1 **Circulating Th17.1 cells as candidate for prediction of therapeutic response to**

2 **abatacept in patients with rheumatoid arthritis: exploratory research**

3 Short title: Prediction of therapeutic response to abatacept using Th17.1 cells

4

5 Shinji Maeda[1]*, Satoshi Osaga[2], Tomoyo Maeda[1], Norihisa Takeda[1], Shin-ya Tamechika[1],

6 Taio Naniwa[1], Akio Niimi[1]

7

8 [1]Department of Respiratory Medicine, Allergy and Clinical Immunology, Nagoya City

9 University Graduate School of Medical Sciences, Nagoya 467-8601, Japan

10 [2]Clinical Research Management Center, Nagoya City University Hospital, Nagoya 467-8601,

11 Japan

12

13

14 *Corresponding author

15 Email: snb51961@med.nagoya-cu.ac.jp (SM)

16

Abstract

T helper 17.1 cells (Th17.1) are highly pathogenic T cells in inflammatory diseases. This study aimed to identify Th cell biomarkers, including the analysis of Th17.1, that predict therapeutic response to abatacept in patients with rheumatoid arthritis. The circulating Th subsets among CD4+ T lymphocytes in 40 patients with rheumatoid arthritis before abatacept treatment were determined using multicolor flow cytometry. All patients received abatacept treatment for 24 weeks, and the change in disease activity score, including 28-joint count C-reactive protein, and the responsiveness of other indices to abatacept treatment were evaluated according the European League Against Rheumatism criteria [good responders, moderate responders, and non-responders]. The correlation between the abatacept responses and the Th subsets (baseline) was analyzed. Logistic regression analysis with inverse probability weighting method was conducted to calculate odds ratio adjusted for patient characteristics. The proportion of baseline Th17.1 cells was significantly lower in patients categorized as good responders than in those categorized as non-good responders (moderate responders and non-responders; $p = 0.0064$). The decrease in 28-joint count C-reactive protein after 24 weeks of abatacept therapy also showed a significant negative correlation with the proportion of Th17.1 cells. The adjusted odds ratio for achieving good response in patients with baseline Th17.1 level below the median value was 14.6 (95% confidence interval, 2.9–72.3; $p = 0.0021$) relative to that in the remaining patients. The proportion of Th17.1 cells at baseline is a good candidate for the prediction of response to abatacept treatment. These novel findings may represent an important step in the pursuit of precision medicine.

Introduction

41   Advances in medicine and pharmaceutical technology have led to tremendous

42   improvements in the treatment of rheumatoid arthritis (RA) [1]. Moreover, research in

43   clinical human immunology has found new autoimmune cells. The development of potent

44   anti-rheumatic drugs, particularly biological products, has helped in the improvement of

45   clinical remission rates [2,3]. The targets of these biologics include both cytokines and T

46   cells, which play key roles in the pathogenesis of RA. The therapeutic effect of abatacept

47   (ABA), a strong inhibitor of T cells [4], has been shown to be equivalent to that of tumor

48   necrosis factor α (TNF-α) inhibitor therapy [5-7].

49   The target lymphocytes of ABA, particularly CD4+ T cells, play a central role in the

50   pathogenesis of RA, particularly in terms of acquired immunity, and in the induction of

51   autoimmune response [4]. Research conducted on autoimmune mice models [8,9] has

52   demonstrated the decisive role of Th17 in the pathogenesis of arthritis and autoimmune

53   diseases. In humans, CCR6, which is a representative surface marker of Th17 [10], is a

54   disease susceptibility gene of human RA [11]. Th17 is also involved in human RA pathology

55   [12] by antigenically producing inflammatory cytokines, such as interleukin (IL)-17A,

56   IL-17F, and IL-22. Unlike mice, these human Th17 cells have subpopulations. In particular,

57   Th17.1 cells that are CD161+ CXC chemokine receptors (CXCR3)+ interferon-γ-producing

58   Th17 cells, also referred to as classical Th1 or extinguish Th17 (ex-Th17) are a subgroup of

59   Th17 cells found in humans [13] and are believed to be the most pathogenic among CCR6+

60   CD4+ T cells [12]. Th17 cells convert to inflammatory Th17.1 cells in an inflammatory

61   milieu induced by cytokines, such as IL-1β, IL-23, TNF-α, and IL-12 [14]. Th17.1 cells have

62   high expression levels of P-glycoprotein/multidrug resistance type 1 (MDR-1) and exhibit

63   glucocorticoid resistance [13]. RA is characterized by joint destruction that is resistant to

64   steroid treatment alone [15]. It is believed that the glucocorticoid resistance of Th17.1 cells is

65   not attributable only to the function of MDR-1, and thus, further elucidation of the

66   mechanism is expected in future. ABA is a strong inhibitor of these pathological T cells.

67   Although the therapeutic efficacy of ABA is well demonstrated, some patients are

68   refractory to ABA treatment. The immune mechanisms that drive the chronicity of synovitis

69   are multifactorial, such as adoptive immune pathways, innate immune pathways, stromal

70   pathways, and systemic pathways [16]. Therefore, the contribution of immune factors other

71   than T cells may influence the efficacy of ABA therapy. Among the various T cell subsets,

72   some decrease in response to ABA treatment, whereas others do not [17]. ABA treatment has

73   been reported to markedly reduce the proportion of T follicular helper cells and slightly

74   decrease Th17 and activated regulatory T cells (Treg) cells. However, few studies have

75   focused on the relationship between Th17.1 cells and therapeutic response to ABA in patients

76   with RA. Moreover, there is a need to identify biomarkers that predict the therapeutic

77   response. Identification of drug-specific biomarkers that predict therapeutic response is a

78   desirable goal in the realm of personalized medicine [1]. Indeed, advances in cell analysis

79   technology has raised prospects for the discovery of novel cellular immunological biomarkers

80   that can predict treatment response in rheumatic diseases [18,19].

81   In the present study, we analyzed the proportion of each Th cell subset, including

82   CXCR3+ Th17 cells (Th17.1), in the peripheral blood of patients with active RA before ABA

83   treatment to explore early cellular biomarkers of response to ABA. We demonstrated the

84   correlation between the proportion of Th17.1 cells and response to ABA therapy and

85   proposed its potential use as a biomarker for predicting therapeutic response.

86   # Materials and Methods

87

88   ## Ethics statement

89   This research was approved by the Ethics Review Committee of the Graduate School

90   of Medicine, Nagoya City University. The study was conducted in compliance with the

91 Declaration of Helsinki. Written informed consent was obtained from all patients.

Participants

93 Patients with RA who fulfilled the classification criteria of the 1987 American

94 College of Rheumatology rheumatoid arthritis classification (revised criteria of classification

95 of RA) [20] and received ABA treatment at the Nagoya City University Hospital between

96 2009 to 2015 were eligible for inclusion. Inclusion criteria were as follows: 1) patients who

97 consented to participate in this research, 2) patients who agreed to provide peripheral blood

98 mononuclear cells (PBMCs) for immune-phenotyping analysis of Th subsets and Treg using

99 multicolor flow cytometry, and 3) patients who did not achieve adequate improvement on

100 previous treatment with at least one conventional synthetic disease-modifying anti-rheumatic

101 drug (DMARDs).

102 Participants received intravenous ABA at 0 weeks, 2 weeks, 4 weeks, and every 4

103 weeks thereafter. The dose of ABA was based on body weight (BW) as follows: 500 mg for

104 patients with a BW of <60 kg and 750 mg for those with a BW of 60 to 100 kg.

105

Cell surface and intracellular staining and flow cytometry analysis

108 After obtaining informed consent, we obtained PBMCs of participants at baseline and

109 at 4 and 24 weeks of ABA treatment. PBMCs were separated by density gradient

110 centrifugation with Ficoll–Paque Plus (GE Healthcare, Uppsala, Sweden) and resuspended in

111 flow cytometry buffer (Hank's Balanced Salt Solution supplemented with 2%

112 heat-inactivated fetal calf serum, 0.05% Sodium Azide, and 0.5% EDTA). Cells were stained

113 for 30 min at 4°C under darkened conditions with the following fluorochrome labeled

114 monoclonal antibodies: anti-CD4-AmCyan (Clone SK3, BD Biosciences, Franklin Lakes, Nj,

115   USA), anti-CD25-APCCy7 (Clone M-A251, BD Biosciences), anti-CD45RA-FITC (Clone

116   HI100, BD Biosciences), anti-Ki67-FITC (Clone B56, BD Biosciences), anti-CD196

117   (CCR6)-PE-Cyanine7 (Clone R6H1, eBioscience, San Diego, CA, USA), anti-CD161-PE

118   (Clone HP-3G10, eBioscience), anti-CCR4-Alexa647 (Clone TG6/CCR4, eBioscience),

