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IL-4 receptor engagement in human neutrophils impairs their migration and extracellular trap formation

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Abstract: Background Type 2 immunity serves to resist parasitic helminths, venoms, and toxins, but the role and regulation of neutrophils during type 2 immune responses are controversial. Helminth models suggested a contribution of neutrophils to type 2 immunity, whereas neutrophils are associated with increased disease severity during type 2 inflammatory disorders, such as asthma. Objective We sought to evaluate the effect of the prototypic type 2 cytokines IL-4 and IL-13 on human neutrophils. Methods Human neutrophils from peripheral blood were assessed without or with IL-4 or IL-13 for (1) expression of IL-4 receptor subunits, (2) neutrophil extracellular trap (NET) formation, (3) migration toward CXCL8 in vitro and in humanized mice, and (4) CXCR1, CXCR2, and CXCR4 expression, as well as (5) in nonallergic versus allergic subjects. Results Human neutrophils expressed both types of IL-4 receptors, and their stimulation through IL-4 or IL-13 diminished their ability to form NETs and migrate toward CXCL8 in vitro. Likewise, in vivo chemotaxis in NOD-scid-Il2rg^{-/-} mice was reduced in IL-4-stimulated human neutrophils compared with control values. These effects were accompanied by downregulation of the CXCL8-binding chemokine receptors CXCR1 and CXCR2 on human neutrophils on IL-4 or IL-13 stimulation in vitro. Ex vivo analysis of neutrophils from allergic patients or exposure of neutrophils from nonallergic subjects to allergic donor serum in vitro impaired their NET formation and migration toward CXCL8, thereby mirroring IL-4/IL-13-stimulated neutrophils. Conclusion IL-4 receptor signaling in human neutrophils affects several neutrophil effector functions, which bears important implications for immunity in type 2 inflammatory disorders.

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1 **Interleukin-4 receptor engagement in human**
2 **neutrophils impairs their migration and extracellular**
3 **trap formation**

4

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18

19 **Abstract**

20 **Background:** Type 2 immunity serves to resist parasitic helminths, venoms and
21 toxins, but the role and regulation of neutrophils during type 2 immune responses is
22 controversial. Helminth models suggested a contribution of neutrophils to type 2
23 immunity, whereas neutrophils are associated with increased disease severity during
24 type 2 inflammatory disorders such as asthma.

25 **Objective:** To evaluate the impact of the prototypic type 2 cytokines interleukin-4
26 (IL-4) and IL-13 on human neutrophils.

27 **Methods:** Human neutrophils from peripheral blood were assessed without or with
28 IL-4 or IL-13 for (i) expression of IL-4 receptor (IL-4R) subunits; (ii) neutrophil
29 extracellular trap (NET) formation; (iii) migration toward CXC chemokine ligand 8
30 (CXCL8) *in vitro* and in humanized mice; (iv) CXC chemokine receptor 1 (CXCR1),
31 CXCR2, and CXCR4 expression; and (v) in non-allergic versus allergic subjects.

32 **Results:** Human neutrophils expressed both types of IL-4Rs and their stimulation via
33 IL-4 or IL-13 diminished their ability to form NETs and migrate toward CXCL8 *in*
34 *vitro*. Likewise, *in vivo* chemotaxis in NOD-*scid-Il2rg*^{-/-} mice was reduced in IL-4-
35 stimulated human neutrophils compared to controls. These effects were accompanied
36 by downregulation of CXCL8-binding chemokine receptors CXCR1 and CXCR2 on
37 human neutrophils upon IL-4 or IL-13 stimulation *in vitro*. *Ex vivo* analysis of
38 neutrophils from allergic patients or exposure of neutrophils from non-allergic
39 individuals to allergic donors' serum *in vitro* impaired their NET formation and
40 migration toward CXCL8, thereby mirroring IL-4/IL-13-stimulated neutrophils.

41 **Conclusion:** Signaling in human neutrophils affects several neutrophil effector
42 functions, which bears important implications for immunity in type 2 inflammatory
43 disorders.

44

45 **Key messages:**

- 46 • IL-4R stimulation on human neutrophils by IL-4 or IL-13 decreased NET
47 formation.
- 48 • Stimulation of IL-4R on human neutrophils downregulated their CXCR1 and
49 CXCR2 and impaired their chemotaxis to CXCL8 *in vitro* as well as in NOD-*scid*-
50 *Il2rg*^{-/-} mice.
- 51 • *In vivo* or *in vitro* exposure of neutrophils to allergic patients' serum decreased
52 CXCR1 and CXCR2 as well as impaired NET formation and migration, thereby
53 mirroring IL-4/IL-13-stimulated neutrophils.

54

55 **Capsule summary:** IL-4 receptor engagement in human neutrophils impairs their
56 formation of extracellular traps and their *in vitro* and *in vivo* chemotaxis.

57

58 **Key words:** allergy; neutrophil; innate immunity; IL-4; IL-13; IL-4 receptor;
59 inflammation.

60

61 **Abbreviations:** IL-4R, IL-4 receptor; NET, neutrophils extracellular trap.

62

63 **Introduction**

64 Neutrophil granulocytes (neutrophils) are the first non-resident immune cells to react
65 to pathogen- or danger-associated stimuli and they rapidly migrate to the site of
66 inflammation. Migration of neutrophils is governed by the expression of the
67 chemokine receptors CXC chemokine receptor 1 (CXCR1) and CXCR2 versus
68 CXCR4. Thus, downregulation of CXCR4 and upregulation of CXCR2 on
69 neutrophils maturing in the bone marrow allows these cells to leave the organ and join
70 the migratory pool of blood neutrophils.¹⁻³ On the contrary, senescent or non-
71 migratory neutrophils upregulate CXCR4. In the target tissue, neutrophils employ
72 different effector functions, including phagocytosis and the secretion of cytotoxic
73 granules and cytokines. Moreover, activated neutrophils can initiate a cellular
74 program leading to the release of neutrophil extracellular traps (NET) to immobilize
75 and kill large microbes.^{4,5} NETs are web-like structures composed of decondensed
76 mitochondrial or nuclear DNA that are associated with modified histone proteins and
77 different antimicrobial peptides, such as neutrophil elastase and myeloperoxidase
78 (MPO). Neutrophils release NETs in response to a range of stimuli, including phorbol
79 12-myristate 13-acetate (PMA), CXCL8 (also termed interleukin-8 [IL-8]), crystals,
80 lipopolysaccharide (LPS), and microorganisms. NETs not only contribute to pathogen
81 defense, but they are also associated with non-infectious disorders, including
82 vasculitis and systemic lupus erythematosus.

83 Neutrophils are predominant in type 1 (or T helper [Th] 1 cell) and type 3
84 (Th17 cell) immune responses. However, the regulation and role of neutrophils during
85 type 2 (or Th2 cell) immune responses remains ill-defined.⁶⁻⁹ Type 2 immune
86 responses evolved to protect the host against large extracellular parasitic helminths,
87 venoms, and toxins by strengthening epithelial barrier defenses and stimulating

88 different immune cell mechanisms.^{10,11} Many of these effects are initiated, driven and
89 maintained by the actions of type 2 cytokines, most notably IL-4 and IL-13, which
90 signal via heterodimeric IL-4 receptors (IL-4R). IL-4 binds and signals via two
91 different IL-4Rs, termed type 1 and type 2 IL-4R, respectively (**Fig. 1A**). The type 1
92 IL-4R consists of a heterodimer made of IL-4R α (also termed CD124) and the
93 common gamma chain (also known as γ_c or CD132). The type 2 IL-4R is composed
94 of CD124 and IL-13R α 1 (also named CD213 α 1). IL-13R α 2 (also termed CD213 α 2)
95 serves as a decoy receptor for IL-13. In addition to IL-4, IL-13 also associates with
96 and signals through the type 2 IL-4R. IL-4 and IL-13 mediate signaling via
97 phosphorylation of Signal Transducer and Activator of Transcription 6 (STAT6),^{11,12}
98 but typically not of STAT5 and STAT3, whereas STAT5 and STAT3 become
99 activated with γ_c cytokines and granulocyte colony-stimulating factor (G-CSF).¹³

100 In type 2 immunity, during certain helminth infections in mice, the recruitment
101 of neutrophils contributed to early containment of the parasite during its migration
102 through the lungs.¹⁴ Yet, in the same model, neutrophils also caused increased
103 damage to the lungs.^{14,15} Considering type 2 inflammation in humans, as typically
104 seen with atopic dermatitis and allergic asthma, some studies have shown that
105 neutrophil responses were attenuated or maybe even suppressed, which has been
106 attributed to reciprocal regulation of type 2 and type 3 inflammatory pathways.^{16,17}
107 However, neutrophils are present in certain severe forms of allergic asthma, which
108 may result in a mixed type 2 and type 3 inflammation,^{6,18-21} and NETs have recently
109 been associated with asthma exacerbations during rhinovirus infection of asthmatics.²²
110 Notably, coding polymorphisms in the human *IL4Ra* gene have been linked to
111 susceptibility and severity of atopy and asthma in patients,²³⁻²⁵ as well as a mixed
112 neutrophilic-eosinophilic type of severe experimental asthma in mice.²⁶ Moreover,

113 recently, mice with a deficiency in IL-4, IL-13 or STAT6 were shown to exhibit a
114 neutrophilic form of experimental asthma upon challenge.²⁷

115 These data suggest that the IL-4R–STAT6 signaling pathway regulates
116 neutrophils in type 2 inflammation, possibly via other, more rapid mechanisms than
117 its involvement in type 2 vs. type 3 immune skewing. With the hypothesis that IL-4
118 could directly affect neutrophils, as recently shown in mice,²⁸ we have investigated in
119 the present study expression and impact on primary human neutrophils of IL-4R
120 signaling following stimulation with the prototypic type 2 cytokines IL-4 and IL-13.

