

Hyperinflammation in Chronic Granulomatous Disease leads to impairment of hematopoietic stem cell functions

Maren Weisser, PhD^a, Uta M. Demel, MSc^{b,c}, Stefan Stein, PhD^a, Linping Chen-Wichmann, PhD^{a1}, Fabien Touzot, MD, PhD^{d,e}, Giorgia Santilli, PhD^f, Stefanie Sujer, MSc^b, Christian Brendel, PhD^{a2}, Ulrich Siler, PhD^g, Marina Cavazzana, MD, PhD^{d,e}, Adrian J. Thrasher, MD, PhD^f, Janine Reichenbach, MD^g, Marieke A.G. Essers, PhD^{b,c}, Joachim Schwäble, MD^{a,h†3} and Manuel Grez, PhD^{a†°}

^aInstitute for Tumor Biology and Experimental Therapy, Georg-Speyer-Haus, Frankfurt, Germany; ^bJunior Research Group ‘Hematopoietic Stem Cells and Stress’, German Cancer Research Center (DKFZ), INF280, Heidelberg, Germany; ^cHeidelberg Institute for Stem Cell Technology and Experimental Medicine (HI-STEM), INF280, Heidelberg, Germany; ^dBiotherapy Department, Necker Children's Hospital, Assistance Publique-Hôpitaux de Paris, Paris, France; ^eParis Descartes – Sorbonne Paris Cité University, Imagine Institute, Paris, France; ^fSection of Molecular and Cellular Immunology, UCL Institute of Child Health, London, United Kingdom; ^gDivision of Immunology, University Children's Hospital, and Children's Research Centre Zürich, Switzerland; ^hDepartment of Medicine, Hematology/Oncology, Goethe University, Frankfurt am Main, Germany

¹Present address: Experimental Cell Therapy and Hematology, Department of Transfusion Medicine, Cell Therapy and Haemostaseology, Ludwig Maximilian University Hospital Munich, Germany

²Present address: Division of Hematology/Oncology, Boston Children's Hospital, Boston, MA, USA

³Present address: German Red Cross Blood Donor Service Baden-Württemberg – Hessen and Institute for Transfusion Medicine and Immunohematology of the Goethe University, Frankfurt, Germany

[°] Corresponding author:

Manuel Grez, PhD

Institute for Tumor Biology and Experimental Therapy

Georg-Speyer-Haus, Paul-Ehrlich-Str. 42-44

60596 Frankfurt, Germany

Tel: +49 69 63395-113; Fax: +49 69 63395-297

Email: grez@gsh.uni-frankfurt.de

[†]Equal contribution

42 This study work was supported by grants from the Deutsche Forschungsgemeinschaft
43 (SFB815), the European Union (FP7 integrated project NET4CGD), the Wellcome Trust and
44 the LOEWE Center for Cell and Gene Therapy Frankfurt funded by the Hessische
45 Ministerium für Wissenschaft und Kunst (HMWK; funding reference numbers: III L 4-
46 518/17.004 (2010) and III L 5- 518/17.004 (2013)). UD, SSu, and MAGE are supported by
47 the SFB873, funded by the Deutsche Forschungsgemeinschaft (DFG) and the Dietmar Hopp
48 Foundation. JR and US were funded by the Gebert Rief Stiftung, programme “Rare Diseases –
49 New approaches” (grant no. GRS-046/10, JR), the Zurich Centre for Integrative Human
50 Physiology (ZIHP, JR), and by the Ettore e Valeria Rossi Foundation. The Georg-Speyer-
51 Haus is funded jointly by the German Federal Ministry of Health (BMG) and the Hessische
52 Ministerium für Wissenschaft und Kunst.

53

54 **Conflict-of-interest disclosure:** The authors declare no competing financial interests.

55

56

57

58 **Abstract**

59 **Background:** Defects in the phagocytic NADPH oxidase 2 (NOX2) function cause chronic
60 granulomatous disease (CGD), a primary immunodeficiency characterized by dysfunctional
61 microbicidal activity and chronic inflammation.

62 **Objective:** To study the effect of chronic inflammation on the hematopoietic compartment in
63 patients and mice with the X-linked form of CGD (X-CGD).

64 **Methods:** We used immunostaining and functional analyses to study the hematopoietic
65 compartment in CGD.

66 **Results:** Analysis of bone marrow (BM) cells from X-CGD patients and mice revealed a
67 dysregulated hematopoiesis characterized by increased numbers of hematopoietic progenitors
68 (HPCs) at the expense of repopulating hematopoietic stem cells (HSCs). In X-CGD patients
69 there was a clear reduction in the proportion of HSCs in BM and peripheral blood and they
70 were also more rapidly exhausted after *in vitro* culture. In X-CGD mice, increased cycling of
71 HSCs, expansion of HPCs and impaired long-term engraftment capacity were found to be
72 associated with high concentrations of proinflammatory cytokines, including interleukin 1
73 beta (IL-1 β). Treatment of wild-type mice with IL-1 β induced enhanced cell cycle entry of
74 HSCs, expansion of HPCs and defects in long-term engraftment, mimicking the effects
75 observed in X-CGD mice. Inhibition of cytokine signaling in X-CGD mice reduced HPC
76 numbers but had only minor effects on the repopulating ability of HSCs.

77 **Conclusions:** Persistent chronic inflammation in CGD is associated with hematopoietic
78 proliferative stress leading to a decrease in the functional activity of HSCs. Our observations
79 have clinical implications for the development of successful autologous cell therapy
80 approaches.

81

82 **Key messages:**

83

84 • Chronic inflammation in X-CGD is associated with dysregulated hematopoiesis and
85 impaired hematopoietic stem cell activity.

86

87 • IL-1 β is increased in X-CGD and induces cycling of hematopoietic stem cells leading
88 to a decrease in HSC function and increased numbers of hematopoietic progenitors
89 with limited repopulating capacities.

90

91

92 **Capsule Summary**

93 Inflammation in X-CGD is associated with increased numbers of HPCs and functionally
94 impaired HSCs as a result of increased cytokine production, including IL-1 β .

95

96

97 **Key Words**

98 Chronic Granulomatous Disease, hyperinflammation, hematopoietic stem cell, dysfunctional
99 hematopoiesis, competitive repopulation assay, engraftment defect, cell cycle, IL-
100 1 β , Anakinra, gene therapy

101

102

103 **Abbreviations**

104 BM: bone marrow

105 CFU: colony forming unit

106 CGD: Chronic Granulomatous Disease

107 CMP/MEP/GMP: common myeloid progenitor/megakaryocyte-erythroid
108 progenitor/granulocyte-macrophage progenitor

109 CRU: competitive repopulating unit

110 HC: healthy control

111 HSC/HPC: hematopoietic stem cell / hematopoietic progenitor cell

112 NADPH: nicotinamide adenine dinucleotide phosphate

113 NOX2: NADPH oxidase 2

114 PB: peripheral blood

115 WT: wild type

116 X-CGD: X-linked CGD

117

118 **Introduction**

119 Chronic Granulomatous Disease (CGD) is a rare inherited primary immunodeficiency
120 characterized by defective antimicrobial activity of phagocytes, resulting in increased
121 susceptibility to recurrent and life-threatening infections.¹⁻⁶ In addition, CGD patients often
122 display augmented inflammatory responses, even in the absence of infectious agents (sterile
123 inflammation), leading to granuloma formation and inflammatory bowel disease.⁷⁻¹⁰
124 Approximately two thirds of all CGD patients have mutations within the X-linked *CYBB* gene
125 (X-CGD), encoding the gp91^{phox} subunit of NOX2. X-CGD mice faithfully reproduce the
126 pathology observed in X-CGD patients.^{7,11}

127 CGD can be cured by allogeneic HSC transplantation, which has been particularly successful
128 in patients with a fully HLA-matched donor in combination with reduced-intensity
129 conditioning.^{12,13} Despite the use of advanced HSC transplantation protocols, cases of low
130 donor chimerism, graft-versus-host disease and graft rejection have been observed.¹⁴⁻¹⁶ Thus,
131 for those patients without suitable HSC donors and for those in critical health conditions,
132 alternative treatment options beyond the standard of care are still required. The transplantation
133 of autologous gene-modified cells is an alternative for the treatment of CGD, and has been
134 implemented predominantly in X-CGD patients.¹⁷⁻²⁰ These clinical trials have provided
135 evidence that gene therapy can offer significant clinical benefit to CGD patients. However,
136 most of the patients lacked significant long-term engraftment of transduced cells.^{21,22}
137 Although many factors may have influenced the engraftment potential of CD34⁺ cells during
138 cell processing, alterations in numbers and/or fitness of HSCs in the CGD inflammatory
139 background could also contribute to the engraftment deficit. We therefore analyzed the
140 influence of chronic inflammation on the HSC compartment in X-CGD. We found a profound
141 defect in HSC content and/or activity in the bone marrow (BM) of both X-CGD patients and
142 mice.

143 The hematopoietic defects observed in X-CGD mice were mainly mediated by IL-1 β and
144 inhibition of IL-1 β signaling suppressed HPC expansion in X-CGD mice but did not revert
145 the functional defects in HSC activity.

146 Thus, chronic inflammation in CGD leads to a dysregulated hematopoietic homeostasis. Our
147 findings may not be limited to CGD, but may also be relevant to other pathologies with
148 sustained chronic inflammation and autoinflammatory processes.²³⁻²⁷

149

150 **Methods**

151 **Patient material**

152 BM and G-CSF mobilized peripheral blood (PB) mononuclear cells were obtained from X-
153 CGD patients (BM: n = 3, median 5.5 years old, range 0.7-8 years; PB: n=4, median: 10 years
154 old, range 4-17 years) and healthy controls (BM: n = 4, median 11 years old, range: 3-14
155 years; PB: n=4, median 29, range 25-37 years) after informed consent and approval by the
156 local ethic committee.

157 **Mice**

158 B6.129S6-*Cybb*^{tm1Din}/J (X-CGD mice, CD45.2), C57BL/6J (WT mice, CD45.2) and B6.SJL-
159 *Ptprc*^a *Pepc*^b/BoyJ (CD45.1) mice were obtained from Charles River Laboratories (Sulzfeld,
160 Germany). Health monitoring was conducted regularly by MFD Diagnostics (Wendelsheim,
161 Germany) according to Federation for Laboratory Animal Science Associations guidelines.
162 Animals with overt infections were not included in the study. Male littermates were used for
163 experiments unless stated otherwise. B6.Cg MyD88^{tm1Aki} mice (MyD88-deficient mice)²⁸
164 were bred at the Animal Facility of the German Cancer Research Center. Animal experiments
165 were approved by the regional council (Regierungspräsidium Darmstadt, Germany).

166 **Hematopoietic Stem Cell assays and Cytokine Arrays**

167 All assays including cell isolation, analysis and sorting, HSC assays, cell cycle analysis,
168 cytokine stimulation experiments, lentiviral transductions, transplantation assays, competitive
169 repopulation assays and cytokine arrays were done according to standard protocols. Detailed
170 methods are provided in the Supplementary Material section in this article's Online
171 Repository.

172 **Statistical Analysis**

173 Statistical significances were calculated by unpaired two-tailed t-tests (Fig 1-2, 4A, C-D, F-G,
174 5C), one-factorial ANOVA with Dunnet's Multiple Comparison Test (Fig 4B), two-factorial
175 ANOVA with Bonferroni posttests (Fig 3A, C, 4E, 5A-B) and three-factorial ANOVA (Fig
176 4H). CRU frequency was calculated with L-Calc Limiting Dilution Analysis Software
177 (StemSoft, Version 1.1). Overall test for differences in CRU frequencies between X-CGD and
178 WT was performed with ELDA: Extreme Limiting Dilution Analysis
179 (<http://bioinf.wehi.edu.au/software/elda/index.html>). Scatter plots indicate mean \pm SD. Bar
180 diagrams show mean + SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

181 **Results**

182 **Increased numbers of hematopoietic progenitors in X-CGD mice**

183 We analyzed the hematopoietic compartment of X-CGD mice and found significantly
184 ($p < 0.05-0.01$) increased levels of LSK ($\text{Lin}^- \text{Sca-1}^+ \text{c-Kit}^+$) cells, myeloid progenitors,
185 common myeloid progenitors (CMPs) and granulocyte-monocyte progenitors (GMPs) in the
186 BM, whereas the percentages of LSK SLAM ($\text{Lin}^- \text{Sca-1}^+ \text{c-Kit}^+ \text{CD150}^+ \text{CD48}^-$), lineage
187 negative (Lin^-) and megakaryocyte-erythroid progenitors (MEPs) cells were unchanged (Fig
188 1, A-E and see Fig E1, A-B, in the Online Repository and data not shown). BM cells from X-
189 CGD animals generated higher numbers of colonies derived from granulocyte-monocyte-
190 progenitors (CFU-GM) than cells from WT mice (see Fig E1, C, in the Online Repository).

