A abrogation of regulatory T cell function deteriorates Rheumatoid Arthritis
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Introduction
Prediction of the course of the disease in patients with rheumatoid arthritis (RA) in the initial stages is difficult partly due to the variable rate of disease progress. In this study, we performed a multidisciplinary approach to early disease prognosis. First, we examined multiple gene expression using DNA micro-array in an attempt to identify the specific genes which lead to a deterioration of the disease process. Secondly, we investigated the correlation between conventional Th1 and Th2 helper T cell, and regulatory T cell (Treg) function using suspension-array analysis (Bio-Plex, Bio-Rad Laboratories, Hercules, CA, USA) in an attempt to measure multiplex cytokines in the sera. Thirdly, specific bindings of antibodies were analyzed with surface and intracytoplasmic staining of lymphocytes. Finally, RT-PCR and real-time PCR analysis was performed. We also evaluated the predictive importance of IFN-γ/IL-10 ratio as a prognostic tool for the patients with early RA.

Materials and methods
Patients: Moderate RA patients were defined as those with Stainbrocker Stage-I and II joint changes, while those with Stainblocker Stage-IV changes were defined as severe RA patients.
DNA micro-array analysis: Peripheral blood lymphocytes were prepared by ficoll and analyzed using Mini-Paque, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradient centrifugation. Total RNA was extracted using ISOGEN (NIPPON GENE CO. LTD., Tokyo, Japan) and then reverse-transcribed into cDNA, labeled with α-32P-dATP and hybridized to DNA micro-array (Atlas Human 1.2 Array III, Clontech Laboratories Inc., Foster City, CA, USA). The expression value of each gene transcript was plotted as a scatter plot.
Suspension-array analysis of serum cytokines: For suspension-array analysis, human 17-plex array system containing 17 dyed and addressed beads were used. Each bead conjugates with monoclonal antibodies specific for 17 different human cytokines, and PE-conjugated anti-cytokine-antibodies to measure the quantity of captured cytokines. Captured cytokines on addressed beads were measured by flowcytometry (Bio-Rad Laboratories, Hercules, CA, USA). At least 200 beads were measured on each cytokine. Data were analyzed by Bio-plex manager software (Bio-Rad Laboratories, Hercules, CA, USA) and the concentration of each cytokine was determined.
Surface and intracytoplasmic staining of lymphocytes: For cytomteric analysis, FITC-conjugated mouse-anti-human-CD4 monoclonal antibody (clone:RPA-T4,LgG1), APC-conjugated mouse-anti-human-CD25 monoclonal antibody (clone:BC96,LgG1), PE-conjugated rat-anti-human-Foxp3 monoclonal antibody (clone: PCH101,LgG2a), and PE-conjugated mouse-anti-human-GITR(AITR) monoclonal antibody (clone:cBioAITR,LgG1) were used. Cells were fixed and permeabilized with eBioscience Fixation/Permeabilization buffer (eBioscience, San Diego, CA, USA), then incubated with the antibodies previously described. After incubation, specific bindings of antibodies were analyzed by FACs can analyzer and Cell Quest software (BD biosciences, San Jose, CA, USA)
RT-PCR and real-time PCR analysis: PCR-reactions were carried out with specific primers for T-bet, GATA-3, Foxp3, GITR, and for β-actin. RT-PCR analysis was achieved using Gene Amp RNA PCR Core Kit (Applied Biosystems, Foster city, CA, USA). RT-PCR products were visualized with ethidium bromide under UV illumination. Real time PCR was achieved using SYBR Prime Script RT-PCR Kit (Takara Bio, Inc., Kyoto, Japan) and analyzed with Applied Biosystems 7900HT Fast Real Time PCR system and SDS software (Applied Biosystems, Foster city, CA, USA). Relative gene expression was determined by comparative Ct method. Statistics: Comparisons between the groups were performed by two-sided t test. Data were presented as means ± S.E. P values<0.05 were considered to be statistically significant.

Results
The result of the DNA micro-array analysis, CD25, known as a specific surface marker on the Treg subset was elevated in moderate- and severe-RA patients. In suspension-array analysis, severe RA patients showed increased production of IFN-γ and suppressed production of IL-10. Interestingly, in moderate RA patients, most of the cytokine productions tested were elevated. Next, we analyzed the balance between several kinds of cytokines, using IL-10 concentration as an internal control. IFN-γ/IL-10 ratio was regarded as Th1/Treg function. This ratio most sensitively indicated the aggravation of the disease from the incipient stage of the disease. In flowcytometry analysis, CD4+CD25+ T cells in moderate RA patients were almost identical to those in healthy-control subjects. However, we observed decreased production of IL-10 and increased production of IFN-γ from the incipient stage of the disease. Then, in the PCR analysis, T-bet, GATA-3 and Foxp3 that are specific for Th1, Th2 and Treg were analyzed. In moderate RA patients, Foxp3 expression required for Treg function was highly suppressed. Further, intracytoplasmic Foxp3 and surface GITR expressions were investigated by flowcytometry. In moderate RA patients, decreased Foxp3 and increased GITR expression were observed.

Discussion
Taking these data together, we conclude that abrogation of CD4+CD25+ regulatory T cell function possibly caused by GITR/GITR-ligand signal transduction pathway aggravates RA. Further, we demonstrated that serum IFN-γ/IL-10 ratio sensitively indicates the progression of the disease, and is a highly predictive prognostic parameter and diagnostic tool for patients with early and more progressive RA.