1 **Ruxolitinib partially reverses functional NK cell deficiency in patients with**

2 ***STAT1* gain-of-function mutations**

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52   **Key Message:** *In vivo* and *in vitro* treatment with the JAK inhibitor ruxolitinib

53   partially restores perforin expression and cytotoxic function in immature CD56^{dim}

54   NK cells from patients with STAT1-gain of function (GOF) mutations.

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56   **Capsule Summary:** Patients with heterozygous gain-of-function *STAT1*

57   mutations develop an NK cell deficiency characterized by impaired perforin

58   expression and poorly functional NK cells that fail to mature. JAK inhibition with

59   ruxolitinib restores perforin expression, thus augmenting NK cell function and

60   providing a possible therapeutic intervention.

61

62   **Key Words:** STAT1 GOF, NK cell deficiency, perforin, JAK inhibition, ruxolitinib,

63   NK cell maturation

64

65   **Abbreviations:** Chronic mucocutaneous candidiasis (CMC), Signal Transducer

66   and Activator of Transcription 1 (STAT1), STAT1 Gain-of-function (STAT1 GOF),

67   Coiled-coil domain (CCD), DNA-binding domain (DBD).

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69

70 **ABSTRACT**

71 **Background:** Natural Killer (NK) cells are critical innate effector cells whose

72 development is dependent on the JAK-STAT pathway. NK deficiency can result

73 in severe or refractory viral infections. Patients with Signal Transducer and

74 Activator of Transcription (STAT)1 gain of function (GOF) mutations have

75 increased viral susceptibility.

76 **Objective:** We sought to investigate NK cell function in STAT1 GOF patients.

77 **Methods:** NK cell phenotype and function were determined in 16 STAT1 GOF

78 patients. NK cell lines expressing patient mutations were generated with

79 CRISPR-Cas9 mediated gene editing. STAT1 GOF NK cells were treated in vitro

80 with ruxolitinib.

81 **Results:** Peripheral blood NK cells from of STAT1 GOF patients had impaired

82 terminal maturation. Specifically, patients with *STAT1 GOF* mutations have

83 immature CD56^{dim} NK cells with decreased expression of CD16, perforin, CD57

84 and impaired cytolytic function. STAT1 phosphorylation was elevated but STAT5

85 was aberrantly phosphorylated in response to IL-2 stimulation. Upstream

86 inhibition of STAT signaling with the small molecule JAK1/2 inhibitor ruxolitinib *in*

87 *vitro* and *in vivo* restored perforin expression in CD56^{dim} NK cells and partially

88 restored NK cell cytotoxic function.

89 **Conclusions:** Properly regulated STAT1 signaling is critical for NK cell

90 maturation and function. Modulation of elevated STAT1 phosphorylation with

91 ruxolitinib is an important option for therapeutic intervention in patients with

92 *STAT1 GOF* mutations.

INTRODUCTION

Natural killer (NK) cells account for approximately 10-15% of all circulating lymphocytes[1] and are important early effectors in the innate immune response to a variety of viral infections.[2] Within peripheral blood, NK cells comprise two phenotypic and functional subsets.[3] CD56^{dim} NK cells are considered to be terminally mature and are the primary mediators of contact-dependent lysis of target cells,[1, 3] whereas the CD56^{bright} NK cells contain less mature NK cell subsets are potent producers of cytokines.[4, 5]

NK cells are derived from CD34^{+} precursors,[3, 6] and their development can be stratified to 5 stages,[7] characterized by specific surface receptors and proliferative and functional capacities.[8, 9] CD56^{bright} NK cells are the minor subset (~10%) of NK cells within peripheral blood.[5] They are characterized by low expression of perforin and high expression of CD94-NKG2A receptors,[7] and a subset of cells retain CD117 (c-Kit) expression.[7, 10] NKG2D, NKp46, CD62L and detectable CD122 are highly expressed.[10-12] These cells are sources of cytokines reflected by high levels of interferon (IFN)-γ and granulocyte monocyte-colony stimulating factor (GM-CSF).[1, 7-10, 13-18] A functional and phenotypic intermediate populations between CD56^{bright} and CD56^{dim} have also been described in healthy donors.[9, 11, 19] NK cell terminal maturation is defined by up-regulation of perforin, CD16, CD57, CD8 and NKp46, and down-regulation of CD94.[7, 11, 15-17] NK cell development and homeostasis requires interleukin (IL)-15,[20, 21] and in both mouse and human systems NK cells do not develop in the absence of IL-15.[22]

115   Classical natural killer deficiency (cNKD) is characterized by the absence

116   of both NK cells and their cytotoxic function,[23-27] whereas functional NK cell

117   deficiency (fNKD) is characterized by normal frequencies of NK cells in

118   peripheral blood with decreased function.[28, 29] There are several immune

119   deficiency diseases that affect NK cell development and/or function. [29] Patients

120   with NKD have increased susceptibility to viral infections, including

121   herpesviruses, varicella zoster, herpes simplex, cytomegalovirus, and human

122   papilloma virus.[23-27, 30] Severe herpes viral infection with decreased NK cell

123   natural cytotoxicity has been reported in patients with loss-of-function mutations

124   in signal transducer and activator of transcription-(STAT) 1,[31] and recently in 8

125   patients with STAT1 gain of function (GOF) mutations.[32]

126   The STAT family includes seven members: STAT1-4, STAT5a and b, and

127   STAT6.[33] Activation through intracellular domains of cytokine receptors, including

128   those for IFN-α/γ, IL-2, IL-4, IL-15, IL-21, and IL-6[34-36] leads to association with

129   Janus kinase (JAK) family members and recruitment and phosphorylation of

130   STAT proteins.[33, 37-40] Phosphorylated STAT proteins form homo- or

131   heterodimers and translocate to the nucleus where they bind to consensus

132   sequences in the promoters of target genes[33, 41]. In addition to roles in

133   development and homeostasis, STAT proteins mediate viral defense in NK

134   cells.[42] Upon IL-2 stimulation, pSTAT5 binds two enhancers located in the 5'

135   region of *PRF1* promoting its transcription;[43] upon IL-6 and IL-12 stimulation this

136   enhancer is bound by pSTAT1 and pSTAT4 respectively[44, 45] STAT5b knockout

137   mice have significantly lower levels of perforin expression at baseline and greatly

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138   decreased NK cell cytolytic function.[46] In humans, STAT5b deficiency is

139   associated with an abnormal NK cell development causing susceptibility to

140   severe viral infections in these patients.[47] Heterozygous GOF mutations in

141   *STAT1* lead to significantly higher levels of phosphorylated STAT1 (pSTAT1) and

142   increased STAT1 response to type I and II interferons.[48] These mutations are

143   mostly located in the coiled-coil (CCD) or DNA-binding (DBD) domains and lead

144   to an excess of pSTAT1-driven target gene transcription.[48-50] Patients with these

145   mutations can develop recurrent or persistent chronic mucocutaneous

146   candidiasis (CMC) or other cutaneous mycosis,[48, 49] staphylococcal infections,

147   disseminated dimorphic fungal infections (*Coccidioides inmitis* and *Histoplasma*

148   *capsulatum*), viral infections and autoimmune disease.[51-54]

149   Our investigations of patients with unexplained significant viral

150   susceptibility identified functional NK cell defects in patients with STAT1 GOF

151   mutations, suggesting that STAT1 is important for human NK cell differentiation

152   and function. In this study, we describe an immature, poorly functioning CD56^{dim}

153   NK cell population with low perforin expression and impaired cytotoxic capacity in

154   patients with STAT1 GOF mutations. Administration of the specific JAK1/2

155   inhibitor ruxolitinib, both *in vitro* and *in vivo*, restored perforin expression in

156   immature CD56^{dim} NK cells and partially restored NK cell cytotoxic function.

157   Together, these data demonstrate effects of ruxolitinib treatment and identify

158   decreased perforin expression and impaired terminal maturation as contributing

159   to functional NK cell defect in STAT1 GOF patients.

160

161   **METHODS**

162   **Study approval**

163   Blood samples were collected from patients and healthy donors with

164   consent under approved protocols from Baylor College of Medicine Institutional

165   Review Board, and National Institutes of Health (NIH). All samples were collected

166   with sodium heparin collection tubes.

167   **Flow cytometry and antibodies.**

168   Peripheral blood mononuclear cells (PBMCs) from healthy donors and

169   patients were purified by Ficoll-Paque Plus density gradient centrifugation (GE

170   Healthcare, Pittsburgh, PA). NK cells from all patients with confirmed *STAT1*

171   *GOF* mutations were studied phenotypically by FACS and evaluated for NK cell

172   activating, adhesion, inhibitory, and maturation markers as well as intracellular

173   cytokines and lytic granule content. Intracellular cytokines were evaluated in cells

174   stimulated with PMA and Ionomycin (Sigma Aldrich, St. Louis, MO) for 6 hours.

