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1 **Glycogen synthase kinase 3 β /CCR6-positive bone marrow cells correlate with**
2 **disease activity in multicentric Castleman disease-TAFRO**

3

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16

17 **Running head:** Disease-associated GSK3 β ⁺CCR6⁺ cells in MCD-TAFRO

18

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25

26

27 **Summary**

28 Multicentric Castleman disease–thrombocytopenia, anasarca, reticulin fibrosis of bone
29 marrow, renal dysfunction and organomegaly (MCD-TAFRO) is an emergent phenotype
30 characterized by lymphoproliferation, fluid collection, hemocytopenia, and multiple
31 organopathy. Although studies have demonstrated an aberrant blood cytokine/chemokine
32 profile referred to as “chemokine storm”, the pathogenesis remains unclear. We aimed to
33 identify pathogenic key molecules, potential diagnostic targets and therapeutic markers in
34 MCD-TAFRO using serum cytokine/chemokine profiles. We performed the targeted
35 cytokine/chemokine multiplex analysis in six cases of MCD-TAFRO with remission or
36 non-remission status. We observed significant changes in serum concentrations of CCL2,
37 CCL5, and Chitinase-3-like-1 in the MCD-TAFRO patients with active state compared to
38 inactive state. Ingenuity pathway analysis revealed that glycogen synthase kinase 3
39 (GSK3) and CCR6, which is expressed in megakaryocytes, were detected as upstream
40 positive regulators for activating MCD-TAFRO status. More GSK3 β +CCR6 $^+$ cells like
41 megakaryocytes were detected in the bone marrow of patients with MCD-TAFRO than in
42 those with systemic lupus erythematosus, MCD-not otherwise specified or autoimmune
43 haemophagocytic lymphohistiocytosis. The cellularity of GSK3 β +CCR6 $^+$ cells was
44 correlated with disease activity, including thrombocytopenia and anaemia. In conclusion,
45 GSK3 β and CCR6 of bone marrow cells were potentially involved in the pathogenesis of
46 MCD-TAFRO and may act as diagnostic targets and therapeutic markers.

47

48 **Keyword**

49 Multicentric Castleman disease-TAFRO; Lymphoproliferative disorders; Chemokine;
50 Bone marrow; Glycogen synthase 3
51

52 **Introduction**

53 Multicentric Castleman disease (MCD) is one of the heterogeneous lymphoproliferative
54 diseases, characterized by generalized lymphadenopathy with histopathologic features of
55 hyaline vascular proliferation and plasma cell infiltration ¹. Among MCD cases, MCD-
56 TAFRO is an emergent phenotype characterized by systemic inflammatory reaction with
57 thrombocytopenia, anasarca, myelofibrosis, renal dysfunction, and organomegaly
58 (TAFRO) ². The clinical symptoms of MCD-TAFRO are often more severe, leading to
59 worse outcomes compared with the other subtype of MCD ³. These clinical conditions
60 and pathological findings overlap with a broad spectrum of other systemic autoimmune
61 diseases. While the aetiology of MCD-TAFRO remains unclear, the proposed etiologic
62 inducers of MCD pathogenesis include autoimmunity ¹, autoinflammation ⁴, neoplasm
63 and infection ⁵. Thus, multiple immune dysregulations involving abnormal
64 cytokine/chemokines profiles may promote MCD pathogenesis ⁶. For example,
65 hypercytokinaemia, including interleukin 6 (IL-6) and vascular endothelial growth factor
66 (VEGF) ^{7,8} was proposed for the pathophysiology of MCD-TAFRO. The symptoms of
67 capillary leak syndrome caused as adverse events of interleukin-2 (IL-2) immunotherapy
68 overlap with MCD-TAFRO, suggesting the possible pathogenic contribution of IL-2 to
69 MCD-TAFRO pathogenesis ⁹. A previous proteomic analysis of six patients with MCD
70 (2 MCD-TAFRO and 4 MCD-not other specified) revealed that multiple chemokine
71 storms associated with MCD flares ¹⁰. Therefore, analysing cytokine/chemokine profiles
72 may be useful for clarifying the pathogenesis of MCD-TAFRO. Here, we report six cases
73 of MCD-TAFRO where we conducted a multiplex cytokine/chemokine analysis to
74 explore pathogenenic molecules, diagnostic targets and therapeutic markers.

75 **Methods**

76 *Clinical information and evaluation of the patients included in this study*

77 All the patients diagnosed via the 2015 proposed diagnostic criteria satisfied the 2019
78 updated diagnostic criteria ^{11,12} (detailed in Supplementary Methods). Cases 1, 2, 5 were
79 diagnosed and managed in Hokkaido University Hospital, and cases 3,4,6 were in
80 Obihiro-Kosei General Hospital. As a previous report described, active/non-remission
81 disease including disease relapse was defined by hypoalbuminemia (< 3.5 g/dL), elevated
82 levels of C-reactive protein (CRP) (> 10 mg/L), anaemia (haemoglobin < 13.5 g/dL),
83 renal insufficiency (creatinine > 1.3 mg/dL), constitutional symptoms, and/or fluid
84 collection in body cavities ¹³. Inactive/remission disease was defined as the absence of
85 any of these symptoms. Disease severity classification scoring was based on the degree of
86 present symptoms including anasarca, thrombocytopenia, fever and/or inflammation and
87 renal insufficiency respectively scored from 0 to 3. Relationship between the score and
88 disease severity was defined as following: 0–4 points, mild disease (Grade 1); 5–6 points,
89 moderate disease (Grade 2), 7–8 points, slightly severe disease (Grade 3); 9–10 points,
90 severe disease (Grade 4); 11–12 points, very severe disease (Grade 5) ¹². Therapeutic
91 response was assessed regarding the NCI-CTCAE ((National Cancer Institute-Common
92 Terminology Criteria for Adverse Events) version 4.0 about 34 MCD-related symptoms
93 and signs ¹⁴. Therapeutic approach was initially started using glucocorticoids (1.0
94 mg/kg/day) including methylprednisolone pulse therapy (500-1000 mg of
95 methylprednisolone for 3 consecutive days). When the patients were unresponsive to the
96 initial treatment, different treatments such as cyclosporine, tacrolimus, tocilizumab,
97 rituximab, intravenous immunoglobulin (IVIg) and thrombopoietin receptor agonist
98 (TPORA) was applied at attending physician's discretion. Supportive cares including
99 blood transfusion, haemodialysis, plasmapheresis and mechanical ventilation were
100 applied properly. Bone marrow in the patients with MCD-TAFRO were biopsied with
101 Original Jamshidi™ (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) in

102 active and inactive disease status. The lymph node specimens were surgically resected for
103 the diagnosis in a clinical setting. At patient's baseline data, interleukin-6 (IL-6) and
104 vascular endothelial growth factor (VEGF) were measured by enzyme immunoassay in
105 clinical laboratory using Human IL-6 QuantiGlo ELISA Kit and Human VEGF
106 Quantikine ELISA Kit (R&D Systems), and their lower limit of reference range were 4.0
107 pg/mL and 38.3 pg/mL, respectively. Also, serum levels of glycogen synthetase kinase
108 (GSK) 3 β and CC chemokine receptor (CCR) 6 were evaluated using Glycogen Synthase
109 Kinase 3 β ELISA Kit (Cloud-clone corp.) and Human CCR6 ELISA Kit (Aviva Systems
110 Biology).

111 For comparison, we extracted the serum and bone marrow specimens of treatment-naïve
112 patients with systemic lupus erythematosus (SLE) ($n = 4$ sera and 5 bone marrows),
113 MCD-not otherwise specified (MCD-NOS) ($n = 2$ sera and 3 bone marrows) and
114 autoimmune haemophagocytic lymphohistiocytosis (HLH) ($n = 4$ sera and 5 bone
115 marrows) diagnosed and managed in Hokkaido University Hospital. The patients with
116 SLE manifested pancytopenia or thrombocytopenia without hemophagocytosis. The
117 patients with autoimmune HLH complicated with mixed connective tissue disease
118 (MCTD), adult-onset Still's disease (AOSD), SLE and dermatomyositis (DM).
119 Ethical approval of conducting the present study was obtained from Institutional Review
120 Board of Hokkaido University Hospital (reference number: 020-0042). This study
121 complied with the Declaration of Helsinki. We obtained the patient informed consents for
122 participation in this study and publication from all the patients included in this study.

123

124 *Cytokine/chemokine multiplex analysis*

125 We performed multiplex cytokine/chemokine beads assays using sera of the patients and
126 a Luminex[®] Assay and a Luminex[®] High Performance Assay (LXSAHM and FCSTM,
127 R&D Systems, Minneapolis, MN, United States of America) analysed with a Luminex[®]
128 200 xPONENT[®] System (Merck KGaA, Darmstadt, Germany) in accordance with the

129 manufacturer's protocols in the laboratory of Institute for Genetic Medicine, Hokkaido
130 University. The measured analytes of cytokines and chemokines included tumour
131 necrosis factor α (TNF α), interferon (IFN)- γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8/C-X-C
132 motif chemokine ligand (CXCL) 8, IL-10, IL12p70, IL-13, IL-17, IL-18, IL-23, IL-1
133 receptor-like 1/ST2, CXCL1/Growth-related oncogene α , CXCL13/B lymphocyte
134 chemoattractant, C-C motif chemokine ligand (CCL) 2/monocyte chemotactic protein-1
135 (MCP-1), CCL3/macrophage inflammatory protein-1 α (MIP-1 α), CCL5/Regulated on
136 Activation, Normal T cell Expressed and Secreted (RANTES), CCL7/MCP3,
137 CCL20/MIP-3, TNF ligand superfamily member 13 (TNFSF13)/A proliferation-inducing
138 ligand (APRIL), TNFSF13B/B cell activating factor belonging to the TNF family
139 (BAFF), Chitinase-3-like-1 (CHI3L1), epidermal growth factor, fibroblast growth factor
140 23 (FGF-23), and VEGF-A. The sera of the patients with MCD-TAFRO were collected at
141 initial onset, remission, or relapse, and during receiving ineffective treatment. The
142 collected sera were centrifuged at 3000 rotations per minute at 4 °C for 15 minutes, and
143 the supernatant was stored at -80 °C until use.

144

145 *Proteomic analysis*

146 The serum cytokines and chemokines data at active disease phase and inactive disease
147 phase were subjected to protein-protein interaction (PPI) analysis using The Search Tool
148 for the Retrieval of Interacting Genes (STRING) database¹⁵. By Ingenuity Pathway
149 Analysis (IPA) (QIAGEN, Hilden, Germany), upstream regulator analysis, disease/toxic
150 functional analysis and regulatory effect analysis were performed using serum cytokines
151 and chemokines data with differential abundance between a phase of active and inactive
152 disease.