119   anti-CD183 (CXCR3)-Pacific Blue (Clone G025H7, BioLegend), anti-forkhead box P3

120   (Foxp3)-PerCP-Cyamine5.5   (Clone   PCH101,   eBioscience),   rat

121   immune(Ig)G2a-PerCP-Cyamine5.5 antibody (eBioscience), and anti-CD45RO-APC (Clone

122   UCHL1, TONBO Biosciences, San Diego, CA, USA). For the intracellular staining of Foxp3

123   and Ki67, the Foxp3-Staining Buffer Set (fixation/permeabilization and permeabilization

124   buffers, eBioscience) was used according to the manufacturer's protocol. Stained cells were

125   washed twice using the flow cytometry buffer and resuspended for analysis using the

126   Canto-II Flow cytometer (BD Bioscience) and the Diva software (BD Bioscience) and

127   analyzed with FlowJo software (Tree Star). We defined the Th subset as follows:

128   Treg, CD4+ CD25+ Foxp3+; nonTreg, CD4+ Foxp3−; Th1, CXCR3+ CCR4− CCR6-

129   nonTreg; Th2, CCR4+ CXCR3− CCR6− nonTreg; Th1&2, CXCR3+ CCR4+ CCR6−

130   nonTreg; Th17, CCR6+ CD161+ CCR4+ CXCR3− nonTreg; and Th17.1, CCR6+ CD161+

131   CXCR3+ CCR4− nonTreg.

132

133   **Clinical assessment and evaluation of therapeutic response**

134   Data pertaining to the following demographic and clinical variables were obtained

135   from the medical records: age, sex, disease duration, use of corticosteroids,

136   disease-modifying anti-rheumatic drugs, non-steroidal anti-inflammatory drugs, tender joint

137   count, swollen joint count, patient global assessment [patient visual analog scale, 0–100 mm],

138   physician global assessment [doctor's visual analog scale, 0–100 mm], C-reactive protein

139   (CRP) level, matrix metalloproteinase-3 (MMP-3) level, rheumatoid factor (RF) level, and

6

140   anti-citrullinated protein/peptide antibody (ACPA) level.

141   Disease activity was assessed by calculating DAS28-CRP for each patient at each

142   visit. The DAS28-CRP was calculated, and patients were categorized into the following four

143   groups: remission, low (LDA), moderate (MDA), or high (HDA) disease activity according

144   to the recommended formula

145   (https://www.das-score.nl/das28/en/difference-between-the-das-and-das28/how-to-measure-t

146   he-das28/how-to-calculate-the-das28/alternative-validated-formulae.html).   Because

147   DAS28-CRP values are reportedly lower than those obtained in the original DAS28

148   assessment using erythrocyte sedimentation rate, a threshold of 4.1 (instead of the original

149   5.1) was used as the cut-off for HDA; 2.7 (instead of 3.2) as the cut-off for low disease LDA;

150   and 2.3 (instead of 2.6) as the cut-off for remission [21]. The therapeutic response to ABA at

151   24 weeks was evaluated using the European League Against Arthritis (EULAR) response

152   criteria

153   (https://www.das-score.nl/das28/en/difference-between-the-das-and-das28/importance-of-das

154   28-and-tight-control/eular-response-criteria.html), with 4.1 and 2.7 used as the thresholds for

155   HDA and LDA, respectively. Briefly, patients were classified into three groups based on their

156   6-month DAS28-CRP and their absolute change from baseline according to the EULAR

157   criteria as no response, moderate response, or good response. A good responder must

158   demonstrate improvement of at least 1.2 units and achieve an absolute DAS28-CRP score of

159   <2.7. A non-responder must demonstrate an improvement of ≤0.6 and have a final

160   DAS28-CRP score of >4.1. Moderate responses fall between these data points. Furthermore,

161   responsiveness to ABA treatment was evaluated using the following indicators: changes in

162   disease activity before and after ABA treatment (ΔDAS28-CRP 0–24 weeks) and the

163   evaluation of disease activity after 24 weeks of ABA treatment (remission, LDA, MDA,

164   HDA).

7

165   After the initiation of ABA therapy, the clinical course was followed up for 24 weeks

166   (every 4 weeks), and the correlation between responses to ABA treatment, RA disease

167   activity, and the baseline proportion of Th subsets among CD4+ T lymphocytes (before

168   treatment) was analyzed.

169

MDR-1 activity assay

171   For the analysis of the MDR-1 activity of T cells, the fluorescent dye rhodamin 123

172   (Rh-123) was used according to the methods reported elsewhere [13]. Briefly, total CD4+ T

173   cells were isolated using the Dynabeads CD4 positive T cell isolation kit (Invitrogen).

174   Purified cells were 95%–98% pure as determined by flow cytometry analysis. Purified T cells

175   in complete medium [DMEM (Gibco) supplemented with 10% FBS, 1% L-glutamine, 1%

176   sodium pyruvate, 1% Hepes, and 1% Pen-Strep (all from Gibco) were loaded with Rh-123

177   (Sigma-Aldrich) at a final concentration of 1 μg/mL for 30 min on ice. Cells were then

178   washed and moved to a 37°C incubator for 2 h. After an efflux period, cells were washed on

179   ice in PBS, stained with surface markers (CD4, CD45RO, CXCR3, CCR6, and CD161), and

180   washed again in PBS, and stained cells were kept on ice prior to flow cytometry analysis.

181   Fluorescence reduction due to the emission of fluorescent dye by MDR-1 was confirmed by

182   flow cytometry. For a negative control, 1 μM cyclosporine A (Sigma-Aldrich) was added to

183   cells immediately before the incubation step.

184

Statistical analysis

186   Mann–Whitney U test was used to assess between-group differences with respect to

187   continuous variables, and Fisher's exact test was used to assess between-group differences

188   with respect to categorical variables. Kruskal–Wallis test was used for the analysis of

189   differences in continuous variables between three groups. Friedman rank sum test and

190   Wilcoxon signed rank test were used to analyze sequential changes in the proportion of each

191   Th subset among CD4+ lymphocytes (0, 4, and 24 weeks) and Ki67 expression in each Th

192   subset (0 and 4 weeks). Spearman rank correlation coefficient was used to assess correlation

193   between two continuous variables. Stepwise variable selection method based on Akaike's

194   Information Criterion, Bayesian information criterion, and p-value was performed to identify

195   the candidate Th subset that predicted ABA response.

196   The enrolled patients (n = 40) were divided into two groups based on the median

197   proportion of Th17.1 cells among CD4+ T cells: Th17.1-lower (n = 20) and Th17.1-higher (n

198   = 20).

199   To minimize the potential confounding effect due to baseline differences in patient

200   characteristics between Th17.1-lower and higher groups, the inverse probability weighting

201   (IPW) method, which is an application of the propensity score [22-24], was applied to

202   compare the DAS28-CRP and good response rate of ABA treatment between the groups.

203   Propensity scores for the IPW method were estimated using multivariate logistic regression

204   analysis with the Th17.1 status (lower or higher) as the dependent variable and using the

205   following baseline characteristics as independent variables: age, sex, DAS28-CRP (baseline),

206   RF, ACPA RA disease duration (years), history of biological DMARDs, prescription of

207   methotrexate (MTX), and glucocorticoids. The discriminative power of the propensity score

208   was quantified by the c statistic corresponding to the area under the receiver operating

209   characteristic curve. Next, each patient background variable was compared under the

210   correction by the IPW method using the weighted Mann–Whitney test, the weighted t-test,

211   and the weighted chi-squared test, and the respective p-values were calculated. The effect of

212   Th17.1 on patient ABA response was evaluated using estimated odds ratios (OR) and 95%

213   confidence intervals (CIs). All calculated p-values were two-sided, and p-values < 0.05 were

214   considered statistically significant for all analyses. Statistical analyses were performed with

9

215   the R-software version 3.3.3 (R Development Core Team, Vienna, Austria) and EZR version

216   1.35 (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [25], which is a

217   graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria).

218   The following R-software packages were used for statistical processing and creation of

219   graphs and tables: survey (version 3.31-5) [26], aod (version 1.3), weights (version 0.85),

220   ggplot2 [27], and corrplot (version 0.77).

221

Results

222

223

Baseline characteristics of patients

224

225   Table 1 shows the baseline demographics and clinical characteristics of the enrolled

226   patients (N = 40). The disease activity of RA was high in the study population (median

227   DAS28-CRP, 4.43; simplified disease activity index, 23.8). ACPA-positive patients

228   accounted for 60% of the study population, and they were relatively older (median age, 70.5

229   years). With respect to use of concomitant drugs, 77.5% of the patients were taking MTX,

230   whereas 60% were taking glucocorticoids. With respect to medication history, only 32.5% of

231   the patients had a history of treatment with biological DMARDs.