175   Brefeldin A (final concentration 10 ug/mL-Sigma Aldrich, St. Louis, MO) was

176   added 3 hours before antibody staining. The cells were fixed and permeabilized

177   with Cytofix/Cytoperm (BD Biosciences). The antibodies were purchased from

178   BD (CD69, FN50; CD16, B73.1; CD244, 2-69; CD11a, HI111; CD11b, ICRF44;

179   CD18, 6.7; CD94, HP-3D9; Perforin, δG9; CD28, L293), BioLegend (CD56,

180   HCD56; CD3, OKT3; CD16, 3G8; CD8, RPA-T8; NKp46, 9E2; DNAM-1, 11A8;

181   NKG2D, 1D11; CD45, HI30; NKp30, P30-15; CD158b, DX27; CD158d, mAb33;

182   CD62L, DREG-56; CD127, A019D5; CD117, 104D2; CD94, DX22; CD34, 581;

183   GM-CSF, BVD2-1C11; TNF-α, Mab11; IFN-γ, 4S.B3; IL-10, JES3-9D7; IL-13,

8

184   JES10-5A2), Beckman Coulter (NKp44, Z231; CD25, B1.49.9; CD2, 39C1.5;

185   CD57, NC1; CD122, CF1), eBioscience (CD158a, HP-MA4; CD27, 0323;

186   CD107a, eBioH4A3), R&D Systems (CD159c, 134591; CD159a, 131411;

187   CD215, 151303), and Invitrogen (Granzyme B, GB11). Data was acquired with

188   LSR-Fortessa (BD) cytometer and analyzed using FlowJo (Tree Star, Ashland,

189   OR, USA). NK cell subsets were identified as $CD56^{bright}CD3^-$ or $CD56^{dim}CD3^-$.

190   The percentage of NK cells positive for the receptor of interest was defined using

191   a corresponding fluorescent minus one (FMO). For ruxolitinib assays, PBMCs

192   and YTS cell lines were incubated for 48 hours in RPMI supplemented medium

193   with 1000 nM of Ruxolitinib (Selleckchem). After this time the cells were

194   recovered, washed and stained for NK cell receptor expression analysis.

195   **Cytotoxicity assays**

196   ADCC and NK cell cytotoxicity were measured with Cr^{51} release assay as

197   previously described.[55] ADCC was evaluated with Raji cell line incubated in the

198   presence or absence of anti-CD20 (Rituximab) (20 μg/mL) and co-cultured with

199   fresh PBMCs for 4 hours at 37°C in 5% CO_2. For natural cytotoxicity, PBMCs

200   from patients and healthy donors were incubated for 4 hours with IL-2 (1000

201   U/mL) and the K562 target cell line. YTS and NK92 cell cytotoxicity was

202   evaluated with K562 cell line using a 10:1 effector to target ratio.

203   **STAT activation assays**

204   STAT1 phosphorylation was measured by flow cytometry after stimulation

205   with IFNα (10 ng/mL-Millipore) for 30, 60, and 120 minutes. STAT5

206   phosphorylation was measured after stimulation with IL-2 (10 ng/mL, Cell

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207 Signaling) for 30. In the final 30 minutes of activation cells were stained with anti-

208 CD3 and anti-CD56 antibodies (Biolegend). After these times the cells were fixed

209 with Fixation Buffer (BD Biosciences) for 10 minutes at 37°, washed with Stain

210 buffer (BD Biosciences) and permeabilized with Perm Buffer III (BD Biosciences)

211 for 30 minutes on ice. Cells were stained with pY701-STAT1 AlexaFluor 488, and

212 pY694-STAT5 Alexa Fluor 647 (BD Phosflow) antibodies. The samples were

213 collected with LSR Fortessa cytometer (BD) and analyzed with FlowJo software

214 (TreeStar, Ashland, Ore, USA).

215 **Proliferation assay**

216   Proliferation was evaluated using CellTrace CFSE Cell Proliferation Assay

217 (Life Technologies, Eugene Oregon). PBMCs were stained with 1 μM of CFSE,

218 stimulated with IL-15 (5 ng/mL) and incubated with RPMI supplemented medium

219 at 37°C in 5% CO_2 for 5 days. The cells were then washed and stained with CD3

220 and CD56 antibodies (BioLegend). CFSE intensity was measured by flow

221 cytometry and proliferation was evaluated in the CD56$^+$CD3$^-$ population.

222 **Cell lines**

223   NK92-dependent interleukin-2 (IL-2) Natural killer cell line is derived from

224 rapidly progressive non-Hodgkin's lymphoma cells, these cells expresses the

225 following surface marker CD2, CD7, CD11a, CD28, CD45, CD54, this cell line

226 not express CD1, CD3, CD4, CD5, CD8, CD10, CD14, CD16, CD19, CD20,

227 CD23, CD34, and HLA-DR.[56] NK92 cells were cultured at 37°C in Myelocult

228 H5100 medium (StemCell technologies) supplemented with

10

229   Penicillin/Streptomycin (Gibco Life technologies) and 100 U/mL IL-2 (Cell

230   Signaling).

231   The natural killer cell lymphoblastic leukemia (YTS) cell line expressing

232   CD56,[57] was cultured at 37°C in RPMI 1640 (Gibco Life Technologies)

233   supplemented with Penicillin/Streptomycin (Gibco Life Technologies), non-

234   essential amino acids (Gibco Life Technologies), 1mM Sodium Pyruvate

235   (Corning Cellgro), 1M HEPES solution (Gibco Life Technologies), and L-

236   Glutamine (Gibco Life Technologies). STAT1 mutant NK cell lines were

237   generated by CRISPR-Cas9 gene editing and performed by nucleofection of 5 ug

238   of GeneArt CRISPR Nuclease Vector plasmid containing the guide sequences

239   ATCACTCTTTGCCACACCATGTTT, ACTGTTGGTGAAATTGCAAGTTTT, and

240   TTCAGCCGCCAGACTGCCATGTTTT (Life Technologies) using the Amaxa

241   nucleofector and Lonza-Kit R. 24 hours post-nucleofection GFP positive cells

242   were sorted. Single clones were expanded and STAT1GOF positive cells were

243   confirmed by flow cytometry.

244   **Statistics.**

245   The statistical significance of differences between healthy donors and

246   patients was determined by unpaired two-tailed Student's *t*-test. Differences

247   between multiple groups were determined by Ordinary one-way ANOVA test.

248   Statistical analyses were conducted using Prism 7.0 (GraphPad Software).

249

250 **RESULTS**

251 **Patients and clinical manifestations.**

252 We evaluated 16 patients with STAT1 GOF disease**.** The male to female

253 ratio was 1:1, and median age was 18.18 years (range: 3-31 years). Two of

254 these patients died from progressive fungal infections. The clinical features are

255 listed in Table 1. *Immune manifestations*. Two patients had IgA deficiency

256 (<7mg/dL). IgG deficiency varied; two patients received IgG replacement. CD4

257 lymphopenia was present in 3 patients. Eleven patients had <0.5% CD4$^+$IL-17$^+$

258 cells (normal range 0.5-2.5%).

259 ***STAT1* mutations**

260 Sequencing of *STAT1* identified heterozygous mutations in each patient.

261 In 8 patients, the mutations were in the coiled-coil domain (CCD) and the

262 remainders were in the DNA-binding domain (DBD) (Table I). Out of eight

263 discrete mutations, seven have been previously reported;[51, 53, 54] c.983A>G

264 p.H328R is novel.

265 *In vitro* STAT1 activation was tested in primary patient NK cells

266 representing 5 mutations (3 CCD and 2 DBD) in response to IFN-α. We observed

267 persistent phosphorylation without appropriate dephosphorylation in all patients

268 NK cells when compared to healthy donors (Fig 1, A). Furthermore, mutations in

269 the DBD had significantly higher levels of activated STAT1 after 120 minutes of

270 stimulation than mutations located in the CCD (Fig 1, B). Interestingly, we

271 observed that patients with mutations in the DBD tend to have a more severe

272 clinical phenotype than patients with CCD mutations (Table I).

273 **Patients with *STAT1 GOF* mutations have decreased NK cell cytotoxic**

274 **function**

275   We performed standard Cr^{51} release assays using PBMCs from 14

276 patients and healthy donor controls using K562 target cells in the presence and

277 absence of exogenous IL-2. We compared the NK cell cytotoxicity activity from

278 each patient with a representative NK cell cytotoxicity from a pool of 10 healthy

279 donors. NK cells from most patients with CCD mutations had residual cytotoxic

280 function that was partially responsive to IL-2 (Fig 2, A; and see Fig E1, A,). In

281 contrast, cells carrying mutations in the DBD had more severe defects in their NK

282 cell cytotoxicity that was not improved by IL-2 (Fig 2, B; and see Fig E1, B). NK

283 cells from 10 patients were incubated with Rituximab-opsonized Raji target cells

284 to assay antibody-dependent cellular cytotoxicity (ADCC). All patients with CCD

285 mutations had residual ADCC (see Fig E2, A,), whereas, ADCC in cells from

286 patients with DBD mutations was more impaired (see Fig E2, B). These data

287 suggest that impairment of NK cell function is affected by the location of the

288 *STAT1* mutation.