191 LSK cells and the number of CFUs derived from spleen and PB were higher in X-CGD mice
192 (Fig 1, *F-G*, and see Fig E1, *D* in the Online Repository). Cell cycle analysis revealed that the
193 frequency of quiescent HSCs in X-CGD BM was significantly ($p < 0.01$) reduced compared
194 to WT (Fig 1, *H-I*), suggesting an increase in the percentage of actively cycling HSCs in X-
195 CGD animals.

196

197 **Reduced numbers of HSCs in human X-CGD BM and mobilized peripheral blood**

198 We analyzed BM from X-CGD patients ($n = 3$) versus age-matched healthy controls ($n = 4$).
199 We found a significant increase in the proportion of committed progenitors ($CD34^+ Lin^-$
200 $CD38^+$) in X-CGD BM at the expense of HSCs ($CD34^+ Lin^- CD38^-$)(Fig 2, *A-B* and see Fig
201 E2, *A*, in the Online Repository). Moreover, we found a shift in the balance of progenitors
202 with the tendency towards less CMPs and MEPs and more GMPs in X-CGD BM when
203 compared to healthy controls (Fig E2, *B-D*, in the Online Repository). Analysis of G-CSF
204 mobilized PB mononuclear cells obtained from X-CGD patients ($n = 3$) revealed a
205 pronounced reduction in the proportion of primitive HSCs ($CD34^+ CD38^- CD90^+$) and early
206 progenitors ($CD34^+ CD38^-$) compared to healthy donors ($n = 3$) (Fig 2, *C-D* and see Fig E3 in
207 the Online Repository). Isolated PB $CD34^+ CD38^{dim} CD90^+$ cells from healthy donors and X-
208 CGD patients were cultured in the presence of early-acting cytokines for 4 days, thus
209 resembling the conditions used in gene therapy settings. At day 4 HSCs and multipotent
210 progenitors accounted for half of the population in the sample derived from healthy donors,
211 while HSCs and MPPs derived from X-CGD patients were rapidly exhausted under these
212 conditions (Fig 2, *E*). More than 85% of the X-CGD cells remaining in the culture were
213 $CD38^+$ late progenitors, a cell population with restricted engraftment capacities. These
214 observations indicate that the alterations in the HSC/HPC pool observed in X-CGD mice are
215 reciprocated in X-CGD patients and suggest a common underlying aetiology.

216

217 **Impaired competitive repopulating ability of HSCs derived from X-CGD mice**

218 To assess the fitness of HSCs/HPCs we monitored the production of CFUs from BM long-
219 term cultures derived from X-CGD or WT animals after 4, 8 or 12 weeks of culture. Cells
220 from X-CGD BM produced a significantly higher number of CFUs than WT cells after
221 4 weeks of culture, consistent with the elevated LSK cell count (Fig 3, A). However, after 12
222 weeks culture the number of X-CGD-derived CFUs was reduced compared to WT, indicating
223 an accelerated exhaustion of the HSC pool. We also analyzed the self-renewal capability of
224 HSCs *in vivo* by exposing mice to serial cytotoxic injury through repeated 5-fluorouracil (5-
225 FU) dosing. This treatment depletes replicating cells and promotes activation of formerly
226 quiescent HSCs. In this assay, the survival of X-CGD mice was impaired compared to WT
227 animals, again suggesting early exhaustion of HSCs (Fig 3, B).

228 Next, we compared the capability of X-CGD and WT HSCs to reconstitute hematopoiesis in
229 transplanted recipients. Lin⁻ cells from either mouse group were differentially labeled using
230 eGFP- or tBFP-encoding lentiviral vectors and equal numbers of gene-marked cells were
231 mixed and transplanted into recipient mice. PB analyses at 2, 4, 8, 12 and 18 weeks showed
232 that in the first weeks after transplantation, the frequency of gene-marked X-CGD cells in PB
233 was higher compared to WT cells. However, gene-marked X-CGD cells were outcompeted by
234 their WT counterparts from week 4 onwards, suggesting a lower frequency of long-term
235 repopulating cells in the X-CGD samples (Fig 3, C).

236 Lastly, we estimated the absolute number of cells with myeloid and lymphoid reconstitution
237 potential in the BM of WT and X-CGD mice by a limiting dilution competitive repopulating
238 unit (CRU) assay with LSK SLAM cells. Short-term repopulation analysis after 4 weeks
239 resulted in similar CRU frequencies in both mouse groups. Upon 8 weeks and 16 weeks after
240 transplantation X-CGD HSCs displayed a reduced (1.8-fold, $p = 0.079$) multilineage
241 contribution to the PB of primary hosts compared to WT HSCs (Fig 3, D). Subsequently,
242 isolated HSCs from animals transplanted with 50 and 250 LSK SLAM cells were used for

243 secondary BM transplantation. Sixteen weeks after secondary transplantation 79% (11/14) of
244 the recipient animals injected with WT BM, but only 22% (4/18) of hosts receiving X-CGD
245 BM showed successful multilineage repopulation in the PB (Fig 3, E). Taken together, our *in*
246 *vitro* and *in vivo* experiments reveal a reduced potential of primitive HSCs derived from X-
247 CGD mice to reconstitute long-term hematopoiesis.

248

249 **No intrinsic defect in HSCs derived from X-CGD mice**

250 To study whether the defects observed for X-CGD HSCs were intrinsic or acquired over time,
251 we analyzed fetal liver HSCs/HPCs. The composition of the fetal liver HSCs/HPCs was
252 comparable for X-CGD and WT mice (see Fig E4, A, in the Online Repository). Competitive
253 transplantations were performed with Lin⁻ fetal liver cells from X-CGD and WT littermates.
254 X-CGD fetal liver HSCs/HPCs were capable of engrafting and maintaining multilineage
255 hematopoiesis to the same extent as WT cells (see Fig E4, B, in the Online Repository). This
256 indicates that the observed deficits in long-term repopulation abilities of adult X-CGD HSCs
257 are indeed acquired over time. As alterations in the BM microenvironment might be involved
258 in the development of the long-term repopulation defect, we performed transplantations of
259 WT (CD45.1) Lin⁻ cells into WT (CD45.2) and X-CGD recipient mice. No significant
260 differences in engraftment and chimerism, response to 5-FU challenge or hematopoietic
261 reconstitution in secondary transplanted animals between both groups were observed (see Fig
262 E4, C-E, in the Online Repository).

263

264 **Increased levels of proinflammatory cytokines in the BM of X-CGD animals**

265 In light of the established proinflammatory phenotype associated with CGD we screened BM
266 fluid from WT and X-CGD mice for chemokine and cytokine levels using protein arrays. A
267 high proportion of cytokines/chemokines (17/40) was significantly ($p < 0.05$) upregulated in
268 the lavage from X-CGD BM (see Fig E5, A and Table E1 in the Online Repository). The

269 levels of C-X-C motif chemokine (CXCL) 9, CXCL10, CXCL13, C-C motif chemokine 5
270 (CCL5) and IL-1 β were increased between 1.8 and 7.8-fold compared to WT. Likewise, the
271 levels of other cytokines known to modulate HSC homeostasis were also altered (M-CSF, IL-
272 3, IL-1 α , TIMP-1), although to a minor extent. Quantification of IL-1 β by ELISA revealed a
273 1.5-fold higher concentration in X-CGD compared to BM lavages from WT mice (Fig 4, A).
274 Next we analyzed the effects of the cytokines and chemokines IL-1 β , CXCL10, CCL5 and
275 CXCL9 on LSK cell proliferation *in vitro*. The stimulation of WT Lin⁻ cells with CXCL10
276 induced a modest increase in the frequency of LSK cells, while CCL5 and CXCL9 had no
277 effect (see Fig E5, B, in the Online Repository). Addition of IL-1 β was sufficient to expand
278 the LSK cell pool 2.3- to 2.4-fold (Fig 4, B). Moreover, upon injection of IL-1 β we observed
279 a significant ($p < 0.01$) increase in the proportion of LSK cells in the BM of WT animals
280 (Fig 4, C). This was associated with cell cycle activation of HSCs, as shown by increased
281 BrdU incorporation into LSK CD150⁺ CD48⁻ CD34⁻ cells (Fig 4, D) and a decrease in the
282 frequency of quiescent HSCs (Fig 4, E).
283 Most likely, the effects of IL-1 β are mediated by IL-1 β binding to its receptor and subsequent
284 activation of downstream signals, as the effects of IL-1 β on HSC activation were absent in
285 mice lacking MyD88, an essential adaptor molecule of IL-1 β signaling (Fig 4, E). In addition,
286 treatment of WT mice with IL-1 β resulted in a higher content of colony forming cells in the
287 bone marrow and increased LSK cell frequencies in the spleen, thus reproducing faithfully the
288 phenotype observed in X-CGD animals (Fig 4, F-G). Lastly, we performed competitive
289 transplantation assays with a mixture of bone marrow cells containing 2×10^6 mononuclear
290 cells obtained from animals treated with IL-1 β and PBS. In all transplanted animals the
291 engraftment of cells derived from the IL-1 β -treated animals was inferior to the engraftment of
292 control cells (Fig 4, H). This data shows conclusively that IL-1 β has detrimental effects on the
293 fitness of the HSC pool.

294 **Blocking inflammatory signals partially reverts the hematopoietic dysfunction in X-**
295 **CGD mice**

296 We treated 3-4 weeks old X-CGD and WT mice intraperitoneally with the IL-1 β receptor
297 antagonist Anakinra or PBS for 14-16 days. Anakinra treatment led to a decrease in the
298 proportion of LSK cells in the BM ($p < 0.05$) and spleen of X-CGD but not WT mice and a
299 decrease in the number of colony forming cells (CFCs) in peripheral blood (Fig 5, *A-B*, and
300 see Fig E6 in the Online Repository). In addition, X-CGD mice were treated with the anti-
301 inflammatory steroid dexamethasone alone or in combination with Anakinra for 14 days.
302 Dexamethasone and dexamethasone + Anakinra led to a decrease in the proportion of LSK
303 cells in the BM ($p < 0.05$) and in the spleen of X-CGD mice, although the later did not reach
304 significance (Fig 5, *C-D*). Also the number of CFCs in the bone marrow of dexamethasone-
305 treated animals was decreased (see Fig E6 in the Online Repository). The combined
306 dexamethasone + Anakinra treatment reduced the IL-1 β concentration in the bone lavages
307 significantly (Fig 5, *E*). In competitive transplantation experiments 4,000 Lin⁻ BM cells from
308 PBS-, Anakinra-, or dexamethasone- treated X-CGD mice (CD45.2) were transplanted
309 together with 10⁵ CD45.1 mononuclear cells in CD45.1 recipients. Analysis of BM and PB
310 chimerism at 19 and 8 weeks after transplantation, respectively showed a slight improvement
311 in engraftment of cells derived from the Anakinra- or dexamethasone-treated animals,
312 although the difference to the recipients of PBS-treated controls did not reach significance
313 (see Fig E6 in the Online Repository). Nevertheless multilineage engraftment was obtained in
314 8 out of 13 recipients transplanted with cells from the dexamethasone-treated group, while
315 only 4 out of 10 recipients of the PBS-treated group showed tri-lineage engraftment above
316 0.1%.