121

122

123 **Materials and Methods**

124 **Human subjects**

125 Following written informed consent, volunteers were recruited for donating blood,
126 which was immediately processed to isolate neutrophils. Serum was also obtained
127 from the same donors for IgE detection using ImmunoCAP250 (Phadia,
128 ThermoFisher). All experiments using human samples were carried out in accordance
129 with the Cantonal Ethical Committee of Zurich (BASEC number 2016-01440).
130 Healthy donors (HD) were defined as subjects with serum IgE levels below 100 kU/L
131 and no history of seasonal or perennial allergies. Allergic donors (AD) were subjects
132 with total serum IgE levels above 100 kU/L (mean 279.7 ± 184.9 kU/L) with a known
133 allergy to grass pollen, tree pollen, or house dust mite, verified by a positive skin
134 prick test or allergen-specific IgE test, and with specific clinical symptoms such as
135 seasonal or perennial rhino-conjunctivitis and asthma. AD were investigated during
136 their allergen season. Absolute neutrophil counts and percentages in peripheral blood
137 were determined by an automated system (Abbott Diagnostics, Santa Clara).

138

139 **Isolation and *in vitro* stimulation of neutrophils**

140 Venous blood samples were collected in ethylenediaminetetraacetic acid (EDTA)
141 tubes (BD Vacutainer®). Neutrophils were purified by HetaSep™ (Stemcell
142 Technologies) followed by negative magnetic selection using the EasySep Direct
143 Human Neutrophil Isolation Kit (Stemcell Technologies). Cell viability (>97%) was
144 assessed by trypan blue exclusion and purity of neutrophil preparations ($96.4 \pm 2.4\%$)
145 was determined by flow cytometry based on CD16 (3G8, BioLegend), CD61 (VI-
146 PL2, BioLegend) and CD66b (G10F5, BioLegend). Purified neutrophils were
147 resuspended in RPMI 1640 supplemented with 1% fetal bovine serum (FBS;

148 ThermoFisher), before stimulation with recombinant human cytokines (used
149 throughout the study), including G-CSF, IL-4, and IL-13 (at 150 ng/mL, unless stated
150 otherwise; PeproTech), for 0, 4, 6, and 24 h, or with serum from either HD or AD,
151 followed by flow cytometry analysis (see below). Viability was measured using the
152 Annexin V Apoptosis Detection kit (BD Biosciences) in combination with propidium
153 iodide (PI).

154

155 **Flow cytometry**

156 Single-cell suspensions of neutrophils were processed for analysis by flow cytometry
157 and stained using phosphate-buffered saline (PBS) with 1% FBS, 2 mM EDTA, and
158 fluorochrome-conjugated monoclonal antibodies against the following human
159 antigens (from BioLegend unless otherwise stated): CD16 (3G8), CD61 (VI-PL2),
160 CD66b (G10F5), CD114 (LMM741), CD124 (G077F6), CD132 (TUGh4), CD162
161 (KPL-1), CD213 α 1 (SS12B), CD213 α 2 (SHM38), CXCR1 (8F1/CXCR1), CXCR2
162 (5E8/CXCR2), and CXCR4 (12G5). For MPO staining, neutrophils were fixed by
163 addition of Fixation Buffer I, followed by permeabilization with Perm Wash Buffer
164 III (BD Phosflow, BD Bioscience). Intracellular staining for MPO (MPO421-8B2)
165 was performed according to manufacturer's instructions. Samples were acquired using
166 a BD LSR Fortessa and analyzed using FlowJo software (Tristar).

167

168 **Assessment of intracellular signaling pathways**

169 Highly-purified neutrophils were stimulated *in vitro* in RPMI medium (RPMI 1640
170 with 1% of FBS) and G-CSF, IL-4, or IL-13 (50 ng/mL) for 0, 2, 5, 15, 30, and 60
171 min, followed by fixation with Fix Buffer I and permeabilization with Perm Buffer
172 III. Intracellular staining for phospho-Y705 of STAT3 (pSTAT3; 13A3-1,

173 BioLegend), phospho-Y694 of STAT5 (pSTAT5; SRBCZX, ThermoFisher),
174 phospho-Y641 of STAT6 (pSTAT6; CHI2S4, ThermoFisher) was performed as
175 previously established.^{28,29} Samples were acquired and analyzed by flow cytometry,
176 as stated above.

177

178 **SDS-PAGE and Western blot analysis**

179 10^7 neutrophils in RPMI 1640 supplemented with 1% FBS were stimulated with G-
180 CSF, IL-4, or IL-13 (50 ng/mL) for 0 and 5 min at 37°C. Subsequently, cells were
181 lysed with 4x Laemmli's buffer (Bio-Rad). Proteins were separated using 4–20%
182 SDS-PAGE mini ready gels (Bio-Rad) and transferred to polyvinylidene difluoride
183 (PVDF) membranes (Bio-Rad). Membranes were labeled with rabbit antibody (Cell
184 Signaling Technology) for pSTAT3 (D3A7), pSTAT5, pSTAT6, total STAT3
185 (79D7), total STAT5 (D206Y), total STAT6 (D3H4), and vinculin. After incubation
186 with the appropriate primary antibody, the blots were developed with horseradish
187 peroxidase-conjugated secondary antibodies by using enhanced chemiluminescence
188 reagents (ThermoScientific) following standard protocols.

189

190 **Analysis of NETs**

191 10^5 neutrophils were seeded on glass coverslips treated with poly-lysine (Sigma) in
192 24-well tissue culture plates and incubated for 6 h at 37°C in 5% CO₂ with medium
193 (RPMI 1640 with 1% of FBS) alone or with G-CSF, IL-4, or IL-13 (150 ng/mL).
194 Subsequently, neutrophils were stimulated with 100 nM PMA (Sigma) for 2 h to
195 induce NET formation. Cells were then fixed with 4% paraformaldehyde for 10 min
196 at room temperature and mounted using ProLong Gold AntiFade with 4',6-diamidino-
197 2-phenylindole dihydrochloride (DAPI; Life Technologies). Cells were imaged using

198 a 40-fold magnification 1.25 NA with an inverted CSLM Leica SP5 confocal
199 microscope (Leica). Every picture consisted of an overlay of 15 stacks of 20 μm .
200 Quantification of NET⁺ neutrophils were done both manually and automatically using
201 ImageJ software. For manual counting, 5 randomly selected areas of the same slide
202 were counted by three independent investigators with two of them blinded to the
203 treatment conditions. For automatic analysis, we used ImageJ software with the
204 DANA plug-in to quantify the area, raw integrated density, aspect ratio, roundness,
205 maximum and minimum brightness, and solidity of each region of interest of DAPI-
206 labeled neutrophils, as previously described.³⁰

207

208 **Transwell migration assay**

209 Transwell migration assays were performed as previously published.^{28,31} In brief,
210 freshly-isolated neutrophils were incubated for 2 h at 37°C in 5% CO₂ with medium
211 (RPMI 1640 containing 1% of FBS) alone or supplemented with 150 ng/mL G-CSF,
212 IL-4, IL-13, or serum from HD or AD. Where indicated, neutrophils were incubated
213 with cytokines, or with serum, or preincubated with rabbit anti-human IL-4R α
214 monoclonal antibody (anti-CD124; Sino Biological) for 1 h before seeding into the
215 upper chamber of a 3 μm transwell (Corning Costar). Neutrophil migration toward
216 CXCL8 (PeproTech), added to the lower chamber, was determined after 120 min by
217 determining neutrophil counts in the lower chamber using flow cytometry counting
218 beads (123count eBeads, ThermoFisher), as previously published.^{32,33}

219

220 **Airpouch mouse model**

221 An airpouch was formed in the back of 2–4-month-old NOD-*scid-Il2rg*^{-/-} (NSG;
222 Charles River Laboratories) mice by subcutaneous injection of 3 mL filtered sterile air

223 on days 0 and 3, as described.²⁸ On day 5, LPS (100 nM) and CXCL8 (100 ng/mL) in
224 2 mL of PBS was injected into the airpouch along with intravenous injection of 10⁷
225 highly-purified human neutrophils pre-incubated for 2–6 h with medium (RPMI 1640
226 medium with 1% FBS) alone or in combination with IL-4 (150 ng/mL), or G-CSF
227 (150 ng/mL). Mice were euthanized after 90 min to collect airpouch infiltrate and
228 spleens. Samples were analyzed by flow cytometry as stated above. Mouse
229 experiments followed the Swiss Federal Veterinary Office guidelines and were
230 approved by the Cantonal Veterinary Office.

231

232 **Statistics**

233 The numbers of samples and subjects used in each experiment are indicated in the
234 figure legends. Data are presented as mean ± standard deviations (SD). *P*-values were
235 calculated using Student's *t*-test, two-way analysis of variance (ANOVA) with
236 Bonferroni multiple comparison test, one-way ANOVA with Tukey's multiple
237 comparisons test, or Friedman test with Dunn's multiple comparisons test as
238 indicated. Statistical analysis was performed with Graph-Pad Prism. Statistical
239 significance was established at $P < 0.05$.

240

241 **Results**

242 **Human neutrophils express functional type 1 and type 2 IL-4Rs**

243 Previous studies found human neutrophils expressed both CD124 and CD132, thus
244 forming type 1 IL-4Rs, whereas CD213 α 1 and CD213 α 2 were absent on these
245 cells.^{34,35} We re-evaluated IL-4R expression on freshly-isolated human neutrophils.
246 Notably, our neutrophil purification method, yielding on average 96.4% pure human
247 neutrophils, did not significantly affect expression levels of CD16, CD66b, IL-4R
248 subunits, crucial chemokine receptors, as well as CD49d, in comparison to the
249 assessment of these molecules on neutrophils in whole blood (**Fig. E1, A to C**). We
250 were able to detect CD124, CD132, and CD213 α 1 on purified human neutrophils
251 right after isolation from whole blood of HD, and expression of these IL-4R subunits
252 further significantly increased upon stimulation of neutrophils for 24 h with G-CSF (P
253 $< .0001$, CD124 for G-CSF vs time 0; $P < .0001$, CD132 for G-CSF vs time 0; $P =$
254 $.0008$, CD213 α 1 for G-CSF vs time 0; **Fig. 1, B to D**). Also, the decoy receptor
255 CD213 α 2 was detectable on neutrophils and increased upon incubation of neutrophils
256 with G-CSF ($P = .0385$, G-CSF vs time 0; **Fig. E1B and E2**). A resting period of 30
257 to 120 min in medium or autologous serum following isolation did not change IL-4R
258 expression (data not shown).

