find that the *1q23* SNP associated with response to etanercept therapy was a strong eQTL in PBMCs (Figure 2A and Figure 3). In an analysis of 679 SNPs for cis-regulated expression of five genes in the region of LD (*SLAMF6*, *CD84*, *SLAMF1*, *CD48*, and *SLAMF7*), we found that rs6427528-*CD84* (and SNPs in LD with it) was the top eQTL of all results (n = 228 subjects; Figure 2A). This SNP was specifically associated with *CD84* expression, and was not an eQTL for other genes in the region (P>0.36 for the other genes).

We replicated our eQTL finding in 132 RA patients with both GWAS data and genome-wide expression data. PBMC expression data were available from RA patients in the Brigham RA Sequential Study (BRASS) and Autoimmune Biomarkers Collaborative Network (ABCoN) collections. We observed a significant association between rs6427528 genotype and *CD84* expression (linear regression adjusted for cohort P = 0.004, rank correlation P = 0.018). The direction of effect was the same as in the PBMC samples from 228 non-RA patients. A combined analysis of RA patients and the non-RA patient eQTL data (described above) yielded rank correlation $P = 3\times10^{-10}$ (n = 360 total individuals).

We searched sequence data to determine if rs6427528, or any of the SNPs in LD with it, were located within conserved, non-coding motifs that might explain the eQTL data. We used HaploReg [16] to examine the chromatin context of rs6427528 and 26 SNPs in LD with it (at $r^2>0.50$). We found that 5 SNPs occur in strong enhancers inferred from chromatin marks (Figure 2B) [17]. Two of these 5 SNPs, rs10797077 and rs6427528 ($r^2 = 0.74$ to each other), are predicted to disrupt transcription factor binding sites, and rs10797077 occurs at a site that shows conservation across mammalian genomes [18]. Figure 2C shows the DNA sequence position weight matrices of the transcription factor binding sites changed by rs10797077 (the minor allele creates a stronger binding site for the AIRE transcription factor) and rs6427528 (the minor allele creates a binding site for KROX and SREBP).

Expression of *CD84* as a biomarker of disease activity and treatment response

Because the genetic data demonstrates that the allele associated with better response is associated with higher *CD84* expression, this suggests that *CD84* expression itself may serve as a useful biomarker of disease activity or treatment response. We tested both hypotheses using PBMC expression data from the BRASS and ABCoN collections. First, we tested if *CD84* expression is associated with cross-sectional DAS, adjusting for age, gender and cohort (Figure 4). We observed a significant inverse association between *CD84* expression and cross-sectional DAS in 210 RA patients (beta = −0.3, P = 0.02, $r^2 = 0.02$). That is, higher *CD84* expression was associated with lower DAS, regardless of treatment.

Second, we tested *CD84* for association with our primary treatment response phenotype, ΔDAS. The sample size for this analysis was smaller than for the cross-sectional analysis, as we required that patients be on anti-TNF therapy and have pre- and post-treatment DAS. We found that *CD84* expression levels showed a non-significant trend towards an association with ΔDAS in 31 etanercept-treated patients (beta = 0.2, $r^2 = 0.002$, P = 0.46) and in all 78 anti-TNF-treated patients (beta = 0.14, $r^2 = 0.004$, P = 0.4). The effect is in the same direction one would predict based on the genetic association at rs6427528: the allele associated with better response is also associated with higher *CD84* expression (Figure 3), and in 31 RA patients, higher *CD84* expression (regardless of genotype) is associated with a larger ΔDAS (i.e., better response; Figure 4).