289 **Impaired terminal maturation of the CD56^{dim} NK cell subset in patients with**

290 ***STAT1 GOF* mutations**

291   NK cell targeted lysis is mediated and enabled by activating and inhibitory

292 receptors on mature NK cells. To determine if the impaired NK cell cytotoxic

293 function found in our patients was associated with a demonstrable NK cell

294 phenotype, we performed flow cytometry for NK cell developmental markers,

295 functional NK cell receptors (activating and inhibitory), intracellular cytokines, and

13

296   lytic granule contents[7, 10, 25, 58]. NK cell subsets were identified as CD56^{bright}CD3$^-$

297   or CD56^{dim}CD3$^-$ (Fig 3, A). We observed lower NK cell frequencies in STAT1

298   GOF patients (4.0±2.5%) (mean±SD) compared to healthy donors (9.7 ± 2.1%).

299   The frequency of CD56^{bright} NK cell subset in peripheral blood from patient and

300   control groups was similar. However, patients with *STAT1 GOF* mutations had a

301   decreased frequency of cells within the CD56^{dim} NK cell subset (2.631±1.774%)

302   in peripheral blood than healthy donors (9.894%, range 7.25-13.7%) (Fig 3, B**)**,

303   suggesting impaired terminal maturation or survival of the CD56^{dim} NK cell

304   subset.

305   In healthy donors, the CD56^{dim} NK cell subset is characterized by high

306   levels of expression of CD16 and perforin and acquisition of CD57 and CD8.[7, 15]

307   Patients with *STAT1 GOF* mutations have abnormal expression with low mean

308   fluorescence intensity (MFI) of CD16, perforin, CD57, CD8 (Fig 3, C; and see Fig

309   E3, A,). STAT1 GOF patients had decreased percentage of CD56^{dim}perforin$^+$ NK

310   cells (70.94 ± 23.4%) and CD56^{dim}CD16$^+$ NK cells (61.03± 13.62%) (see Fig E3,

311   B) compared to healthy donors ((91.44 ± 3.602%), (95.55 ± 3.588%))

312   respectively. Patient NK cells also had higher expression of receptors associated

313   with the CD56^{bright} subset, including CD94, CD117, CD94, and NKG2A (Fig 3, D

314   and E3, B). While the CD56^{bright} subset in NK cells from patients with *STAT1*

315   *GOF* mutations had higher levels of granzyme B and lower levels of TNF-α (see

316   Fig E3, C**),** the CD56^{dim} subset appears to have impaired terminal maturation.

317   These findings suggest that the CD56^{dim} subset in STAT1 GOF patients is

318   immature and potentially non-functional.

14

319 ***STAT1 GOF* mutations lead to decreased perforin expression and cytotoxic**

320 **capacity in NK cell lines**

321   To further delineate the effect of STAT1 dysregulation on NK cell

322 phenotype and function, we modeled two of the DBD mutations (E353K and

323 T385M) in YTS NK cell lines using CRISPR-Cas9 mediated gene editing. While

324 expression of total STAT1 was not affected by either mutation in either cell line

325 (data not shown), both mutant cell lines showed enhanced STAT1

326 phosphorylation in response to IFN-α stimulation compared with WT cells (Fig 4,

327 A). To further elucidate the abnormality seen in the primary cells, we looked at

328 select developmental markers in this cell line. The expression of CD94 and

329 NKG2A was increased in the mutant cell lines compared to WT (Fig 4, B).

330 Furthermore, we detected decreased perforin expression of YTS STAT1 mutant

331 cell lines compared to WT (Fig 4, B). To determine whether these phenotypic

332 differences corresponded with an effect on function, we performed Cr^{51} release

333 cytotoxicity assays and observed that the cytotoxic capacity of YTS STAT1

334 mutants was decreased more than 4-fold compared with the WT (Fig 4, C).

335 Similarly, expression of the CCD M202I mutation in a different NK cell line, NK92,

336 led to an analogous functional phenotype (Fig 4, D and see Fig E4) with

337 decreased perforin expression and reduced NK cell cytotoxic function (Fig 4, D).

338 This phenotype was consistent with our results from primary patient cells, as the

339 effect of CCD mutations was milder than that of the DBD mutations and define

340 the impact of patient-derived mutant STAT1 GOF on NK cell perforin expression

341 and maturation signatures.

342   **STAT1 GOF mutations impair STAT5 phosphorylation in NK cells**

343   The functional assays were performed in patients with mutations in the

344   CCD (patients 2, 4, 5, 6, 7, and 8) and DBD (patients 11, 15, and 16).

345   Considering the vital STAT-related role of IL-15 in NK cell development and

346   homeostasis, we evaluated the proliferative capacity of NK cells from STAT1

347   GOF patients in response to IL-15 stimulation. All patients tested had significantly

348   decreased proliferation compared to healthy donors, independent of the affected

349   domain (Fig 5, A).

350   Significant crosstalk between STAT signaling molecules exists, including

351   antagonistic binding to common enhancer elements. In particular, the competitive

352   binding of STAT3 and STAT5 to a common 5' perforin enhancer has been

353   demonstrated in T lymphocytes, dendritic cells, and cancer cell lines.[18, 43, 59] On

354   other hand, STAT5 induces perforin expression in response to IL-2.[43] To

355   determine whether the delayed dephosphorylation of STAT1 affects the

356   activation of STAT5 and leads to the significantly decreased perforin expression

357   in NK cells from patients with STAT1 GOF mutations, we evaluated STAT5

358   activation after IL-2 stimulation. We observed significantly decreased STAT5

359   activation in response to IL-2 in CD56^{dim} NK cells from the CCD and DBD

360   mutations (Fig 5, B). Interestingly, patients with DBD mutations had lower levels

361   of perforin compared with CCD patients (Fig 5, C). The lower levels of

362   phosphorylated STAT5 in DBD patients after 30 minutes of stimulation

363   corresponds with the degree of impairment of perforin expression in STAT1 GOF

364   NK cells (Fig 5, B and C).

365   To determine whether low NK cell proliferation with IL-15 stimulation and

366   low STAT5 activation in response to IL-2 was due to abnormal IL-2 and IL-15

367   receptor expression, we measured their expression by flow cytometry. We found

368   normal receptor expression for IL-2 and IL-15 in healthy donors and affected

369   subjects (data not shown). Therefore, the degree of impairment of NK cell

370   proliferation and perforin expression in STAT1 GOF NK cells tracks with the

371   degree of impairment in STAT5 activation.

372   **Ruxolitinib treatment restores perforin expression and cytotoxicity in**

373   **CD56^{dim} NK cells from STAT1 GOF patients**

374   Recently, three studies reported the efficacy of JAK1/2 pathway inhibition

375   by ruxolitinib in patients with STAT1 GOF mutations. Constitutive hyper-

376   phosphorylation of STAT1 in these patients was inhibited while STAT3

377   phosphorylation was not affected. Importantly, ruxolitinib treatment led to

378   improvement of CMC and autoimmunity.[60-62] We sought to determine whether

379   ruxolitinib affected the phenotype of NK cells from patients with STAT1 GOF

380   mutations. We evaluated the NK cell phenotype and function from three patients

381   (3, 9, and 11) before and during oral ruxolitinb treatment. We observed higher

382   levels of CD56^{dim}Perforin$^+$ NK cells after treatment with restoration of perforin

383   expression on CD56^{dim} NK cells during ruxolitinib treatment (Fig 6, A and see Fig

384   E5, A), accompanied by a 5–fold increase in NK cytotoxicity in response to IL-2

385   in patient 9 and a 2-fold increase in patient 11 (Fig 6, B). In addition, the ADCC

386   from patient 11 showed a 2-fold increase (see Fig E5, C). Increased perforin

387   expression accompanied improved NK cytotoxic function. We did not observe an

17

388 increase in NK cytotoxicity in patient 3 likely do to the short duration of treatment

389 when the assay was performed (Fig E5, B). Patient 9 and 11 had been on

390 ruxolitinib for at least 3 months when the post-ruxolitinib assays were performed,

391 whereas, Patient 3 was treated for only 2 weeks. Perforin restoration is

392 immediate while improvement in cytotoxicity comes with a longer duration of

393 therapy. Weinacht et al[62] reported a patient with clinical and laboratory

394 improvement after a year on ruxolitinib treatment. Taking our experiences

395 together, it appears at least 3 months of therapy with Ruxolitinib is needed to

396 demonstrate improvement in function.