232   **Table 1. Clinical characteristics of patients at baseline.**

Patient characteristics	Overall n = 40 Median [IQR], or, (%)

Age, year old	70.5 [60.8, 74.6]
Sex: male/female, (%)	8/32 (20.0/80.0)
Disease duration (years)	4.2 [1.5, 15.9]
DAS28-CRP	4.43 [4.02, 5.01]
SDAI score	23.8 [20.3, 28.8]
CRP, mg/dl	1.02 [0.48, 2.27]
MMP-3 (ng/ml)	167.7 [100.2, 290.2]
ACPA (negative/positive, (%))	16/24 (40.0/60.0)
negative, (%)	16 (40.0)
Low positive, (%)	5 (12.5)
High positive, (%)	19 (47.5)
RF (negative/positive, (%))	14/26 (35.0/65.0)
negative, (%)	14 (35.0)
Low positive, (%)	9 (22.5)
High positive, (%)	17 (42.5)
Concomitant methotrexate, n (%)	31 (77.5)
MTX mg (mean (sd))	9.4 (2.9)
Concomitant glucocorticoid, n (%)	24 (60.0)
prednisolone, mg (mean, (sd))	5.8 (3.6)
Concomitant tacrolimus, n (%)	3 (7.5)
Concomitant NSAIDs, n (%)	20 (50.0)
Biologic DMARDs naïve, n (%)	27 (67.5)
Pulmonary complications associated with RA, n (%)	18 (45.0)

233

234   This table shows patient baseline demographics. Data are presented as median [IQR,

235   interquartile range], mean [SD], or frequency [%].

236   DAS28-CRP, disease activity score 28-joint count C-reactive protein; SDAI, simplified

237   disease activity index; CRP, C-reactive protein; NSAIDs, non-steroidal anti-inflammatory

238   drugs; MMP-3, matrix metallo-proteinase 3; ACPA, anti-citrullinated protein antibody; RF,

239   rheumatoid factor; MTX, methotrexate; DMARDs, disease modified anti-rheumatic-drug;

240   Low positive, less than 3 times normal upper limit among positive; High positive, more than

241   3 times the normal upper limit.

242   Between-group differences with respect to median values determined using the Mann–

243   Whitney U test, whereas those with respect to percentage values were determined using the

244   Fisher's exact test.

245

Characterization of Th17.1 in patients with RA

247   We analyzed the subtype of peripheral blood T cells before and four weeks after ABA

248   treatment (Fig 1a); furthermore, in each cell group, Ki67 expression was determined by flow

249   cytometry as a cell proliferation marker.

250   Th17.1 was the smallest subset of CD4+ cells (median 1.17%; interquartile range

251   0.71–1.93) (S1 Fig). Next, the expression of MDR-1, one of the major features of Th17.1,

252   was confirmed using Rh-123 (Fig 1b). The results showed that MDR-1 was highly expressed

253   only in Th17.1 cells and not in the Th1 and Th17 cells, as reported so far. The expression rate

254   of CD28, an inhibitory target in ABA treatment, in the Th17.1 cells was as high as that in the

255   others (>99%) (Fig 1c).

256

257   **Fig 1. Characteristic of circulating Th17.1 cells in patients with rheumatoid arthritis**

258 **(RA)**. a. Flow cytometry plots explaining the gating strategy for the identification of

259 peripheral blood Treg, Th1, Th2, Th1&2, Th17, and Th17.1 subpopulations. CD4+ T cell

260 subsets in the peripheral blood of adult patients with RA were analyzed using flow

261 cytometry. b. The MDR1 activity of indicated Th subset assessed using multicolor flow

262 cytometry with rhodamine 123 (Rh-123). Total CD4+ T cells isolated from peripheral blood

263 were labeled with Rh123. After a 1-h efflux period at 37°C in the presence of vehicle

264 (Dimethyl sulfoxide) or MDR1 inhibitors (cyclosporine A), cells were stained with

265 antibodies to CCR6, CXCR3, and CD45RO, and Rh-123 efflux of each Th subsets was

266 analyzed using flow cytometry. Data shown are flow cytometry plots representing three

267 independent experiments performed on cells isolated from different donors with RA. c.

268 Proportion of cells with CD28 expression within the indicated CD4+ T cell subpopulations of

269 patients with RA. Data are representative of at least three independent experiments.

270

271 ## Difference between early changes in proliferation status of Th

272 ## subsets

273   Changes in the proportions of Th subset among CD4+ T cells before ABA treatment

274 and 4 weeks after treatment were confirmed to evaluate the effect of ABA treatment on each

275 Th subset. However, noticeable changes were not observed (Fig 2a). Therefore, we next

276 analyzed the expression of Ki67 in the cells to confirm the early effects of ABA on each Th

277 cell subset (Fig 2b). Before ABA treatment, the proportion of Ki67 positive cells among each

278 Th subset was different; particularly, the expression rate in Th17.1 cells was remarkably

279 lower than that in the other subsets (S2 Fig, Figs 2b and 2c). In contrast, the expression rate

280 of Ki67 in Treg cells was relatively higher, which suggests that Treg is active during cell

281 proliferation in patients with RA. Next, the Ki67 positivity rate for each Th subset was

282 determined after ABA treatment for 4 four weeks and compared with the baseline. In only 4

283   weeks, the proportion of Ki67 positive cells was significantly reduced in all subsets other

284   than the Th17.1 cells (Fig 2c). The change in Ki67 expression in Th17.1 cells was not

285   statistically significant (p = 0.39).

286

287   **Fig 2. Early change in cell proliferation state of each Th subset by ABA treatment.**

288   Peripheral blood mononuclear cells of patients with RA were obtained at baseline and at 4

289   and 24 weeks of abatacept treatment (0 and 4 weeks, n = 40; 24 weeks, n = 29).

290   a. Sequential changes in the proportion of each T cell subset among CD4 T cells in peripheral

291   blood induced by ABA treatment. Data were analyzed using Friedman rank sum test.

292   b. Flow cytometry plot showing the frequency of Ki67 expression in the indicated CD4+ Th

293   cells in patients with RA assessed using intracellular staining of Ki67 antigen and analyzed

294   using multicolor flow cytometry.

295   c. Sequential changes in the proportion of Ki67 expression in each Th subsets induced by

296   ABA treatment (0 and 4 weeks, n = 40).

297   Data were analyzed using Wilcoxon signed rank test.

298

Therapeutic response to ABA and baseline Th17.1

300   ABA treatment was continued for 24 weeks, and the progress of disease activity in

301   each patient and the responsiveness to ABA treatment were evaluated. Subsequently, we

302   analyzed the correlation between ABA response and the Th subset at baseline. A remarkable

303   finding was that the proportion of baseline Th17.1 cells among CD4+ T cells in good

304   responders was significantly lower than that in poor responders (p = 0.0064) (Fig 3a). In

305   contrast, no significant difference was observed with respect to the other Th subsets.

306   The attenuation of disease activity (ΔDAS28-CRP) after 24 weeks of ABA treatment

307   also showed a significant negative correlation with Th17.1 (both percentage and absolute

14

308   number) (Fig 3b).

309

310   **Fig 3. Clinical significance of Th17.1 levels in abatacept treatment response.** ABA

311   treatment was continued for 24 weeks. Subsequently, correlation between ABA response and

312   the Th subset at baseline was analyzed. The following indicators were used to evaluate

313   response to ABA treatment: changes in DAS28-CRP scores from baseline at 24 weeks after

314   ABA treatment (ΔDAS28-CRP 24 weeks), disease activity evaluation after 24-week ABA

315   treatment [remission and low (LDA), moderate (MDA), and high (HDA) disease activity],

316   and treatment response evaluation using the EULAR response criteria [good responder (GR),

317   moderate responder (MR), and non-responder (NR)]. a. ABA treatment response after 24

318   weeks was evaluated as GR (n = 14), MR (n = 13), and NR (n = 13). The proportion of

319   indicated Th subsets among CD4 + lymphocytes at baseline in each group was plotted and

320   displayed as box plot. b. Scatter plot shows the ratio or absolute number of Th17.1 cells at

321   baseline and ΔDAS28-CRP 24 weeks. Regression line (red line) and 95 CI of the regression

322   line (gray zone) are also shown in the plot. c. d. Patients were stratified into three groups

323   (low, intermediate, and high) based on tertiles of the proportion of Th17.1. Hundred

324   percentage stacked bar chart shows the EULAR response rate (c) and the disease activity

325   score (d) at 24 weeks after the initiation of ABA treatment in three groups. Data were

326   analyzed using the Kruskal–Wallis and Mann–Whitney tests for between-groups comparisons

327   with Bonferroni correction (a), Spearman's rank correlation coefficient (b), and Fisher's

328   exact test and Bonferroni correction for paired comparisons (c, d).