153

154 *Immunohistochemistry*

155 We used the healthy volunteer's specimens of bone marrow (Human bone marrow
156 Paraffin Tissue Section, HP-704, Zyagen) as a healthy control.
157 The specimens were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned
158 at 5-10 μm . The sections were deparaffinized with xylene and dehydrated with ethanol.
159 Heat-induced antigen retrieval was performed with citrate sodium. The sections were
160 rinsed with TBS-0.1% Tween and blocked with 5% BSA and 0.3% Triton X-100 in PBS
161 for 1h. Primary antibodies were diluted in antibody buffer containing as 2% BSA and
162 0.3% Triton X-100 as follows: Rabbit anti-human GSK 3 β (1:100, HPA028017, Sigma-
163 Aldrich), Rat anti-human CCR 6 (1:200, clone: 18B9E6, Novus biologicals), Mouse anti-
164 human CD61 (1:25, clone: VI-PL2, LSBio) and Mouse anti-human CD163 (1:50, clone:
165 EDHu-1, Novus Biologicals), and incubated overnight at 4 °C. Secondary Alexa-
166 conjugated antibodies (Invitrogen) were added at 1:200 in the antibody buffer for 2 h at
167 room temperature. Slides were mounted in ProLongTM Diamond Antifade Mountant with
168 DAPI (ThermoFisher Scientific) and imaged using the Keyence inverted fluorescence
169 microscope, BZ-9000 (Keyence Japan, Osaka, Japan). All quantifying analyses for
170 fluorescent positive cells were performed by a blinded observer (Tomoya S) to the
171 patients' clinical data using Image J software (National Institutes of Health, Bethesda,
172 Maryland, USA). We performed correlation analyses using the results of the
173 quantification and clinical data.

174

175 *Statistical analysis*

176 We used analysis of variance (ANOVA) to compare the values of unpaired continuous
177 variables with groups. For multiple comparisons, we applied Holm-Sidak's method.
178 Wilcoxon signed-rank test was used for paired comparisons of continuous variables.
179 Correlations were determined using Pearson's product-moment correlation coefficient.
180 We used JMP® Pro 13 software (SAS Institute Inc., Cary, NC, USA) and GraphPad
181 Prism® version 7.04 (GraphPad Software, San Diego, CA, USA) for statistical analyses.

182 When the p-value was below 0.05, the results were defined as statistically significant. All
183 statistical tests were two-sided.
184

185 **Results**

186 *Significant changes of serum cytokines and chemokines*

187 Clinical information and treatment courses of six MCD-TAFRO patients (cases 1-6) are
188 summarized in Table 1, Supplementary Methods and Fig. S1. To elucidate the
189 pathogenesis, diagnostic targets and therapeutic markers for MCD-TAFRO, we performed
190 a multiplex cytokine/chemokine analysis using the MCD-TAFRO patients' sera in this
191 case series. A total of the 20 samples were analysed and three were collected in initial
192 onset without treatment and six in remission under cyclosporine A (CsA) therapy except
193 the case 6 under tocilizumab treatment (Fig. 1A, B). The multiplex analysis results were
194 as follows; there were no samples in which IL-17 and IL-23 were detected. The average
195 serum values of ILs had absence of the trend among each case regardless of the disease
196 activity status (Table S1). Meanwhile, the changes in the levels of some analytes
197 including IL1RL1/ST2, CCL2/MCP-1, CCL3/MIP-1 α , CCL5/RANTES, CCL7/MCP-3,
198 CCL20/MIP-3, TNFSF13/APRIL, CHI3L1 and FGF-23 showed a pattern between the
199 remission state and the non-remission state (Fig. 1C). Among these, the average levels of
200 CCL2/MCP-1, CCL5/RANETS significantly increased in the remission compared with
201 the non-remission, and CHI3L1 significantly decreased after remission. Indeed, the
202 serum levels of CCL2/MCP-1 and CCL5/RANTES were elevated the most during
203 remission in all the cases. Also, serum FGF-23 levels were most decreased in the
204 remission state of all the patients (Fig. S2A-F). Thus, the serum levels of some
205 chemokines and growth factors were definitely affected by treatment and disease activity
206 status, possibly becoming new therapeutic biomarker in MCD-TAFRO.

207

208 *Predicted activation of glycogen synthase kinase 3 β and CCR6 in non-remission state of*
209 *MCD-TAFRO*

210 Next, we performed a PPI analysis using the ratio of average serum levels of multiplexed
211 cytokines and chemokines in non-remission state of MCD-TAFRO to those in remission.

212 PPI network for the cytokines and chemokines showed some sort of connection with each
213 analyte (Fig. 2A). The upstream regulator analysis demonstrated that 21 molecules were
214 predicted as significant upstream activators or inhibitors with Z-score >2.0 in non-
215 remission stated MCD-TAFRO. The predicted upstream activators with high activation
216 Z-score (>2.0) included CCR6 and GSK3 (Fig. 2B). RIPK1, SMAD3, CTNNB1,
217 IL20RB, EGR1 and EGR2 were included as the predicted upstream inactivators with low
218 activation Z-score (<2.0) in non-remission MCD-TAFRO. The disease and toxic function
219 analysis predicted the significant inhibition of leucocytosis, haematopoiesis and antigen
220 presenting cells development, and the increased probability of developing
221 glomerulonephritis (Fig. 2C, D). Regulatory effect analysis revealed that the predicted
222 states of the regulators including EGR2, GSK3, IL20RB and EGR1 were consistent with
223 the measured status of target molecules in dataset such as CCL2/MCP-1,
224 CCL5/RANTES, IL-8/CXCL8, TNF- α and VEGF-A, and were expected to have impacts
225 on diseases and functions such as development of phagocytes, glomerulonephritis,
226 leucocytosis and development of antigen presenting cells (Fig. S3). Thus, proteomic
227 analysis using serum multiplex cytokine/chemokine level would lead the candidates
228 related to the disease activity of MCD-TAFRO.

229

230 *Highly expressed GSK3 β and CCR6 in bone marrow of MCD-TAFRO, correlating with*
231 *systemic inflammation*

232 We evaluated the protein expression of GSK and CCR6 in bone marrow and lymph node
233 of the patients with MCD-TAFRO and a healthy control via immunohistochemistry.
234 Human GSK3 has two isoforms, α and β , and The Human Protein Atlas (available from
235 <https://www.proteinatlas.org/>)¹⁶, a public protein database, demonstrated that GSK3 α
236 highly expresses even in bone marrow and lymph node of healthy human, but not GSK3 β
237 (Fig. S4A). Previous reports demonstrated GSK3 β as a regulator of megakaryocytic

238 differentiation and platelet production in bone marrow^{17,18}. Therefore, we assessed the
239 expression of GSK3 β isoform.

240 We identified GSK3 β and CCR6-positive cells in bone marrow of MCD-TAFRO patients
241 while these cells were not detected in the healthy control. These GSK3 β /CCR6-double
242 positive cells included relatively large cells and a part of cells were multinucleid, which
243 suggest these cells are megakaryocytes. Compared to effectively treated state of MCD-
244 TAFRO, GSK3 β or CCR6-positive cells, and GSK3 β /CCR6-double positive cells were
245 significantly increased in untreated or ineffectively treated state (Fig. 3A and Fig. S4B).

246 Next, we investigated the nature of these cells using immunostaining of CD61 and
247 CD163, which are cell markers for megakaryocytes and monocytes/macrophages,
248 respectively. In the bone marrows of patients with MCD-TAFRO, a part of
249 GSK3 β /CCR6-double positive cells were colocalized with CD61, but not CD163 (Fig.
250 4B), suggesting that the cells were of the megakaryocyte lineage. Furthermore, the
251 percentage of GSK3 β /CCR6-double positive cells was correlated with disease grade of
252 MCD-TAFRO, platelet count and haemoglobin level, but not serum CRP level (Fig. 3C
253 and Fig. S4D). Regarding 34 MCD-related symptoms and signs evaluated by NCI-
254 CTCAE (National Cancer Institute-Common Terminology Criteria for Adverse Events)
255 version 4.0 (Table S2)¹⁴, the cellularity of GSK3 β /CCR6-double positive cells in bone
256 marrow of the patients with MCD-TAFRO was significantly correlated with the grades of
257 the symptoms and signs such as fatigue, dyspnoea, generalized oedema and the total
258 score (Fig. S4D). Ascites was tended to correlate, but the symptoms including fever and
259 pleural effusion had no significant correlation with GSK3 β /CCR6-double positive cells
260 (Fig. 3C and Fig. S4D). Comparing with disease controls such as SLE without HLH and
261 MCD-NOS, GSK3 β ⁺CCR6⁺ cells were detected specifically in treatment-naïve patients
262 with MCD-TAFRO (Fig. 4D). Although haemophagocytosis was not observed in bone
263 marrows of the MCD-TAFRO patients, we investigated the effect of inflammation for
264 these cells using bone marrow samples of patients with autoimmune HLH.

265 GSK3 β ⁺CCR6⁺ cells were also detected in autoimmune HLH secondary to MCTD,
266 AOSD, SLE and DM, but the percentage of these cells in the bone marrow was less than
267 those with treatment-naïve MCD-TAFRO (Fig. 4D).

268 We finally measured serum levels of GSK3 β and CCR6 in MCD-TAFRO and the other
269 diseases including MCD-NOS, SLE and autoimmune HLH. No GSK3 β was detected in
270 all the serum samples of our study. Serum CCR6 level of MCD-TAFRO was not elevated
271 compared with the other diseases (Fig. S4E), suggesting that the GSK3 β /CCR6-double
272 positive cells were specifically regulated in bone marrows of MCD-TAFRO.

273

274 Thus, the GSK3 β /CCR6-double positive cells in bone marrow would be involved in the
275 pathogenesis of MCD-TAFRO, a novel diagnostic target and a therapeutic marker in
276 MCD-TAFRO.
277

278 **Discussion**

279 We investigated the transition of serum cytokine/chemokine levels based on MCD-
280 TAFRO case series to evaluate novel molecules for the pathogenesis. This study revealed
281 that the levels of chemokines and growth factors such as CCL2/MCP-1, CCL5/RANTES
282 and CHI3L1 were significantly changed by treatment in MCD-TAFRO. GSK3 β and
283 CCR6, which were predicted as the upstream activators, were highly and specifically
284 expressed in the bone marrow of active MCD-TAFRO, positively correlating with its
285 disease activity and laboratory findings associated with thrombocytopenia and anaemia.