259 To investigate whether neutrophils expressed functional IL-4Rs resulting in
260 phosphorylated STAT6 (pSTAT6), we performed time-course experiments in purified
261 human neutrophils upon stimulation with IL-4 or IL-13, and G-CSF as a control. G-
262 CSF resulted in strong and significant activation of STAT3 ($P < .0001$) and STAT5
263 ($P = .0031$) in human neutrophils 5 min after stimulation, whereas such treatment did
264 not affect pSTAT6 levels (**Fig. 1E**). On the other hand, IL-4 and IL-13 significantly

265 activated STAT6 in human neutrophils, inducing a sharp peak in pSTAT6 5 min after
266 cytokine stimulation ($P < .0001$, for both IL-4 and IL-13 at 5 min vs time 0), which
267 declined thereafter (**Fig. 1E**). However, pSTAT3 and pSTAT5 expression did not
268 change significantly following IL-4 or IL-13 stimulation (**Fig. 1E**). These findings
269 were further confirmed by Western blot analysis (**Fig. E3**).

270 Together, these results demonstrate the presence of functional type 1 and type
271 2 IL-4Rs on human neutrophils.

272

273 **IL-4R engagement impairs formation of NETs in human neutrophils**

274 To evaluate the effects of IL-4 and IL-13 on neutrophil effector functions we assessed
275 NET formation upon stimulation with PMA (**Fig. 2**). Compared to medium or G-CSF,
276 IL-4- or IL-13-conditioned neutrophils produced significantly less NETs, as assessed
277 by manual counting ($P < .0001$, Medium vs IL-4; $P = .0001$, Medium vs IL-13; $P <$
278 $.0001$, G-CSF vs IL-4 or IL-13) and by automatic analysis of DNA area ($P < .0001$,
279 Medium vs IL-4 or IL-13; $P < .0001$, G-CSF vs IL-4; $P = .0037$, G-CSF vs IL-13;
280 **Fig. 2, A and B**). Moreover, the NETs of IL-4-/IL-13-stimulated neutrophils were of
281 different characteristics featuring enlarged and less dense nuclei next to groups of
282 round and condensed nuclei (**Fig. 2A**). These phenotypic differences were also
283 responsible for the slight but non-significant difference in manual counting vs
284 automatic analysis of NETs in medium-conditioned neutrophils (**Fig. 2B**). These data
285 establish that IL-4R stimulation significantly affects NET formation in human
286 neutrophils.

287

288 **IL-4 and IL-13 inhibit chemotaxis of human neutrophils to CXCL8 *in vitro***

289 To gain further insight into the effects of IL-4R stimulation on human neutrophils, we
290 assessed *in vitro* migration of neutrophils using a transwell migration assay. When
291 freshly-isolated human neutrophils were pre-incubated for different times without
292 (Medium) or with IL-4 (**Fig. 3, A and B**) or IL-13 (**Fig. 3, C and D**), IL-4-/IL-13-
293 stimulated neutrophils showed decreased migration toward CXCL8 (which binds
294 CXCR1 and CXCR2)³, with significant and consistent reduction in chemotaxis at 60
295 ($P = .0014$ [in cell counts] and $P = .0043$ [in %] for IL-4 vs Medium; $P < .0001$ and P
296 $= .0042$, for IL-13 vs Medium) and 120 min ($P = .0029$ and $P = .0004$ for IL-4 vs
297 Medium; $P < .0001$ and $P < .0001$ for IL-13 vs Medium; **Fig. 3, A to D**). Next, we
298 assessed the migration of human neutrophils toward different concentrations of
299 CXCL8 pre-incubating the human neutrophils with IL-4 (**Fig. 3E**) or IL-13 (**Fig. 3F**).
300 While 10 to 100 ng/mL CXCL8 exerted potent chemotactic activity on control
301 neutrophils (medium), pre-incubation with IL-4 or IL-13 significantly decreased
302 neutrophil chemotaxis at all CXCL8 concentrations of 1 ng/mL and higher ($P =$
303 $.0360$, IL-4 vs Medium for 1 ng/mL; $P = .0263$, IL-4 vs Medium for 10 ng/mL; $P =$
304 $.0076$, IL-4 vs Medium for 100 ng/mL; $P = .0198$, IL-13 vs Medium for 1 ng/mL; $P =$
305 $.0377$, IL-13 vs Medium for 10 ng/mL; $P = .0240$, IL-13 vs Medium for 100 ng/mL;
306 $P = .0204$, IL-13 vs Medium for 1000 ng/mL; **Fig. 3, E and F**). Finally, human
307 neutrophils were incubated with different concentrations of either IL-4 or IL-13
308 followed by chemotaxis toward a set concentration of CXCL8 ($P = .0004$, 150 ng/mL
309 of IL-4 vs Medium; $P = .0002$, 150 ng/mL of IL-13 vs Medium). Both IL-4 and IL-13
310 significantly inhibited neutrophil migration in a dose-dependent manner (**Fig. 3, G**
311 **and H**).

312

313 **IL-4R signaling hampers migration of human neutrophils in humanized mice**

314 To investigate whether IL-4R signaling inhibited the migration of human neutrophils
315 *in vivo*, we challenged NSG mice harboring human neutrophils. To this end, we
316 induced a sterile inflammation in form of an airpouch on the back of NSG mice by
317 repeated injection of sterile air on days 0 and 3, followed by administration of the
318 neutrophil chemoattractants CXCL8 and LPS into the airpouch and adoptive transfer
319 of pre-conditioned human neutrophils via tail vein injection on day 5 (**Fig. 4A**). Mice
320 were sacrificed 90 min after adoptive transfer of neutrophils to avoid loss of
321 neutrophils, and we analyzed the airpouch infiltrate by flow cytometry by gating on
322 human CD45-positive (and mouse CD45-negative) CD16⁺ CD66b⁺ cells (**Fig. 4B**). In
323 the airpouch, the counts of human neutrophils were 4.2 times higher in the control
324 group (Medium) compared to IL-4 ($P = .0247$; **Fig. 4C**). Conversely, the counts of
325 infiltrating human neutrophils in spleen was comparable in both groups of mice (**Fig.**
326 **4C**). Moreover, we compared G-CSF to IL-4, which confirmed that IL-4 pre-
327 conditioning significantly affected migration of human neutrophils into to the
328 airpouch ($P = .0214$), but not the spleen (**Fig. 4D**).

329

330 **IL-4R engagement modulates chemokine receptors on human neutrophils**

331 The aforementioned data demonstrate that IL-4R engagement hampers the migration
332 of human neutrophils *in vitro* and *in vivo*. These actions could, at least in part, result
333 from changes in expression of CXCR1, CXCR2 and CXCR4. We assessed expression
334 of these chemokine receptors in human neutrophils upon *in vitro* stimulation with IL-
335 4 or IL-13. As previously mentioned, the isolation method did not affect the
336 expression of these chemokine receptors (**Fig. E1C**). Upon stimulation with either IL-
337 4 or IL-13, human neutrophils significantly downregulated CXCR1 ($P = .0077$, IL-4

338 vs Medium; $P = .0031$, IL-13 vs Medium) and CXCR2 ($P = .0238$, IL-4 vs Medium;
339 $P = .0170$, IL-13 vs Medium) compared to incubation in medium (**Fig. 5A**). In
340 contrast, CXCR4 expression did not significantly change in IL-4-/IL-13-conditioned
341 neutrophils (**Fig. 5A**). The changes in CXCR1 and CXCR2 were not due to apoptosis,
342 as shown by annexin V and PI staining, which remained below 5% during the
343 incubation period (**Fig. 5B**). Collectively, IL-4-/IL-13-stimulated neutrophils adopt a
344 chemokine receptor pattern akin to bone marrow-sessile, non-migratory cells.

345

346 **Neutrophils of allergic individuals resemble IL-4-/IL-13-stimulated cells**

347 The effects of IL-4R signaling on human neutrophils suggest that, in type 2
348 inflammatory disorders, neutrophil functions could be affected. To investigate this
349 hypothesis, we compared freshly-isolated neutrophils from healthy donors (HD) to
350 patients with known active allergies (see Materials and Methods). Counts and
351 percentages of neutrophils in peripheral blood of allergic donors (AD) were
352 comparable to that of HD (**Fig. 6A**). However, neutrophils from AD exhibited
353 differences in several functional markers, including a significant decrease in CD16 (P
354 $= .0071$, AD vs HD; **Fig. 6B**). Moreover, neutrophils from AD showed significantly
355 lower expression of CXCR1 ($P = .0012$, AD vs HD) and CXCR2 ($P < .0001$, AD vs
356 HD), as well as a tendency toward higher CXCR4 levels (**Fig. 6C**). Thus, neutrophils
357 of AD were reminiscent of IL-4-/IL-13-conditioned cells.

358 Moreover, the expression levels of MPO in neutrophils from AD were
359 significantly lower compared to HD ($P = .0002$; **Fig. 6D**). MPO contributes to
360 antimicrobial activity and NET formation.³⁶ In line with this phenotypic change,
361 neutrophils from AD showed significantly impaired NET formation ($P < .0001$, AD
362 vs HD) compared to the ones isolated from HD upon stimulation with PMA (**Fig. 6E**).

363 Overall, human neutrophils from AD resemble IL-4-/IL-13-stimulated
364 neutrophils in terms of migratory phenotype and NET formation.