329

330   To assess the clinical relevance of the correlation between Th17.1 cells and ABA

331   response, we divided the patients into two groups (lower and higher) using the median Th

332   17.1 proportion (/CD4+) as cut-off (Table 2), and into three groups (lowest, intermediate, and

333   highest) based on tertiles. ABA treatment response in each group was analyzed. Treatment

334   response was significantly different in the three groups (p = 0.019) (Fig 3c). The treatment

335   response in the Th17.1-lower (lowest) group was significantly better than that in the higher

336   (highest) group (S3a–S3b Figs). On analysis of the trend of disease activity at 24 weeks, a

337   lower proportion of Th17.1 cells at baseline was associated with a higher percentage of

338   remission (Fig 3d). Remission rates in the Th17.1-lower and lowest groups were 55% and

339   57.1%, respectively, and no remission was observed in the higher and highest groups.

340

341   **Table 2. Clinical characteristics of the Th17.1-lower and Th17.1-higher patients at**

342   **baseline**

Patient characteristics	Th17.1 / CD4+ T cells		
	Lower n = 20	Higher n = 20	p
Age, year old	70.5 [60.8, 74.2]	71.0 [63.4, 74.7]	1.00
Sex: male/female, (%)	6/14 (30.0/70.0)	2/18 (10.0/90.0)	0.24
Disease duration (years)	3.4 [0.9, 6.4]	6.7 [2.5, 19.1]	0.07
DAS28-CRP	4.50 [3.77, 4.91]	4.43 [4.22, 5.02]	0.71
SDAI score	22.8 [18.5, 28.7]	24.3 [21.6, 29.2]	0.31
CRP, mg/dl	1.18 [0.57, 2.52]	0.82 [0.48, 2.16]	0.81
MMP-3 (ng/ml)	206.3 [104.6, 316.1]	152.2 [94.8, 267.8]	0.50
ACPA (negative/positive, (%))	9/11 (45.0/55.0)	7/13 (35.0/65.0)	0.75
negative, (%)	9 (45.0)	7 (35.0)	
Low positive, (%)	3 (15.0)	2 (10.0)	0.67
High positive, (%)	8 (40.0)	11 (55.0)	
RF (negative/positive, (%))	7/13 (35.0/65.0)	7/13 (35.0/65.0)	1.00
negative, (%)	7 (35.0)	7 (35.0)	
Low positive, (%)	5 (25.0)	4 (20.0)	1.00
High positive, (%)	8 (40.0)	9 (45.0)	
Concomitant methotrexate, n (%)	17 (85.0)	14 (70.0)	0.45
MTX mg (mean (sd))	10.2 (2.5)	8.3 (3.1)	0.07
Concomitant gulucocorticoid, n (%)	13 (65.0)	11 (55.0)	0.75

16

prednisolone, mg (mean, (sd))	5.5 (4.3)	6.1 (3.0)	0.72
Concomitant tacrolimus, n (%)	0 (0.0)	3 (15.0)	0.23
Concomitant NSAIDs, n (%)	10 (50.0)	10 (50.0)	1.00
Biologic DMARDs naïve, n (%)	14 (70.0)	13 (65.0)	1.00
Pulmonary complications associated with RA, n (%)	7 (35.0)	11 (55.0)	0.34

343 Enrolled patients (n = 40) were stratified into two groups based on the median proportion of

344 Th17.1 cells among CD4+ T cells: Th17.1-lower (n = 20) and Th17.1-higher (n = 20). The

345 table shows clinical features and differences of patient subgroups of Th17.1-lower and

346 Th17.1-higher at baseline. Data presented as median [IQR, interquartile range] or mean [SD],

347 or frequency [%].

348 DAS28-CRP, disease activity score 28-joint count C-reactive protein; SDAI, simplified

349 disease activity index; CRP, C-reactive protein; NSAIDs, non-steroidal anti-inflammatory

350 drugs; MMP-3, matrix metallo- proteinase 3; ACPA, anti-citrullinated protein antibody; RF,

351 rheumatoid factor; MTX, methotrexate; DMARDs, disease modified anti-rheumatic-drug;

352 Low positive, less than 3 times normal upper limit among positive; High positive, more than

353 3 times the normal upper limit.

354 Between-group differences with respect to median values determined using Mann–Whitney

355 U test, and those with respect to percentage values determined using Fisher's exact test.

356

357 We assessed whether changes in objective biomarkers of arthritis [serum CRP,

358 MMP-3)] after ABA treatment for 24 weeks were different between the Th17.1 lower and

359 higher groups. A significant reduction in serum CRP (S4a Fig) and MMP-3 levels (the

360 significant high-titer class) was observed in the Th17.1-lower group (S4b and S4c Figs).

361

362 **Fig 4. Prediction of therapeutic response to ABA based on the proportion of Th 17.1 at**

363 **baseline.** a. The difference in ABA therapeutic response between Th17.1-lower (binary by

364   median) and Th17.1-higher after adjustment of patient background factors using inverse

365   probability weighting (IPW). The size of the balloon plot indicates the weighting using the

366   IPW method in each case. The red lines indicate the weighted mean (horizontal line) and SD

367   (vertical line) after IPW adjustment. Black lines indicate non-weighted mean (horizontal line)

368   and SD (vertical line). Data were analyzed using the weighted (red) or non-weighted (black)

369   *t*-test. b. The adjusted odds ratio for achievement of GR with baseline Th17.1-lower relative

370   to Th17.1-higher. Logistic regression analysis using the IPW method to calculate the odds

371   ratio adjusted for patient characteristics. Forest plot shows unadjusted and adjusted odds

372   ratios and 95% CI and p-value.

373   ABA, abatacept; IPW, inverse probability weighting; SD, standard deviation; OR, odds ratio;

374   CI, confidence interval

375

376   Subsequently, we performed receiver operating characteristic curve analysis to

377   determine the optimal threshold level of Th17.1 associated with good response or remission

378   at 24 weeks (S5 Fig). A cut-off level of 1.09% (Th17.1 cells/CD4+ cells) was associated with

379   79.2% sensitivity and 81.2% specificity for GR and 75.9% sensitivity and 100% specificity

380   for remission.

381

382   **Th17.1 and patient background factors**

383   Differences in the clinical features between the Th17.1-lower and Th17.1-higher

384   groups at baseline were evaluated; however, no significant differences were observed. Next,

385   because CD4+ T cells play an important role in the pathogenesis of RA, we assessed the

386   correlation between various patient characteristics, disease activity, Th subset, and ABA

387   therapeutic response using Spearman's rank correlation coefficient (S6 Fig). The baseline

388   disease activity (DAS28-CRP baseline) showed a strong correlation with serum CRP, ACPA,

18

389 RF, and MMP-3 levels. In the Th subset, although baseline disease activity showed a

390 negative correlation with Treg, no significant correlation was observed with other Th subsets.

391 The disease duration of RA showed a positive correlation between Th1&2, Th17, and Th17.1

392 cells. In the Th subset analysis, Th17.1 showed a strong correlation with Th1 and Th17.

393 Analysis of the correlation between patient characteristics and ABA response revealed a

394 strong correlation of ΔDAS28-CRP (0–24 weeks) with baseline DAS28-CRP and age.

395 However, none of the patient characteristics showed a significant correlation with EULAR

396 response criteria and disease activity after 24 weeks (S6 Fig, S1 Table). Therefore, in this

397 study, we found no meaningful association between the background characteristics of

398 patients and therapeutic response to ABA. However, baseline levels of Th17.1 and Th17

399 subsets showed a significant association with all three indices of ABA response, and baseline

400 Th1 level was significantly associated only with disease activity after 24 weeks. Among these

401 three Th subsets, the Th17.1 subset showed the most significant association with ABA

402 response.

403 Among the patient background factors and Th subsets, we selected and narrowed

404 down the candidate variables to construct an optimal model for prognostic prediction using

405 stepwise variable selection in multivariate analysis (S2 Table). In all multivariate analyses,

406 only Th17.1 was selected and showed the most significant association after adjustment for

407 potential confounders. Given the limited number of cases for adjusting confounding factors

408 by multivariate analysis, we used the IPW method to reduce the number of confounders and

409 to analyze the adjusted effect of baseline Th17.1 on ABA therapeutic response.–The c

410 statistic, the discriminative power of propensity score (PS) for Th17.1-lower group was 0.735

411 (95% CI 0.576–0.894). With IPW, all patient background factors that are shown in S3 Table

412 were more evenly adjusted between Th17.1-lower and higher, including RA disease duration,

413 which was not significantly different but tended to correlate. Of note, even after adjustment

19

414   for different covariate distributions for both groups, there was a significant difference

415   between Th17.1-lower and higher groups with respect to ΔDAS28-CRP (0–24 weeks) and

416   disease activity after 24 weeks (DAS28-CRP 24 weeks) (Fig 4a). The effect of Th17.1-lower

417   on ABA good response as compared to that of Th17.1-higher was evaluated by estimated OR

418   with 95% CIs, after adjustment by IPW. In this study, good responders and patients with low

419   disease activity after 24 weeks were equivalent. As a result, in the Th17.1-lower group, OR

420   for achieving good response was 14.6 (95% CI, 2.9–72.3; p = 0.0021) (Fig 4b, S4 Table).