Replication of genetic data in a small, multi-ethnic cohort

Since most of the samples available to us as part of our international collaboration were included in our GWAS, few additional samples were available for replication. In addition, the remaining samples available to us were from different ethnic backgrounds. Nonetheless, we sought to replicate the associations of rs6427528 with ΔDAS in these additional samples. We genotyped 139 etanercept-treated patients from a rheumatoid arthritis registry in Portugal (Reuma.pt) and 151 etanercept-treated patients from two Japanese collections (IORRA, n = 88 patients on etanercept and Kyoto University, n = 63 on etanercept). Replication sample sizes, clinical data and results for these

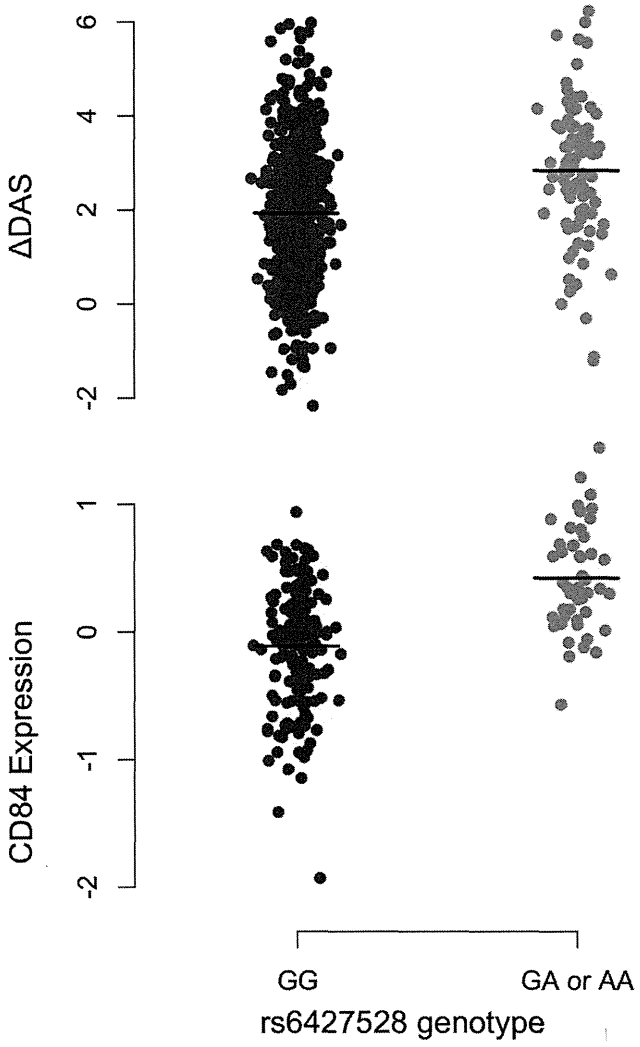


Figure 3. *1q23/CD84* genotype association plots for ΔDAS and *CD84* gene expression. Shown are ΔDAS in our GWAS in etanercept-treated patients (top panel, n = 733; n = 634 with the GG genotype and n = 99 with the GA or AA genotype) and *CD84* expression in our eQTL results (bottom panel, n = 228 non-RA patients; n = 178 with the GG genotype and n = 50 with the GA or AA genotype). The rare-allele homozygous genotype AA was observed four times in our ΔDAS GWAS and was pooled with the heterozygous GA genotype for this figure; AA homozygotes were not observed in the *CD84* eQTL data. Association analyses reported in the text regressed phenotype (ΔDAS, $P = 8 \times 10^{-8}$; *CD84* expression, $P = 1 \times 10^{-11}$) on minor-allele dosage (range 0–2).
doi:10.1371/journal.pgen.1003394.g003