365

366 **Human neutrophils stimulated with serum from allergic individuals show**
367 **similarity with IL-4/IL-13 stimulated cells**

368 To further evaluate whether soluble components of AD serum can affect neutrophils,
369 we incubated freshly-isolated neutrophils from HD with serum from HD or AD.
370 Compared to incubation with HD serum, AD serum severely affected the ability of
371 HD neutrophils to form NETs, as measured by counting ($P < .0001$) and assessment
372 of DNA area ($P = .0056$; **Fig. 7A**). Moreover, the migration of HD neutrophils toward
373 100 ng/mL CXCL8 was significantly reduced upon incubation with AD serum ($P <$
374 $.0001$), and concomitant use of an anti-CD124 monoclonal antibody was able to
375 partially restore their migration ($P = .0033$, HD neutrophils preincubated with anti-
376 CD124 vs Ctrl followed by AD serum; **Fig. 7B**), indicating that IL-4 and/or IL-13 in
377 AD serum significantly affected the HD neutrophils. These functional changes in HD
378 neutrophils incubated with AD serum were accompanied by a significant decrease of
379 CXCR1 ($P < .0001$) and CXCR2 ($P = .0004$), whereas CXCR4 remained unchanged
380 (**Fig. E4**).

381 Overall, serum from allergic individuals alters the neutrophil chemotaxis and
382 NET formation, thereby mimicking the effects of IL-4 and IL-13.

383

384 **Discussion**

385 Our data demonstrate that the prototypic type 2 cytokines IL-4 and IL-13 adversely
386 affect several functional properties of human neutrophils. As a result, neutrophils
387 appear desensitized toward CXCL8-mediated chemotaxis *in vitro* and *in vivo* as well
388 as to stimulation-induced NET formation. Such IL-4R-mediated conditioning of
389 neutrophils results in a phenotype characterized by low CXCR1 and CXCR2 and
390 decreased MPO expression. These phenotypic and functional characteristics are also
391 observed in freshly-isolated neutrophils from allergic subjects as well as upon
392 incubation of HD neutrophils with AD serum.

393 Our data suggest that human neutrophils stimulated by their IL-4Rs enter an
394 activation or differentiation state that differs from neutrophils freshly isolated from
395 peripheral blood of healthy individuals. The question arises whether IL-4/IL-13-
396 conditioned neutrophils represent senescent, aged, exhausted or apoptosis-prone cells.
397 We have not observed increased apoptosis of IL-4/IL-13-conditioned neutrophils.
398 Also, unlike senescent or aged neutrophils, showing decreased CD16 and increased
399 CXCR4 and NET formation,^{1,37} CXCR4 expression remained unchanged in IL-4/IL-
400 13-conditioned neutrophils, although CD16 was slightly decreased, and they showed a
401 deficiency in NET formation, which is contrary to what was observed for senescent
402 and aged neutrophils.

403 In terms of NET formation, IL-4/IL-13-conditioned neutrophils could present
404 with a delayed type of NET formation or, alternatively, IL-4R signaling could skew
405 neutrophils toward suicidal vs. vital NET formation.³⁸⁻⁴⁰ Although we did not observe
406 a kinetic delay in NET formation or increased apoptosis in IL-4/IL-13-conditioned
407 neutrophils, we cannot formally exclude these possibilities. We prefer to consider IL-
408 4/IL-13-conditioned neutrophils as "alternatively-activated" neutrophils, similar to

409 their IL-4/IL-13-conditioned counterparts in macrophages.¹² "N2" neutrophils have
410 been described in the context of tumor-infiltrating pro-tumorigenic neutrophils.⁴¹
411 Whether IL-4/IL-13-conditioned neutrophils resemble N2 neutrophils is an interesting
412 question for future studies.

413 Steady-state and induced levels of IL-4Rs on neutrophils will determine their
414 susceptibility to IL-4 and IL-13. We found that freshly-isolated human neutrophils
415 from whole blood expressed significant levels of type 1 and type 2 IL-4Rs. This
416 finding slightly contrasts with a previous publication describing only the presence of
417 type 1 IL-4Rs on human neutrophils.³⁵ Yet, another publication reported the absence
418 of type 1 IL-4Rs but the expression of type 2 IL-4Rs on mouse neutrophils.²⁸ These
419 differences might indicate discrepancies between human and mouse neutrophils, and
420 they might result from different neutrophil preparation methods, such as the
421 preincubation of neutrophils with autologous serum to prevent nonspecific binding via
422 Fc receptors.³⁵ Importantly, IL-4R expression on human neutrophils was further
423 increased by certain stimuli, most notably G-CSF. This result is consistent with what
424 has been reported in mice.²⁸ Also, Toll-like receptor (TLR) ligands and common
425 vaccine adjuvants, including LPS, alum and incomplete Freund's adjuvant, have been
426 shown in mice to upregulate CD124 on CD11b⁺ myeloid cells, which include
427 monocytes and neutrophils.⁴² G-CSF and TLR ligands are typically released upon
428 inflammation and infection, indicating that such conditions increase the neutrophils'
429 sensitivity to IL-4 and IL-13.

430 IL-4/IL-13-mediated conditioning of neutrophils, as described here, could play
431 a role in shaping the different asthma phenotypes, including eosinophilic and
432 neutrophilic asthma.^{17,43,44} Thus, production of IL-4 and/or IL-13 and the ensuing IL-
433 4R engagement could contribute to the eosinophilic variant of asthma by inhibiting

434 the recruitment of neutrophils into the tissues. This suggestion is in line with mouse
435 data showing that eosinophilic asthma is dependent on IL-4, IL-13, and STAT6,
436 whereas lack of these factors favors neutrophilic asthma in mice.²⁷ Also, this
437 hypothesis fits with publications reporting that type 2 and type 3 immune signatures
438 are mutually exclusive in asthma patients.¹⁷ Conversely, it is known that rhinovirus
439 infection can result in neutrophil activation and release of NETs along with disease
440 exacerbation in asthmatic subjects.^{6,22} Moreover, certain *IL4Ra* genotypes predispose
441 to a mixed eosinophilic-neutrophilic asthma presentation.²³⁻²⁶ These considerations
442 suggest that IL-4R-mediated conditioning of neutrophils can be overwritten by
443 stronger stimuli, such as an infection, or by interference with normal IL-4R signaling.

444 For eosinophilic asthma, IL-4R-interfering biologic agents should not only
445 dampen type 2 inflammation and improve disease^{45,46} but also re-invigorate
446 neutrophils thereby strengthening anti-pathogen immunity. This has so far not been
447 investigated. Contrarily, the use of IL-4R-stimulating molecules could be used in
448 diseases with predominant neutrophilic inflammation, an idea that has been tested in
449 both humans and mice. In psoriasis, a skin and joint disease characterized by
450 prominent neutrophil infiltration, a small proof-of-concept clinical trial showed that
451 administration of recombinant IL-4 improved skin disease, which the authors
452 interpreted as a skewing from Th1 to Th2 cell responses.⁴⁷ In mice and rats, provision
453 of recombinant IL-4 improved experimental arthritis,^{48,49} which in the collagen-
454 induced DBA/1 mouse model is known to rely on IL-1 β and G-CSF-dependent
455 neutrophil activity.^{50,51} However, translation and use of recombinant IL-4 in humans
456 is currently hampered by the very short *in vivo* half-life and dose-dependent toxic
457 adverse effects of IL-4, which might be improved by provision of long-acting IL-4
458 formulations.⁵²

459 Collectively, our data demonstrate that IL-4R engagement in human
460 neutrophils antagonizes several effector functions, which might open possibilities to
461 interfere with this pathway in allergic and neutrophilic disorders.

462

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472 **Author contribution**

473 D.I. designed and performed the experiments, analyzed the data and wrote the
474 manuscript. F.R. performed the experiments. M.R. collected the human samples,
475 analyzed confocal microscopy data and wrote the manuscript. C.E. performed
476 Western blot analysis. A.G.A.K. collected human serum from healthy and allergic
477 donors. J.W. and D.L. gave scientific input. O.B. designed and analyzed experiments,
478 supervised the study, and wrote the manuscript with input from all the authors.

479

480

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643
644

645 **Figure legends**

646 **Figure 1. Human neutrophils express functional type 1 and type 2 IL-4**
647 **receptors. A,** Schematic representation of the type 1 and type 2 IL-4R. **B to D,**
648 Expression of IL-4R subunits on human neutrophils of healthy donors (HD).
649 Representative histograms of CD124 (**B**), CD132 (**C**), and CD213 α 1 (**D**) after
650 isolation (T0; black lines) or following stimulation for 24 h with granulocyte colony-
651 stimulating factor (G-CSF; blue lines). Fluorescence minus one (FMO) values are
652 represented by histograms filled with grey color. Bars represent the geometric mean
653 fluorescence intensity (GMFI) of the indicated receptor subunits at T0 (grey bars) or
654 upon 24 h stimulation with G-CSF (blue bars). Data are presented as mean \pm standard
655 deviation (SD) of $n = 15$ donors. Significance of the differences between groups was
656 calculated using Student's *t*-test. **(E)** STAT pathways activated by G-CSF, IL-4, or IL-
657 13. Phosphorylated STAT3 (pSTAT3; top row), pSTAT5 (middle row), and pSTAT6
658 (bottom row) in human neutrophils upon stimulation for the indicated times with G-
659 CSF (left column), IL-4 (middle column) or IL-13 (right column). Diagrams represent
660 MFI of pSTATs at indicated time points. Small squares show representative flow
661 cytometry histograms of pSTATs at 0 and 5 min. Data are shown as mean \pm SD and
662 are representative of $n = 10$ independent experiments using unrelated donors.
663 Statistical significance was calculated using Friedman test with Dunn's multiple
664 comparisons test; *ns*, Not significant.