421   The proportion of Th17.1 cells among CD4+ T cells at baseline was a good predictor of ABA

422   treatment response.

423

Discussion

425   In the present study, we found that baseline Th17.1 levels may be a prognostic predictor of

426   ABA treatment in patients with RA. Th17 also correlated with ABA therapeutic response;

427   however, Th17.1 showed a stronger correlation with ABA therapeutic response. Among Th

428   cells that cause antigen-specific responses, the identification of cells that correlate with ABA

429   therapeutic response is the most novel finding of this study.

430   A key novelty aspect of this study was the inclusion of analysis of cell surface

431   CD161, intracellular forkhead box P3 (Foxp3), and Ki 67 in flow cytometry analysis.

432   Although CD161 is not included as a surface marker in the international standard human

433   immune-phenotyping method [28], it is particularly important as a surface marker of

434   pathogenic Th17 and Th17.1 subsets. In addition, CCR6 and CCR4 are also expressed in

435   Treg; therefore, intracellular Foxp3 was stained to precisely exclude regulatory T cells.

436   Analysis of the expression of Ki67 allowed us to capture the early change in cell proliferation

437   status of each Th subset induced by ABA treatment. When compared with the reported

438   standard method, there are certain limitations in the interpretation of this research method and

20

439   results; however, it is important to analyze Th17.1 more precisely.

440   In this study, Th17.1 showed a significant correlation with the patient background

441   characteristics such as disease duration. Even after adjustment for patient background

442   characteristics using the IPW method, the baseline proportion of Th17.1 subset significantly

443   predicted ABA responsiveness. For the identification of cellular immunological biomarkers

444   that predict therapeutic response by flow cytometric analysis, adjustment for patient

445   background characteristics is important because target cells themselves may interact with

446   patient background factors other than therapeutic effect. However, because of the limited

447   number of samples owing to the handling of living cells and the associated cost burden, it is

448   difficult to adjust for confounding factors using multivariate analysis. In such settings, the use

449   of the IPW method to adjust for patient background characteristics is a well-accepted

450   practice.

451   Previous studies have found only few factors that adequately predict the therapeutic

452   response to ABA in RA treatment. These include CRP [29], presence or absence of MTX

453   combination, history of use of other biologics [30], positivity for ACPA and RF [31], and age

454   [32]. In this study, no significant correlation was observed between these known baseline

455   patient characteristics and the proportion of peripheral blood Th17.1 cells. None of the

456   known patient characteristics was found to predict ABA treatment response. Furthermore, the

457   following lymphocyte-related indices, which are independent of Th17.1, were reported as

458   prognostic factors: proportion of terminally differentiated effector memory cells among CD8

459   T cells [33] and the proportion of CD28 negative T cells [34,35]. However, these lymphocyte

460   subsets were not analyzed in this study. Therefore, the correlation between these known

461   lymphocytes and Th17.1 was not clarified, which is a study limitation.

462   Our data also showed interesting results pertaining to intracellular Ki67 expression in

463   each Th subset. The expression level of Ki67 in Th17.1 cells at baseline was significantly

21

464   lower than that in the other subsets. Moreover, the positivity rate of Ki67 in Th subsets,

465   except for Th17.1, decreased markedly in the early stage of ABA treatment. These findings

466   suggest that Th17.1 is less susceptible to ABA with respect to cell proliferation. Considering

467   that the mechanism of action of several immunosuppressive drugs (such as DMARDs)

468   involves the inhibition of lymphocyte proliferation, immunosuppressive drugs are less likely

469   to target Th17.1 because Th17.1 is non-proliferating in active patients with RA undergoing

470   conventional synthetic therapy with DMARDs.

471   In contrast to Th17.1 cells, the difference in Treg levels did not predict the response to

472   ABA treatment despite the difference in Treg proportions in the patients at baseline.

473   Immunological mechanisms of RA pathology, such as the disruption of autoimmune

474   tolerance, onset and persistence of inflammation, and joint destruction, are extremely

475   complex. Even in the contribution of T cells to the persistence of inflammation, the

476   importance of the involvement of dysfunction in the suppression (regulatory) side, such as

477   Treg, and the existence of inflammatory T cells that can resist Treg suppression is also

478   conceivable. Treg expresses cytotoxic T-lymphocyte antigen-4 (CTLA-4) [36], which is an

479   immunosuppressive functional molecule common to both Treg and ABA. It was inferred that

480   the inactivation of the immune system by CTLA-4-Ig strongly supplemented the difference in

481   the amount of Treg and the influence of endogenous Treg for the arthritis condition will be

482   reduced. In contrast, when inflammatory cells that are resistant to suppression by these

483   CTLA-4 molecules are present at baseline, these levels are likely to affect disease activity

484   after the ABA treatment.

485   These results pose a simple question of whether Th17.1 has a causal relationship with

486   treatment resistance. In previous reports, Th17.1 cells were described as the most pathologic

487   inflammatory cells among the CCR6 positive T cells [12]. Both Th17 and Th17.1 cells are

488   present in the synovial fluid of patients with RA; however, Th17.1 cells are particularly more

489   abundant in the synovial fluid than in the peripheral blood [37]. Compared with Th1 and

490   Th17, Th17.1 cells produce more types of pro-inflammatory cytokines, such as IL-17A,

491   granulocyte macrophage colony-stimulating factor, interferon-γ, and TNF-α, which are

492   associated with rheumatoid inflammatory conditions. More specifically, treatment with the

493   neutralizing   antibody   of   the   inflammatory   cytokine   granulocyte   macrophage

494   colony-stimulating factor, which is strongly produced by Th17.1, is effective in RA [38].

495   Therefore, we envisage a certain role of Th17.1 in the activity of RA even during ABA

496   treatment in non-responders. CTLA-4 molecule is a strong negative regulator of T cell

497   immune response [39,40] and plays the central role in Treg's regulatory function [36]. More

498   interestingly, it was recently reported that ex-Th17, which has the same phenotype as Th17.1,

499   is not restricted by Treg suppression [41]. Based on these facts and the results of this study, it

500   is suggested that Th17.1 cells play a role in disrupting immune tolerance by CTLA-4 and

501   Treg. Currently, it is possible to induce clinical remission in some patients with RA using

502   biological DMARDs or targeted synthetic DMARDs. However, when targeting immunologic

503   remission, immunological factors as therapeutic targets still remain unknown. Immunological

504   remission can be achieved by the inactivation or suppression of inflammation-sustaining

505   pathological factors, such as autoreactive-T-cells, via endogenous immune regulatory

506   mechanisms, such as CTLA-4 or Treg. Although further biological studies are needed to

507   assess whether Th17.1 is resistant to the repression of CTLA-4-Ig, our study suggests that

508   Th17.1 cells serve as potential novel therapeutic targets for achieving immunologic

509   remission.

510

Conclusions

512   The present study demonstrated that the proportion of circulating Th17.1 cells showed

513   differences in immunological quality that determine the therapeutic response to ABA in

23

514   patients with RA. A vast array of anti-rheumatic drugs is currently available for the treatment

515   of RA. The identification of the most appropriate drug for individual patients is a key

516   imperative to achieve early improvement. The identification of Th17.1 as a good candidate

517   biomarker of the therapeutic response to ABA may represent an important step in the pursuit

518   of precision medicine.

519

Author contributions

521   SM conceived, designed the study, performed data analysis, and wrote the manuscript. SO

522   contributed to determining statistical methods for data analysis and statistical programming

523   and interpretation and assisted in the preparation of the manuscript. SM and TM performed

524   all flow cytometric analyses, and SM and TT conducted an experiment of MDR-1 activity

525   assay. ST helped with obtaining patient's blood samples. All other authors have contributed

526   to data collection and interpretation and have critically reviewed the manuscript. All authors

527   have approved the final version of the manuscript.

528

Acknowledgements

533

References

535   1. Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. Lancet. 2016;388: 2023-2038. doi:

536   10.1016/S0140-6736(16)30173-8

537   2. Felson DT, Smolen JS, Wells G, Zhang B, Van Tuyl LHD, Funovits J, et al. American

538   College of Rheumatology/European League Against Rheumatism provisional definition of

539   remission in rheumatoid arthritis for clinical trials. Arthritis Rheum. 2011;63: 573-586. doi:

540   10.1002/art.30129.

541   3. Van Tuyl LHD, Vlad SC, Felson DT, Wells G, Boers M. Defining remission in rheumatoid

542   arthritis: results of an initial American College of Rheumatology/European League Against

543   Rheumatism consensus conference. Arthritis Rheum. 2009;61: 704-710. doi:

544   10.1002/art.24392.