317

318

319 In summary our findings suggest that pro-inflammatory cytokines are responsible for the
320 increased HPC pool and dysfunctional HSCs in X-CGD (Fig 5, F).

321

322 **Discussion**

323 Our data show that hematopoiesis is dysregulated in X-CGD. Both the quality and quantity of
324 HSCs are affected, as demonstrated by the low proportion of primitive HSCs/HPCs present in
325 BM cells from X-CGD patients and the low repopulation efficiency of murine X-CGD BM
326 cells. Our observations add to the recent report by Panch et al., demonstrating a significant
327 reduction in the number of G-CSF mobilized CD34⁺ cells in X-CGD patients.²⁹ Similar to our
328 findings, the deficit in CD34⁺ mobilization was directly associated with parameters linked to
329 inflammation.

330 Inflammation is known to promote HSC proliferation and orchestrates HSC/HPC egress to the
331 blood stream via a variety of mechanisms.³⁰⁻³⁶ Inflammatory signals lead to augmented NF-
332 κ B activity, transcription of proinflammatory cytokines and activation of the inflammasome
333 resulting in caspase-1-dependent secretion of the proinflammatory cytokines IL-1 β and IL-
334 18.³⁷⁻³⁹ In CGD patients, increased activity of caspase-1 and elevated release of IL-1 β and
335 other proinflammatory cytokines by activated mononuclear cells contributes to dysregulated
336 inflammatory responses, even in the absence of clinical infection.^{7-9,40-48} In view of the
337 compelling evidence linking inflammation with defects in HSCs, it is not surprising that
338 similar defects are observed in the HSC compartment of CGD patients and mice.

339 We found that a series of proinflammatory mediators were upregulated in samples derived
340 from BM of X-CGD animals partially overlapping with those previously identified in a cDNA
341 microarray analysis on isolated X-CGD monocytes.⁴⁹ In particular, we found increased IL-1 β
342 levels in BM of X-CGD mice, a pleiotropic proinflammatory modulator of other cytokines,
343 adhesion molecules and enzymes which also influences hematopoietic cell differentiation.^{50,51}

344 IL-1 β activates HSCs, which are known to express IL-1 receptors⁵² and induces HSCs to exit
345 from quiescence. Cell cycle induction by IL-1 β has been previously demonstrated in human
346 CD34⁺ CD38⁻ acute myeloid leukemia cells, leading to the downregulation of the cell cycle
347 inhibitor p21, impaired self-renewal and reduced reconstitution potential.⁵³ Similarly, reduced
348 repopulation capacity has been shown for LSK cells upon treatment of mice with IL-1 α ,
349 which also triggers signaling through the IL-1 receptor.⁵⁴

350 We propose a model, in which a functional defect in X-CGD HSCs develops over time due to
351 persistent exposure of HSCs/HPCs to inflammatory cytokines and chemokines. Chronic
352 inflammation has been linked to enhanced cell cycle entry of HSCs, impaired repopulation
353 ability and HSC exhaustion.^{35,36,55} Treatment of CGD patients with the IL-1 receptor
354 antagonist Anakinra led to suppression of inflammation-related colitis in one study but only
355 partial responses in others.^{41,46,56} In our studies, treatment of X-CGD mice with Anakinra
356 alone resulted in reconstitution of HPCs levels and in combination with dexamethasone to a
357 reduction of IL-1 β secretion. Thus, proactive treatment of CGD patients with anti-
358 inflammatory drugs might therefore reduce chronic sterile inflammation in CGD and could
359 also have an impact on the quantity of HSCs available for autologous gene therapy. In terms
360 of clinical practice, our results implies that large numbers of CD34⁺ cells need to be collected
361 for genetic modification from X-CGD patients undergoing gene therapy. Furthermore the
362 decrease in the percentage of CD34⁺/CD38⁻ in the peripheral blood of G-CSF-mobilized X-
363 CGD patients and the almost complete loss of primitive HSCs (CD34⁺CD38⁻CD90⁺ cells)
364 after a 4 days culture period in the presence of cytokines raises concerns as to current
365 expansion protocols and warrants the development of alternative mobilization and cell culture
366 strategies to avoid premature cell differentiation and lastly graft failure. Finally, anti-
367 inflammatory treatment of X-CGD patients prior to collection of CD34⁺ cells might improve
368 the ratio between HSCs and progenitors in the graft and consequently may increase the
369 proportion of transduced cells with engrafting potential. Ultimately these measurements may

370 lead to successful engraftment of gene corrected cells in CGD patients enrolled in a gene
371 therapy program.

372

373 **Acknowledgments**

374 We thank Hana Kunkel, Tefik Merovci (both GSH, Frankfurt, Germany), Maeva Joigneaux
375 and Laure Caccavelli (both Necker Children's Hospital, Paris, France) and Christian Brandts
376 and The Duy Nguyen (both University Hospital, Frankfurt, Germany) for technical help and
377 reagents. Thanks also to Kerstin Kaufmann and Hind Medyouf (both GSH, Frankfurt,
378 Germany) and Christian Wichmann (Ludwig Maximilian University Hospital, Munich,
379 Germany) for advice and critical reading of the manuscript. We thank Gaby Schneider
380 (Institute for Mathematics, Goethe University Frankfurt, Germany) for her help with the
381 statistical analyses.

382

383

384 References

- 385 1. Roos D. The genetic basis of chronic granulomatous disease. *Immunol Rev* 1994;
386 138:121-57.
- 387 2. Segal AW. The NADPH oxidase and chronic granulomatous disease. *Mol Med Today*
388 1996; 2:129-35.
- 389 3. Segal BH, Leto TL, Gallin JJ, Malech HL, Holland SM. Genetic, biochemical, and
390 clinical features of chronic granulomatous disease. *Medicine (Baltimore)* 2000; 79:170-200.
- 391 4. Seger RA. Chronic granulomatous disease: recent advances in pathophysiology and
392 treatment. *Neth J Med* 2010; 68:334-40.
- 393 5. Dinayer MC. Chronic granulomatous disease and other disorders of phagocyte
394 function. *Hematology Am Soc Hematol Educ Program* 2005:89-95.
- 395 6. Bianchi M, Niemiec MJ, Siler U, Urban CF, Reichenbach J. Restoration of anti-
396 *Aspergillus* defense by neutrophil extracellular traps in human chronic granulomatous disease
397 after gene therapy is calprotectin-dependent. *J Allergy Clin Immunol* 2011; 127:1243-52 e7.
- 398 7. Morgenstern DE, Gifford MA, Li LL, Doerschuk CM, Dinayer MC. Absence of
399 respiratory burst in X-linked chronic granulomatous disease mice leads to abnormalities in
400 both host defense and inflammatory response to *Aspergillus fumigatus*. *J Exp Med* 1997;
401 185:207-18.
- 402 8. Rosenzweig SD. Inflammatory manifestations in chronic granulomatous disease
403 (CGD). *J Clin Immunol* 2008; 28 Suppl 1:S67-72.
- 404 9. Schappi MG, Jaquet V, Belli DC, Krause KH. Hyperinflammation in chronic
405 granulomatous disease and anti-inflammatory role of the phagocyte NADPH oxidase. *Semin*
406 *Immunopathol* 2008; 30:255-71.
- 407 10. Magnani A, Brosselin P, Beaute J, de Vergnes N, Mouy R, Debre M, et al.
408 Inflammatory manifestations in a single-center cohort of patients with chronic granulomatous
409 disease. *J Allergy Clin Immunol* 2014; 134:655-62 e8.

- 410 11. Petersen JE, Hiran TS, Goebel WS, Johnson C, Murphy RC, Azmi FH, et al.
411 Enhanced cutaneous inflammatory reactions to *Aspergillus fumigatus* in a murine model of
412 chronic granulomatous disease. *J Invest Dermatol* 2002; 118:424-9.
- 413 12. Seger RA. Modern management of chronic granulomatous disease. *Br J Haematol*
414 2008; 140:255-66.
- 415 13. Gungor T, Teira P, Slatter M, Stussi G, Stepensky P, Moshous D, et al. Reduced-
416 intensity conditioning and HLA-matched haemopoietic stem-cell transplantation in patients
417 with chronic granulomatous disease: a prospective multicentre study. *Lancet* 2013; 383:436-
418 48.
- 419 14. Oshrine B, Morsheimer M, Heimall J, Bunin N. Reduced-Intensity Conditioning for
420 Hematopoietic Cell Transplantation of Chronic Granulomatous Disease. *Pediatr Blood Cancer*
421 2015; 62:359-61.
- 422 15. Ahlin A, Fasth A. Chronic granulomatous disease - conventional treatment vs.
423 hematopoietic stem cell transplantation: an update. *Curr Opin Hematol* 2015; 22:41-5.
- 424 16. Cole T, Pearce MS, Cant AJ, Cale CM, Goldblatt D, Gennery AR. Clinical outcome in
425 children with chronic granulomatous disease managed conservatively or with hematopoietic
426 stem cell transplantation. *J Allergy Clin Immunol* 2013; 132:1150-5.
- 427 17. Bianchi M, Hakkim A, Brinkmann V, Siler U, Seger RA, Zychlinsky A, et al.
428 Restoration of NET formation by gene therapy in CGD controls aspergillosis. *Blood* 2009;
429 114:2619-22.
- 430 18. Kang EM, Choi U, Theobald N, Linton G, Long Priel DA, Kuhns D, et al. Retrovirus
431 gene therapy for X-linked chronic granulomatous disease can achieve stable long-term
432 correction of oxidase activity in peripheral blood neutrophils. *Blood* 2010; 115:783-91.
- 433 19. Kang HJ, Bartholomae CC, Paruzynski A, Arens A, Kim S, Yu SS, et al. Retroviral
434 gene therapy for X-linked chronic granulomatous disease: results from phase I/II trial. *Mol*
435 *Ther* 2011; 19:2092-101.

- 436 20. Ott MG, Schmidt M, Schwarzwaelder K, Stein S, Siler U, Koehl U, et al. Correction
437 of X-linked chronic granulomatous disease by gene therapy, augmented by insertional
438 activation of MDS1-EVI1, PRDM16 or SETBP1. *Nat Med* 2006; 12:401-9.
- 439 21. Stein S, Ott MG, Schultze-Strasser S, Jauch A, Burwinkel B, Kinner A, et al. Genomic
440 instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene
441 therapy for chronic granulomatous disease. *Nat Med* 2010; 16:198-204.
- 442 22. Grez M, Reichenbach J, Schwable J, Seger R, Dinauer MC, Thrasher AJ. Gene
443 therapy of chronic granulomatous disease: the engraftment dilemma. *Mol Ther* 2011; 19:28-
444 35.
- 445 23. Feldman N, Rotter-Maskowitz A, Okun E. DAMPs as mediators of sterile
446 inflammation in aging-related pathologies. *Ageing Res Rev* 2015; Jan 29;doi:
447 10.1016/j.arr.2015.01.003. [Epub ahead of print].
- 448 24. Geiger H, Denking M, Schirmbeck R. Hematopoietic stem cell aging. *Curr Opin*
449 *Immunol* 2014; 29:86-92.
- 450 25. Sauce D, Larsen M, Fastenackels S, Pauchard M, Ait-Mohand H, Schneider L, et al.
451 HIV disease progression despite suppression of viral replication is associated with exhaustion
452 of lymphopoiesis. *Blood* 2011; 117:5142-51.
- 453 26. Makipour S, Kanapuru B, Ershler WB. Unexplained anemia in the elderly. *Semin*
454 *Hematol* 2008; 45:250-4.
- 455 27. Dinarello CA, Simon A, van der Meer JW. Treating inflammation by blocking
456 interleukin-1 in a broad spectrum of diseases. *Nat Rev Drug Discov* 2012; 11:633-52.
- 457 28. Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, et al. Targeted
458 disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity*
459 1998; 9:143-50.
- 460 29. Panch SR, Yau YY, Kang EM, De Ravin SS, Malech HL, Leitman SF. Mobilization
461 characteristics and strategies to improve hematopoietic progenitor cell mobilization and