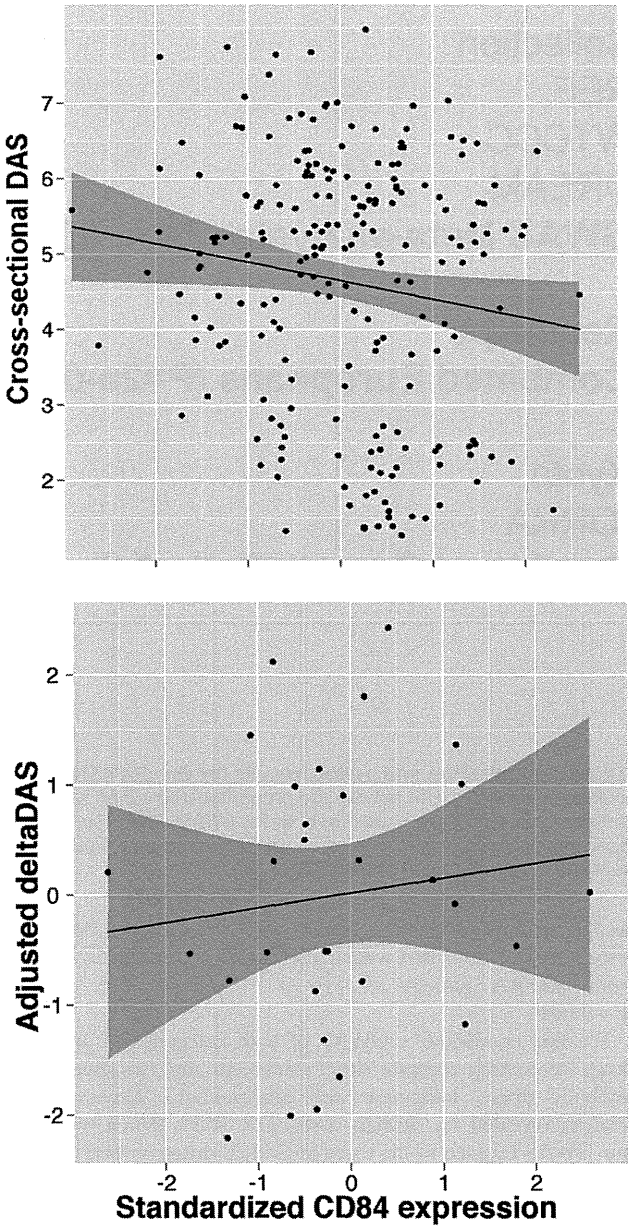


Figure 4. *CD84* expression level and clinical features. Analyses are shown in RA patients from the BRASS and ABCoN registries, for baseline DAS (top panel, n = 210; $R^2 = 0.02$, p = 0.02) and ΔDAS (bottom panel, n = 31; $R^2 = 0.001$, p = 0.46). Best-fit linear regression lines are shown in black, with shaded regions showing linear regression model (slope and intercept) 95% confidence intervals. *CD84* expression levels were quantile normalized, and ΔDAS values were adjusted for age, gender and baseline DAS.
doi:10.1371/journal.pgen.1003394.g004

two SNPs are shown in Table S4. Based on the observed effect size in the GWAS and observed allele frequency in the replication samples, we had 32% power to replicate this finding in the Portuguese samples and 17% power to replicate this finding in the Asian samples at $P<0.05$. The same association analysis as for GWAS was carried out: linear regression assuming an additive genetic model and using ΔDAS as phenotype, adjusted for baseline DAS. Replication results are shown in Figure 5.

While the SNPs fail to replicate in these patient collections at $P<0.05$, the direction of effect is the same in the Portuguese and Kyoto replication samples as in our GWAS. In a combined analysis limited to subjects of European-ancestry (GWAS data and Portuguese replication samples), rs6427528 remained highly suggestive ($P = 2 \times 10^{-6}$). Including the Japanese subjects, the overall GWAS+replication combined meta-analysis P-value remained suggestive ($P = 5 \times 10^{-4}$).