397 Interestingly, while the expression of perforin increased, CD16, CD8, and

398 CD57 expression was not affected (see Fig E5, D). Despite the dramatic

399 increase in perforin and improved NK cell cytotoxicity, the course of the fungal

400 infection was so advanced in patient 11 when treatment was started that her

401 disease was not ameliorated and she eventually died. However, on acyclovir

402 prophylaxis, she did not have any breakthrough herpes infections. Patient 9

403 experienced significant improvement in his enteropathy and chronic norovirus.

404 His stool output decreased from 3 liters of stool per day to 2-3 loose stools 4

405 weeks after treatment began, and then 1-2 formed stools per day by 3 months

406 post treatment.

407 To determine whether ruxolitinib had a direct effect on perforin expression,

408 we evaluated the STAT5 phosphorylation after stimulation with IL-2 in $CD56^{dim}$

409 NK cells. Our findings showed a lower level of STAT5 phosphorylation in $CD56^{dim}$

410 NK cells with IL-2 stimulation (Fig 6, C), which also corresponds with low perforin

18

411   expression in pre-treatment samples. After ruxolitinib treatment, we observed

412   higher levels of STAT5 activation in post-treatment sample from patient 9 and 11

413   (Fig 6, D). These results demonstrate that decreased activation of STAT1

414   through JAK inhibition affects the activation of STAT5 and, in turn, perforin

415   expression. To compliment the studies on the *in vivo* effects of ruxolitinib

416   treatment over perforin expression, we incubated PBMCs isolated from another

417   patient (Pt 13) for 48 hours with 1 μM of ruxolitinib. Perforin expression was

418   partially restored in CD56^{dim} NK cells (Fig 6, E); whereas, the levels of perforin

419   observed in CD56^{dim} NK cells from healthy donors were not affected. Similar

420   results were observed using the STAT1 GOF NK cell line (see Fig E5, E). Thus,

421   inhibition of JAK1/2 signaling *in vivo* and *in vitro* leads toward normalized STAT1

422   phosphorylation and restoration of NK cell function in STAT1 GOF NK cells.

423   While the maturation defect in STAT1GOF NK cells is not rescued, direct

424   modulation of STAT1 phosphorylation with ruxolitinib can partially remediate poor

425   NK cell function in these patients.

426

427 **DISCUSSION**

428 Patients with *STAT1 GOF* mutations have impaired IL-17A and IL-17F

429 immunity, associated with CMC.[48, 49, 53] However, impaired IL-17 immunity does

430 not fully explain the susceptibility to viral infections.[52, 54] *STAT1* loss-of-function

431 mutations have been reported with impaired NK cell activity and CMV infection.[31]

432 The association of herpesvirus infection with NK cell deficiency led us to

433 investigate the phenotype and function of NK cells in patients with *STAT1 GOF*

434 mutations. We have identified that excess STAT1 phosphorylation owing to these

435 mutations is associated with impaired NK cell terminal maturation, decreased

436 expression of the critical cytolytic effector molecule perforin, and impaired

437 cytolytic function. Importantly, these effects can be partially ameliorated *in vivo*

438 and *in vitro* by treatment with the JAK1/2 inhibitor ruxolitinib.

439 We found that the STAT1 GOF-associated CD56^{dim} NK cell subset have

440 an immature phenotype, characterized by aberrant expression of CD94, CD117,

441 NKG2A, molecules associated with the CD56^{bright} subset, as well as low levels of

442 CD16, perforin, CD57 and CD8. While a similar developmentally intermediate

443 population typically makes up between 20% and 40% of CD56^{dim} NK cells in

444 healthy donors,[11, 19, 63] it was the majority (80%) of CD56^{dim} NK cells found within

445 the peripheral blood of STAT1 GOF patients. Notably, The STAT1 GOF CD56^{dim}

446 population is characterized by low levels of CD16 and perforin; two important

447 components of the cytotoxic capacity to eliminate infected cells.

448 Interestingly, DNA-binding domain mutations generally conferred a more

449 profound NK cell defect than those in the coiled-coil domain. The *STAT1* domain

450   mutated is important in the severity of the NK cell dysfunction in STAT1 GOF

451   patients. However, expression of both the coiled-coil and DNA-binding domain

452   mutations in NK cell lines equally affected perforin expression and cytotoxic

453   capacity, suggesting that additional factors may dictate the effect of *STAT1*

454   mutation on NK cell function. This variability in phenotype has also recently been

455   reported by Tabellini et al., who describe a cohort of STAT1 GOF patients with

456   normal perforin and CD16 expression.[32]

457   STAT5 phosphorylation and signaling, including IL-15 mediated

458   proliferation, are impaired in STAT1 GOF. STAT5's unique role in NK cell

459   proliferation and cytotoxic activity suggests that its impairment may cause at

460   least some of the NK cell maturation defect seen in STAT1 GOF patients.[18, 46] In

461   addition, the binding of *STAT5* to the 5' enhancer in the *PRF* gene likely

462   contributes specifically to the down-regulation of perforin in patient NK cells. The

463   re-expression of perforin upon reduction of STAT1 phosphorylation suggests that

464   this is not a hardwired developmental defect. The mechanism of suppression of

465   STAT5 by excess STAT1 phosphorylation is unclear. STAT1-induced SOCS-

466   mediated negative regulation of STAT5 is one possibility, and the recent

467   description of increased SOCS1 and SOCS3 expression in STAT1 GOF patient

468   NK cells supports this hypothesis.[32] The lack of rescue of overexpression of

469   CD94/NKG2A and CD117 on CD56^{dim} NK cells following ruxolitinib treatment

470   suggests that these markers are not directly modulated by excess STAT1

471   signaling.

472   With the onset of genetic testing in primary immunodeficiency

473   disease(PID), we can now choose therapies that modulate a specific target.[64]

474   For example, abatacept is now successfully used to treat CTLA-4 haplo-

475   insufficiency as well as other directed cytokine and biologic therapies for a wide

476   range of PIDs. [65] Autoimmune disease in STAT3 GOF disease was successfully

477   treated with IL-6R blockade.[66] Treatment with the JAK 1/2 inhibitor ruxolitinib in a

478   patient with refractory candida esophagitis led to resolution of many symptoms

479   within a 5-month period.[60] Increases in IL-17 production in STAT1 GOF patients

480   following treatment with ruxolitinib was also associated with reduced burden of

481   CMC, reduced hyperresponsiveness to type I and II interferons, normalized T_H1

482   and follicular T helper responses, and had remission of the autoimmune

483   mediated cytopenias.[61, 62] Our results demonstrate that perforin expression and

484   NK cell cytotoxic capacity are partially restored following *in vivo* treatment,

485   suggesting that ruxolitinib may improve NK cell function, but its effect on other

486   lymphoid and myeloid elements must be assessed to determine the therapeutic

487   utility of ruxolitinib in STAT1 GOF disease.

488   In conclusion, STAT1 GOF mutations cause a distinct NK cell phenotype

489   likely owing to an impairment of NK cell terminal maturation. These mutations

490   cause poor perforin expression and this NK cell population has decreased or

491   absent NK cell cytotoxic activity. Importantly, perforin expression and NK cell

492   function are restored by JAK inhibition with ruxolitinib. These data indicate that

493   STAT1 has critical roles in NK cell development and maturation, through direct

494   mediation of cytotoxic mediators and through modulation of other mediators,

495   including STAT5.

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497

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709 **Figure Legends**

710 **Table I.** Clinical manifestations and STAT1 mutations. CMC, Chronic

711 Mucocutaneous Candidiasis; CMV, Cytomegalovirus; HSV, Herpes simplex

712 virus; IPEX, Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked;

713 VZV, Varicella zoster virus; TB, *Tubercle bacillus*: M, male; F, Female; R, patient

714 with ruxolitinib treatment; +, died.

715

716 **FIG 1.** STAT1 GOF mutations lead to delayed dephosphorylation in NK cells.

717 Dephosphorylation in NK cells stimulated with IFN-α for the indicated periods in

718 healthy donors, **A**, patients with mutations into the Coiled-coil domain (CCD;

719 patients 2, 3, 4, 5, 6, 7, and 8) or DNA-binding domain (DBD; patients 9, 13, and

720 15). **B**, levels of STAT1-activated after 120 minutes of stimulation. HD, healthy

721 donor; Pt; patient. The statistical significance was analyzed using the Ordinary

722 one-way ANOVA test ***$p<0.001$, ****$p<0.0001$.

723

724 **FIG 2.** Impaired NK cell cytotoxic capacity in patients with *STAT1 GOF*

725 mutations. NK cell cytotoxicity was measured by Cr^{51} release in the presence or

726 absence of 1000 U/mL of IL-2. **A**, patients with Coiled-coil domain (CCD)

727 mutations; **B,** patients with DNA-binding domain (DBD) mutations. HD, healthy

728 donor; Pt; patient.