- 462 collection in patients with chronic granulomatous disease and severe combined
463 immunodeficiency. *Transfusion* 2015; 55:265-74.
- 464 30. Rodriguez S, Chora A, Goumnerov B, Mumaw C, Goebel WS, Fernandez L, et al.
465 Dysfunctional expansion of hematopoietic stem cells and block of myeloid differentiation in
466 lethal sepsis. *Blood* 2009; 114:4064-76.
- 467 31. Zhang P, Nelson S, Bagby GJ, Siggins R, 2nd, Shellito JE, Welsh DA. The lineage-c-
468 Kit+Sca-1+ cell response to *Escherichia coli* bacteremia in Balb/c mice. *Stem Cells* 2008;
469 26:1778-86.
- 470 32. Singh P, Yao Y, Weliver A, Broxmeyer HE, Hong SC, Chang CH. Vaccinia virus
471 infection modulates the hematopoietic cell compartments in the bone marrow. *Stem Cells*
472 2008; 26:1009-16.
- 473 33. Baldrige MT, King KY, Goodell MA. Inflammatory signals regulate hematopoietic
474 stem cells. *Trends Immunol* 2011; 32:57-65.
- 475 34. Griseri T, McKenzie BS, Schiering C, Powrie F. Dysregulated hematopoietic stem and
476 progenitor cell activity promotes interleukin-23-driven chronic intestinal inflammation.
477 *Immunity* 2012; 37:1116-29.
- 478 35. Essers MA, Offner S, Blanco-Bose WE, Waibler Z, Kalinke U, Duchosal MA, et al.
479 IFN α activates dormant haematopoietic stem cells in vivo. *Nature* 2009; 458:904-8.
- 480 36. Essers MA, Trumpp A. Targeting leukemic stem cells by breaking their dormancy.
481 *Mol Oncol* 2010; 4:443-50.
- 482 37. Chen GY, Nunez G. Sterile inflammation: sensing and reacting to damage. *Nat Rev*
483 *Immunol* 2010; 10:826-37.
- 484 38. Henao-Mejia J, Elinav E, Strowig T, Flavell RA. Inflammasomes: far beyond
485 inflammation. *Nat Immunol* 2012; 13:321-4.
- 486 39. Strowig T, Henao-Mejia J, Elinav E, Flavell R. Inflammasomes in health and disease.
487 *Nature* 2012; 481:278-86.

- 488 40. Brown JR, Goldblatt D, Buddle J, Morton L, Thrasher AJ. Diminished production of
489 anti-inflammatory mediators during neutrophil apoptosis and macrophage phagocytosis in
490 chronic granulomatous disease (CGD). *J Leukoc Biol* 2003; 73:591-9.
- 491 41. Meissner F, Seger RA, Moshous D, Fischer A, Reichenbach J, Zychlinsky A.
492 Inflammasome activation in NADPH oxidase defective mononuclear phagocytes from
493 patients with chronic granulomatous disease. *Blood* 2010; 116:1570-3.
- 494 42. van de Veerdonk FL, Smeekens SP, Joosten LA, Kullberg BJ, Dinarello CA, van der
495 Meer JW, et al. Reactive oxygen species-independent activation of the IL-1beta
496 inflammasome in cells from patients with chronic granulomatous disease. *Proc Natl Acad Sci*
497 *U S A* 2010; 107:3030-3.
- 498 43. Segal BH, Han W, Bushey JJ, Joo M, Bhatti Z, Feminella J, et al. NADPH oxidase
499 limits innate immune responses in the lungs in mice. *PLoS One* 2010; 5:e9631.
- 500 44. Rubartelli A. Redox control of NLRP3 inflammasome activation in health and disease.
501 *J Leukoc Biol* 2012; 92:951-8.
- 502 45. Zeng MY, Pham D, Bagaitkar J, Liu J, Otero K, Shan M, et al. An efferocytosis-
503 induced, IL-4-dependent macrophage-iNKT cell circuit suppresses sterile inflammation and is
504 defective in murine CGD. *Blood* 2013; 121:3473-83.
- 505 46. de Luca A, Smeekens SP, Casagrande A, Iannitti R, Conway KL, Gresnigt MS, et al.
506 IL-1 receptor blockade restores autophagy and reduces inflammation in chronic
507 granulomatous disease in mice and in humans. *Proc Natl Acad Sci U S A* 2014; 111:3526-31.
- 508 47. Baroja-Mazo A, Martin-Sanchez F, Gomez AI, Martinez CM, Amores-Iniesta J,
509 Compan V, et al. The NLRP3 inflammasome is released as a particulate danger signal that
510 amplifies the inflammatory response. *Nat Immunol* 2014; 15:738-48.
- 511 48. Geering B, Stoeckle C, Conus S, Simon HU. Living and dying for inflammation:
512 neutrophils, eosinophils, basophils. *Trends Immunol* 2013; 34:398-409.

- 513 49. Brown KL, Bylund J, MacDonald KL, Song-Zhao GX, Elliott MR, Falsafi R, et al.
514 ROS-deficient monocytes have aberrant gene expression that correlates with inflammatory
515 disorders of chronic granulomatous disease. *Clin Immunol* 2008; 129:90-102.
- 516 50. Fibbe WE, Hamilton MS, Laterveer LL, Kibbelaar RE, Falkenburg JH, Visser JW, et
517 al. Sustained engraftment of mice transplanted with IL-1-primed blood-derived stem cells. *J*
518 *Immunol* 1992; 148:417-21.
- 519 51. Garlanda C, Dinarello CA, Mantovani A. The interleukin-1 family: back to the future.
520 *Immunity* 2013; 39:1003-18.
- 521 52. McKinstry WJ, Li CL, Rasko JE, Nicola NA, Johnson GR, Metcalf D. Cytokine
522 receptor expression on hematopoietic stem and progenitor cells. *Blood* 1997; 89:65-71.
- 523 53. Yang J, Ikezoe T, Nishioka C, Nobumoto A, Yokoyama A. IL-1beta inhibits self-
524 renewal capacity of dormant CD34(+)/CD38(-) acute myelogenous leukemia cells in vitro and
525 in vivo. *Int J Cancer* 2013; 133:1967-81.
- 526 54. Yonemura Y, Ku H, Hirayama F, Souza LM, Ogawa M. Interleukin 3 or interleukin 1
527 abrogates the reconstituting ability of hematopoietic stem cells. *Proc Natl Acad Sci U S A*
528 1996; 93:4040-4.
- 529 55. Nagamachi A, Nakata Y, Ueda T, Yamasaki N, Ebihara Y, Tsuji K, et al. Acquired
530 deficiency of A20 results in rapid apoptosis, systemic inflammation, and abnormal
531 hematopoietic stem cell function. *PLoS One* 2014; 9:e87425.
- 532 56. Hahn KJ, Ho N, Yockey L, Kreuzberg S, Daub J, Rump A, et al. Treatment With
533 Anakinra, a Recombinant IL-1 Receptor Antagonist, Unlikely to Induce Lasting Remission in
534 Patients With CGD Colitis. *Am J Gastroenterol* 2015; 110:938-9.
- 535
- 536

537 **Figure Legends**

538 **FIG 1. Increased HPC pool and HSC activation in X-CGD mice.**

539 **A-B**, Number of LSK cells and FACS plots showing Lin⁻ cells from the BM of WT and X-
 540 CGD mice (n = 22). **C-E**, Frequency of myeloid progenitors, CMPs and GMPs in BM (n = 6).
 541 **F**, Colony formation by PB-derived cells (n = 18). **G**, Frequency of LSK cells in spleen
 542 (n = 9). **H-I**, Frequency of quiescent Ki67^{neg} LSK SLAM cells from the BM and
 543 representative FACS plots showing the cell cycle distribution (n = 10). Error bars show mean
 544 ± SD. *, p < 0.05; **, p < 0.01.

545

546 **FIG 2. X-CGD patients have fewer HSCs/HPCs in BM and PB.**

547 **A-B**, Frequency of Lin⁻ CD34⁺CD38^{+/-} cells in the BM of aged-matched healthy controls
 548 (HC, n = 4) and X-CGD patients (n = 3) and representative FACS plots. **C-D**, Frequency of
 549 CD34⁺CD38^{+/-} cells and CD34⁺CD38⁻CD90⁺ HSCs in G-CSF-mobilized PB mononuclear
 550 cells (n = 4). **E**, Representative FACS plots showing the distribution of HSCs/HPCs after 4
 551 days culture of sorted PB CD34⁺CD38^{dim}CD90⁺ cells from one HC and two X-CGD patients.
 552 Error bars show mean + SD. *, p < 0.05.

553

554 **FIG 3. HSCs/HPCs from X-CGD mice show faster exhaustion during hematopoietic**
 555 **reconstitution than WT cells.**

556 **A**, Colony formation by BM cells after long-term culture (n ≥ 9). **B**, Kaplan-Meier survival
 557 curves after serial 5-FU challenge (n = 6). **C**, Proportion of eGFP⁺ and tBFP⁺ donor-derived
 558 PB cells two to 18 weeks after competitive transplantation of gene-marked WT and X-CGD
 559 cells (n = 6). **D**, CRU assays with sorted HSCs. Shown is the frequency ± SE (n = 3). **E**,
 560 Percentage of multilineage reconstituted mice transplanted with BM cells obtained from D
 561 (n ≥ 14). Data are provided as mean + SD. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

562 **FIG 4. IL-1 β is increased in the BM of X-CGD mice and induces HSC activation, HPC**
563 **expansion and reduces HSC long-term engraftment.**

564 **A**, IL-1 β protein levels in bone lavages (n = 13). **B**, LSK cell frequency of IL-1 β -treated WT
565 Lin⁻ cells (n = 6). **C-D**, Frequency of LSK cells and BrdU incorporation in LSK SLAM
566 CD34⁻ cells in the BM of WT mice after IL-1 β or PBS treatment (C: n = 7; D: n = 3). **E**, Cell
567 cycle distribution of LSK SLAM CD34⁻ cells in IL-1 β -treated WT and MyD88-deficient
568 mice (n = 3). **F-G**, Frequency of BM-derived CFUs and spleen LSK cells in IL-1 β -treated
569 WT mice (n = 3). **H**, PB chimerism 16 weeks after competitive transplantation of cells from
570 IL-1 β or PBS-treated animals (n = 4). Error bars show mean \pm SD. *, p < 0.05; **, p < 0.01;
571 ***, p < 0.001.

572

573 **FIG 5. Blocking inflammatory signals restores HPC levels in X-CGD mice.**

574 **A-B**, Frequency of LSK cells in the BM (A) and spleen (B) of WT and X-CGD mice after
575 treatment with PBS or Anakinra (n = 3-4). **C-D**, Frequency of LSK cells in the BM (C) and
576 spleen (D) of X-CGD mice after treatment with PBS or Dexamethasone +/- Anakinra (n = 4).
577 **E**, IL-1b concentration in bone lavages from the mice in (C-D). Error bars show mean \pm SD.
578 *, p < 0.05; **, p < 0.01. **D**, Proposed model of HSC/HPC exhaustion in X-CGD BM.