286

287 There are a few studies exploring the pathogenesis and therapeutic approaches of MCD-
288 TAFRO from the aspect of proteome. The first report using flowcytometry-based
289 multiplex assay from Japan revealed that the serum CXCL10 level of MCD-TAFRO was
290 significantly higher than that of MCD-NOS and healthy controls ¹⁹. Also, a high-
291 throughput protein quantifying analysis demonstrated that multiple chemokines including
292 CXCL13, CCL21 and CCL23 were significantly high level during MCD-TAFRO flare
293 compared with the remission state ¹⁰. Our study could not demonstrate the elevated level
294 of serum CXCL13 in non-remission MCD-TAFRO. However, the levels of multiple
295 chemokines including CCL2/MCP-1, CCL3/MIP-1 α , CCL5/RANTES, CCL7/MCP-3
296 and CCL20/MIP-3 had a certain tendency between remission state and non-remission
297 state. Surprisingly, CCL2/MCP-1 and CCL5/RANTES levels were elevated via
298 successful treatment using CsA or tocilizumab. Thus, the dysregulation of chemokines
299 could have a prominent role for developing the pathogenesis of MCD-TAFRO.

300

301 Our analysis demonstrated GSK3 β and CCR6 as the candidates of positive regulators in
302 MCD-TAFRO. CCR6 is a CC chemokine receptor protein and its ligand is CCL20/MIP-
303 3 α . CCR6 is expressed on various immune cells including T cells, B cells, dendric cells,
304 NK cells and even bone marrow megakaryocytes. Megakaryocytes express several

305 chemokine receptors including CXCR4, CCR6, CCR8, CCR5 and CCR2²⁰. Indeed, our
306 immunohistochemical findings showed that CCR6-positive cells in bone marrows are
307 relatively large and multinucleid, suggesting CCR6-positive megakaryocytes.
308 Furthermore, a part of these cells expressed CD61, a cell surface marker of
309 megakaryocytes, suggesting that the cells are of megakaryocyte lineage. However, the
310 function of CCR6 signalling in megakaryocytes remains to be established.

311

312 On the other hand, GSK3 is a serine/threonine kinase and the phosphorylation permits
313 many biological activities such as glycogen metabolism, cell signalling and cellular
314 transport²¹. GSK3 β , one of the GSK isoforms, is reportedly associated with a variety of
315 diseases such as diabetes, malignancy, inflammatory disorders, Alzheimer's disease. The
316 present study demonstrated that the percentage of GSK3 β /CCR6-double positive cells in
317 bone marrow, which was assumed to be megakaryocytes, was correlated with platelet
318 counts and haemoglobin level. Previous reports revealed that GSK3 β inhibition promotes
319 megakaryocytic survival and proliferation by thrombopoietin-induced Akt signalling¹⁷,
320 and thrombopoiesis from megakaryocytes¹⁸. Moreover, in our study, we focused on
321 GSK3 β /CCR6-double positive cells in bone marrow. Taken together, these cells would
322 be megakaryocytes with impaired cell function such as proliferation and platelet
323 production. Furthermore, GSK3 β negatively regulates CCL5/RANTES production from
324 inflammatory cells and this chemokine increases proplatelet formation of
325 megakaryocytes^{22,23}. Not limited to hematopoietic cells, previous proteomic research
326 also revealed activated CD8⁺ T cells and PI3K/Akt/mTOR signalling activation in IL-6
327 inhibitor-resistant MCD-TAFRO with active disease¹³, also indicating the association of
328 GSK3 β signalling. Moreover, GSK3 β facilitates pathogenic Th17 polarization and
329 inhibition of GSK3 β promotes the polarization of regulatory T cells²⁴⁻²⁸. GSK3 β also
330 inhibits IL-8 production²⁹, which is compatible with our results of decreasing IL-8
331 signalling in MCD-TAFRO with non-remission state.

332

333 In this study, the patients with autoimmune HLH had GSK3 β /CCR6-double positive cells
334 in the bone marrow. Especially, SLE-HLH patients demonstrated higher percentage of
335 GSK3 β /CCR6-double positive cells, indicating that inflammatory process induced these
336 cells to some degree. Indeed, patients with SLE showed clinical manifestations such as
337 thrombocytopenia, serositis, fever and splenomegaly similar to those in MCD-TAFRO ³⁰.
338 It is thus biologically plausible that specific pathological environment called chemokine
339 storm is associated with the development of MCD-TAFRO, and it would not merely
340 impair hematopoietic cells like megakaryocyte in bone marrow, but also affect various
341 cells in multiple organs, leading to a variety of clinical manifestations. GSK3 β signalling
342 pathway would be unitarily associated with some parts of the pathogenesis in MCD-
343 TAFRO.

344

345 We acknowledge several limitations in this study. Because this study is case series
346 analysis, the number of patients was small. Targeted multiplex cytokines/chemokines
347 assay would also produce biased interpretation of the results. Although all the case except
348 one achieved remission by CsA treatment, heterogeneous therapeutic regimen may
349 impact the cytokine/chemokine profiles. However, under this circumstance, there were
350 the tendencies for the transition of some chemokines' concentrations and
351 immunohistochemical findings corresponding to remission or non-remission status. We
352 believe that our preliminary results would be worth further analysing for verification.

353

354 In conclusion, proteomic analysis using multiplex serum cytokine/chemokine results
355 detected GSK3 β and CCR6 as upstream positive regulators in MCD-TAFRO with active
356 disease. The cellularity of GSK3 β /CCR6-double positive cells in bone marrow correlated
357 with disease severity of MCD-TAFRO. Although further functional research for the

358 MCD-TAFRO pathogenesis was required, GSK3 β and CCR6 would be a diagnostic
359 molecules and novel therapeutic targets of MCD-TAFRO.
360

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370

371 **Author contributions**

372 NA conducted data analyses, contributed to study design, clinical data, sample collection
373 and writing the manuscript. Michihito K contributed to study design, data interpretation,
374 and writing the manuscript. Michihiro K and NO contributed to clinical data and sample
375 collection. Tomoya S contributed to data analysis. MY, Taiki S, KK, YS, YF, Masaru K
376 and KO contributed to clinical data and sample collection. RH and MM contributed to
377 data collection and analysis. TA contributed to clinical data, data interpretation and
378 writing the manuscript.

379

380 **Conflict of interest**

381 None declared.

382

383 **Supporting information**

384 Additional supporting information may be found online in the Supporting Information
385 section at the end of the article.

386 **Supplementary Methods**

387 **Fig S1.** Clinical course and relevant data of a multicentric Castleman disease-
388 TAFRO cases in this study.

389 **Fig S2.** Changes of the serum cytokine and chemokine levels in the clinical
390 course of the patients with MCD-TAFRO.

391 **Fig S3.** Regulator effects analysis of the predicted upstream regulators in
392 Ingenuity Pathway Analysis.

393 **Fig S4.** Additional images of immunohistochemistry with the correlation analyses
394 between the quantifying result of immunohistochemistry and clinical data.

395 **Table S1.** Multiplex cytokines/chemokines analysis results in the six MCD-
396 TAFRO patients.

397 **Table S2.** Multicentric Castleman's disease signs and symptoms by NCI-CTCAE,
398 version 4.0
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- 483
484

485 **FIGURE LEGENDS**

486 **Fig. 1. Multiplex cytokine/chemokine analysis of the MCD-TAFRO patients**

487 (A) Schema of the disease state and therapeutic approaches at the time of blood
488 collection. Thick vertical arrow indicates the timing of blood collection. The disease state
489 is described above the horizontal thin arrow of timeline and the details of therapeutic
490 agents are described below. Clinical courses on solid timeline are shown in the present
491 study, but those on dashed timeline are not. (B) Shema of multiplex cytokine/chemokine
492 analysis using collected sera from the MCD-TAFRO patients and described 28 analytes.
493 (C) Paired individual trajectories of change in serum cytokine/chemokine levels between
494 non-remission state and remission state of the MCD-TAFRO patients ($n = 6$). * $p < 0.05$,
495 Wilcoxon signed-rank test was used for paired comparisons.

496 Abbreviations: CsA, cyclosporine A; IVIg, intravenous immunoglobulin; MMF,
497 mycophenolate mofetil; mPSL, methylprednisolone; PSL, prednisolone; RTX, rituximab;
498 TAC, tacrolimus, TCZ, tocilizumab.

499

500 **Fig. 2. Proteomic analysis using the results of multiplex cytokine/chemokine data of**
501 **the MCD-TAFRO patients**

502 (A) Protein-protein interaction network using the ratio of average serum
503 cytokine/chemokine concentration in non-remission state to remission state of MCD-
504 TAFRO patients, constructed by STRING (Search Tool for the Retrieval of Interacting
505 Genes) database version 11.0. Edges represent protein-protein associations
506 corresponding to its colouring described in the legend. The halo colour is based on the
507 rank of the protein in the full set of input values. Red halo shows the high rank, blue halo
508 shows the low rank, and grey halo shows the other medium rank. (B-D) Volcano plot
509 displaying (B) predicted upstream regulators, (C) associated diseases and functions, and
510 (D) predicted toxic function by IPA for the non-remission state to the remission state.
511 The vertical axis (y-axis) corresponds to the value of \log_{10} (p-value), and the horizontal

512 axis (x-axis) displays the activation Z-score. The red dots represent the predictably
513 activated items; the blue dots represent the inhibited items. Positive x-values represent
514 predictably activated and negative x-values represent predictably inhibited. Orange
515 horizontal line denotes p-value of 0.05 (prior to logarithmic transformation), a significant
516 threshold.

517

518 **Fig. 3. Expression of glycogen synthase kinase 3 β and CCR6 in bone marrow of the**
519 **MCD-TAFRO patients, correlating with disease activity and laboratory findings**

520 (A) Immunohistochemical images of bone marrow overlaid with glycogen synthase
521 kinase 3 β (GSK3 β , magenta) and CCR6 (green). The magnified panel shows the area
522 which arrow indicates. Scale bars, 50 μ m. The percentages of GSK3 β -positive cells,
523 CCR6-positive cells and GSK3 β /CCR6-double-positive cells in bone marrow of MCD-
524 TAFRO with remission state ($n = 2$), MCD-TAFRO with non-remission ($n = 7$), a
525 healthy control are quantified and shown graphically. Data are mean \pm s.e.m. (B)
526 Representative immunohistochemical images in colocalization analysis of GSK3 β /CCR6-
527 double-positive cells with CD61 and CD163 (red) in MCD-TAFRO patients. The
528 magnified panel with separated colours shows the area which arrow indicates. Scale bars,
529 50 μ m. (C) Correlations between GSK3 β /CCR6-double-positive cells in bone marrow of
530 MCD-TAFRO patients and their clinical data. Dot plot shows individual cases and blue
531 colour indicates the patient with remission state; red with non-remission state. Statistical
532 analysis was performed by Pearson's product-moment correlation coefficient. (D)
533 Representative immunohistochemical images of bone marrow in patients with MCD-
534 NOS ($n = 3$), systemic lupus erythematosus (SLE) ($n = 5$) and autoimmune
535 haemophagocytic lymphohistiocytosis (HLH) secondary to mixed connective tissue
536 disease (MCTD), adult-onset Still's disease (AOSD), dermatomyositis (DM) and SLE for
537 GSK3 β and CCR6. * $p < 0.05$, **** $p < 0.0001$, ANOVA with post-hoc Holm-Sidak

538 method. Scale bar, 50 μm . Small panel represents the area indicated by arrows. Data are

539 mean \pm s.e.m.