545   4. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med. 2007;365:

546   2205-2219. doi: 10.1056/NEJMra1004965.

547   5. Kremer JM, Westhovens R, Leon M, Di Giorgio E, Alten R, Steinfeld S, et al. Treatment of

548   rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig.

549   N Engl J Med. 2003;349: 1907-1915. doi: 10.1056/NEJMoa035075.

550   6. Kremer JM, Genant HK, Moreland LW, Russell AS, Emery P, Abud-Mendoza C, et al.

551   Effects of abatacept in patients with methotrexate-resistant active rheumatoid arthritis: a

552   randomized trial. Ann Intern Med. 2006;144: 865-876. doi:

553   10.7326/0003-4819-144-12-200606200-00003.

554   7. Maxwell LJ, Singh JA. Abatacept for rheumatoid arthritis: A cochrane systematic review. J

555   Rheumatol. 2010;37: 234-245. doi: 10.3899/jrheum.091066.

556   8. Hirota K, Hashimoto M, Yoshitomi H, Tanaka S, Nomura T, Yamaguchi T, et al. T cell

557   self-reactivity forms a cytokine milieu for spontaneous development of IL-17+ Th cells that

558   cause autoimmune arthritis. J Exp Med. 2007;204: 41-47. doi: 10.1084/jem.20062259.

559   9. Hirota K, Yoshitomi H, Hashimoto M, Maeda S, Teradaira S, Sugimoto N, et al. Preferential

560   recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid

561   arthritis and its animal model. J Exp Med. 2007;204: 2803-2812. doi: 10.1084/jem.20071397.

562   10. Bettelli E, Korn T, Oukka M, Kuchroo VK. Induction and effector functions of T(H)17 cells.

563   Nature. 2008;453: 1051-1057. doi: 10.1038/nature07036.

25

564   11. Kochi Y, Okada Y, Suzuki A, Ikari K, Terao C, Takahashi A, et al. A regulatory variant in
565   CCR6 is associated with rheumatoid arthritis susceptibility. Nat Genet. 2010;42: 515-519.
566   doi: 10.1038/ng.583.

567   12. Paulissen SMJ, van Hamburg JP, Dankers W, Lubberts E. The role and modulation of CCR6+
568   Th17 cell populations in rheumatoid arthritis. Cytokine. 2015;74: 43-53. doi:
569   10.1016/j.cyto.2015.02.002.

570   13. Ramesh R, Kozhaya L, McKevitt K, Djuretic IM, Carlson TJ, Quintero MA, et al.
571   Pro-inflammatory human Th17 cells selectively express P-glycoprotein and are refractory to
572   glucocorticoids. J Exp Med. 2014;211: 89-104. doi: 10.1084/jem.20130301.

573   14. van Hamburg JP, Tas SW. Molecular mechanisms underpinning T helper 17 cell
574   heterogeneity and functions in rheumatoid arthritis. J Autoimmun. 2018;87: 69-81. doi:
575   10.1016/j.jaut.2017.12.006.

576   15. Chikanza IC, Kozaci DL. Corticosteroid resistance in rheumatoid arthritis: molecular and
577   cellular perspectives. Rheumatology. 2004;43: 1337-1345. doi:
578   10.1093/rheumatology/keh333.

579   16. Firestein GS, McInnes IB. Immunopathogenesis of rheumatoid arthritis. Immunity. 2017;46:
580   183-196. doi: 10.1016/j.immuni.2017.02.006.

581   17. Nakayamada S, Kubo S, Yoshikawa M, Miyazaki Y, Yunoue N, Iwata S, et al. Differential
582   effects of biological DMARDs on peripheral immune cell phenotypes in patients with
583   rheumatoid arthritis. Rheumatology. 2018;57: 164-174. doi: 10.1093/rheumatology/kex012.

584   18. Ermann J, Rao DA, Teslovich NC, Brenner MB, Raychaudhuri S. Immune cell profiling to
585   guide therapeutic decisions in rheumatic diseases. Nat Rev Rheumatol. 2015;11: 541–551.
586   doi: 10.1038/nrrheum.2015.71.

587   19. Falconer J. Toward cellular biomarkers for rheumatoid arthritis. Rheumatology (Oxford).
588   2018; 57: 10–11. doi: 10.1093/rheumatology/kex283.

589   20. Arnett FC, Edworthy SM, Bloch DA, Mcshane DJ, Fries JF, Cooper NS, et al. The American
590   Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis.
591   Arthritis Rheum. 1988;31: 315-324.

26

592   21. Inoue E, Yamanaka H, Hara M, Tomatsu T, Kamatani N. Comparison of Disease Activity
593   Score (DAS)28-erythrocyte sedimentation rate and DAS28-C-reactive protein threshold
594   values. Ann Rheum Dis. 2007;66: 407-409. doi: 10.1136/ard.2006.054205.

595   22. Austin PC. The performance of different propensity score methods for estimating marginal
596   hazard ratios. Stat Med. 2013;32: 2837-2849. doi: 10.1002/sim.5705.

597   23. Joffe MM, Rosenbaum PR. Invited commentary: propensity scores. Am J Epidemiol.
598   1999;150: 327-333. doi: 10.1093/oxfordjournals.aje.a010011.

599   24. Nagafuji K, Matsuo K, Teshima T, Mori S, Sakamaki H, Hidaka M, Ogawa H, et al.
600   Peripheral blood stem cell versus bone marrow transplantation from HLA-identical sibling
601   donors in patients with leukemia: a propensity score-based comparison from the Japan
602   Society for Hematopoietic Stem Cell Transplantation registry. Int J Hematol. 2010;91:
603   855-864. doi: 10.1007/s12185-010-0581-1.

604   25. Kanda Y. Investigation of the freely available easy-to-use software "EZR" for medical
605   statistics. Bone Marrow Transplant. 2013;48: 452-458. doi: 10.1038/bmt.2012.244.

606   26. Lumley T. Analysis of complex survey samples. J Stat Soft. 2004;9: 1-19. doi:
607   10.18637/jss.v009.i08.

608   27. Wickham H. ggplot2: elegant graphics for data analysis. New York: Springer-Verlag New
609   York; 2009.

610   28. Maecker HT, McCoy JP, Nussenblatt R. Standardizing immunophenotyping for the Human
611   Immunology Project. Nat Rev Immunol. 2012;12: 191-200. doi: 10.1038/nri3158.

612   29. Kubo S, Saito K, Hirata S, Fukuyo S, Yamaoka K, Sawamukai N, et al. Abatacept inhibits
613   radiographic progression in patients with rheumatoid arthritis: a retrospective analysis of
614   6 months of abatacept treatment in routine clinical practice, The ALTAIR Study. Mod
615   Rheumatol. 2014;24: 42-51. doi: 10.3109/14397595.2013.854051.

616   30. Harigai M, Ishiguro N, Inokuma S, Mimori T, Ryu J, Takei S, et al. Postmarketing
617   surveillance of the safety and effectiveness of abatacept in Japanese patients with rheumatoid
618   arthritis. Mod. Rheumatol. 2016;26: 491-498. doi: 10.3109/14397595.2015.1123211.

27

619   31. Gottenberg JE, Ravaud P, Cantagrel A, Combe B, Flipo RM, Schaeverbeke T, et al. Positivity

620   for anti-cyclic citrullinated peptide is associated with a better response to abatacept: data from

621   the "Orencia and rheumatoid arthritis" registry. Ann Rheum Dis. 2012;71: 1815-1819. doi:

622   10.1136/annrheumdis-2011-201109.

623   32. Leffers HC, Ostergaard M, Glintborg B, Krogh NS, Foged H, Tarp U, et al. Efficacy of

624   abatacept and tocilizumab in patients with rheumatoid arthritis treated in clinical practice:

625   results from the nationwide Danish DANBIO registry. Ann Rheum Dis. 2011;70: 1216-1222.

626   doi: 10.1136/ard.2010.140129.

627   33. Piantoni S, Colombo E, Tincani A, Airò P, Scarsi M. Predictive factors of abatacept therapy

628   discontinuation in patients with rheumatoid arthritis. Clin Rheumatol. 2016;35: 1065-1069.

629   doi: 10.1007/s10067-016-3185-1.

630   34. Scarsi M, Ziglioli T, Airo' P. Baseline numbers of circulating CD28-negative T cells may

631   predict clinical response to abatacept in patients with rheumatoid arthritis. J Rheumatol.

632   2011;38: 2105-2111. doi: 10.3899/jrheum.110386.

633   35. Scarsi M, Zanotti C, Chiarini M, Imberti L, Piantoni S, Frassi M, et al. Reduction of

634   peripheral blood T cells producing IFN-γ and IL-17 after therapy with abatacept for

635   rheumatoid arthritis. Clin Exp Rheumatol. 2014;32: 204-210.