579

Figure No.1

Click here to download Figure No.: Fig1-REVISED.pptx

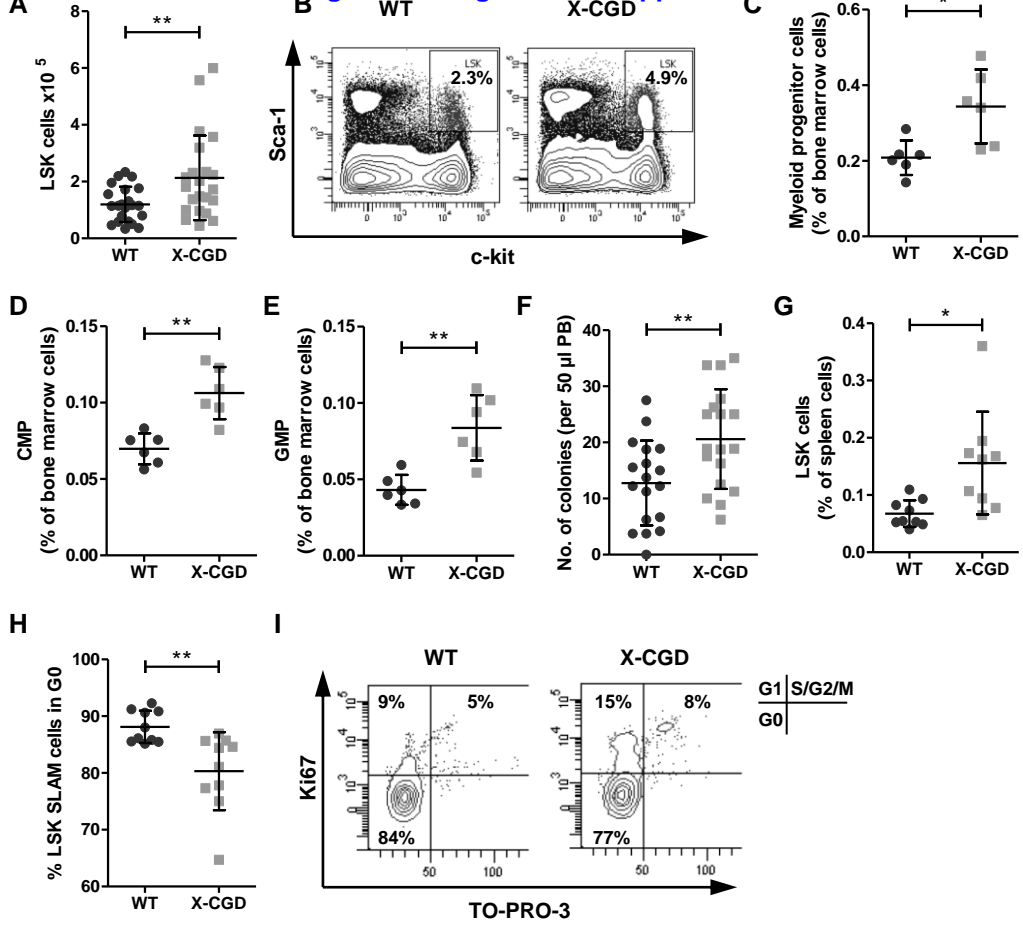


Figure No.2

[Click here to download Figure No.: Fig2-REVISED.pptx](#)

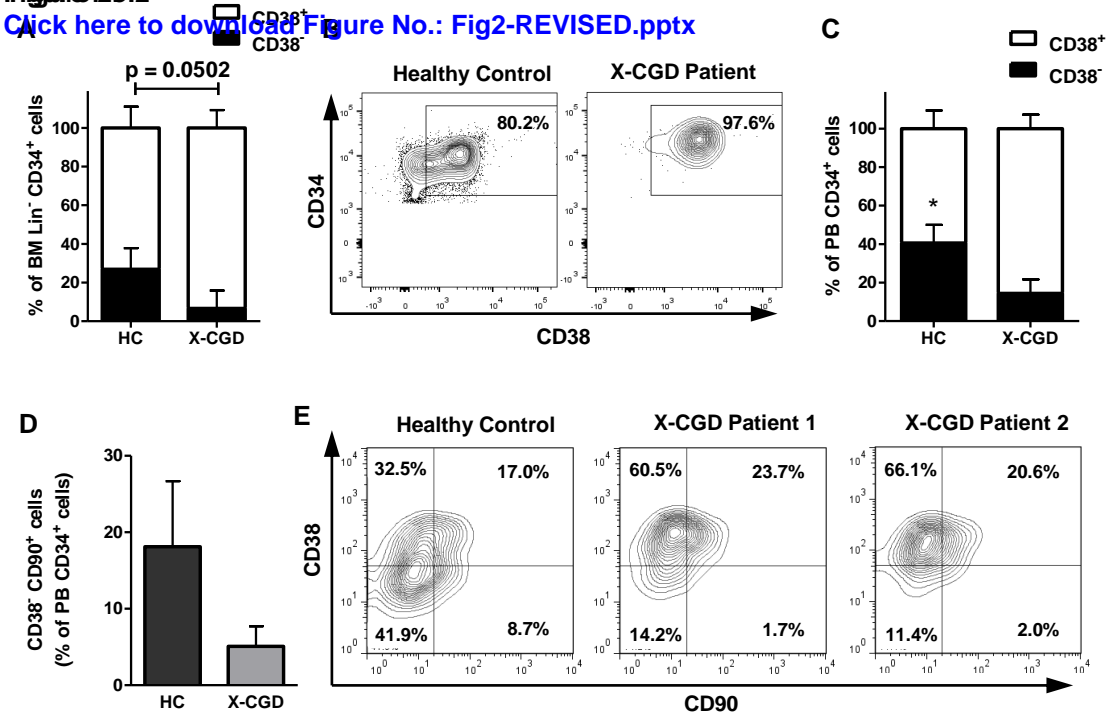
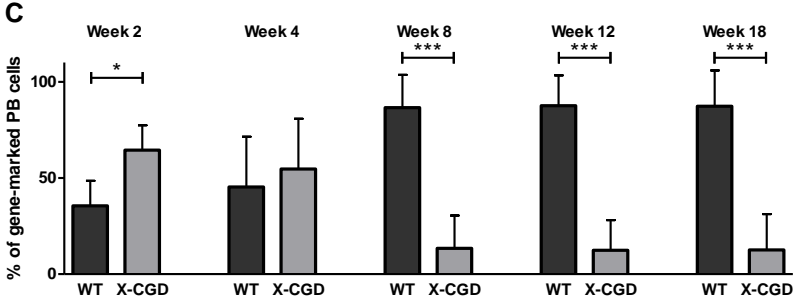
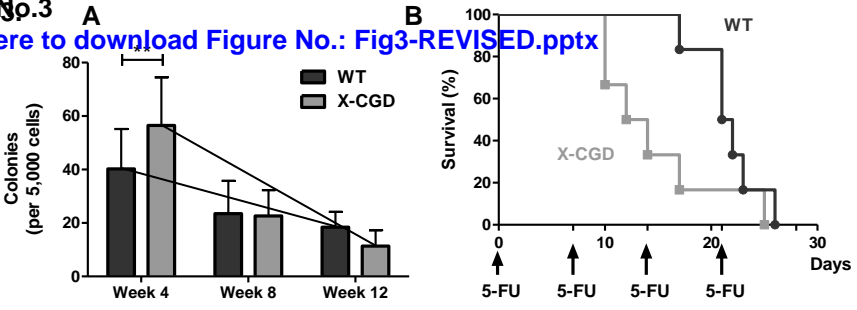


Figure No.3

[Click here to download Figure No.: Fig3-REVISED.pptx](#)



D

Competitive repopulation ability of LSK SLAM cells					
No. of Lin ⁻ c-Kit ⁺ Sca-1 ⁺ CD48 ⁻ CD150 ⁺ cells injected	No. of mice reconstituted/ tested (%) with WT cells		No. of mice reconstituted/ tested (%) with X-CGD cells		
2	1/13	(7.7)	1/15	(6.7)	
10	4/14	(28.6)	1/12	(8.3)	
50	12/15	(80.0)	8/11	(72.7)	
250	14/15	(93.3)	9/11	(81.8)	
CRU frequency:	1 in 46 (1 in 35 – 1 in 59)		1 in 85 (1 in 63 – 1 in 114)		

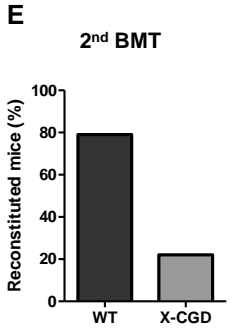


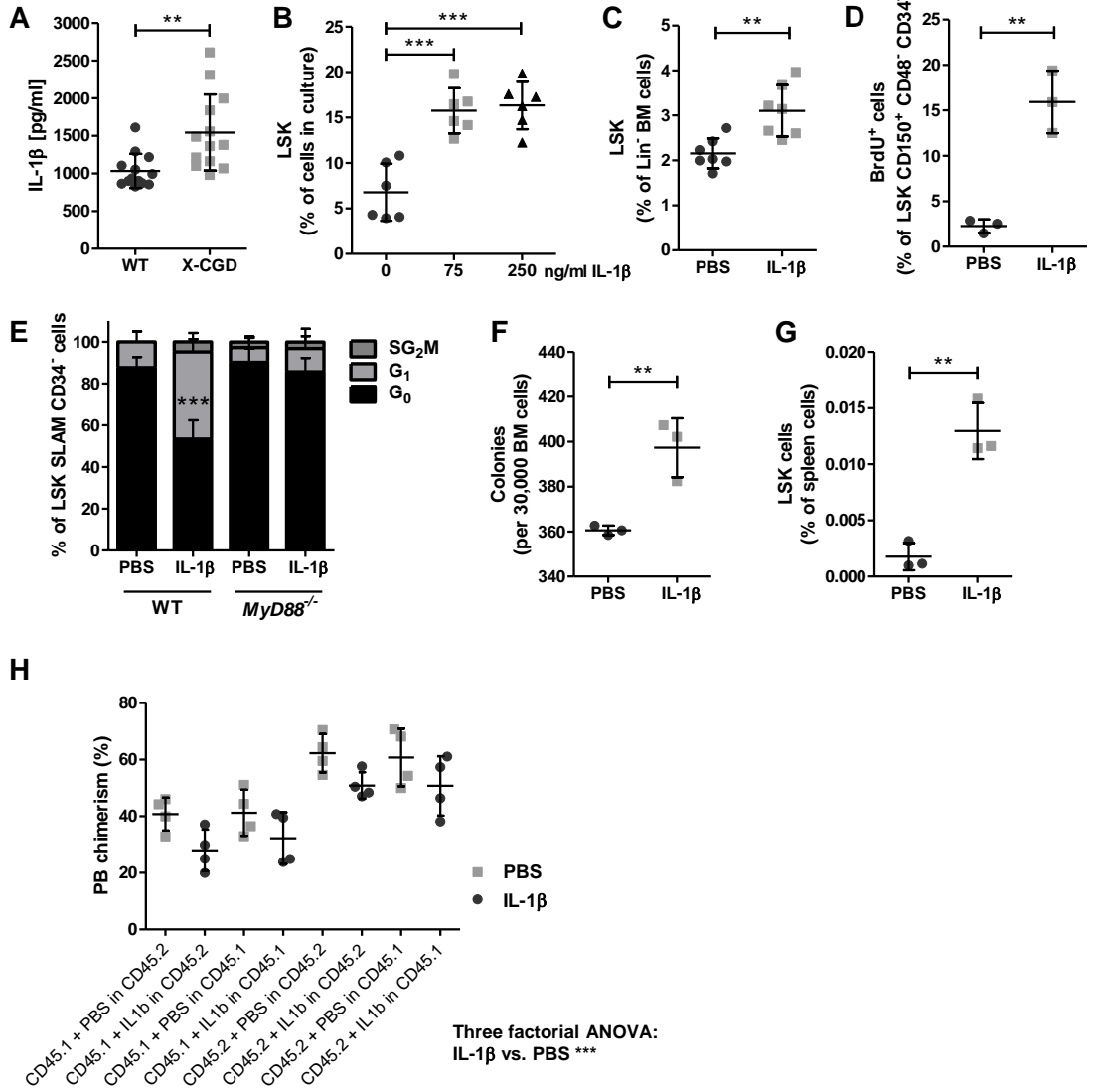
Figure No. 4[Click here to download Figure No.: Fig4-REVISED.pptx](#)

Figure No. 5 - Unmarked
 Click here to download Figure No. - Unmarked: Figure 5 Revision_2.pptx