540

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542 TABLES

Table 1. Characteristics and disease/treatment history at disease new-onset or flare for MCD-TAFRO 1-6 patients

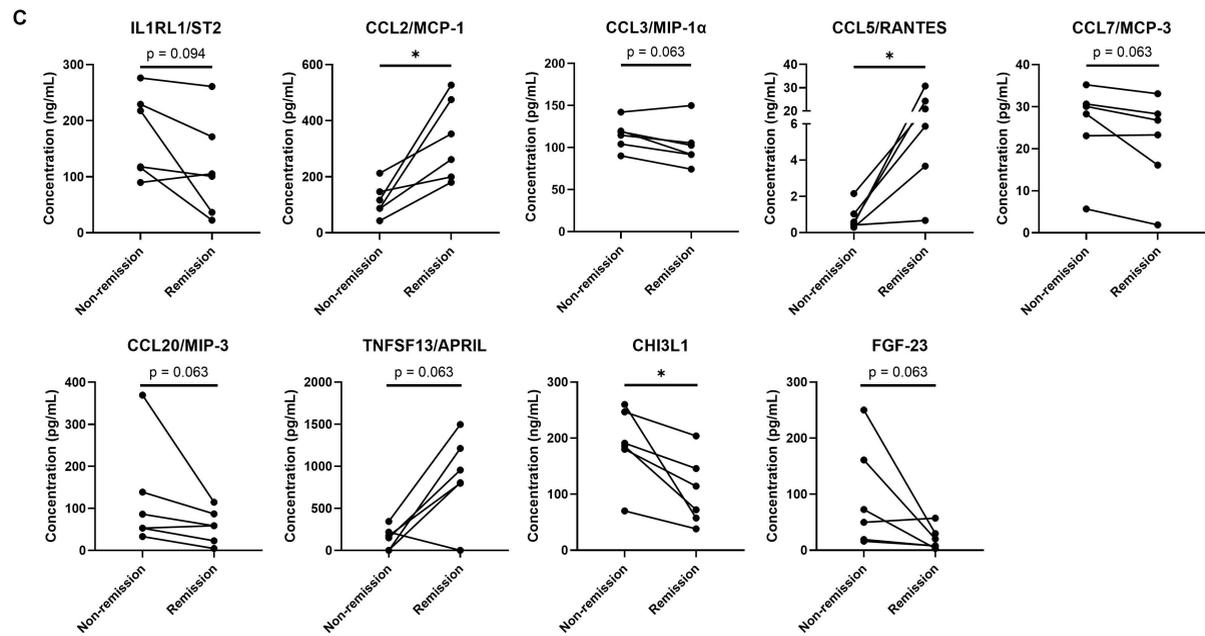
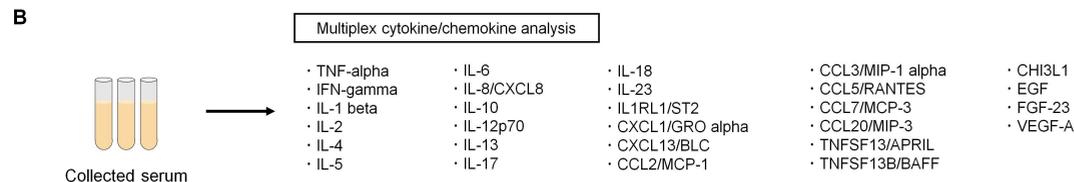
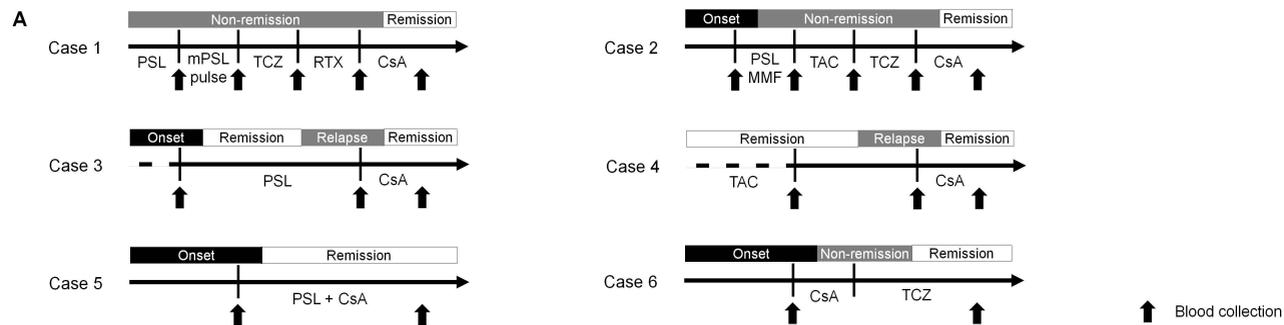
Patient number of MCD-TAFRO	1	2	3	4	5	6
Demographics and diagnosis						
Disease new-onset or flare	New	New	Flare	Flare	New	New
Sex	F	F	M	F	F	F
Age at disease onset or flare	43	47	64	67	62	66
Multicentric lymphadenopathy	+	+	+	+	+	+
Lymph node pathology	N.D.	N.D.	N.D.	Mixed variant subtype	Hypervascular subtype	N.D.
Bone marrow pathology	Reticulin myelofibrosis Increased number of megakaryocytes with slight hyperplasia	Reticulin myelofibrosis Increased number of megakaryocytes with slight hyperplasia	Reticulin myelofibrosis Increased number of megakaryocytes	N.D.	N.D.	No specific findings
Disease severity grade	3	4	2	2	3	3
Clinical characteristics and laboratory findings [reference range] at disease onset or flare						
Fever	+	+	-	-	+	+
Oedema	+	+	+	+	+	+
Anasarca	+	+	+	+	+	+
Organomegaly	+	+	+	+	+	+
Haemoglobin (g/dL) [11.6-14.8]	8.4	6.8	9.9	13.6	11.8	9
Platelet count ($\times 10^4/\mu\text{L}$) [15.8-34.8]	4.4	3.9	5.2	7.2	6.4	2.5
Albumin (g/dL) [4.1-5.1]	1.3	2.6	3.2	3.5	4.0	2.1

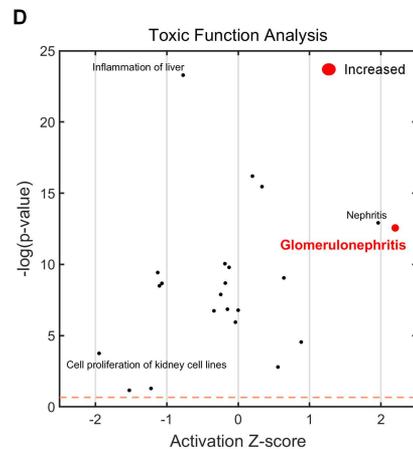
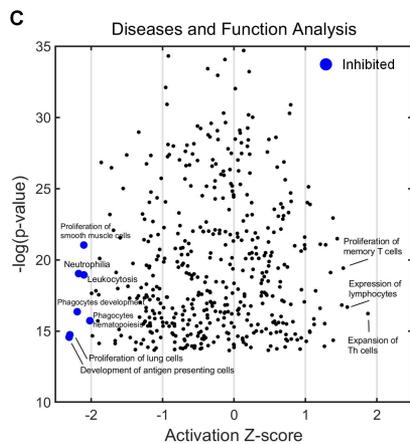
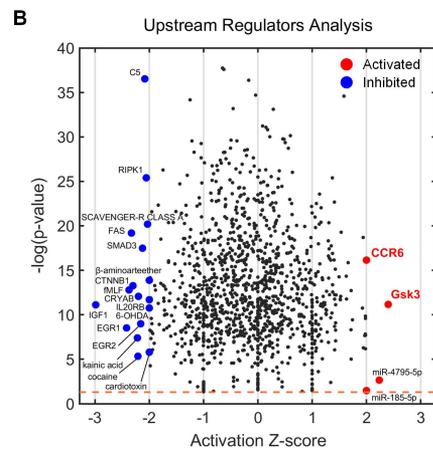
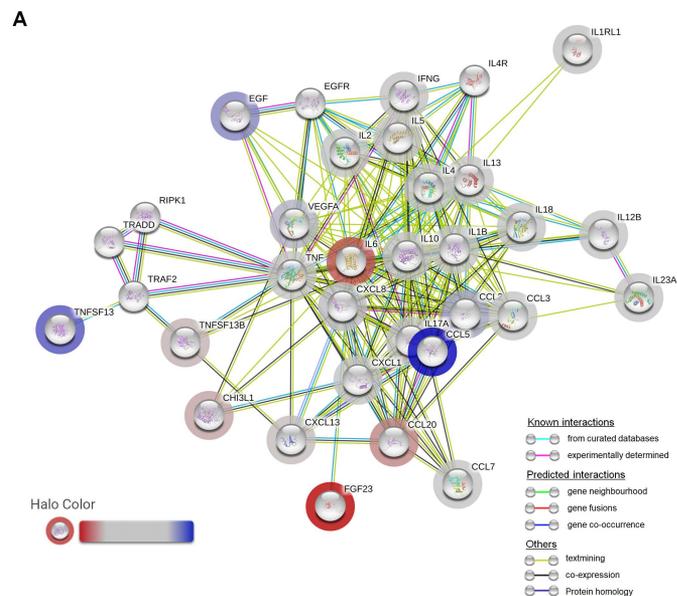
Immunoglobulin G (mg/dL) [861-1747]	1631	1336	540	650	2269	1588
Immunoglobulin G4 (mg/dL) [4-108]	48	60.1	N.D.	16.6	266	34.9
Creatinine (mg/dL) [0.46-0.79]	1.14	1.00	1.37	1.26	0.83	1.10
Haematuria	-	+	2+	-	+	+
Proteinuria (g/g•Cr)	2.17	3.74	0.40	0.18	5.61	0.92
Alkaline phosphatase (U/L) [106-322]	432	347	168	188	529	334
C-reactive protein (mg/L) [<1.4]	185.8	55.6	28.2	11.2	2.3	107
Procalcitonin (ng/mL) [<0.50]	5.07	1.81	0.82	1.3	N.D.	2.72
Soluble interleukin-2 receptor (U/mL) [<459]	1691	659	1070	1889	812	834
Interleukin-6 (pg/mL) [<4.0]	8.4	25	6.8	48.1	N.D.	86.1
Vascular endothelial growth factor (pg/mL) [<38.3]	253	118	208	357	N.D.	981
Rheumatoid factor (IU/mL) [<15]	11.7	16.6	-	3	-	77
Complement 3 (mg/dL) [73-138]	94	115	88	107	101	103
Complement 4 (mg/dL) [11-31]	26	24	22	48.1	27	18
50% hemolytic complement activity (U/mL) [31.6-57.6]	54.1	78.7	60	23	62	60
Antinuclear antibody	1:1280 (Speckled, Cytoplasmic)	1:1280 (Speckled)	1:40 (Speckled)	1:80 (Homogeneous, Speckled)	1:320 (Homogeneous,	1:80 (Speckled)