665

666 **Figure 2. Inhibition of NET formation by IL-4 or IL-13 stimulation *in vitro*.** **A,**
667 Freshly-isolated HD blood neutrophils were directly assessed (Ctrl) or stimulated for
668 2 h with 100 nM phorbol 12-myristate 13-acetate (PMA) in medium alone (Medium;
669 RPMI 1640 with 1% of FBS) or medium plus granulocyte colony-stimulating factor
670 (G-CSF; 150 ng/mL), IL-4 (150 ng/mL) or IL-13 (150 ng/mL). Neutrophils were
671 stained with DAPI and analyzed by confocal microscopy at 40-fold (40x) and 80-fold
672 (80x) magnification. Experiments were repeated using $n = 10$ unrelated donors. Scale
673 bar 100 μm . **B,** Bar histograms represent NET⁺ neutrophils treated as in **A**, including
674 direct assessment (Ctrl) or upon 2 h PMA stimulation with medium alone (Medium;
675 black filled bar), medium containing G-CSF (150 ng/mL; blue open bar), medium
676 containing IL-4 (150 ng/mL; red filled bar), or medium containing IL-13 (150 ng/mL;
677 blue filled bar). Neutrophils were stained with DAPI and analyzed by confocal
678 microscopy, followed by manual counting of NET⁺ neutrophils (left panel) or
679 automatic quantification of the DNA area (μm^2) using the DANA plug-in for ImageJ
680 (right panel). Data are shown as mean \pm SD of at least $n = 10$ independent
681 experiments with unrelated donors. Significance of the differences between groups
682 was calculated using one-way ANOVA; *ns*, Not significant.
683

684 **Figure 3. Human neutrophil chemotaxis is inhibited by IL-4 and IL-13. A to D,**
685 Migration of freshly-isolated HD neutrophils toward a set concentration of CXCL8
686 (100 ng/mL) at different time points (**A** and **C**) or following stimulation for 60 min
687 and 120 min (left and right panels, respectively, **B** and **D**) with medium (black),
688 medium plus IL-4 (150 ng/mL; red; **A** and **B**), or medium plus IL-13 (150 ng/mL;
689 blue; **C** and **D**). **E** and **F**, Chemotaxis of freshly-isolated HD neutrophils toward
690 titrated concentrations of CXCL8 following stimulation for 2 h with set
691 concentrations of IL-4 (150 ng/mL, red; **E**) or IL-13 (150 ng/mL, blue; **F**). **G** and **H**,
692 Migration of freshly-isolated HD neutrophils toward a set concentration of CXCL8
693 (100 ng/mL) after 2 h of stimulation with titrated concentrations of IL-4 (red filled
694 bars; **G**) or IL-13 (blue filled bars; **H**). Shown are counts (**A**, **C**, **E**, and **F**) or
695 percentages (**B**, **D**, **G**, and **H**) of migrated neutrophils. Data are presented as mean \pm
696 SD of three independent experiments with $n = 10-12$ unrelated donors. Significances
697 between the groups were calculated by one-way ANOVA (**A**, **C**, and **E** to **H**) or
698 Student's *t*-test (**B** and **D**).
699
700

701 **Figure 4. Migration of human neutrophils *in vivo*.** **A**, Experimental setup of the
702 airpouch in NOD-*scid-Il2rg*^{-/-} (NSG) mouse model. **B**, Representative flow cytometry
703 analysis of human (h) CD45⁺ mouse (m) CD45⁻ cells (left panel) and hCD16⁺
704 hCD66b⁺ neutrophils (right panel) in the airpouch. **C**, Bars represent the counts of
705 human neutrophils, on the total of CD45⁺ human cells, preconditioned for 2 h with
706 medium (RPMI 1640 containing 1% of FBS, black bars; *n* = 8) or with IL-4 (150
707 ng/mL, red filled bars; *n* = 10) in the airpouch (left panel) or spleen (right panel). **D**,
708 Counts of human neutrophils preconditioned for 6 h with G-CSF (150 ng/mL, blue
709 empty bars; *n* = 6) or with IL-4 (red filled bars; *n* = 6) collected from the airpouch
710 (left panel) or spleen (right panel). Data are presented as mean ± SD of five (**C**) or
711 three (**D**) independent experiments using unrelated HD. Significance of the
712 differences between groups was calculated using Student's *t*-test; *ns*, Not significant.
713

714 **Figure 5. Modulation of CXC chemokine receptors CXCR1 and CXCR2 on**
715 **human neutrophils. A,** Expression of CXC chemokine receptors CXCR1, CXCR2,
716 and CXCR4 on HD neutrophils following stimulation for 6 h with medium (RPMI
717 1640 containing 1% of FBS, black filled bars), IL-4 (red filled bars), or IL-13 (blue
718 filled bars). Shown are the GMFI of indicated CXC chemokine receptors. Data are
719 shown as mean \pm SD of $n = 10$ donors. Significance of the differences between groups
720 was calculated using one-way ANOVA. **B,** Representative flow cytometry analysis of
721 annexin V and propidium iodide (PI) staining on HD neutrophils treated as in **A.**
722 Numbers in plot indicate the percentage. Experiments were repeated $n = 10$ times with
723 independent, unrelated donors; *ns*, Not significant.
724
725

726 **Figure 6. Human neutrophils from healthy versus allergic individuals. A to D,**
727 Freshly-isolated neutrophils from HD (black open bars) and allergic donors (AD; blue
728 filled bars) were assessed for percentages and counts (**A**; HD $n = 20$, AD $n = 24$),
729 CD16 GMFI (**B**; HD $n = 42$, AD $n = 24$), CXC chemokine receptors CXCR1,
730 CXCR2, and CXCR4 GMFI values (**C**; HD $n = 50$, AD $n = 20$), and MPO GMFI (**D**;
731 HD $n = 29$, AD $n = 16$). In **A**, bar histogram represents neutrophil percentages (left Y
732 axis) and symbols the absolute neutrophil counts (right Y axis) in HD (black bar and
733 circle) and AD (blue filled bar and square). Data in **B** to **D** are presented as GMFI
734 mean \pm SD of different and unrelated donors. **E**, NET formation in HD ($n = 10$) or
735 AD ($n = 11$) neutrophils after stimulation with 100 nM of PMA for 2 h. Neutrophils
736 were stained with DAPI and analyzed by confocal microscopy (left panel), followed
737 by manual counting of NET⁺ neutrophils (middle panel) or automatic quantification
738 of the DNA area (μm^2) using the DANA plug-in for ImageJ (right panel). Scale bar is
739 100 μm . Data are presented as mean \pm SD of several donors. Significance of the
740 differences between groups was calculated using Student's *t*-test; *ns*, Not significant.
741
742

743 **Figure 7. Serum of allergic patients affects NET formation and migration of**
744 **healthy human neutrophils. A,** NET formation in HD neutrophils stimulated for 6 h
745 with HD serum or AD serum, followed by stimulation with PMA (100 ng/mL) for 2 h
746 and assessment. Neutrophils were stained and analyzed as described in **Fig. 6E**. Scale
747 bar is 200 μ m. Data are presented as mean \pm SD of $n = 10$ unrelated donors. **B,**
748 Chemotaxis of HD neutrophils toward a set concentration of CXCL8 (100 ng/mL)
749 following stimulation for 2 h with HD serum (10% in RPMI 1640; grey bars) or AD
750 serum (10% in RPMI 1640; red bars) together with an isotype-matched antibody
751 (Ctrl; filled bars) or an anti-human CD124 antibody (5 μ g/mL; Anti-CD124; striped
752 bars). Shown are percentages of migrated neutrophils. Data are presented as mean \pm
753 SD of $n = 10$ unrelated donors. Significance of the difference between groups was
754 calculated using Student's *t*-test (**A**) or one-way ANOVA (**B**); *ns*, Not significant.