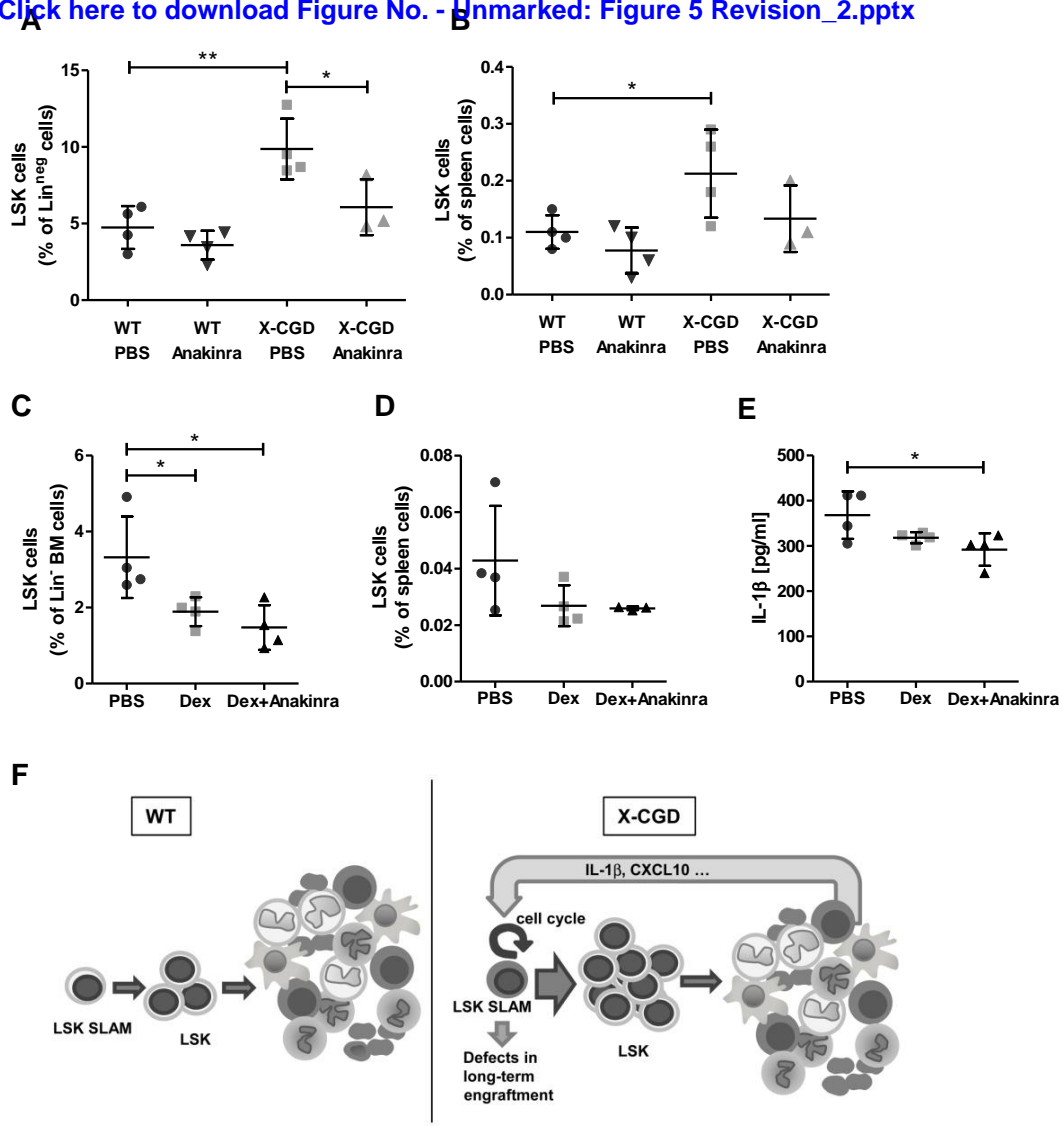


TABLE E1. Increased cytokine and chemokine levels in bone marrow fluid from X-CGD mice compared to WT controls as determined by protein arrays.

Cytokine / chemokine	Fold change	P-value*
	<u>X-CGD</u> WT	
CXCL9	7.8	0.046
CCL5	3.9	0.009
CXCL10	3.3	0.018
CXCL13	2.7	0.002
IL-1Ra	1.8	0.004
IL-1 β	1.8	0.000
TREM-1	1.7	0.005
CCL2	1.6	0.000
CCL3	1.5	0.013
M-CSF	1.3	0.005
IL-3	1.2	0.039
TIMP-1	1.2	0.001
IL-1 α	1.2	0.019
CXCL2	1.1	0.016
CCL4	1.1	0.001
IL-6	1.1	0.013
IL-5	1.1	0.030

*P values from unpaired two-tailed t-tests.

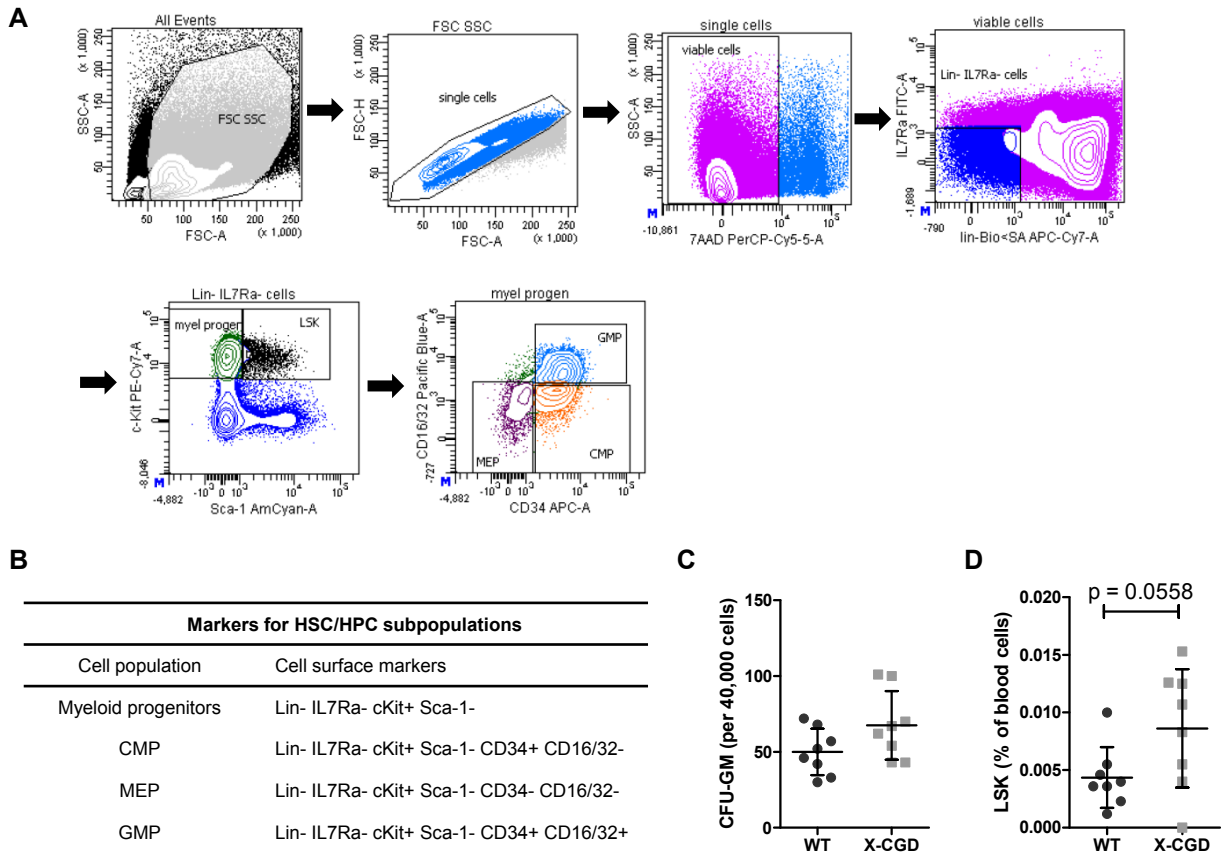
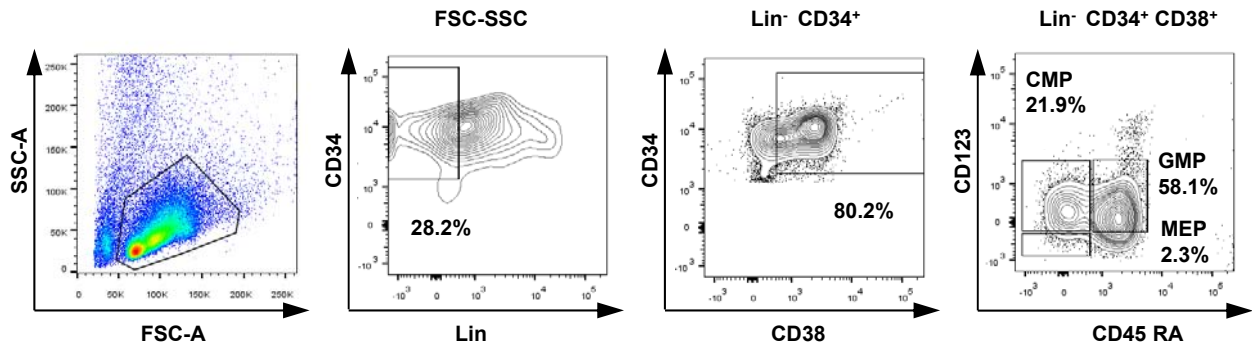
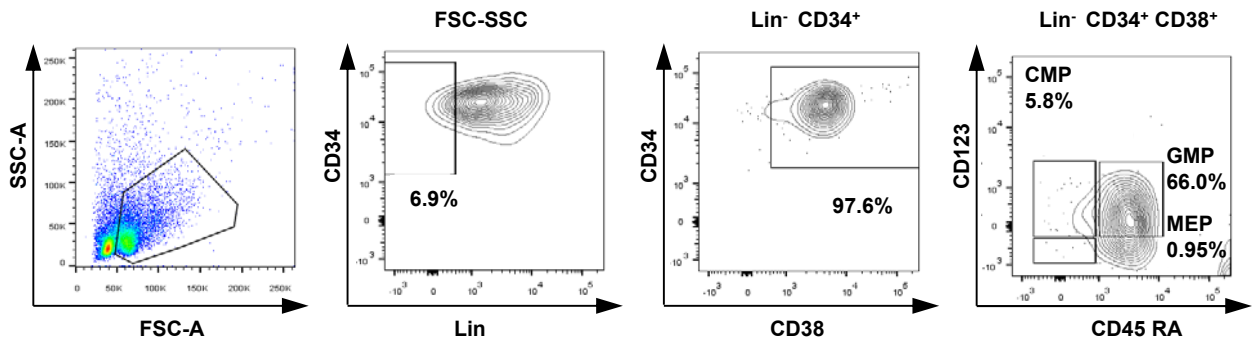


FIG E1. Analysis of the hematopoietic compartment in X-CGD and WT mice. **A**, Gating strategy used to identify HPC subpopulations in the bone marrow. **B**, Markers for myeloid progenitors, common myeloid progenitors (CMP), megakaryocyte-erythroid progenitors (MEP) and granulocyte-monocyte progenitors (GMPs) according to Challen et al., Cytometry A. 2009 Jan; 75(1):14-24. **C**, Number of CFU-GM in bone marrow samples from WT and X-CGD animals ($n = 8$). **D**, Frequency of LSK cells in peripheral blood of WT and X-CGD animals ($n = 8$). Statistical significance was calculated by unpaired two-tailed t-test.