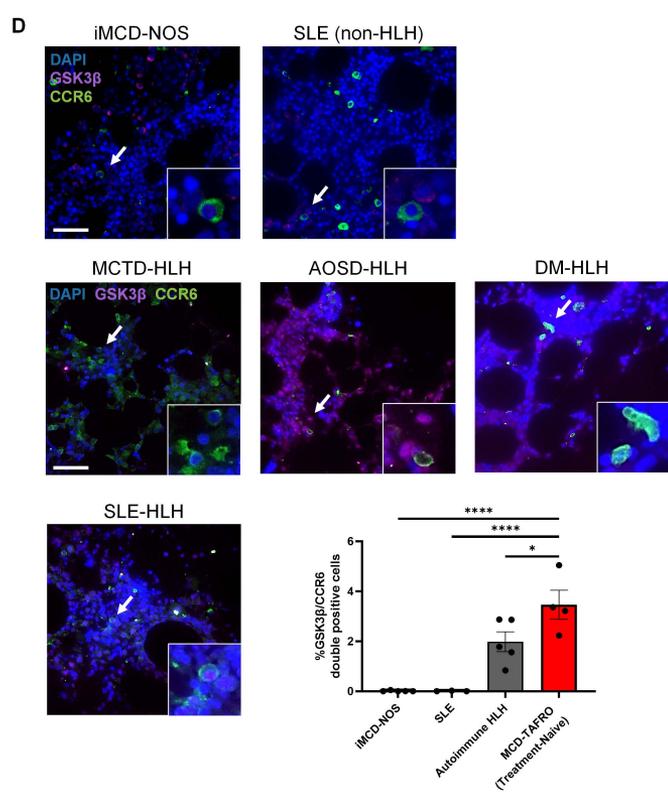
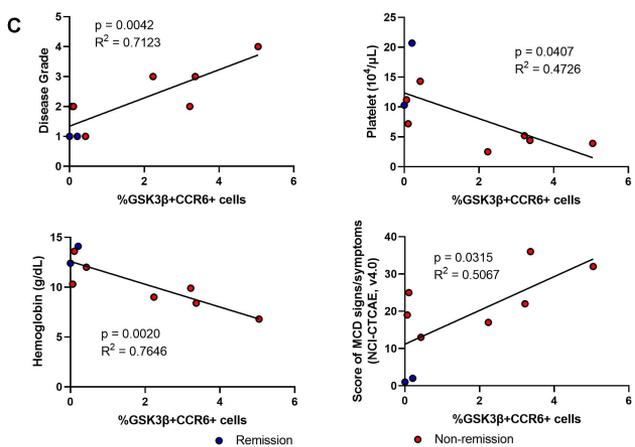
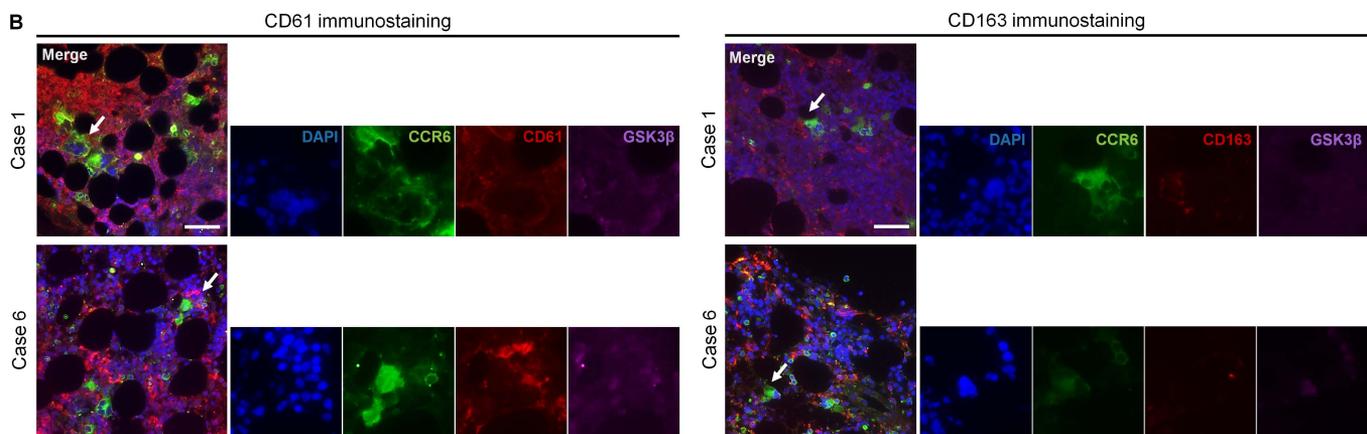
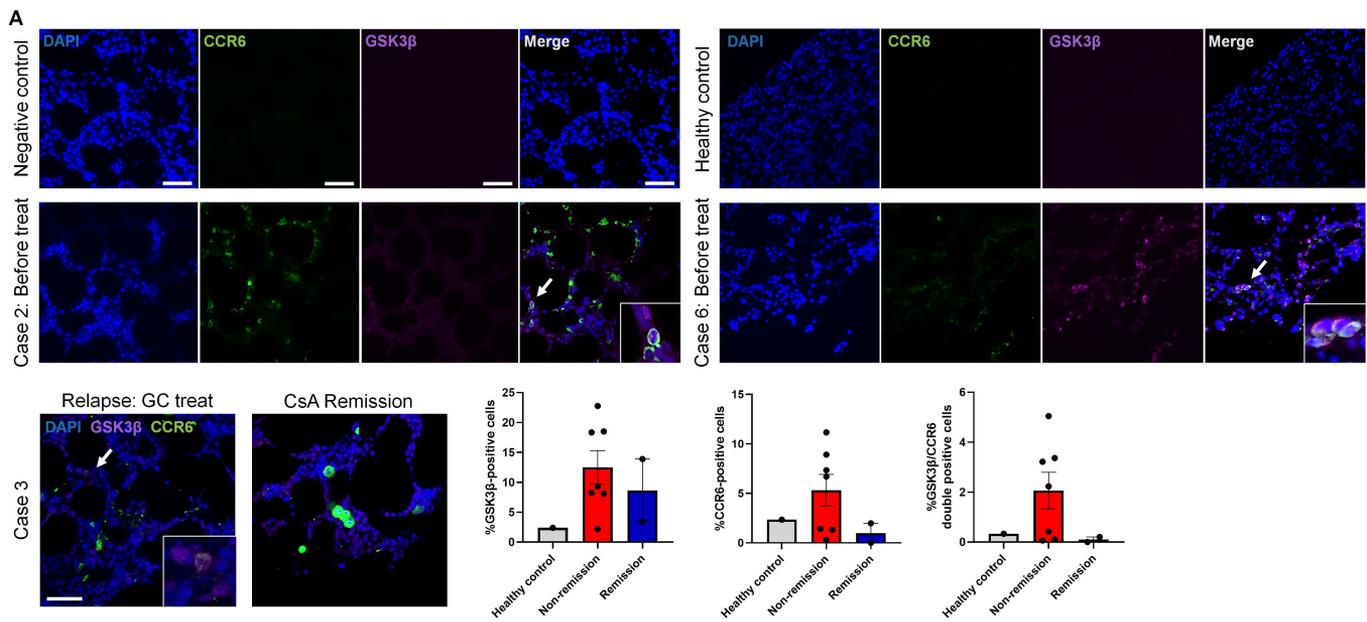
Disease-specific autoantibodies	anti-Ro (132.3 U/mL) anti-La (48.4 U/mL) anti-thyroperoxidase (11.47 U/mL) anti-mitochondria M2 (132 Unit) Lupus anticoagulant (1.41)	anti-U1-RNP (178.3 U/mL) anti-thyroglobulin (2757.23 U/mL) anti-thyroperoxidase (732.58 U/mL) Lupus anticoagulant (1.27)	-	anti-Ro (129.4 U/mL) anti-La (31.0 U/mL)	Speckled, Nucleolar)	-	anti-double stranded DNA (21.4 U/mL) anti-Ro (101.0 U/mL)
Treatment history							
Pretreatment	N.A.	N.A.	PSL 10 mg/day	PSL 12.5 mg/day, TAC	N.A.	N.A.	N.A.
Experienced treatment	N.A.	N.A.	PSL	mPSLpulse, PSL, TAC, RTX, TCZ	N.A.	N.A.	N.A.
Therapeutic agents for initial onset or flare	mPSLpulse, PSL, TCZ, RTX, CsA	mPSLpulse, PSL, MMF, TAC, TCZ, CsA	mPSLpulse, PSL dose-increase, TAC, IVIg, CsA	PSL dose-increase, RTX, CsA, IVIg	PSL, CsA	mPSLpulse, PSL, Intravenous CsA, IVIg, TCZ	
Treatment efficacy	+	+	+	+	+	+	
Effective agents	CsA	CsA	CsA	CsA, IVIg	PSL, CsA	TCZ	
Adverse events	-	-	-	-	-	+, CsA-induced thrombotic microangiopathy	

543 Abbreviations: CsA, cyclosporine A; IVIg, intravenous immunoglobulin; MMF, mycophenolate mofetil; mPSL, methylprednisolone;

544 PSL, prednisolone; RTX, rituximab; TAC, tacrolimus, TCZ, tocilizumab.







SUPPLEMENTAL MATERIALS

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Supplementary Methods

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Supplementary Figure S2: Changes of the serum cytokine and chemokine levels in the clinical course of the patients with MCD-TAFRO

Supplementary Figure S3: Regulator effects analysis of the predicted upstream regulators in Ingenuity Pathway Analysis

Supplementary Figure S4: Additional images of immunohistochemistry with the correlation analyses between the quantifying result of immunohistochemistry and clinical data

Supplementary Table S1. Multiplex cytokines/chemokines analysis results in the six MCD-TAFRO patients

Supplementary Table S2: Multicentric Castleman's disease signs and symptoms by NCI-CTCAE, version 4.0

Supplementary Methods

Diagnosis of multicentric Castleman disease-TAFRO (MCD-TAFRO)

A diagnosis of multicentric Castleman disease-TAFRO (MCD-TAFRO) requires all three major categories and at least two of four minor categories: major categories included anasarca, thrombocytopenia and systemic inflammation, and minor categories included Castleman disease-like features on lymph node biopsy, reticulin myelofibrosis, organomegaly, and progressive renal insufficiency. Exclusion of malignancies, autoimmune disorders, infectious diseases, POEMS (Polyneuropathy, Organomegaly, Endocrinopathy, Monoclonal protein, Skin changes) syndrome, hepatic cirrhosis, and thrombotic thrombocytopenic purpura/haemolytic uremic syndrome is required for a diagnosis of MCD-TAFRO ¹.