729

730 **FIG 3.** CD56^{dim} NK cells have an immature phenotype in patients with *STAT1*

731 *GOF* mutations. NK cell phenotype from healthy donors and STAT1 GOF

31

732   patients were analyzed by flow cytometry. **A,** NK cell subsets were defined as

733   CD56^{bright}CD3^{−} cells or CD56^{dim}CD3^{−}. **B,** representative graph shows the

734   frequencies of CD56^{bright}CD3^{−} and CD56^{dim}CD3^{−} NK cells subsets. **C**,

735   representative graphs shows mean fluorescence intensity (MFI) for each NK cell

736   receptor expressed by CD56^{dim} NK cells. Each blue circle represents a healthy

737   donor and a red box represents a STAT1 GOF patient. Horizontal bars represent

738   means and the vertical bars indicate the standard deviation. The statistical

739   significance was analyzed using the unpaired Student's *t*-test. Results are

740   representative of 3 independent experiments. **p<0.01, ***p<0.001,

741   ****p<0.0001.

742

743   **FIG 4.** *STAT1 GOF* mutations affect the cytotoxic capacity and perforin

744   expression in NK cell lines. DNA-binding domain mutations E353K and T385M

745   were expressed in the YTS cell line using CRISPR-Cas9 gene editing. **A,** STAT1

746   hyper-phosphorylation in response to IFN-α was measured by intracellular flow

747   cytometry using pY701-STAT1 antibody. **B,** the phenotype of YTS STAT1 GOF

748   cell lines was measured by flow cytometry. **C,** NK cell cytotoxicity was analyzed

749   against K562 target cells using standard Cr^{51} release protocol. **D,** STAT1 hyper-

750   phosphorylation in response to IFN-α; perforin, NKG2A, and CD94 expression,

751   and cytotoxic capacity measured in NK92 STAT1-M202I mutant cell line. MFI*,*

752   Mean fluorescence intensity; WT, wild type. Results are representative of 3

753   independent experiments. * *p<0.05.*

754

755 **FIG 5.** Decreased STAT5 activation in CD56^{dim} NK cells from STAT1 GOF

756 patients. **A,** NK cell proliferation from healthy donors and STAT1 GOF patients in

757 presence of IL-15 (5 ng/mL). **B,** phospho-STAT5 levels in CD56^{dim} NK cells

758 stimulated with IL-2 (10 ng/ml) for 30 minutes. Each point represents the mean

759 fluorescence intensity for each individual healthy donor or patients. **C**, perforin

760 expression levels in CD56^{dim} NK cells from patients with CCD or DBD mutations.

761 Statistical significance was analyzed using the Ordinary one-way ANOVA *test*. *

762 *p*<0.05, **p*<0.01, ***p*<0.001, ****p*<0.0001. HD, healthy donors; MFI*,* Mean

763 fluorescence intensity; CCD, Coiled-coil domain (patients 2, 4, 5, 6, 7, and 8);

764 DBD,DNA-binding domain (patients 11, 15, and 16). Results are representative

765 of 2 independent experiments for each patient.

766

767 **FIG 6.** Ruxolitinib restores perforin expression in primary NK cells*.* **A,**

768 percentages of CD56^{dim}Perforin^{+} NK cells and perforin expression on CD56^{dim} NK

769 cell subset pre- and post-treatment. **B**, natural cytotoxicity was performed with

770 Cr[51] release protocol with freshly isolated PBMC from patient 9, and 11 and a

771 respective healthy donor and was tested against K562 target cells in the

772 presence or absence of 1000 U/mL of IL-2. STAT5 activation in CD56^{dim} NK cells

773 after stimulation with IL-2, **D**, patients 3, 9, and 11 (pre-treatment) and **D**, in

774 CD56^{dim} NK cells from patient 9 and 11post-treatment. **E**, PBMCs from Pt 13

775 were incubated *in vitro* for 48 hours with or without 1 μM of ruxolitinib and the

776 perforin expression was measured in CD56^{dim} NK cells. The statistical

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777   significance was analyzed using the Ordinary one-way ANOVA test * $p<0.05$,

778   **$p<0.01$, ***$p<0.001$.

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FIG E1. Natural cytotoxicity was measured in the presence or absence of 1000 U/mL of IL-2. **A,** patients with CCD mutations; **B,** patients with DBD mutations. HD, healthy donor; Pt; patient.

FIG E2. Antibody-dependent cellular cytotoxicity (ADCC) was performed using Raji target cells in the presence or absence of Rituximab (20 ug/mL) in patients with **A**, CCD and **B,** DBD mutations. HD, healthy donor; Pt; patient.

FIG E3. NK cell subsets phenotype in patients with *STAT1 GOF* mutations. **A**, representative perforin expression plots in total NK cells. Positive **B**, CD56^{dim} or **C**, CD56^{bright} NK cells for each receptor. Each blue circle represents a healthy donor and a red box represents a STAT1 GOF patient. Horizontal bars represent means and the vertical bars indicate the standard deviation. The statistical significance was analyzed using the unpaired Student's *t*-test. Results are representative of 3 independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

FIG E4. NK cell receptors expression in NK92 STAT1-M202I cell line. The expression was measured by flow cytometry, the expression was compared with WT cell line and respective fluorescence minus one (FMO). Each histogram showed the FMI for each cell line.

823   **FIG E5.** Ruxolitinib restore the perforin expression in STAT1 GOF NK cell lines.

824   **A**, perforin expression in CD56^{dim} NK cell from patient 3 pre- and post-treatment.

825   **B**, natural cytotoxicity from patient 3 and a respective healthy donor in the

826   presence or absence of 1000 U/mL of IL-2. **C**, ADCC from patient 11 performed

827   using Raji target cells in the presence or absence of Rituximab (20 ug/mL) before

828   and during her treatment. **D**, CD56^{dim} phenotype in patients with oral ruxolitinib

829   treatment, **E**, perforin expression in YTS STAT1 GOF NK cell lines after 48 hrs of

830   stimulation with 1 μM of ruxolitinib. Statistical significance was analyzed using the

831   Ordinary one-way ANOVA *test*. * *p*<0.05, ***p*<0.01, *****p*<0.0001.

832

833

Fig 1 (2-column)

pSTAT1 (pY701)

Fig 2 (2-column)

A

R274W (CCD)

Q285K (CCD)

Y289C (CCD)

B

H328R (DBD)