636   36. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4

637   control over Foxp3+ regulatory T cell function. Science. 2008;322: 271-275. doi:

638   10.1126/science.1160062.

639   37. Penatti A, Facciotti F, De Matteis R, Larghi P, Paroni M, Murgo A, et al. Differences in

640   serum and synovial CD4+ T cells and cytokine profiles to stratify patients with inflammatory

641   osteoarthritis and rheumatoid arthritis. Arthritis Res Ther. 2017;19: 103. doi:

642   10.1186/s13075-017-1305-1.

643   38. Behrens F, Tak PP, Østergaard M, Stoilov R, Wiland P, Huizinga TW, et al. MOR103, a

644   human monoclonal antibody to granulocyte–macrophage colony-stimulating factor, in the

645   treatment of patients with moderate rheumatoid arthritis: results of a phase Ib/IIa randomised,

646   double-blind, placebo-controlled, dose-escalation trial. Ann Rheum Dis. 2015;74: 1058-1064.

647   doi: 10.1136/annrheumdis-2013-204816.

648   39. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al.

649   Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science. 1995;

650   270: 985-988. doi: 10.1126/science.270.5238.985.

651   40. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of

652   CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction,

653   revealing a critical negative regulatory role of CTLA-4. Immunity. 1995;3: 541-547. doi:

654   10.1016/1074-7613(95)90125-6.

655   41. Basdeo SA, Cluxton D, Sulaimani J, Moran B, Canavan M, Orr C, et al. Ex-Th17

656   (nonclassical Th1) cells are functionally distinct from classical Th1 and Th17 cells and are

657   not constrained by regulatory T cells. J Immunol. 2017;198: 2249-2259. doi:

658   10.4049/jimmunol.1600737.

659

660

29

661

Supporting information

664

665 **S1 Fig. The proportion of cells with Ki67 expression among circulating CD4+ Th**

666 **subsets in patients with RA**PBMCs from RA (n = 26, before abatacept treatment) were

667 stained for CD4, CXCR3, CCR4, CD161, CCR6, CD25, Foxp3, and Ki67 mAbs and

668 analyzed using flow cytometry. The percentage of Ki67+ cells in the indicated Th subsets are

669 shown by box plot. Data were analyzed using Kruskal–Wallis test followed by the Mann–

670 Whitney U test using Bonferroni correction.

671

672 **S2 Fig. Change in cell proliferation state of Th17 and Th17.1 subsets by ABA treatment.**

673 The graph shows changes in the proportion of cells with Ki67 expression among each Th

674 subset induced by ABA treatment at various time-points (0, 4, and 24 weeks, n = 29).

675 Data were analyzed using Friedman rank sum test. Wilcoxon signed rank test with

676 Bonferroni correction was used for post-hoc paired comparisons.

677

678 **S3 Fig. Th17.1 level and successive changes in disease activity score.**

679 **a.** The line graph shows the transition of the disease activity (DAS28-CRP) of RA in the Th

680 17.1-lower and Th 17.1-higher groups before and after ABA treatment (at 4, 12, and 24

681 weeks). P-values (vs. Th17.1-higher) were determined with Mann–Whitney U test using

682 Bonferroni correction. **b**. 100% stacked bar chart shows successive changes in DAS28-CRP

683 in the Th17.1-lower, Th17.1-intermediate, and Th17.1-high groups before and after ABA

684 treatment (at 4, 12, and 24 weeks).

685 ABA, abatacept; DAS28-CRP, disease activity score 28-joint count C-reactive protein; REM,

686 remission; LDA, low disease activity; MDA, moderate disease activity; HDA, high disease

30

687   activity.

688

689   **S4 Fig. Th17.1 level and successive changes in CRP and MMP-3 levels.**

690   **a. b.** The line graphs show the transition of serum C-reactive protein (CRP) and

691   metalloproteinase-3 (MMP-3) of rheumatoid arthritis in Th 17.1-lower and Th 17.1-higher

692   groups before and after ABA treatment (at 4, 12, and 24 weeks). P-values (Th17.1-lower vs.

693   Th17.1-higher) were determined using the Mann–Whitney U test.

694   **c**. 100% stacked bar chart shows MMP-3 titer (normal, moderate, and high) in Th17.1-low

695   and Th17.1-high groups after ABA treatment at 24 weeks. P-values (Th17.1-lower vs.

696   Th17.1-higher) were determined using Fisher's exact test.

697   ABA, abatacept; CRP, C-reactive protein; MMP-3, metalloproteinase-3; Normal, within

698   normal limit; Moderate titer, less than 3 times normal upper limit; High titer, more than 3

699   times normal upper limit.

700

701   **S5 Fig. Estimation of Th17.1 cut-off value at baseline to predict ABA therapeutic**

702   **response using ROC curve.**

703   **a.** ROC curve showing a cut-off Th17.1 (% in CD4+) level of 1.1% discriminated between

704   GR and non-GR (MR or NR) at 24 weeks, with 79.2% sensitivity and 81.2% specificity.

705   **b.** ROC curve showing a cut-off Th17.1 level of 1.1% discriminated between REM and

706   non-REM at 24 weeks, with 75.9% sensitivity and 100% specificity.

707   ROC, receiver operating characteristic; AUC, area under the curve; GR, good response; MR,

708   moderate response; NR, no response; REM, remission.

709

710   **S6 Fig.** Correlation coefficient matrix plot shows the correlation (Spearman's correlation

711   coefficient, ρ) of patient background factors, indicated T cell subset at baseline, and ABA

31

712   therapeutic response indicators with significance levels (p-value).

713

714   **S1 Table. Differences in baseline clinical characteristics between EULAR-GR and**

715   **non-GR patients.**

716   **S2 Table**. **Exploratory analysis for optimal Th subset as the predictor of ABA treatment**

717   **response using multivariate analysis.**

718   **S3 Table. Adjusted patient characteristics of Th17.1-lower and Th17.1-higher patients**