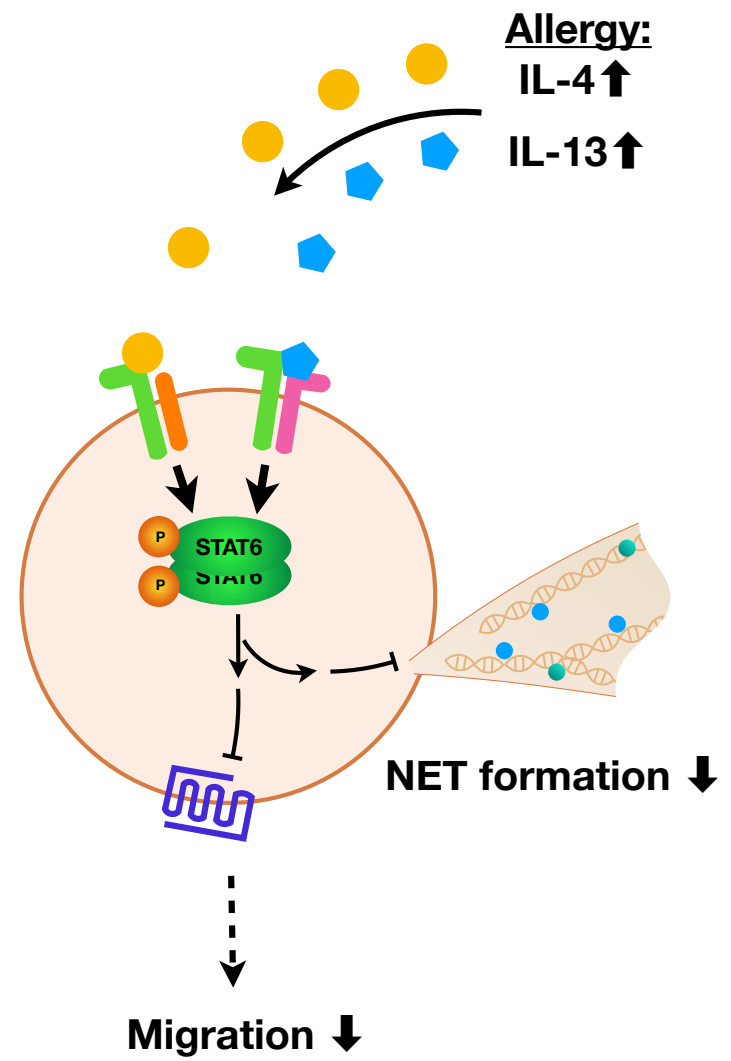
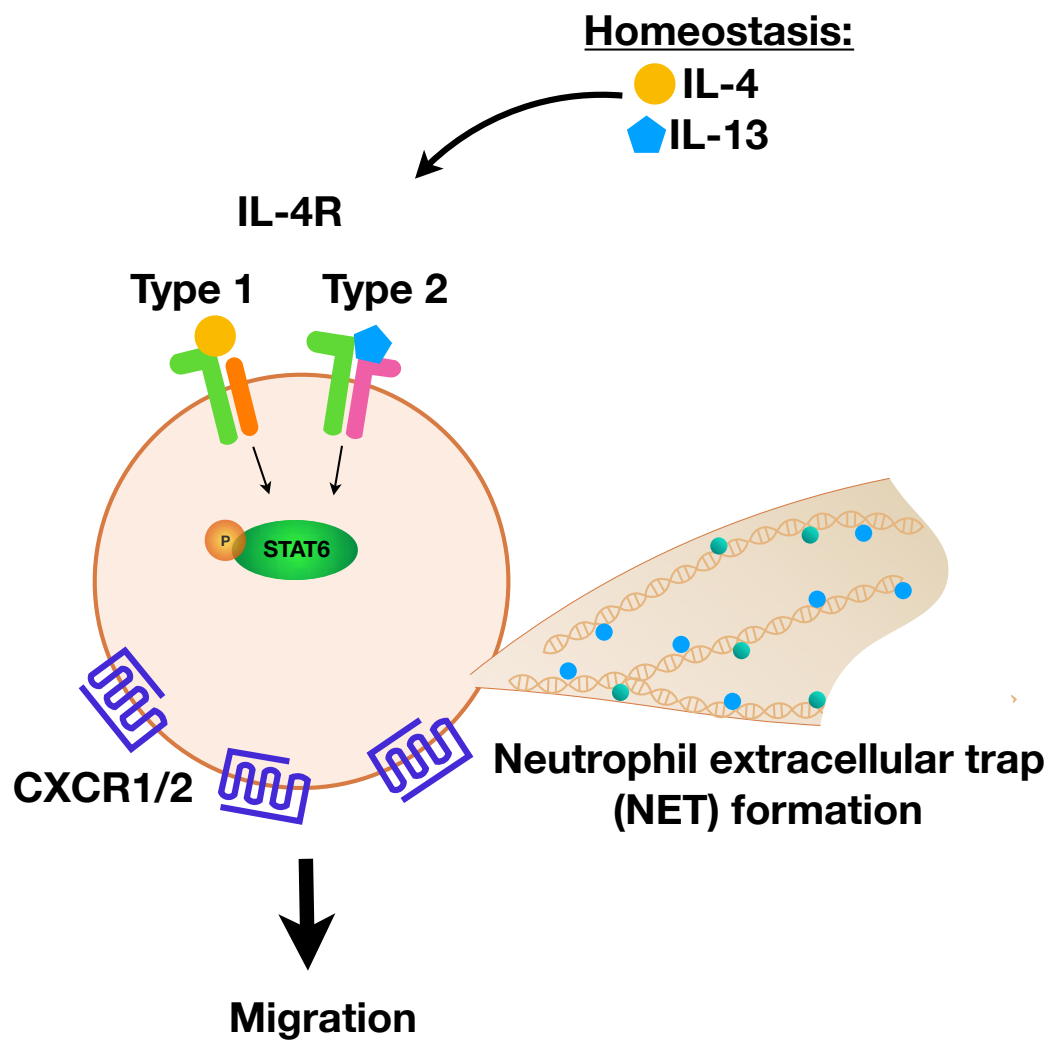


Fig. 1

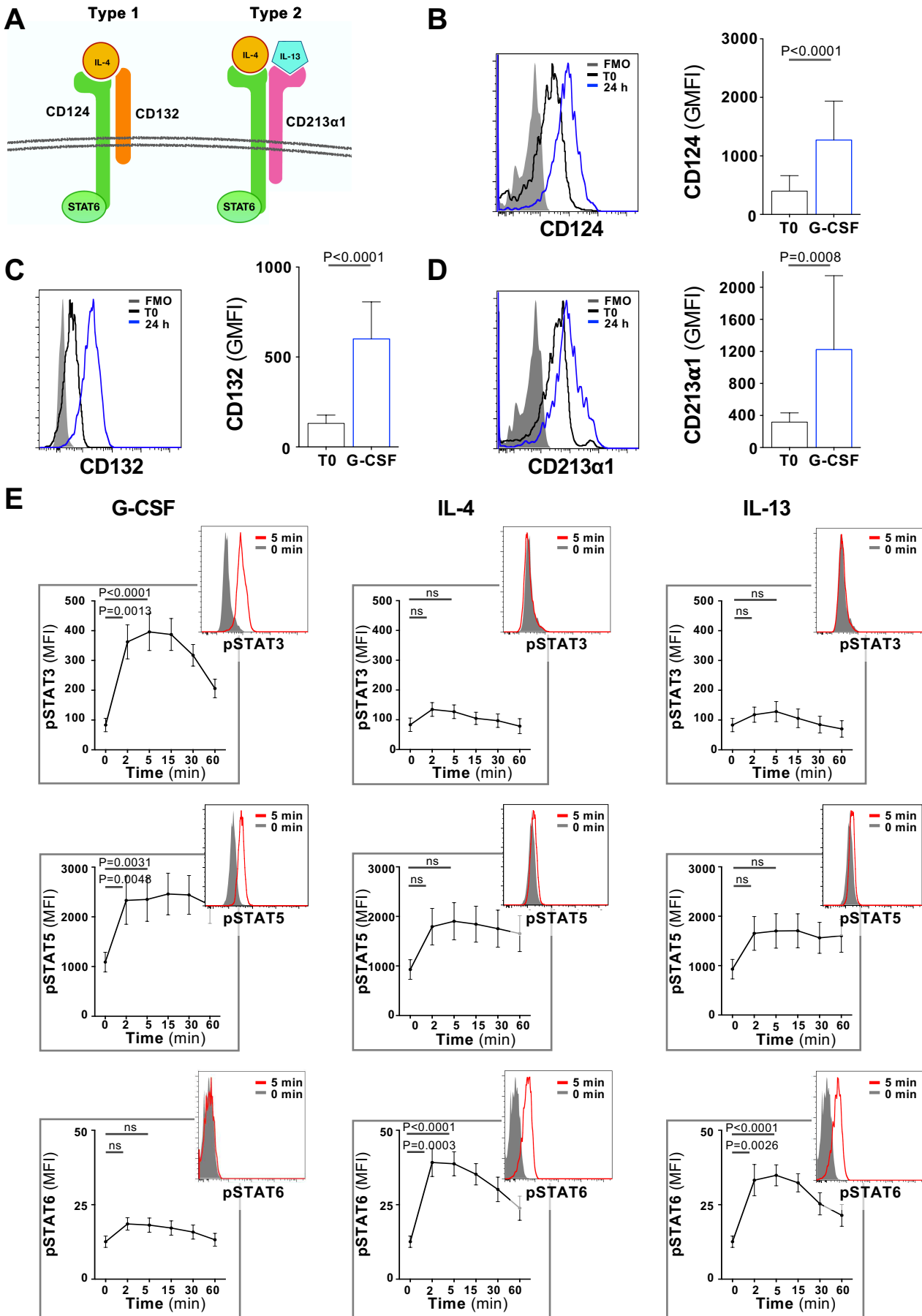
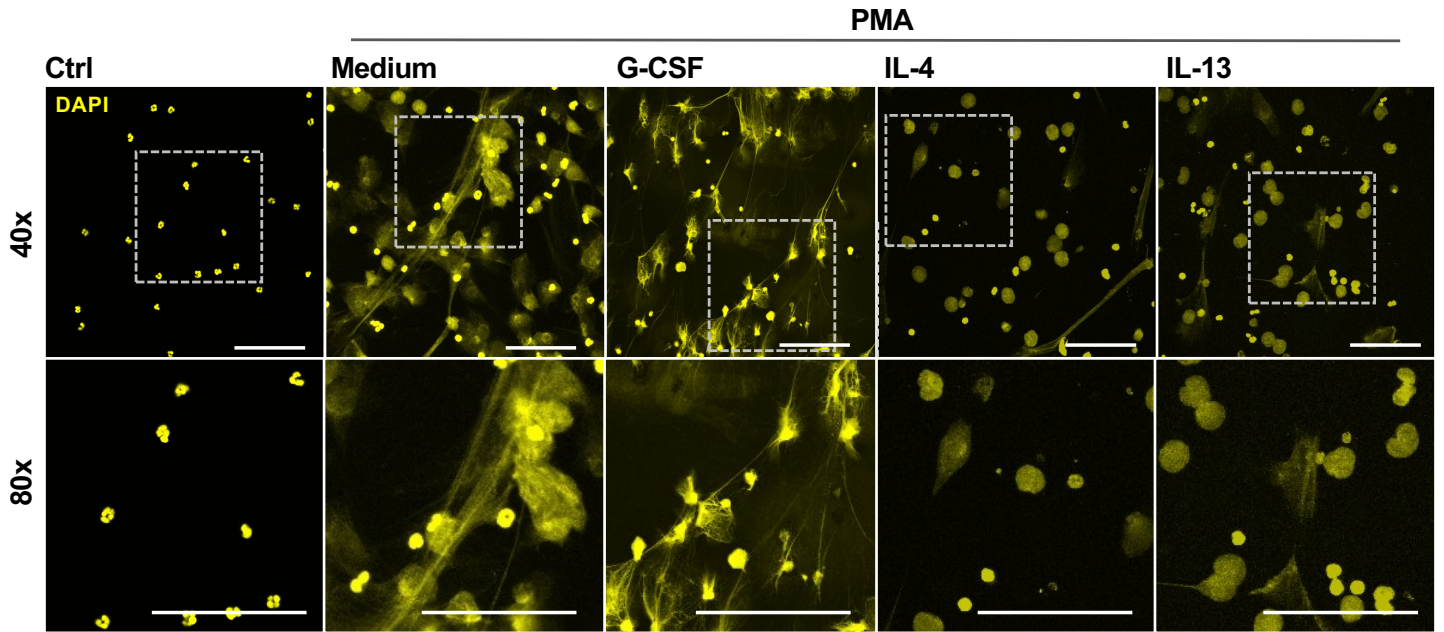