E353K (DBD)
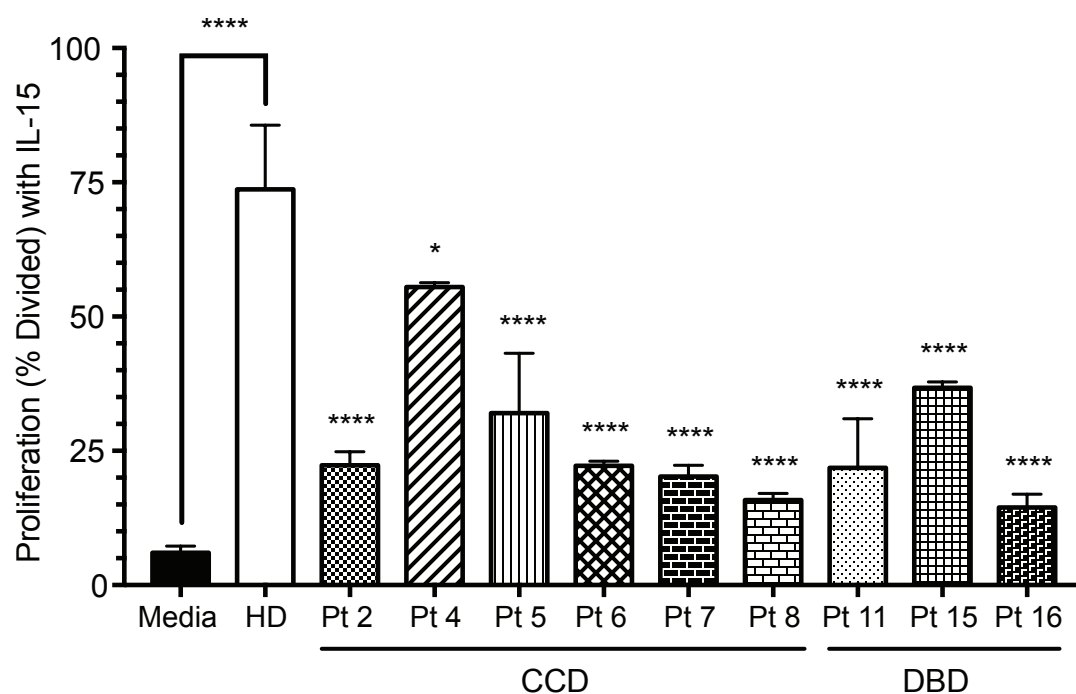
T385M (DBD)
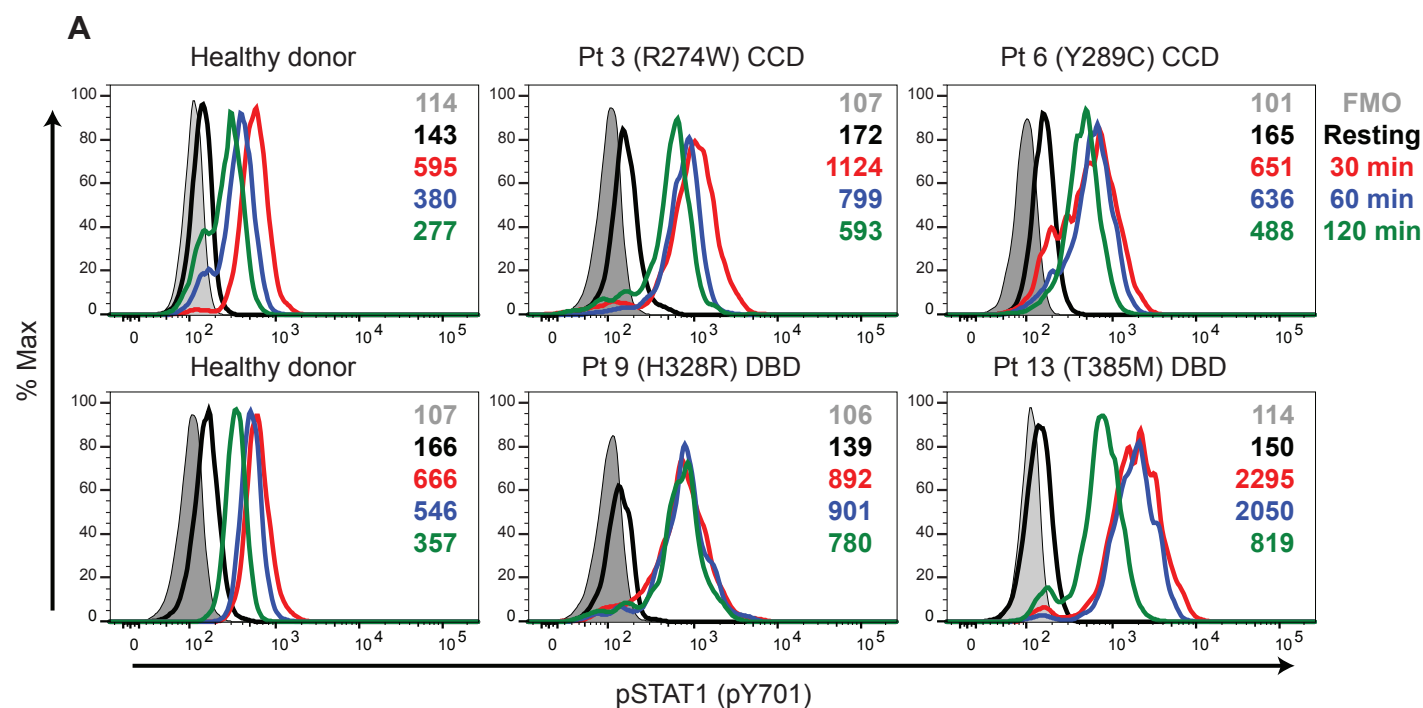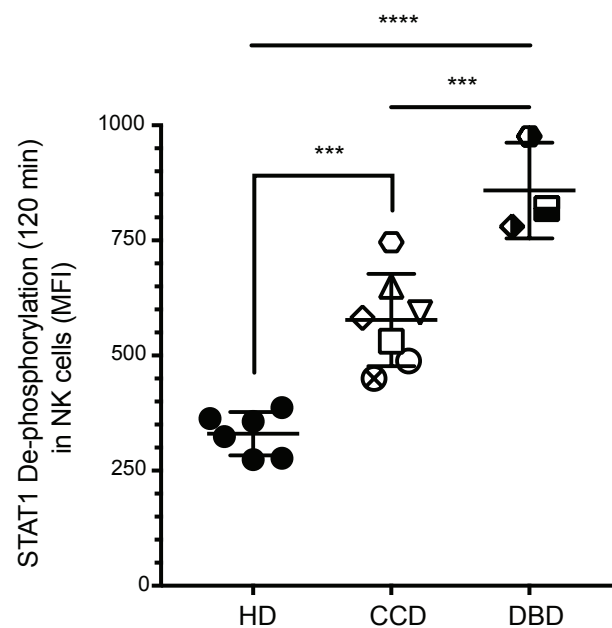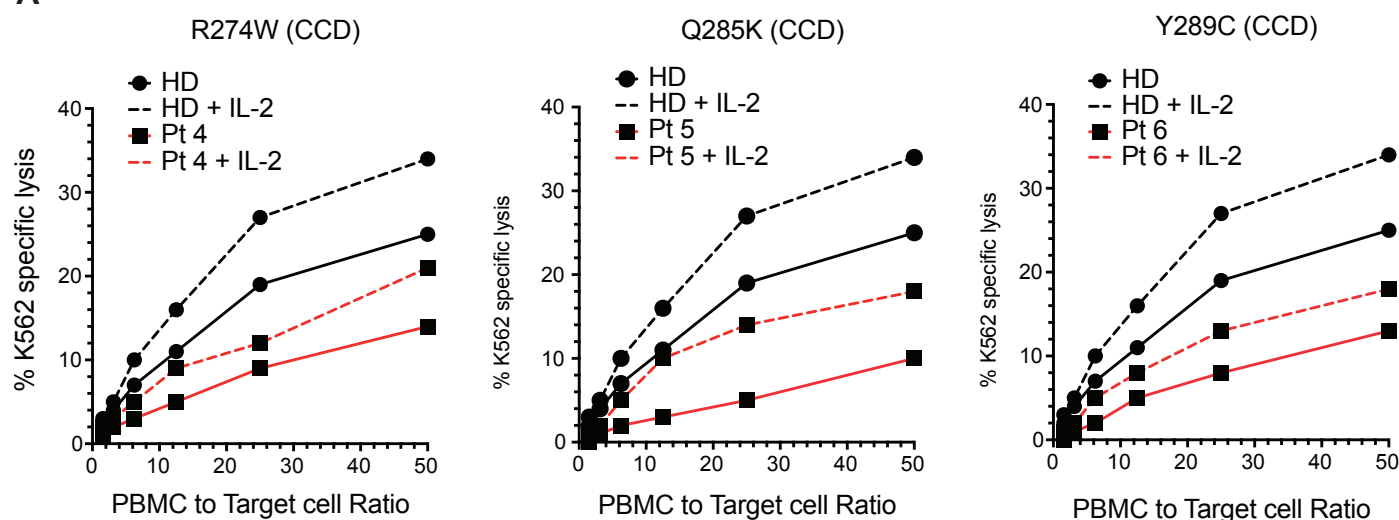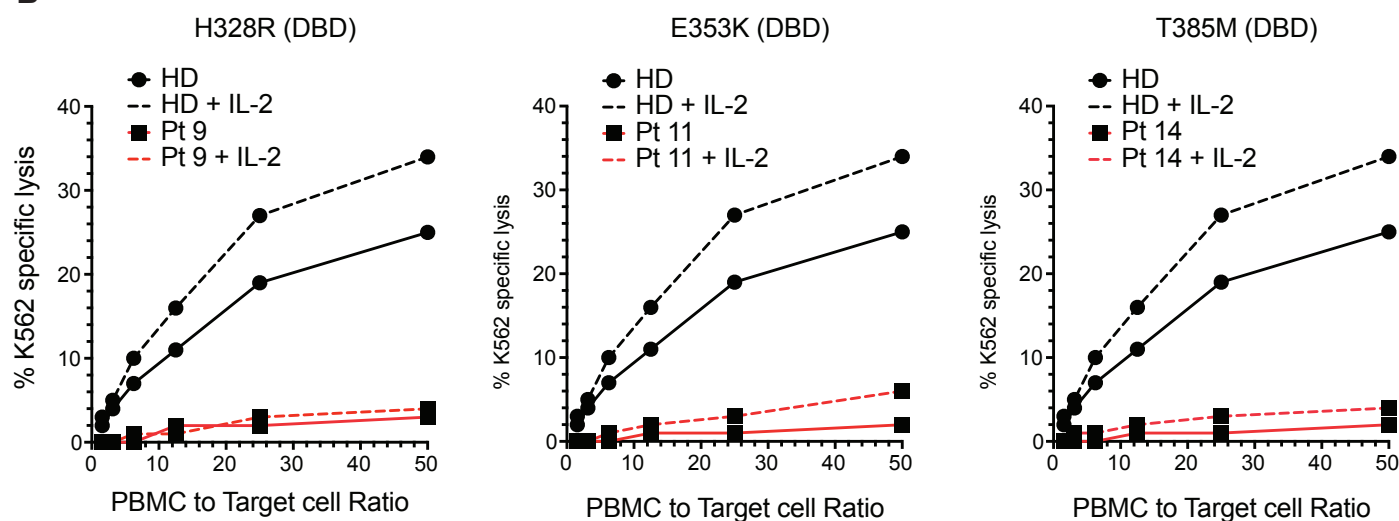
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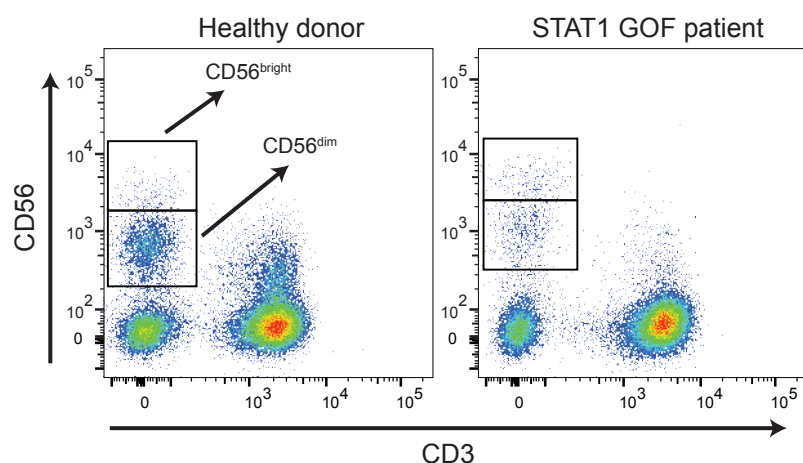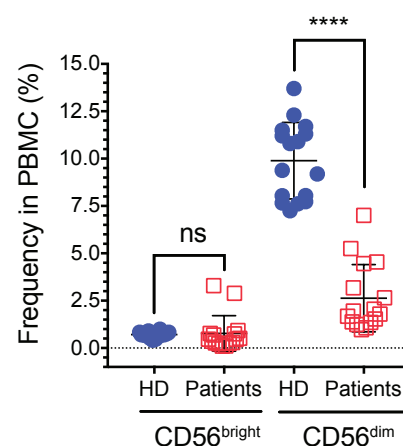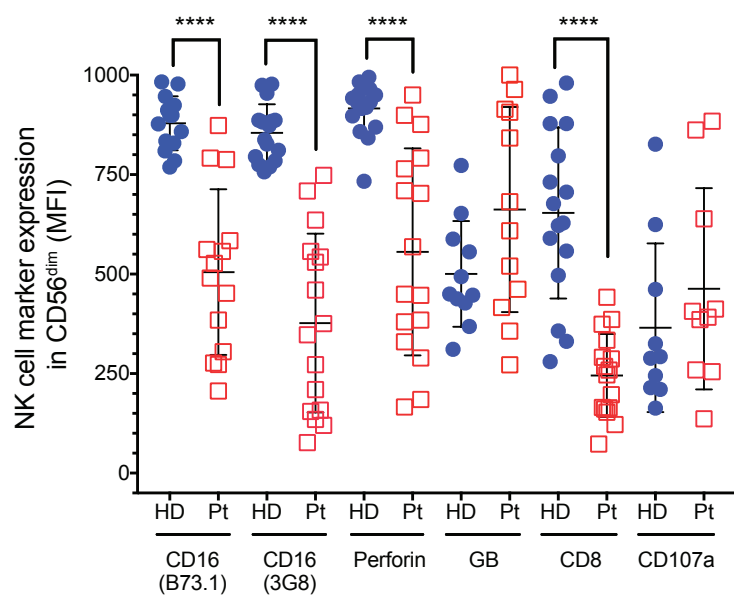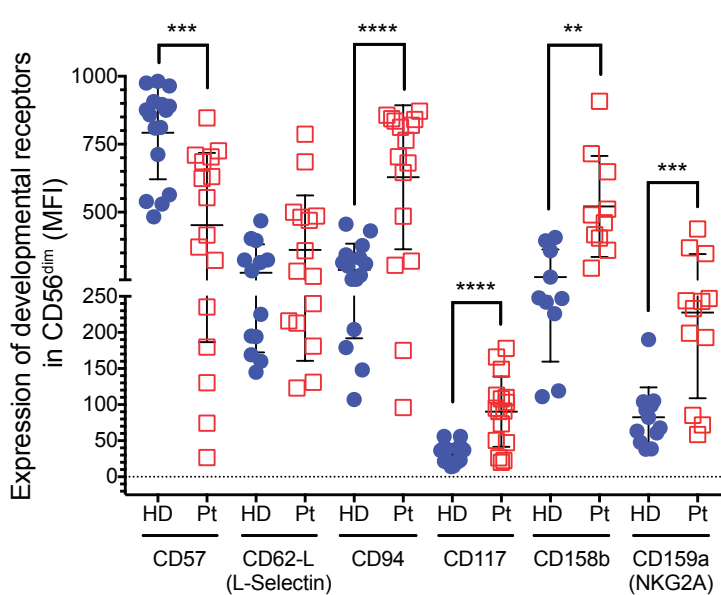
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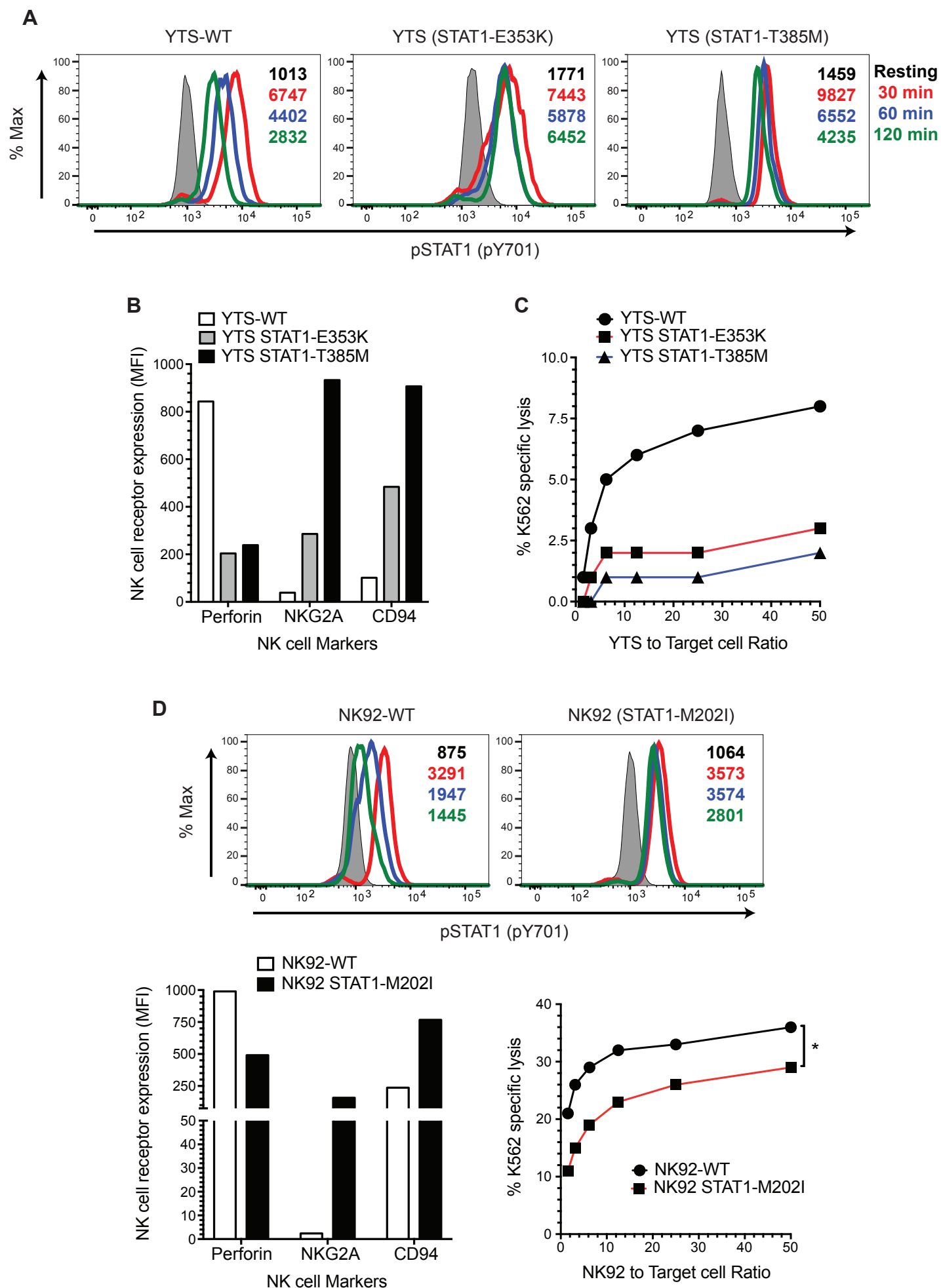
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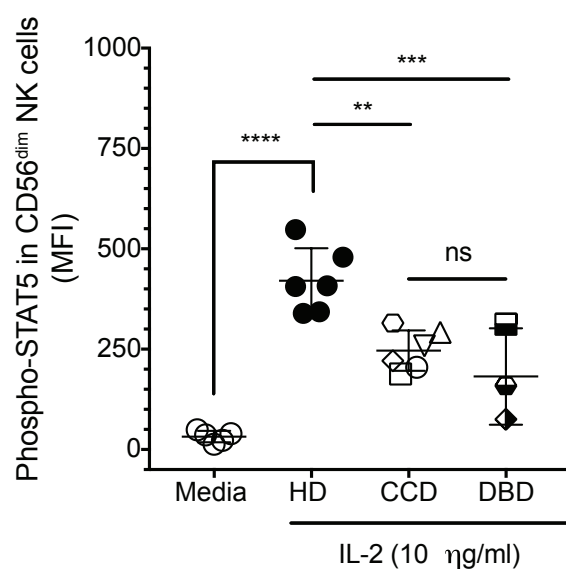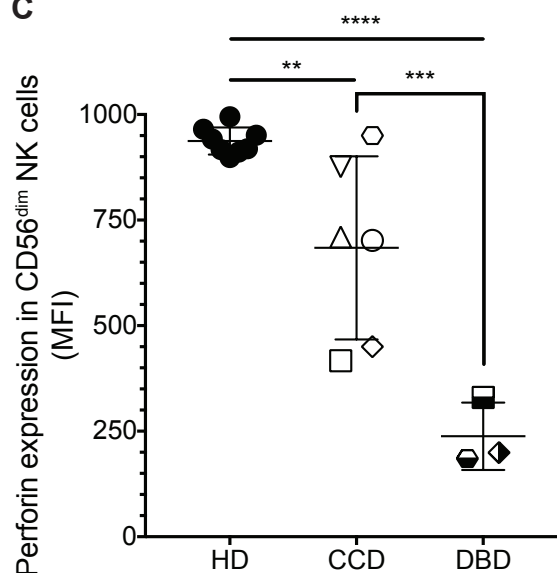
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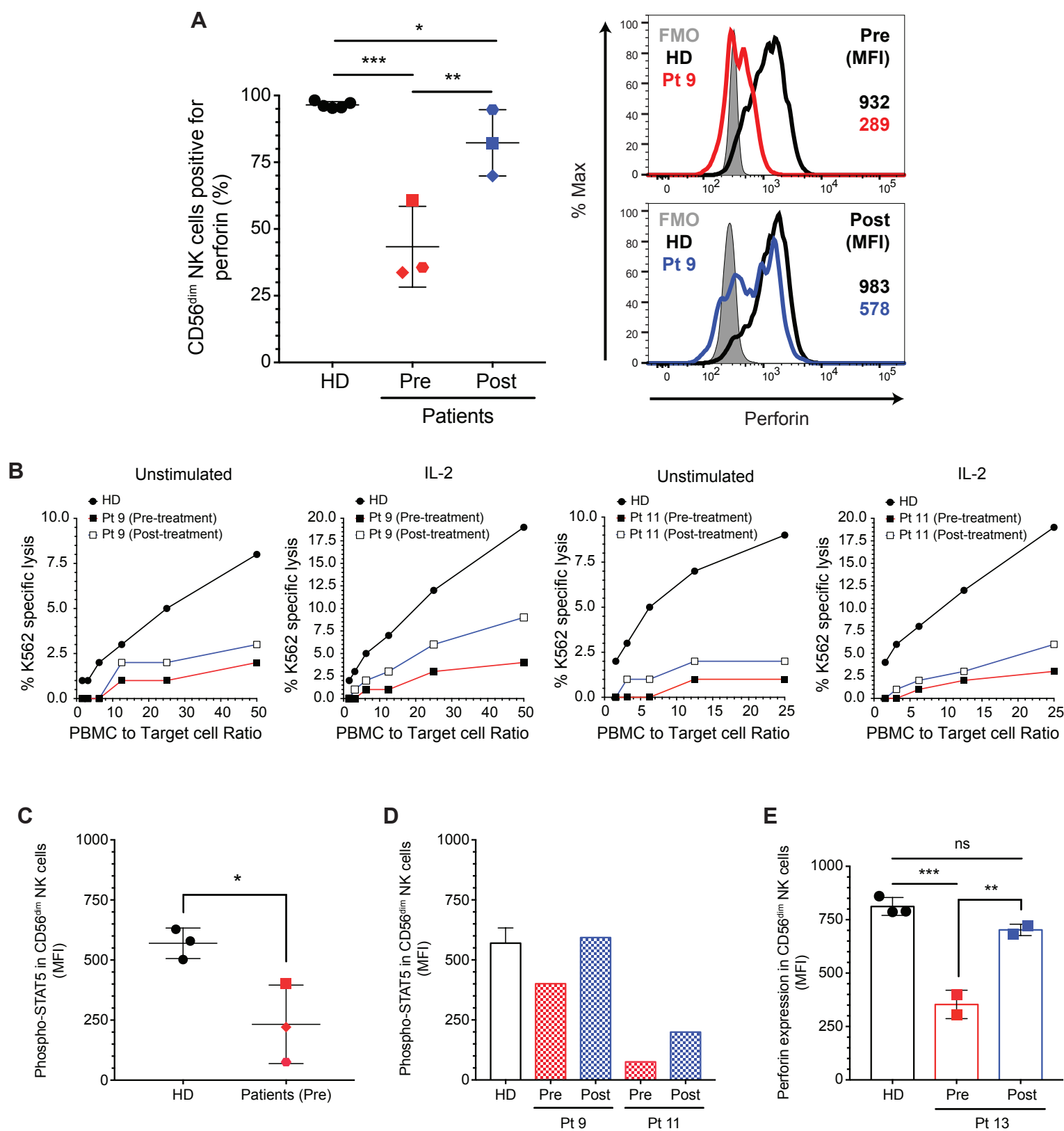
Patient Gender/Age (y)	Mutation	CMC	Other clinical manifestations
Coiled-coil domain mutations			
Patient 1 F/15	c.606G>A p.M202I	CMC	TB pneumonia Cryptococcal pneumonia
Patient 2 M/3	c.820C>T p.R274W	CMC	Influenza infection Adenopathy