Discussion

Here we present the largest GWAS to date on anti-TNF therapy response in 2,706 RA patients. We find a significant association at the *1q23/CD84* locus in 733 etanercept treated patients ($P = 8 \times 10^{-8}$), but not in RA patients treated with drugs that act as a monoclonal antibody to neutralize TNF (infliximab or

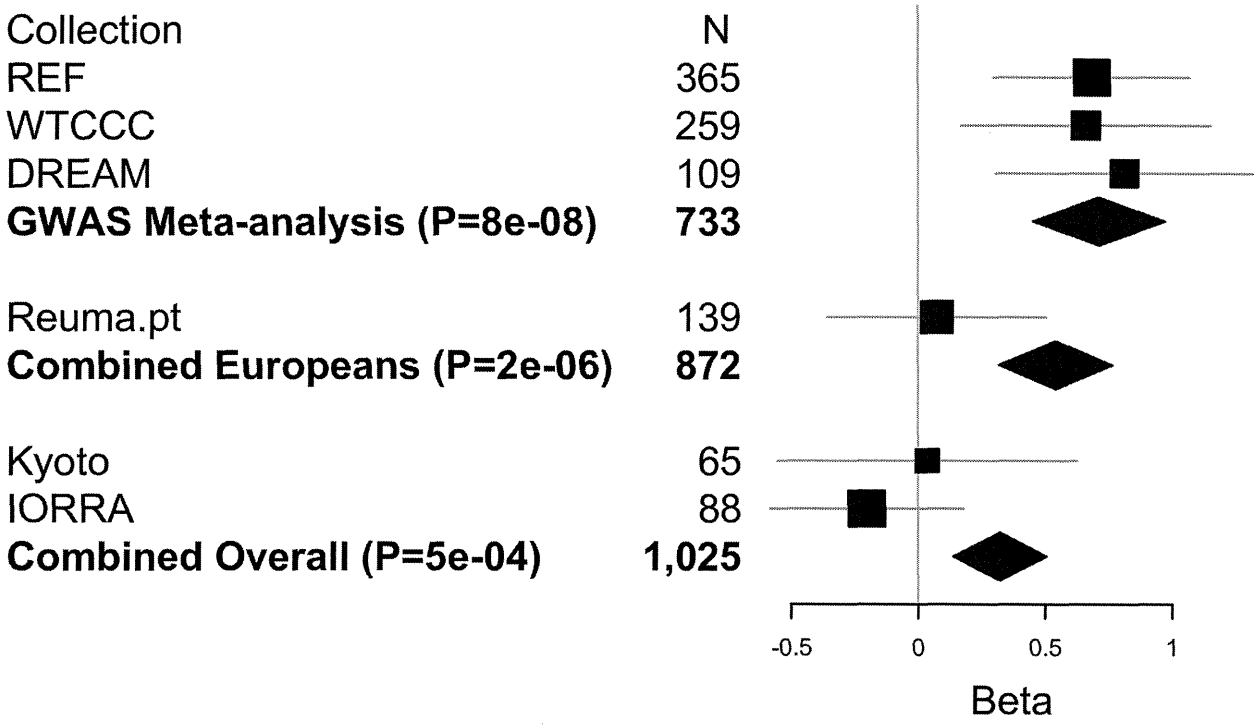


Figure 5. Replication and overall results for the *CD84* SNP rs6427528. Forest plot shows each cohort, sample size and linear regression beta coefficient estimates with symbol size proportional to cohort sample size and thin horizontal lines showing beta 95% CIs. Inverse variance weighted meta-analysis results are shown in bold for GWAS, GWAS+European (Portuguese) replication samples, and for GWAS+European+Asian (Japanese) replication samples, with diamond widths indicating beta 95% CIs.
doi:10.1371/journal.pgen.1003394.g005

adalimumab). The allele associated with a larger ΔDAS (i.e., better response) was associated with higher *CD84* expression in PBMCs from non-RA patients ($P = 1 \times 10^{-11}$) and in RA patients ($P = 0.004$).