Case descriptions of six MCD-TAFRO patients

The case 1 is a 48-year-old woman manifesting chronic sicca symptoms with anti-Ro/La antibodies, leading to a diagnosis of primary Sjögren's syndrome (PSS) (Fig. S1A). Because she presented no other manifestations related to SS, she was followed up without treatment. However, eight months after the diagnosis, she developed point-like subcutaneous bleedings. Laboratory findings revealed severe thrombocytopenia and positive anti-*Helicobacter pylori* antibody. Therefore, she was diagnosed with PSS associated-autoimmune thrombocytopenia and was treated with prednisolone (1.0 mg/kg) along with antibiotic therapy for *H. pylori*. However, she subsequently manifested high fever and abdominal swelling with pain. Although she was treated with broad-spectrum antibiotics, she showed little improvement. Therefore, she was referred to our hospital. A restudy of the laboratory findings showed elevated serum levels of C-reactive protein (CRP) and renal dysfunction. Computed tomography (CT) demonstrated pleural effusion, ascites, splenomegaly, and mild systemic lymphadenopathy (Fig. S1B). Although dry tap was observed on bone marrow aspiration (BMA), BM biopsy revealed megakaryocytic hyperplasia with mild myelofibrosis (Fig. S1C). We diagnosed the patient with MCD-TAFRO, and administered methylprednisolone pulse therapy, tocilizumab, and thrombopoietin receptor

agonist (TPORA); however, these were ineffective (Fig. S1B). Subsequently, she was treated with rituximab (375 mg/m²/week, four times), and her condition transiently ameliorated. Finally, after the administration of cyclosporine A (CsA), she sustainably recovered (Fig. S1B).

The case 2 is a 47-year-old female farmer manifesting Raynaud's phenomenon (RP) with a positive anti-nuclear antibody and anti-U1 ribonucleoprotein antibody (Fig. S1D)². Because she demonstrated no symptoms associated with systemic lupus erythematosus (SLE), systemic sclerosis, and inflammatory myopathies, she did not meet the diagnostic criteria of mixed connective tissue disease. She was treated only with vasodilators for RP. Seven years later, she developed acute abdominal pain, and the gallbladder wall thickening and stone were detected by ultrasound, leading to the diagnosis of acute cholecystitis. Although she received fasting therapy and antibiotics, she did not achieve an improvement. She subsequently developed fever, dyspnoea, and abdominal distention, and she was transferred to our unit. Laboratory investigations revealed anaemia, thrombocytopenia, hypalbuminaemia, renal failure (serum creatinine 5.4 mg/dL), and systemic inflammation. Hypocomplementemia was not observed. Urinalysis showed haematuria and nephrotic-ranged proteinuria with active urine sediments. CT revealed bilateral pleural effusion, massive ascites, splenomegaly, and slight lymphadenopathy. The patient was initially diagnosed with SLE from the evidence of serositis, nephritis, hematopoietic injury, and positive autoantibodies. Although we administered methylprednisolone pulse therapy followed by prednisolone (1.0 mg/kg), mycophenolate mofetil, and tacrolimus, which is one of calcineurin inhibitors binding FK506 binding protein, with transient haemodialysis, her condition was worsening. We performed BM biopsy for a re-evaluation of the disease state, leading the discovery of mild myelofibrosis with hyperplasia of slightly atypical, multinuclear megakaryocytes. We diagnosed her with MCD-TAFRO. Despite tocilizumab therapy and TPORA, her manifestations did not improve. After the administration of CsA, the patient finally achieved full recovery.

The case 3 is a 64-year-old man presenting with generalized oedema (Fig. S1E). The blood test showed thrombocytopenia, renal impairment, and elevated soluble IL-2 receptor (sIL-2R) level. CT scan demonstrated pleural/peritoneal effusion, abdominal lymphadenopathy. BM biopsy revealed hypercellular marrow with myelofibrosis. He was first diagnosed with autoimmune thrombocytopenia and treated with prednisolone of 60 mg daily, improving his conditions. However, when prednisolone was tapered to 10 mg daily, generalized oedema flared up. Re-examination of laboratory data showed anaemia, thrombocytopenia and mild renal dysfunction. Mild elevated level of serum CRP and interleukin (IL)-6, and marked elevation of plasma vascular endothelial growth factor (VEGF) level were observed. Antinuclear antibody was positive, but the disease-specific autoantibodies were negative. Whole-body CT demonstrated body fluid cavity effusion with hepatosplenomegaly and BM biopsy revealed aggregated megakaryocytes and mild reticulin fibrosis (Fig. S1F). We diagnosed the patient with MCD-TAFRO, and administered methylprednisolone pulse therapy with tacrolimus and intravenous immunoglobulins (IVIg). However, the patient did not respond. We switched tacrolimus to CsA, and then the patient fully recovered. A follow-up of BM biopsy revealed decreases of megakaryocytes and reticulin fibrosis (Fig. S1F).

The case 4 was a 67-year-old woman complaining of fever, cough and oedema resistant with antibiotics (Fig. S1G). Laboratory data showed thrombocytopenia and renal impairment. CT demonstrated anasarca and hepatosplenomegaly. Lymph node biopsy revealed atrophic germinal centres, increased interfollicular vascularity and plasmacytosis like CD-like pathology (Figure 1H). She was diagnosed with MCD-TAFRO. Although he was treated with methylprednisolone pulse therapy, rituximab and tocilizumab, she did not respond to treatment and demonstrated respiratory failure due to massive pleural effusion, requiring mechanical ventilation in the intensive care unit. She recovered after tacrolimus administration. However, the disease flared up when prednisolone was tapered to 12.5 mg daily. Laboratory test demonstrated prominent elevated levels of serum IL-6 and positivity of antinuclear antibody and anti-Ro antibody. CT

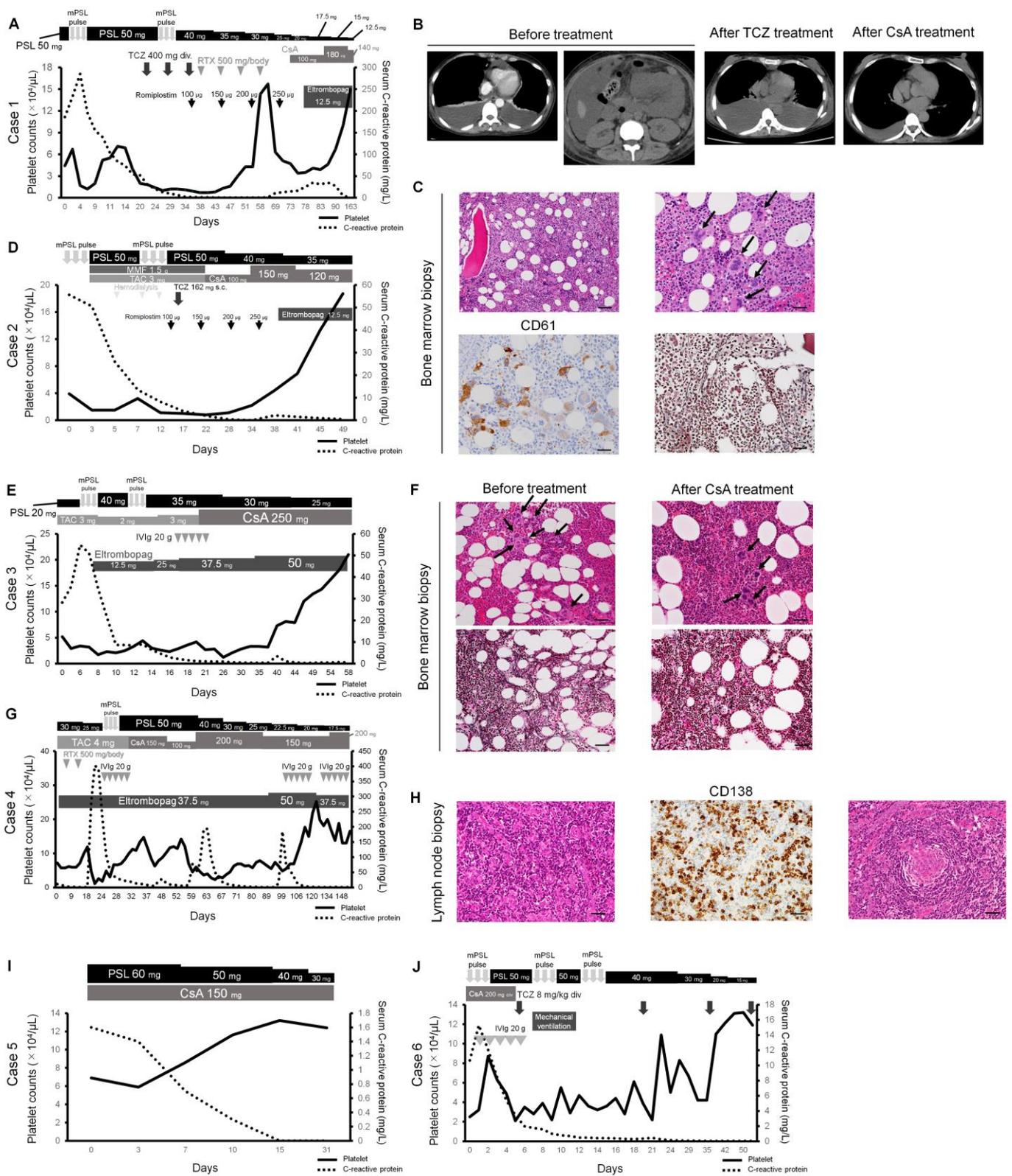
scan showed pleural and peritoneal effusion, and hepatosplenomegaly. Although we added a high dose of prednisolone and rituximab on tacrolimus, these drugs did not improve her symptoms. We administered IVIg therapy and switched tacrolimus to CsA, subsequently improving her conditions.