719   **by IPW.**

720   **S4 Table**. **Logistic regression analysis using the IPW method to calculate odds ratio**
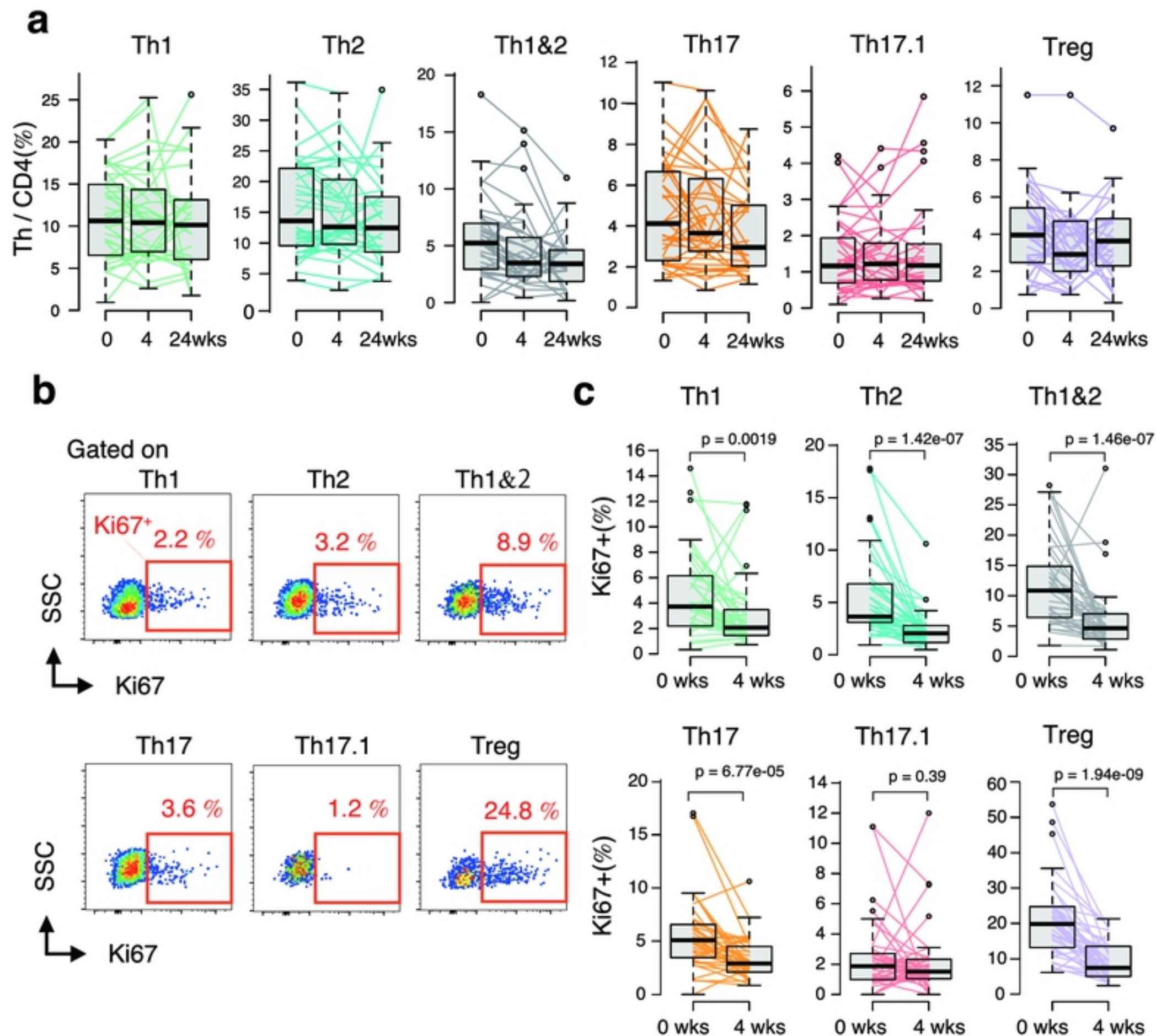
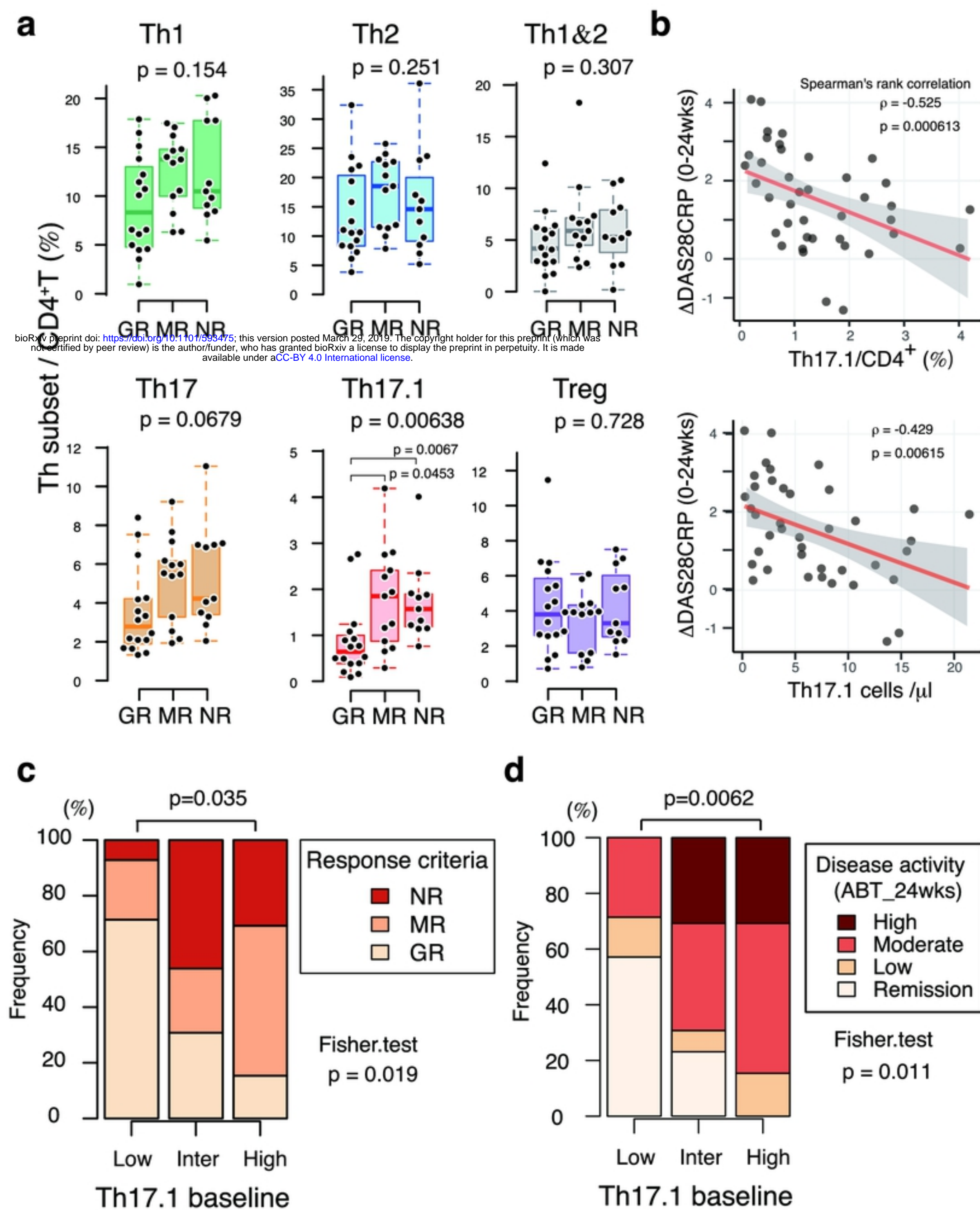721   **adjusted for patient characteristics**.
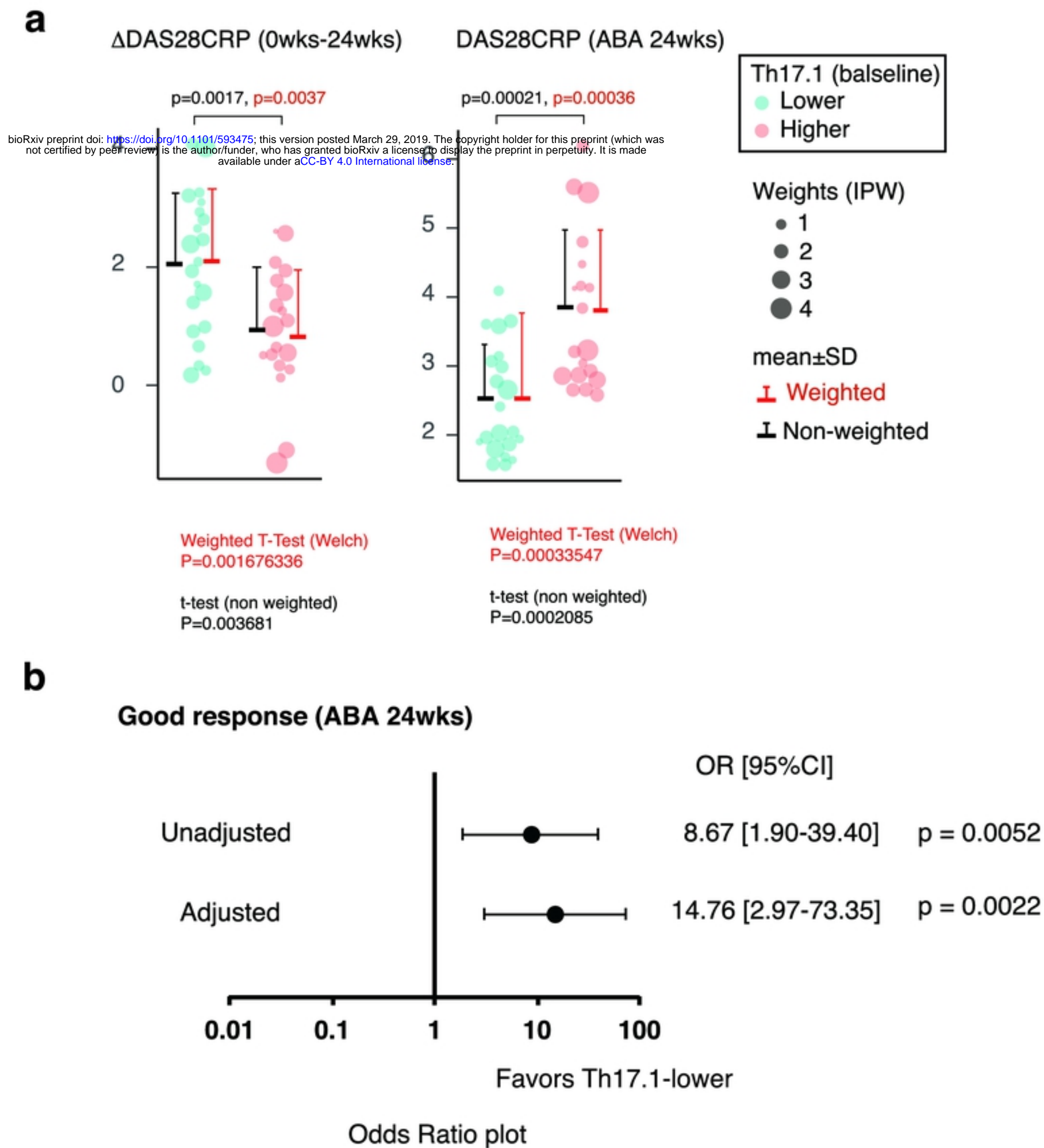
722

Figure 1

Figure 2

Figure 3

Figure 4