We first conducted a GWAS of both categories of anti-TNF drugs (the soluble receptor drug, etanercept, and two monoclonal antibody drugs, infliximab and adalimumab). However, this analysis revealed no strongly associated SNPs. When we subset our GWAS by each of the three individual drugs, several SNPs in the *1q23* locus were highly significant in etanercept-treated patients, and SNPs in three other loci (*10p15*, *5q35* and *2q12*) were associated in infliximab or adalimumab subset analyses. Furthermore, the top SNPs for each analysis (Table S3) showed little correlation across the three anti-TNF drugs. This simple observation suggests that genetic control of treatment response may be different for different drugs. This finding is consistent with the clinical observation that RA patients who fail one anti-TNF drug may still respond to a different anti-TNF drug, albeit at lower rates of response [19]. If confirmed in larger samples and more comprehensive analyses, then this could have major implications for how physicians prescribe these drugs.

The most significant finding from our GWAS was a set of equivalent SNPs in LD with each other from the *1q23* locus in etanercept-treated RA patients (Figure 1 and Figure 2A). While the top SNP did not reach genome-wide significance in predicting treatment response, it did reach genome-wide significance as an eQTL in PBMCs ($P = 1 \times 10^{-11}$; Figure 2A). This finding indicates that the SNP (or another variant in LD with it) is indeed biologically functional in a human tissue that is important in the immune response. Two SNPs, rs10797077 and rs6427528, disrupt transcription factor binding sites, and represent excellent candidates for the causative allele to explain the effect on *CD84* expression (Figure 2C).

Our findings suggest that *CD84* genotype and/or expression could be a biomarker for etanercept treatment response among individuals of European ancestry. The genetic and expression data predict that *CD84* expression should be positively associated with treatment response (i.e., higher expression is associated with better response; Figure 3). While we did not observe a significant association between *CD84* levels and ΔDAS, we did observe a trend consistent with this prediction (Figure 4). Importantly, we note that power was extremely limited with the small sample sizes for which we had *CD84* expression as well as drug response data ($n = 31$ RA patients treated with etanercept).

The *CD84* gene is a compelling candidate for immune response, belonging to the CD2 subset of the immunoglobulin superfamily. It has been implicated in T-cell activation and maturation [20]. CD84 localizes to the surface of CD4+ and CD8+ T cells, and acts as a costimulatory molecule for IFN-gamma secretion [21]. *CD84* is also expressed in B-cells, monocytes and platelets. *CD84* has not been previously implicated in genetic studies of RA risk, disease activity, disease severity, or treatment response.

A limitation of our study is the small sample size available for replication ($n = 290$ etanercept-treated patients), and the lack of replication observed for the top *CD84* SNP (rs6427528) among patients of Portuguese and Japanese ancestry. The simplest explanation is that our original observation in the GWAS data represents a false positive association. However, the eQTL and gene expression data argue against this possibility. Explanations for a false negative finding in our replication collections include: (1) lack of power, especially if the effect size observed in the GWAS represents an over-estimate of the true effect size (the Winner's Curse) – we estimate that we had 32% and 17% power (at $P = 0.05$) to detect an association in the Portuguese and Japanese sample collections, respectively; (2) clinical heterogeneity, which is

always a possibility in pharmacogenetic studies, especially those conducted in different countries; and (3) ethnic differences, including different patterns of LD between the underlying causative allele (which is as yet unknown) and marker SNPs tested in our study. We did observe subtle differences in local patterns of LD between Asians and Europeans using genetic data from the 1000 Genomes Project (Figure S5). We note that the rs6427528 minor allele A has a frequency of ~5–10% in European and East Asian populations, and ~50% in the African YRI population (HapMap2 and 1000 Genomes); therefore, it may be of interest to test African American samples in replication.

What are the options for increasing sample size in pharmacogenetic studies, thereby providing an opportunity to replicate our *CD84* genetic and expression findings? While it might seem trivial to collect more samples through traditional registries, this is extremely challenging for phenotypes pertaining to treatment efficacy. To underscore this point, we highlight our study design, where we organized samples and clinical data from 16 different collections across 7 different countries in order to obtain the samples for the current study. Going forward, non-traditional strategies to collect biospecimens linked with clinical data (e.g., online registries, electronic medical records) may be required to achieve clinical collections of sufficient size to discover pharmacogenomic predictors of efficacy.