The case 5 was a 62-year-old woman presenting with generalized oedema (Fig. S1J). Laboratory findings showed thrombocytopenia, mild renal dysfunction and positive antiulcer antibody. Urinalysis demonstrated haematuria and nephrotic range proteinuria with active urine sediments. CT showed pleural and peritoneal effusion, and systemic lymphadenopathy. Her symptoms were resistant to levofloxacin treatment. Cervical lymph node biopsy revealed atrophy of the germinal centre and hyaline vascular proliferation with histiocyte infiltration. We diagnosed the patient with MCD-TAFRO and, administered prednisolone (1.0 mg/kg daily) and CsA. The patient subsequently recovered fully.

The case 6 was a 66-year-old woman manifesting with fever and dyspnoea (Fig. S1I). Although she was treated with antibiotics, progressive symptoms including thrombocytopenia, renal insufficiency, mild cervical lymphadenopathy and body cavity fluid collection with hepatosplenomegaly were observed. Urine analysis showed proteinuria and haematuria. Laboratory test demonstrated marked elevation of IL-6 and VEGF level, and positive autoantibodies including rheumatoid factor, antinuclear antibody, anti-double-stranded DNA antibody and anti-Ro antibody. She was diagnosed with MCD-TAFRO. We administered methylprednisolone pulse therapy, continuous intravenous CsA. However, her condition was worsening, and laboratory findings revealed severe microangiopathic haemolytic anaemia with thrombocytopenia, liver/renal failure. We diagnosed the patient with CsA-induced thrombotic microangiopathy. We discontinued CsA and administered tocilizumab and as-needed fresh frozen plasma. Although transient mechanical ventilation for respiratory failure was required, her condition was gradually improved and finally recovered.

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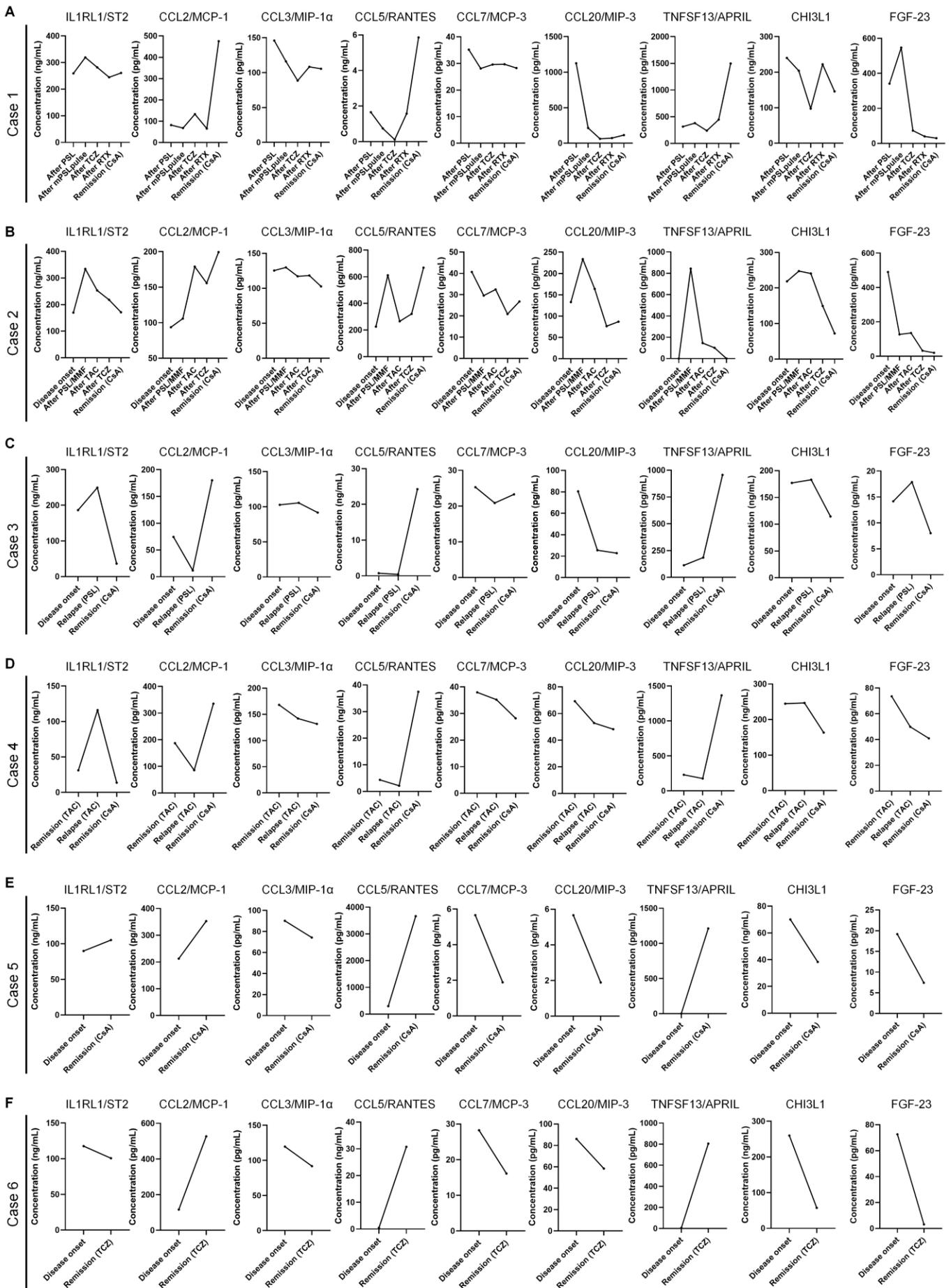


Supplementary Figure S1. Clinical course and relevant data of a multicentric Castleman disease-TAFRO cases in this study

(A, D, E, G, I and J) Timeline of clinical course and laboratory data of the presented six cases of multicentric Castleman disease-TAFRO (MCD-TAFRO). (B) Computed tomography (CT)

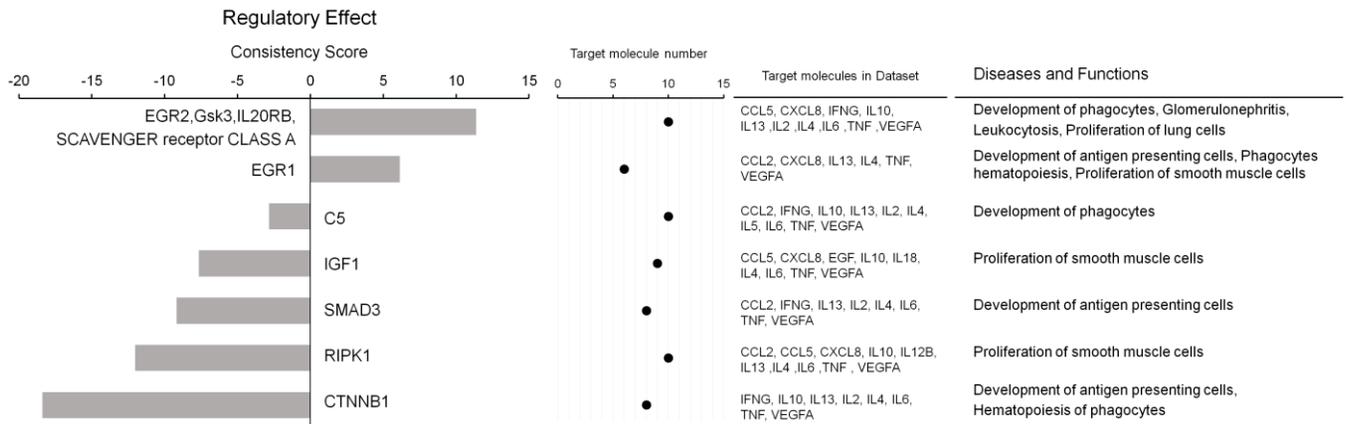
findings of the case 1 corresponding to therapeutic course. (C) Pathology of bone marrow biopsy in the case 1. (Upper) Arrow indicates megakaryocyte, hematoxylin-eosin staining. (Lower left) CD61 immunostaining, a marker of megakaryocytes. (Lower right) Gitter staining for reticulin fibers. Scale bar, (upper left and lower) 50 μm , (upper right) 20 μm . (F) Ameliorating pathology of bone marrow biopsy in the case 2 corresponding to clinical course. (Upper) Arrow indicates megakaryocyte, hematoxylin-eosin staining. (Lower) Gitter staining for reticulin fibers. Scale bar, 50 μm . (H) Pathology of lymph node biopsy in the case 4. (Left) Marked infiltration of plasma cells, hematoxylin-eosin staining. (Center) CD138 immunostaining, a marker of plasma cells. (Right) Hyalinization and remarkable vascular proliferation of germinal centers, hematoxylin-eosin staining. Scale bar, (left and right) 50 μm , (center) 20 μm .

Abbreviations: CsA, cyclosporine A; IVIg, intravenous immunoglobulin; MMF, mycophenolate mofetil; mPSL, methylprednisolone; PSL, prednisolone; RTX, rituximab; TAC, tacrolimus, TCZ, tocilizumab.