Fig. 2

A



B

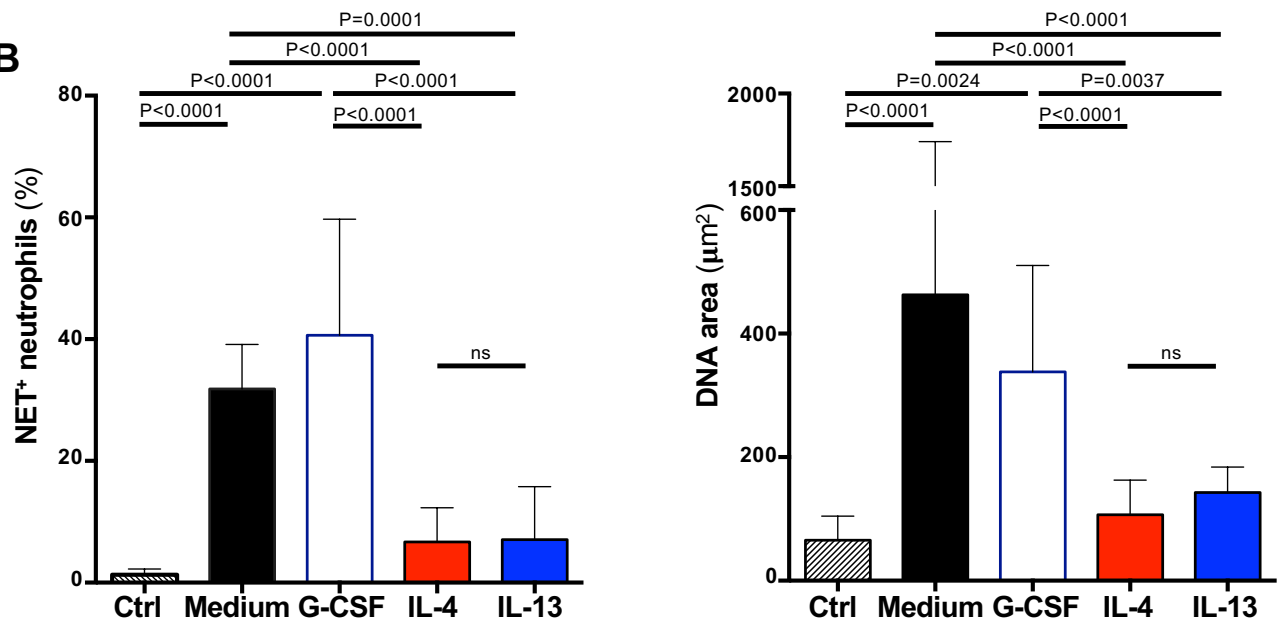


Fig. 3

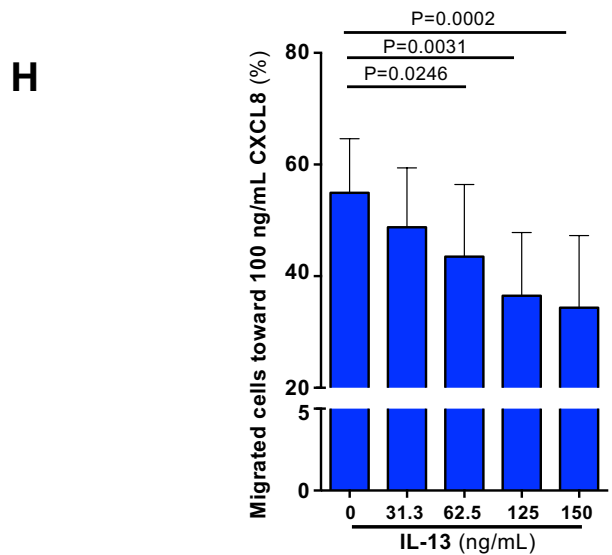
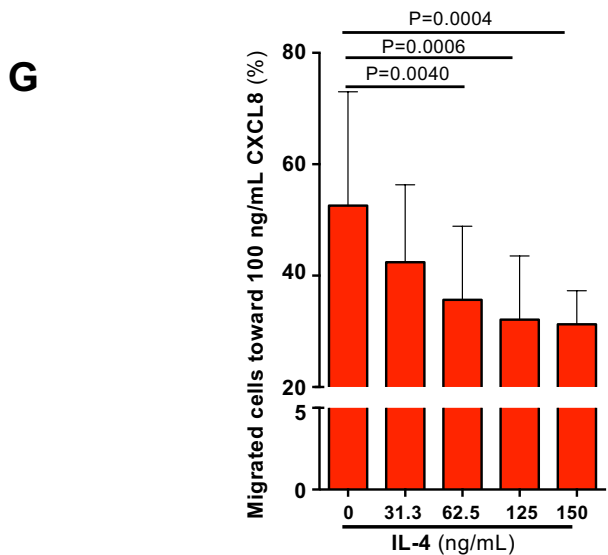
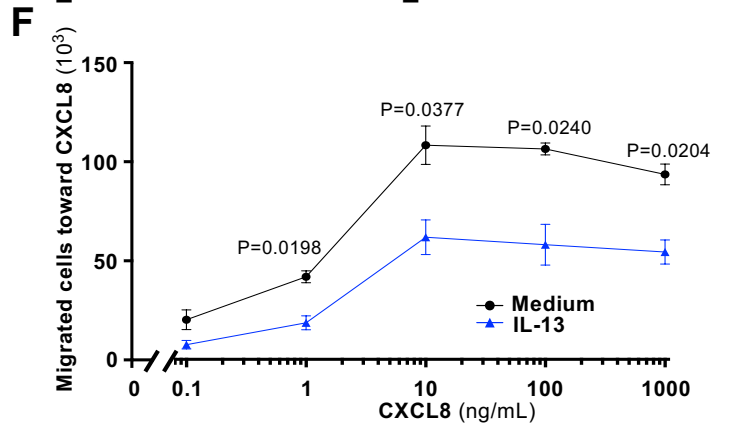
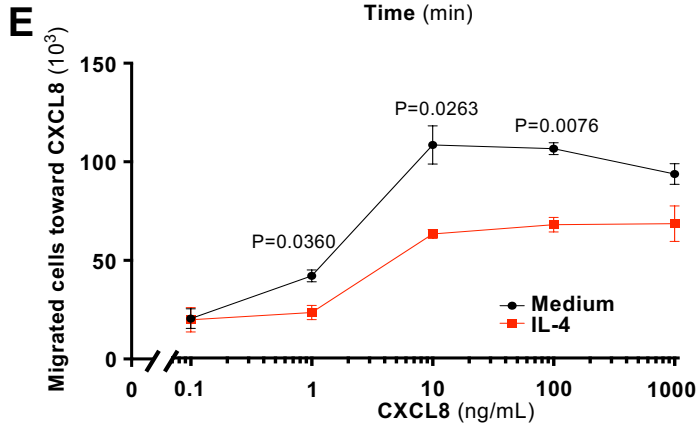
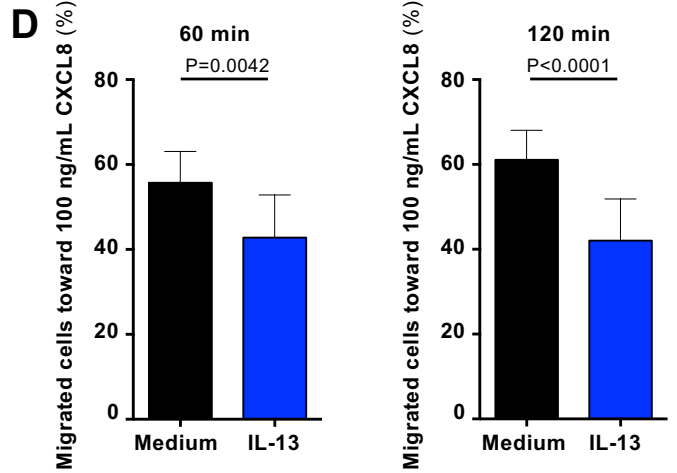
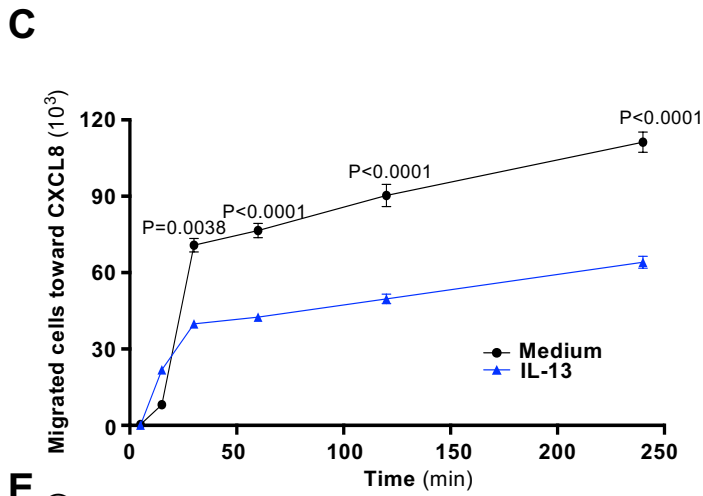
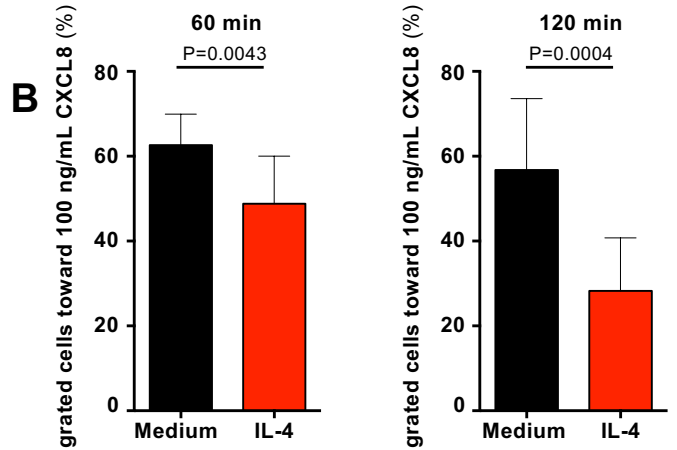
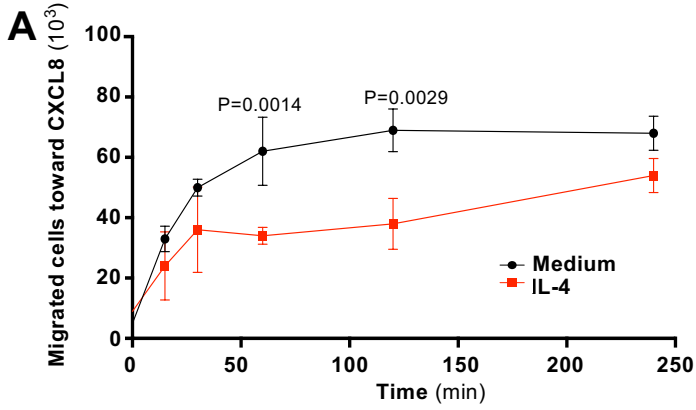
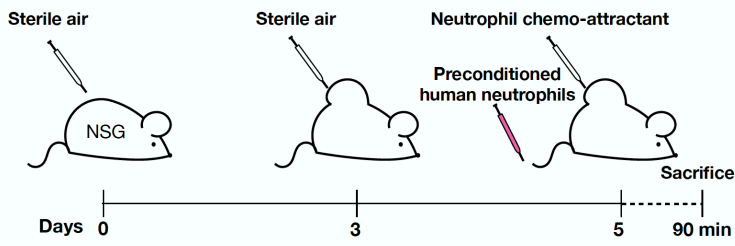
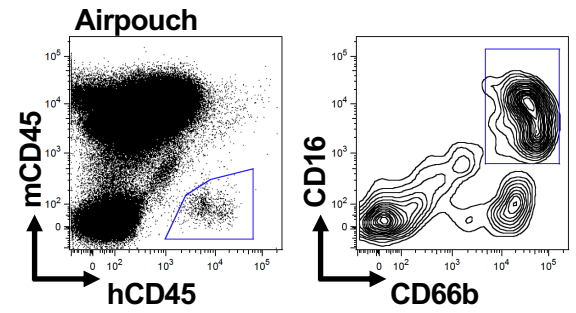