In conclusion, we conducted the largest GWAS to date for response to anti-TNF therapy in RA patients. Our genetic and expression data suggest that *CD84* genetic variants and/or expression levels could be developed as predictive biomarkers for etanercept treatment response in RA patients of European ancestry.

Methods

Samples and clinical data

All patients met 1987 ACR criteria for RA, or were diagnosed by a board-certified rheumatologist. In addition, patients were required to have at least moderate disease activity at baseline (DAS>3.2). All patients gave their informed consent and all institutional review boards approved of this study. A total of 13 collections from across 5 countries were included in GWAS [11,12,13,22]: Autoimmune Biomarkers Collaborative Network (ABCoN) from the U.S. (N = 79); the Genetics Network Rheumatology Amsterdam (GENRA, N = 53); the Dutch Behandelstrategieen voor Rheumatoide Arthritis (BeSt, N = 85); the U.K. Biological in Rheumatoid arthritis Genetics and Genomics Study Syndicate (BRAGGSS, N = 140); the U.S. Brigham Rheumatoid Arthritis Sequential Study (BRASS, N = 55); the Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA, N = 298); the Immunex Early Rheumatoid Arthritis study (eRA N = 57); the Swedish Karolinska Institutet study (KI, N = 77); the Netherlands collection from Leiden University Medical Center (LUMC, N = 43); and the U.S. Treatment of Early Aggressive RA (TEAR, N = 109). We refer to these collections as the American College of Rheumatology Research and Education Foundation (REF) collection, as funding for GWAS genotyping was provided by the "*Within Our Reach*" project. We included additional samples from BRAGGSS (N = 595) [12]; the Dutch Rheumatoid Arthritis Monitoring registry (DREAM) in the Netherlands, and the ApotheekZorg (AZ) database (which facilitates the Dutch distribution of adalimumab; N = 880) [23,24], together referred to as DREAM; and the French Research in Active Rheumatoid Arthritis (ReAct, N = 272) [25].

Additional samples were collected for replication of SNPs in the *1q23* locus. These included the Rheumatic Diseases Portuguese Register (Reuma.pt, N = 378) from the Portuguese Society of Rheumatology (SPR), which captures more than 90% of patients treated with biological therapies and managed in rheumatology departments across Portugal [26]. Additional replication samples (N = 374) of East Asian ancestry were included from the IORRA and Kyoto University Hospital registries, part of the Japanese Genetics and Allied research in Rheumatic diseases Networking consortium (GARNET) [27].

Clinical data were collected in each cohort, including disease activity scores at baseline and at least one time point after treatment, gender, age, methotrexate use, as well as autoantibody status (RF or CCP). The composite disease activity scores for 28 joints (DAS28) included laboratory values for erythrocyte sedimentation rate (ESR) for most samples and C-reactive protein (CRP) for 191 samples in the REF collection (ABCoN, BRASS and eRA cohorts). DAS28 values were available at baseline and at 3–12 months after initiating anti-TNF therapy. Our primary phenotype was defined as ΔDAS = baseline DAS - end DAS, and responder status was also determined according to EULAR criteria for start and end DAS [15]. Clinical variables were assessed for association with phenotype in multivariate linear or logistic regression models for both the ΔDAS and EULAR responder-status phenotypes. Clinical variables that were significant in these analyses were retained as covariates in genetic association tests, except for methotrexate co-therapy. Including a covariate for methotrexate co-therapy reduced sample size substantially due to missing clinical data, so results were compared for our primary analysis and a secondary analysis with the covariates (and with reduced sample size) and the results were verified not to be impacted (not shown).