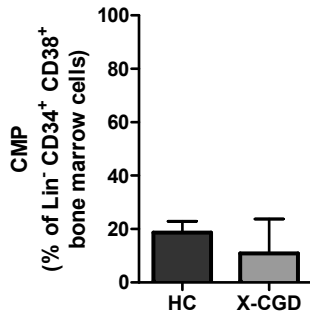
A Healthy control bone marrow (7 year old donor)



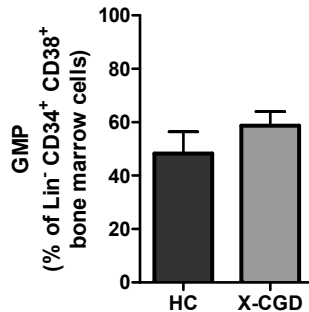
X-CGD patient's bone marrow (8 years old)



B



C



D

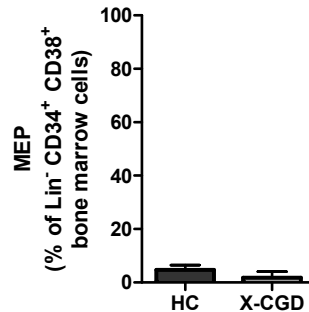
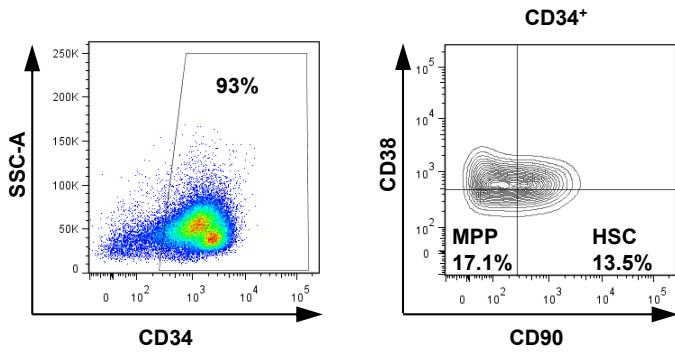


FIG E2. X-CGD patients have fewer HSCs/HPCs (CD38⁻ CD34⁺ Lin⁻ cells) in the bone marrow and the distribution of CD38⁺ CD34⁺ Lin⁻ committed progenitor cells differs from healthy controls. A, Representative FACS plots from bone marrow of a healthy donor and an aged-matched X-CGD patient showing the gating strategy to identify CMPs, GMPs and MEPs. HSCs/HPCs are within the CD38⁻ CD34⁺ Lin⁻ population (gate not shown), while committed progenitors (CD38⁺ CD34⁺ Lin⁻ cells) can be further separated into CMPs (CD38⁺ CD34⁺ Lin⁻ CD123⁺ CD45RA⁻), GMPs (CD38⁺ CD34⁺ Lin⁻ CD123⁺ CD45RA⁺) and MEPs (CD38⁺ CD34⁺ Lin⁻ C123⁻ CD45RA⁺). **B-D,** Frequency of CMPs, GMPs and MEPs within the committed progenitor cell population in the bone marrow of X-CGD patients (n = 3) and age-matched healthy controls (HC) (n = 4) as determined by flow cytometry.

A PB from a healthy control donor upon apheresis



PB from an X-CGD patient upon apheresis

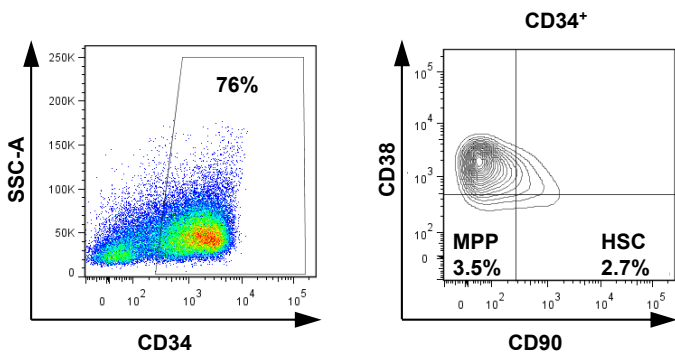


FIG E3. X-CGD patients contain less CD34⁺ CD38⁻ HSCs/HPCs in the peripheral blood (PB) upon apheresis than healthy controls. A, Representative FACS plots of G-CSF-mobilized PB mononuclear cells obtained from a healthy control (HC) and an X-CGD patient. HSCs are defined as CD34⁺ CD38⁻ CD90⁺ cells.

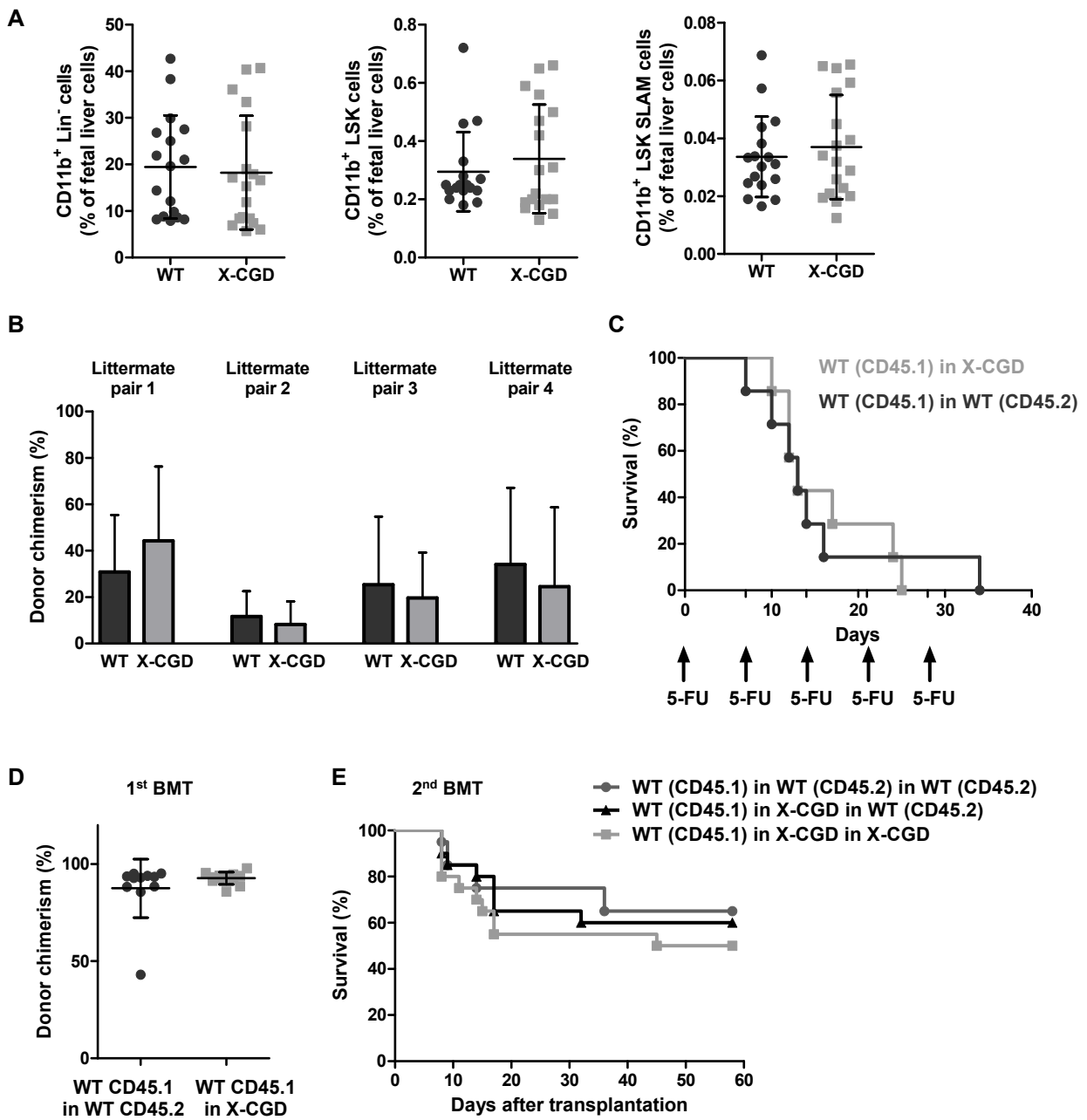


FIG E4. HSCs/HPCs from X-CGD fetal liver at E15.5 are present at the same frequencies and have the same reconstitution capacity as WT cells. **A**, Frequency of CD11b⁺ Lin⁻, CD11b⁺ LSK and CD11b⁺ LSK SLAM cells in the fetal liver of WT and X-CGD mice (n ≥ 17). **B**, Competitive repopulation experiment using Lin⁻ fetal liver cells from WT and X-CGD mice. The graphic shows exemplarily the result of PB reconstitution at week 16 after transplantation. **C-E**, X-CGD stromal cells are not impaired in their capability to support HSC functions. **C**, Survival of 5-FU-treated X-CGD or WT mice transplanted with WT (CD45.1) mononuclear cells (n = 7). **D**, Donor (CD45.1) chimerism in PB of transplanted WT and X-CGD mice 16 weeks after transplantation (n ≥ 11). **E**, Kaplan-Meier survival curves of secondary hosts transplanted with mononuclear cells derived from the animals shown in 4D (n = 20). Error bars show SD.

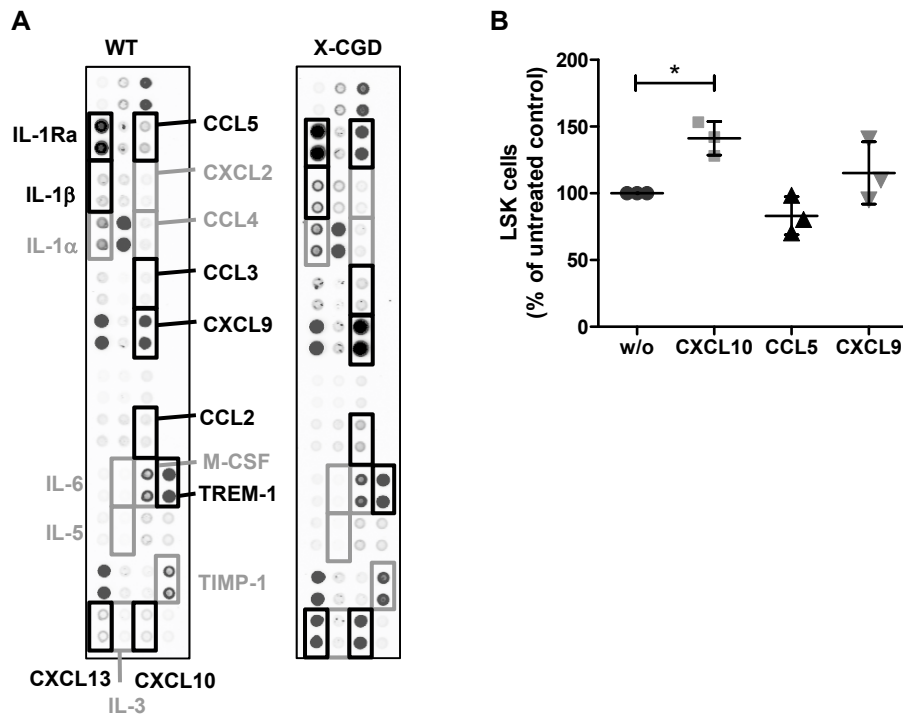


FIG E5. Cytokine levels are increased in bone lavages from X-CGD compared to WT mice and CXCL10 increases LSK cell frequency *in vitro*. **A**, Representative cytokine array using bone lavages from WT and X-CGD mice. Gray frames indicate significant differences ($p < 0.05$) between the littermate samples, black frames show cytokines upregulated ≥ 1.5 -fold in X-CGD compared to WT lavages. Examination of the mice did not reveal any overt infections linked to these results. No differences in white blood counts ($16.0 \times 10^3/\mu\text{l} \pm 1.2$ vs. $14.0 \times 10^3/\mu\text{l} \pm 1.3$), PB lymphocyte counts ($11.8 \times 10^3/\mu\text{l} \pm 1.0$ vs. $9.7 \times 10^3/\mu\text{l} \pm 0.5$), or mean spleen weight (107 ± 5 mg vs. 110 ± 16 mg) were found for the WT versus X-CGD mice used for this analysis, respectively. **B**, LSK cell frequency of WT Lin⁻ cell after 3 days of culture with 250 ng/ml CXCL10, CCL5 or CXCL9 ($n = 3$). Statistical significance was analyzed by one-factorial ANOVA with Dunnet's Multiple Comparison Test. Error bars show mean \pm SD. *, $p < 0.05$