Patient 3[R] F/31	c.820C>T p.R274W	CMC	Papillary thyroid CA Skin squamous cell CAs Diabetes Mellitus type I Recurrent oral/vaginal aphthous ulcers Sinopulmonary bacterial infections Skin and soft tissue infections Hypertension
Patient 4 F/10	c.820C>T p.R274W	CMC	Thrush Growth failure Vulvar erythema Mild erythematous facial rash Persistent vaginal discharge
Patient 5 M/5	c.853C>A p.Q285K	CMC	Growth failure *M. fortuitum* lymphadenitis Sinopulmonary bacterial infections Skin and soft tissue bacterial infections Rectal lesions
Patient 6 M/5	c.866A>G p.Y289C	CMC	CMV viremia HSV gingivostomatitis Recurrent thrush *P. jirovecii* pneumonia
Patient 7 M/8	c.866A>G p.Y289C	None	None
Patient 8 F/30	c.866A>G p.Y289C	CMC	None
DNA-binding domain mutations			
Patient 9[R] M/7	c.983A>G p.H328R	None	IPEX-like enteropathy Diabetes mellitus type I Hypothyroidism Growth failure *M. fortuitum* lymphadenitis Chronic sinopulmonary infections Chronic norovirus enteritis
Patient 10 F/21	c.986C>T p.P329L	CMC	HSV stomatitis
Patient 11[+, R] F/22	c.1057G>A p.E353K	None	Oral herpes Osteomyelitis with spinal cord lesion by coccidioidomycosis diseminated Persistent tinea capitis and kerion by *T. tonsurans*
Patient 12 F/27	c.1057G>A p.E353K	CMC	*T. tonsurans* hand granuloma
Patient 13 M/15	c.1154C>T p.T385M	CMC	Selective IgA deficiency Recurrent sinuopulmonary disease Bronchiectasis *S. aureus* and *S. pneumoniae* infections
Patient 14[+] F/16	c.1154C>T p.T385M	CMC	IPEX-like Enteropathy Herpes zoster infection VZV infection CMV infections Mild eczema Hepatosplenomegaly Bronchiectasis Generalized osteopenia Aneurysms
Patient 15 M/28	c.1154C>T p.T385M	CMC	VZV infections Histoplasmosis Bronchiectasis Single cervical node with *M. fortuitum*
Patient 16 M/30	c.1154C>T p.T385M	CMC	Recurrent HSV infections Molluscum contagiosum Enteropathy Sinuopulmonary bacterial infections Bronchiectasis Diabetes mellitus type I