Genotyping and data processing

A total of eleven genotyping batches were processed separately. (1) BRASS samples were genotyped using Affymetrix 6.0 chip [28]; (2) WTCCC samples were genotyped on Affymetrix 500K chip [12]. All other cohorts were genotyped using Illumina platform arrays (see Table 1). Our American College of Rheumatology Research Education Fund (REF) collection was made up of smaller cohorts from throughout North America and Europe, including BRASS samples. Also included in REF: (3) ABCoN [13] and (4) EIRA [29] were separately genotyped on the Illumina 317K genotyping array; (5) eRA on the Illumina 550K chip; and (6) GENRA, BeSt, BRAGGSS (a subset of N = 53 samples), KI and LUMC were genotyped in one batch, and (7) BRAGGSS (N = 87) and TEAR were genotyped in a second batch, both using Illumina 660k chips, at the Broad Institute (8–10). DREAM and AZ samples were genotyped in three batches, one on 550K chip and two on 660K chips (manuscript in preparation), and (11) ReAct samples were genotyped on Illumina OmniExpress chips. Quality control (QC) filtering was done in each genotyping batch, including filtering individuals with >5% missing data, and filtering SNPs with >1% missing data, minor allele frequency (MAF) <1% and Chi-squared test of Hardy Weinberg equilibrium $P_{HWE}<10^{-5}$. We then used individual-pairwise identity-by-state estimates to remove occasional related and potentially contaminated samples. Data processing and QC were performed in PLINK [30]. Principal Components Analysis (PCA) was performed using EIGENSTRAT [31] (default settings) on the combined dataset using 20,411 SNPs genotyped across all datasets. Ethnicity outliers including all individuals of non-European decent were identified and removed, and the first three eigenvectors were used as covariates in GWAS.

Imputation was conducted on each of eleven datasets separately, using the IMPUTE v1 software [32] and haplotype-phased

HapMap Phase 2 (release 22) European CEU founders as a reference panel. Imputation of BRASS and EIRA was previously reported [28,33], and we followed the same imputation procedures for the remaining datasets. Imputation yielded posterior genotype probabilities as well as imputation quality scores at SNPs not genotyped with a minor allele frequency ≥1% in HapMap CEU. We removed imputed SNPs with imputation 'info' scores <0.5 or MAF <1% in any of the datasets.

Expression profile and eQTL data

Gene expression levels were quantified using mRNA derived from peripheral blood mononuclear cells (PBMCs) using Affymetrix Human Genome U133 Plus 2.0, for 255 multiple sclerosis patients in the Comprehensive Longitudinal Investigation of MS at the Brigham and Women's Hospital [34], either untreated (N = 83) or treated with interferon-beta (N = 105) or glatiramer acetate (N = 67). The raw intensity values were subject to quality control based on the recommended pipeline available in the simpleaffy and affyPLM R Bioconductor packages, and were then normalized using GCRMA (N = 228). The data are available on the Gene Expression Omnibus website (GSE16214). Expression levels for 17,390 probes mapping to 9,665 Ensembl transcripts were adjusted for confounding factors including age, gender, drug and batch using principle components and Bayesian factor analysis [35], and used in eQTL association analyses. Genotype data were collected on the Affymetrix 550K GeneChip 6.0 platform as a part of a previously published study [36]. Allelic dosages from imputed data (HapMap Phase II CEU samples; >2 million SNPs, MACH imputation quality >0.1 and MAF> = 0.05) were used for association analysis. *Cis*-eQTLs were identified +/−1 Mb of transcription start sites (TSS) in the *1q23* locus region. Significance was evaluated by 10,000 permutations per gene, and false discovery rates were calculated based on cis-eQTL analyses in the total of 9,665 genes [37].