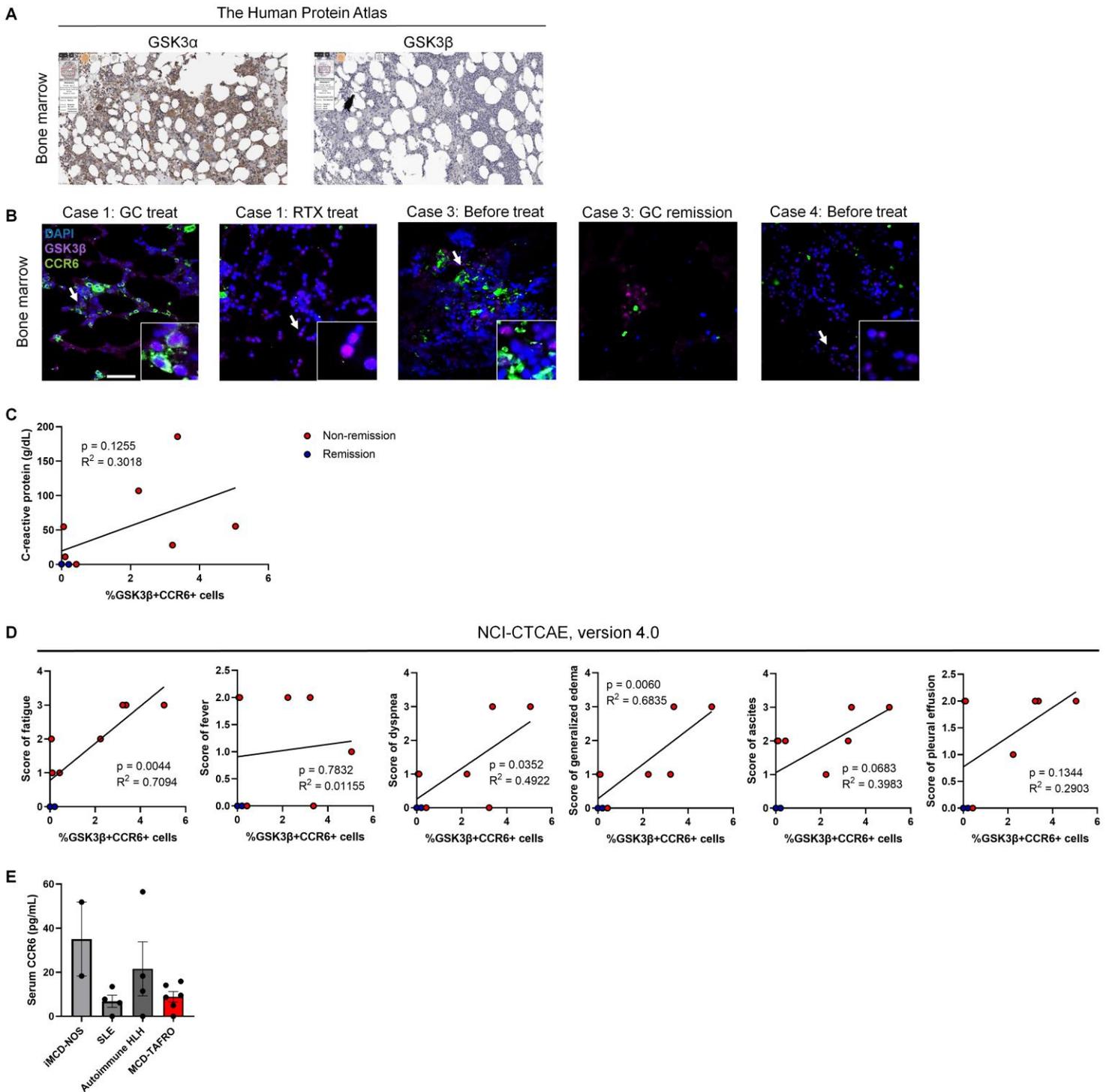
Supplementary Figure S2. Changes of the serum cytokine and chemokine levels in the clinical course of the patients with MCD-TAFRO

(A-E) Measured values of serum cytokines and chemokine as to therapeutic regimen in the case 1 to 6 with MCD-TAFRO.



Supplementary Figure S3. Regulator effects analysis of the predicted upstream regulators in Ingenuity Pathway Analysis

Each predicted upstream regulator is described with Consistency Score, which is calculated for each regulator effect network. The higher scores are awarded to networks that are directionally consistent and those means that most of the pathways from the regulators to target molecule (cytokines and chemokine in this case) to disease/function, are consistent with the predicted status of the regulators, the observed direction of the targets' expression in the dataset and the expected effects on the downstream disease and toxic function. Regulator effects analysis were performed by Ingenuity Pathway Analysis.



Supplementary Figure S4. Additional images of immunohistochemistry with the correlation

analyses between the quantifying result of immunohistochemistry and clinical data

(A) Glycogen synthase kinase (GSK) 3 α and 3 β protein expression in the human bone marrow published in The Human Protein Atlas (<http://www.proteinatlas.org>). (B) Immunohistochemical images of bone marrow overlaid with GSK3 β (magenta) and CCR6 (green). The magnified panel

shows the arrow-indicated area. Scale bars, 50 μm . (C, D) Additional correlation analyses between GSK3 β /CCR6-double-positive cells in bone marrow of MCD-TAFRO patients and their clinical data. Dot plot shows individual cases and blue color indicates the patient with remission state; red with non-remission state. Pearson's product-moment correlation coefficient was used for statistical analysis. (E) Serum C-C chemokine receptor (CCR) 6 levels in patients with MCD-not otherwise specified (NOS) ($n = 2$), systemic lupus erythematosus (SLE) ($n = 4$) and autoimmune haemophagocytic lymphohistiocytosis (HLH) ($n = 4$) secondary to mixed connective tissue disease, adult-onset Still's disease, dermatomyositis and MCD-TAFRO ($n = 6$). ANOVA with post-hoc Holm-Sidak method. Data are mean \pm s.e.m.

Supplementary Table S1. Multiplex cytokines/chemokines analysis results in the six MCD-TAFRO patients

Analytes	Case 1		Case 2		Case 3		Case 4		Case 5		Case 6	
	Non-remission	Remission										
TNF-alpha	2.01	0.06	2.04	3.71	2.10	1.93	1.75	4.41	0.30	0.00	0.00	0.00
IFN-gamma	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.93	0.00	0.00	0.00
IL-2	0.05	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
IL-4	2.39	3.96	0.83	2.18	0.75	3.41	3.41	1.12	0.00	4.89	3.66	0.75
IL-5	0.13	0.21	0.15	0.15	0.16	0.11	0.15	0.15	0.18	0.08	0.34	0.15
IL-6	39.91	5.84	12.56	5.18	0.00	0.00	0.00	0.00	3.47	0.00	15.08	21.91
IL-8/CXCL8	17.84	13.32	6.59	6.13	14.39	52.31	21.88	21.59	1.30	9.31	3.97	39.92
IL-10	0.00	0.00	2.35	1.43	0.00	0.00	0.00	0.50	0.00	0.00	0.00	2.10
IL-12p70	0.00	0.00	5.69	0.00	0.00	0.00	395.49	326.73	0.00	0.00	0.00	0.00
IL-13	0.00	0.00	5.33	0.00	6.66	0.00	277.07	332.04	0.00	0.00	13.32	0.00
IL-18	251.62	190.70	215.25	205.57	311.39	175.70	121.75	302.09	87.47	141.40	101.00	211.06
IL1RL1/ST2	276.22	261.03	229.26	171.29	217.83	36.57	115.98	22.50	89.83	105.17	117.73	100.69
CXCL1/GRO												
alpha	140.74	99.21	63.12	32.69	71.60	233.18	117.65	183.83	0.00	6.30	87.22	98.51
CXCL13/BLC	225.62	309.61	617.99	174.93	1252.88	230.08	848.75	328.23	550.07	210.17	522.61	1188.36

CCL2/MCP-1	87.16	475.24	146.78	199.49	43.13	180.16	85.95	261.44	212.36	352.86	116.66	526.99
CCL3/MIP-1												
alpha	114.62	105.46	118.69	102.66	104.06	91.66	142.04	149.86	90.15	74.20	119.64	91.82
CCL5/RANTE												
S	1.03	5.86	0.42	0.67	0.58	24.30	2.16	20.90	0.29	3.66	0.47	30.70
CCL7/MCP-3	30.62	28.25	30.07	26.79	23.09	23.28	35.22	33.07	5.66	1.89	28.25	16.12
CCL20/MIP-3	369.39	114.70	138.69	86.92	52.97	22.85	52.92	58.75	33.10	4.58	86.11	58.38
TNFSF13/APR												
IL	344.52	1496.30	218.19	0.00	149.14	955.71	175.78	797.97	0.00	1212.14	4.50	804.71
TNFSF13B/B												
AFF	1760.91	2606.52	1160.43	592.57	1824.83	383.08	942.95	551.86	1020.97	231.15	1233.08	410.91
CHI3L1	190.96	145.88	185.70	72.12	180.02	114.40	246.72	204.05	70.06	38.27	260.03	57.48
EGF	4.19	15.86	0.29	0.00	0.63	108.06	56.78	45.35	0.00	7.94	0.00	18.69
FGF-23	250.20	29.70	160.97	20.07	16.02	8.01	49.85	57.18	19.16	7.42	72.64	3.08
VEGF-A	401.26	823.13	43.49	42.95	112.52	330.16	43.75	12.07	3.84	527.56	46.54	50.00

The concentration unit for IL1R1/ST2, CCL5/RANTES and CHI3L1, ng/mL; the others, pg/mL

The average serum values of cytokines and chemokines corresponding to the disease activity status are described.

Abbreviations: tumor necrosis factor, TNF; interferon, IFN; interleukin, IL; C-X-C motif chemokine ligand, CXCL; IL-1 receptor-like 1, IL1RL1; Growth-related oncogene, GRO; B lymphocyte chemoattractant, BLC; C-C motif chemokine ligand, CCL; monocyte chemotactic protein, MCP; macrophage inflammatory protein, MIP; Regulated on Activation, Normal T cell Expressed and Secreted, RANTES; TNF ligand superfamily member 13, TNFSF13; A proliferation-inducing ligand, APRIL; B cell activating factor belonging to the TNF family, BAFF; Chitinase-3-like-1, CHI3L1; epidermal growth factor, EGF; fibroblast growth factor, FGF-23; vascular endothelial growth factor, VEGF

Supplementary Table S2. Multicentric Castleman's disease signs and symptoms by NCI-CTCAE, v4.0

Type of MCD signs and symptoms

NCI-CTCAE, version 4.0 adverse event

General MCD-related

1. Fatigue
2. Malaise
3. Hyperhidrosis
4. Night sweats
5. Fever
6. Weight loss
7. Anorexia
8. Tumor pain
9. Dyspnea
10. Pruritis

Autoimmune phenomena

11. Autoimmune disorder
12. Immune system disorder

Fluid retention

13. Generalized edema
14. Edema face
15. Edema limbs
16. Edema trunk
17. Genital edema

18. Localized edema
19. Neck edema
20. Periorbital edema
21. Capillary leak syndrome
22. Ascites
23. Pleural effusion
24. Pericardial effusion
25. Peripheral motor neuropathy
26. Peripheral sensory neuropathy
27. Nervous system disorder, other
28. Rash acneiform
29. Rash maculo-papular
30. Papulopustular rash
31. Purpura
32. Skin hyperpigmentation
33. Skin induration
34. Skin disorder, other

Neuropathy

Skin disorders

Abbreviations: NCI-CTCAE, v4.0, National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0.