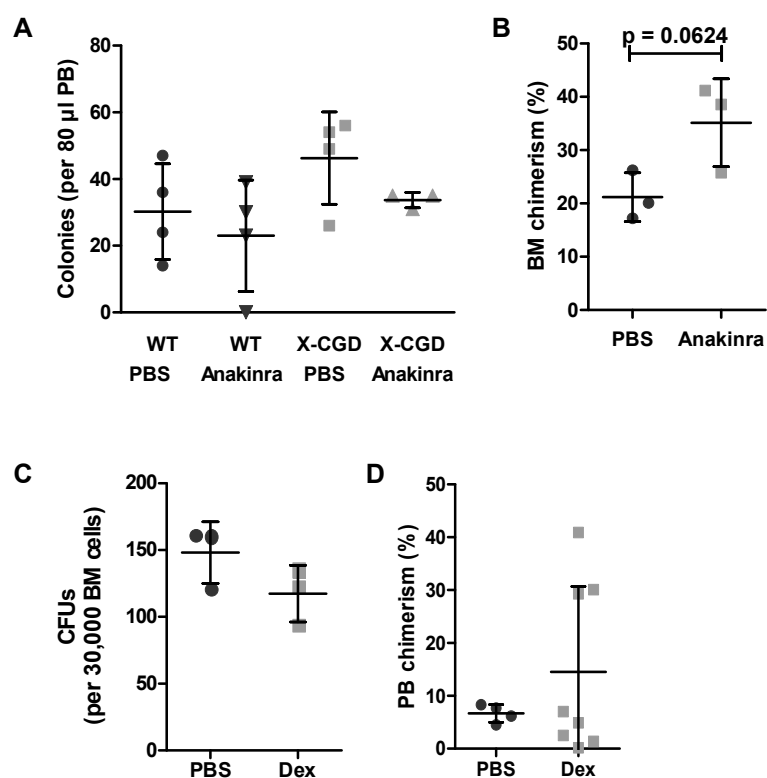


FIG E6. Blocking IL-1 β signaling reduces the number of colony forming cells in bone marrow and peripheral blood, but has only minor effects on HSC engraftment.

A, WT and X-CGD mice were injected intraperitoneally with PBS or Anakinra (25 μ g/g body weight) twice a day for 14-16 days and the frequency of colony-forming cells was measured in the peripheral blood (PB). **B**, Bone marrow (BM) chimerism in CD45.1 mice 19 weeks after transplantation of 4,000 Lin⁻ BM cells obtained from PBS- or Anakinra-treated X-CGD mice (CD45.2) together with 10^5 CD45.1 mononuclear competitor cells ($n = 3$). **C-D**, X-CGD mice were treated with Dexamethasone (Dex, 2 μ g/g body weight) or PBS daily for 14 days. **C**, BM cells were assayed for the frequency of colony-forming units (CFUs) ($n = 3$). **D**, Lin⁻ BM cells from treated donors were used for competitive transplantation as described in (B). PB chimerism is depicted 8 weeks after transplantation ($n \geq 10$). Only animals with tri-lineage engraftment above 0.1% are shown. Error bars show mean \pm SD.

Supplementary Data

Methods

Cell Isolation, Flow Cytometry and Cell Sorting.

For isolation of BM cells from mice humeri, tibiae, femurs and hips were dissected and flushed with PBS. Lineage depletion was performed with Lineage Cell Depletion Kit (MiltenyiBiotec, BergischGladbach, Germany) or Easy Sep Biotin Selection Kit (StemCell Technologies, Köln, Germany). For isolation of fetal liver HSCs the livers were dissected at E15.5 and triturated to single cell suspension. After genotyping, cells were incubated with biotinylated antibodies against CD3, CD19, B220, CD41, Gr-1 and Ter119 and lineage depleted like BM samples.

PB from tail vein was collected in EDTA tubes, underwent erythrocyte lysis (Pharm Lyse buffer, BD Biosciences, Heidelberg, Germany) and staining for flow cytometry. Spleen samples were prepared by mincing the organ through a cell strainer and erythrocyte lysis as described for PB.

For isolation of human CD34⁺ cells we used the Lineage Cell Depletion Kit (MiltenyiBiotec) as recommended by the manufacturer. Aliquots were kept frozen in CryoStore freezing medium (StemCell Technologies) until use or analyzed immediately after isolation. Cells were analyzed and sorted on a FACSCanto II, LSR Fortessa and FACS Aria (all: BD Biosciences) using the following antibodies: CD3 (500A2), human CD90 (5E10), human CD123 (7G3) (all: BD Biosciences), CD11b (M1/70), CD19 (eBio1D3), CD34 (RAM34), human CD34 (4H11), human CD38 (HIT2), CD41 (eBioMWReg30), CD45.1 (A20), human CD45RA (HI100), CD48 (HM48-1), CD127 (A7R34), B220 (RA3-6B2), c-Kit (2B8), Gr-1 (RB6-8C5), Sca-1 (D7), Ter-119 (all: eBioscience, Frankfurt, Germany), CD16/32 (93),

CD45.2 (104), CD150 (TC15-12F12.2) (all: BioLegend, Fell, Germany). Streptavidin eFluor450 was obtained from eBioscience. FACSDiva and FlowJo software were used for analysis.

Cell Cycle Analysis.

Ki67-FITC (clone B56) intracellular staining was performed according to BD Biosciences protocols, then cells were treated with RNase A (60 µg/ml) and stained with 0.5 µM TO-PRO-3 (both: Life Technologies, Darmstadt, Germany) or Hoechst33342 (Molecular Probes, Darmstadt, Germany). For IL-1 β stimulation and *in vivo* BrdU-labeling mice received 2.5 µg IL-1 β or PBS 20 h before BM harvest followed by BrdU intraperitoneally (18 mg/kg) 14 h before analysis. BrdU incorporation was detected using the FITC BrdU Flow Kit (BD Biosciences).

Colony Formation Assays.

CFU and long-term culture assays were performed according to the manufacturer's protocols using MethoCult GF M3434 and MyeloCult M5300 (both: StemCell Technologies).

5-FU Treatment of mice.

Weight-matched WT and X-CGD littermates or mice from WT (CD45.1) transplantations in WT (CD45.2) or X-CGD mice were injected weekly with 150 µg/g 5-fluorouracil (5-FU, Sigma-Aldrich, Taufkirchen, Germany). Neomycin (1.67 mg/ml) was added to drinking water. Mice were monitored and sacrificed in case of > 20% loss of body weight. Survival rates of the littermates were recorded.

Lentiviral Transduction.

Lentiviral vectors encoding for eGFP and tBFP were kindly provided by Christian Brendel, Georg-Speyer-Haus, Frankfurt. Vectors were produced as previously described.^{E1} Transduction was performed as in Brendel et al.^{E2}, but cells were prestimulated with 300 ng/ml mSCF, 300 ng/ml mTPO, 100 ng/ml mFlt3-Ligand, 60 ng/ml mIL-3.

Transplantation of murine cells.

Lethally irradiated (9.5 Gy) mice were transplanted via tail vein injections. Unless stated otherwise, cells were transplanted into WT (CD45.1) mice. For CRU assays 2, 10, 50 or 250 sorted LSK SLAM cells were transplanted with 10^5 competitor mononuclear cells (MNCs) from WT (CD45.1) mice. PB of transplanted recipients was analyzed after 16 weeks by flow cytometry and mice with $\geq 1\%$ donor-derived cells in the T-, B- and myeloid cell compartment were scored positive for donor cell engraftment. CRU frequency was calculated with the L-Calc software (Stem Cell Technologies). For secondary transplants, 10^5 BM MNCs from the first recipients transplanted with 50 or 250 LSK SLAM cells were transplanted with an equal number of WT (CD45.1) MNCs. Criteria for reconstitution were the same as above. For fetal liver experiments, 10^4 Lin⁻ fetal liver cells and 2×10^5 adult WT (CD45.1) MNCs were transplanted into irradiated WT (CD45.1) recipient mice. WT and X-CGD Lin⁻ fetal liver cells were obtained from littermate pairs. For assays with gene-marked cells, Lin⁻ cells from WT or X-CGD animals were transduced and 1:1 mixtures of transduced cells containing 70%-90% eGFP and tBFP-expressing cells were transplanted. For analysis of stromal cell effects, MNCs from WT (CD45.1) mice were transplanted into WT or X-CGD recipient mice. For 5-FU treatment, 5×10^5 WT (CD45.1) BM MNCs were injected and treatment was initiated 8 weeks after bone marrow transplantation (BMT). For serial BMTs

2 x 10⁵ WT (CD45.1) BM MNCs were transplanted. After 16 weeks 2 x 10⁵ BM MNCs from the first recipients were transplanted into secondary WT or X-CGD hosts. Survival rates of secondary recipients were monitored as during 5-FU treatment.

Cytokine Arrays and IL-1 β ELISA.

BM lavage was obtained by flushing the hind limbs of WT and X-CGD mice with 0.5 ml PBS. Cells were removed by centrifugation and the lavage was frozen in aliquots at -80°C. BM lavage (0.35 ml) was used for the Proteome Profiler Mouse Cytokine Array Panel A (R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer's instructions and detected with the Odyssey Infrared Imaging System using IRDye 800CW Streptavidin. Signal quantification was performed with the Odyssey Application Software 2.1.12. Bone lavage samples were also analyzed with the RayBio Mouse IL-1 β ELISA Kit (RayBiotech, Norcross, GA, USA) according to the manual.

Cytokine stimulation experiments

Sorted PB CD34⁺, CD38^{dim} and CD90⁺ cells (500.000 cells/ml) were cultured in X-VIVO20 (Lonza, Walkersville, MD, USA), 1% HSA, hIL3 20ng/ml, hTPO 100ng/ml, hFlt-3L 300ng/ml, hSCF 300ng/ml for 4 days.

For the murine *in vitro* experiments 10⁵ Lin⁻ cells were cultured in StemSpanSFEM (StemCell Technologies) + TPO + SCF (both 100 ng/ml), antibiotics and 75 ng/ml or 250 ng/ml IL-1 β or 250 ng/ml CCL5, CXCL9 or CXCL10 (all: murine cytokines; PeproTech, Rocky Hill, NJ, US) or PBS. LSK cell frequency was determined after 3 days by FACS. For the *in vivo* experiments 250 ng IL-1 β (LSK cells in BM, CFUs) or 2.5 μ g IL-1 β were injected into WT mice 12 h (LSK cells in BM, CFUs), 20 h (BrdU labeling and cell cycle analysis) or 44 h (LSK cells in spleen) prior to sacrifice. To analyze the effects of IL-1 β treatment *in vivo*

on the engraftment capacity of HSCs upon transplantation, CD45.1 and CD45.2 WT mice were injected intraperitoneally with 250 ng IL-1 β or PBS 5x every other day. Subsequently, MNCs were isolated and 2 x 10⁶ MNCs from differently treated mice with distinguishable CD45 markers were mixed and transplanted into both CD45.1 and CD45.2 recipients. PB chimerism was analyzed 16 weeks after transplantation.

Anakinra and Dexamethasone treatment of mice

Anakinra (Kineret, SOBI, Stockholm, Sweden) treatment of X-CGD and WT mice consisted of two intraperitoneal injections per day at a concentration of 25 μ g/g body weight for 14-16 days. Dexamethasone (Sigma-Aldrich) with/without Anakinra treatment of X-CGD mice was performed by daily intraperitoneal injections of 2 μ g/g body weight Dexamethasone +/- 10 μ g/g body weight Anakinra for 14 days. For subsequent BM transplantation, Lin⁻ cells were isolated from treated X-CGD mice and transplanted together with CD45.1 MNCs as competitors into CD45.1 WT recipients (4000 Lin⁻ cells + 10⁵ MNCs per mouse). BM chimerism was detected 19 weeks after transplantation.

References

- E1. Santilli G, Almarza E, Brendel C, et al. Biochemical correction of X-CGD by a novel chimeric promoter regulating high levels of transgene expression in myeloid cells. *Mol Ther.* 2011;19(1):122-132.
- E2. Brendel C, Hanseler W, Wohlgensinger V, et al. Human miR223 promoter as a novel myelo-specific promoter for chronic granulomatous disease gene therapy. *Hum Gene Ther Methods.* 2013;24(3):151-159.