Additional expression profile data were available for subsets of samples that were part of two cohorts in our GWAS. Expression data from patients enrolled in the BRASS registry have been previously published [38]. Expression data were collected on Affymetrix Gene Chip U133 Plus 2 microarrays. BRASS patients had either cross-sectional expression data (n = 132, assayed at the time the patient was enrolled in BRASS) or pre- and post-treatment expression data (n = 17 samples, 8 treated with etanercept). Of these, n = 87 patients had expression and GWAS data. For patients with pre- and post-treatment data, we used the "baseline" pre-treatment expression data for cross-sectional analysis. In ABCoN, 65 RA patients (n = 23 treated with etanercept) had both pre- and post-treatment expression data, as well as ΔDAS clinical data [39], and n = 45 patients had expression and GWAS data. As with BRASS, we use the "baseline" pre-treatment expression data for cross-sectional analysis. For ABCoN expression profile data were collected on Illumina Human WG6v3 microarrays and were quantile normalized according to Illumina recommended protocols. Within both BRASS and ABCoN, expression data were normalized to the mean and standard deviation within each collection. For prospective analyses of expression data and ΔDAS, we combined BRASS and ABCoN to include 31 etanercept-treated patients and 78 anti-TNF-treated patients.

Statistical analyses

In our primary GWAS analysis, we tested each SNP for association with ΔDAS using linear regression adjusted for baseline DAS and the first 3 PCA eigenvectors in each collection. In our secondary GWAS analysis, we modeled SNPs predicting EULAR good response *versus* EULAR non-response using logistic regression, again adjusting for start-DAS value and the first three eigenvectors. Association analysis was done using SNPTEST [32] assuming an additive genetic model. Genomic control λ_{GC} values [40] for genotyped SNPs only and all SNPs were calculated, and no inflation or deflation was observed in the distributions of association test results. We then conducted inverse variance-weighted meta-analysis to combine results across the four datasets, and conducted Cochran's Q tests for heterogeneity using the β coefficients [41]. We further divided samples into 3 subsets according to drug (etanercept, infliximab or adalimumab). GWAS analysis for each group followed the same analysis procedure. Meta-analysis and heterogeneity tests were conducted using SAS. Expression analyses utilized linear regression or Spearman rank correlation, also using SAS. We tested for effects of cohort, age, gender and concurrent methotrexate, and results are shown using significant covariates as indicated.

Supporting Information

Figure S1 Quantile–quantile (QQ) plots for ΔDAS and response analysis, with genomic control λ_{GC} values.
(TIF)

Figure S2 GWAS results for the good response versus non-response phenotype. Shown are strengths of association (−Log10 P-value) for each SNP versus position along chromosomes 1 to 22. A) All samples (n = 1,708). B) Etanercept-treated patients (n = 472). C) Infliximab-treated patients (n = 599). D) Adalimumab-treated patients (n = 636).
(TIF)

Figure S3 Forest plot of replication results for the CD84 SNP rs6427528, in patients treated with anti-TNF drugs other than etanercept (infliximab & adalimumab).
(TIF)

Figure S4 Forest plot of CD84 result in patients treated with etanercept, subset by all collections.
(TIF)

Figure S5 Patterns of linkage disequilibrium (LD) at the CD84 locus in HapMap. Shown patterns of LD for CEU (top panel) and CHBJPT (bottom panel).
(TIF)

Table S1 Sample information for each of thirteen clinical batches.
(DOC)

Table S2 Clinical multivariate model for the ΔDAS phenotype.
(DOC)

Table S3 GWAS results for all SNPs achieving $P<10^{-6}$ from any analysis.
(XLS)

Table S4 Sample and clinical data summary for replication samples.
(DOC)

Acknowledgments

We thank Lars Klareskog and Lars Alfredsson for their contribution to the EIRA cohort, and the Swedish Rheumatology Register and Biologics Register (ARTIS) for providing follow-up data for the patients in the EIRA and KI study groups. We thank participants in the Biologics in Rheumatoid Arthritis Genetics and Genomics Study Syndicate (BRAGGSS).