Fig. 4

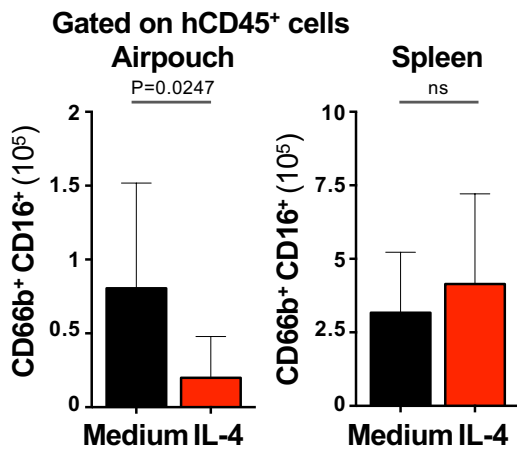
A



B



C



D

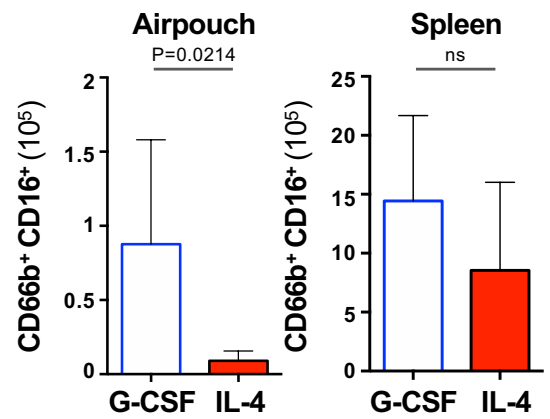
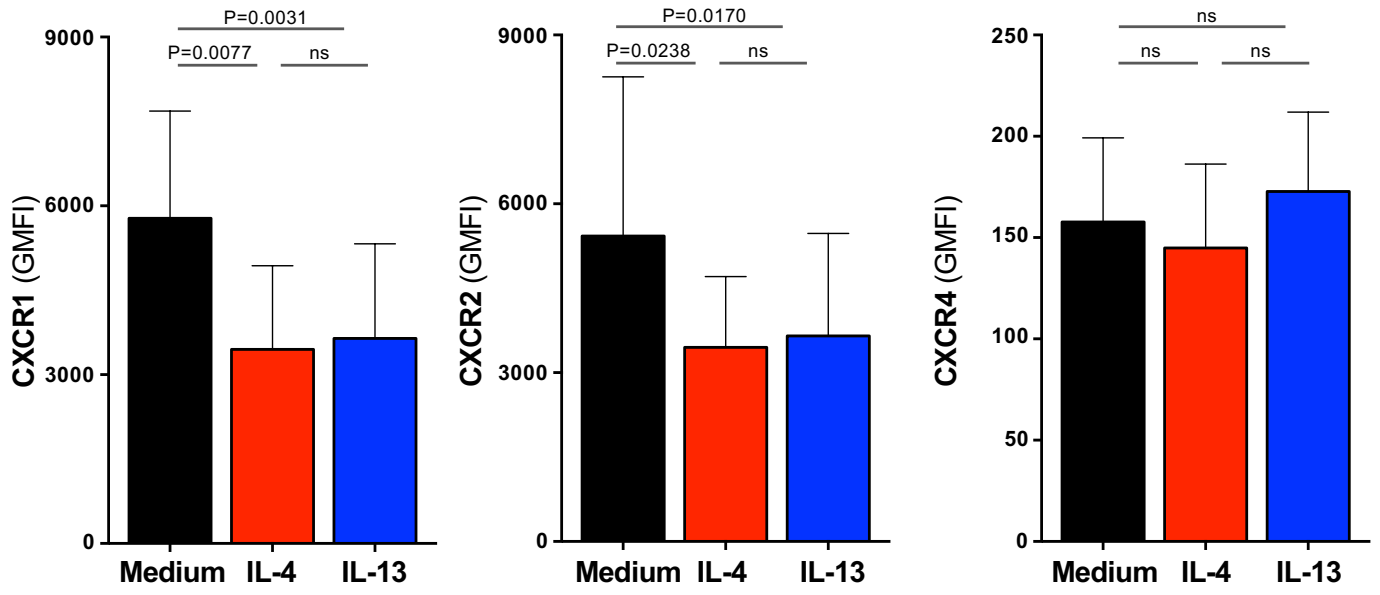


Fig. 5

A



B

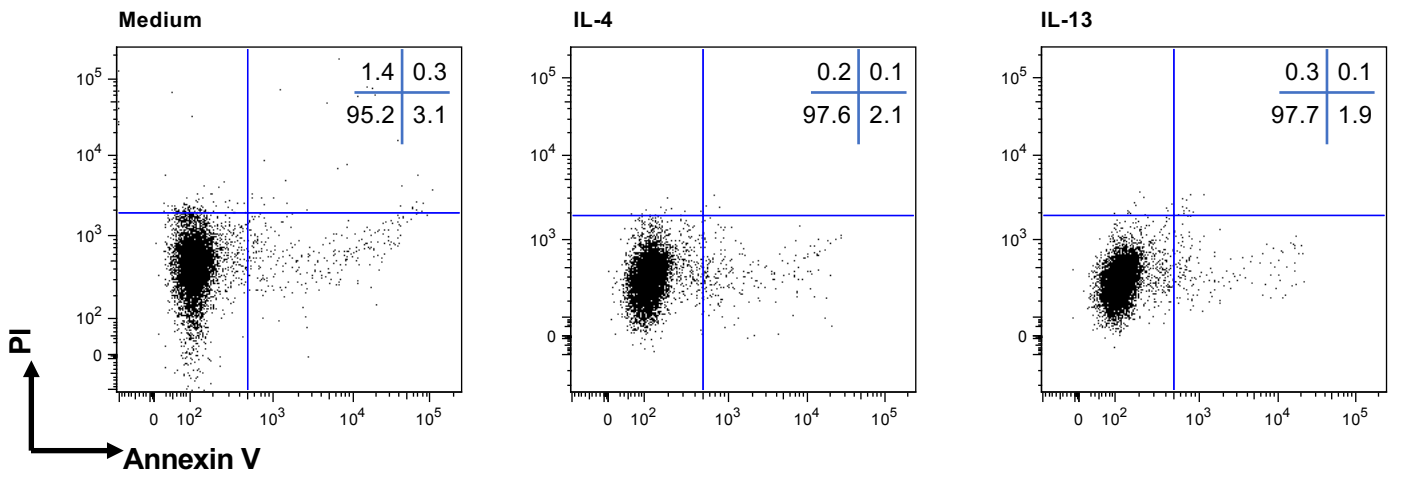


Fig. 6

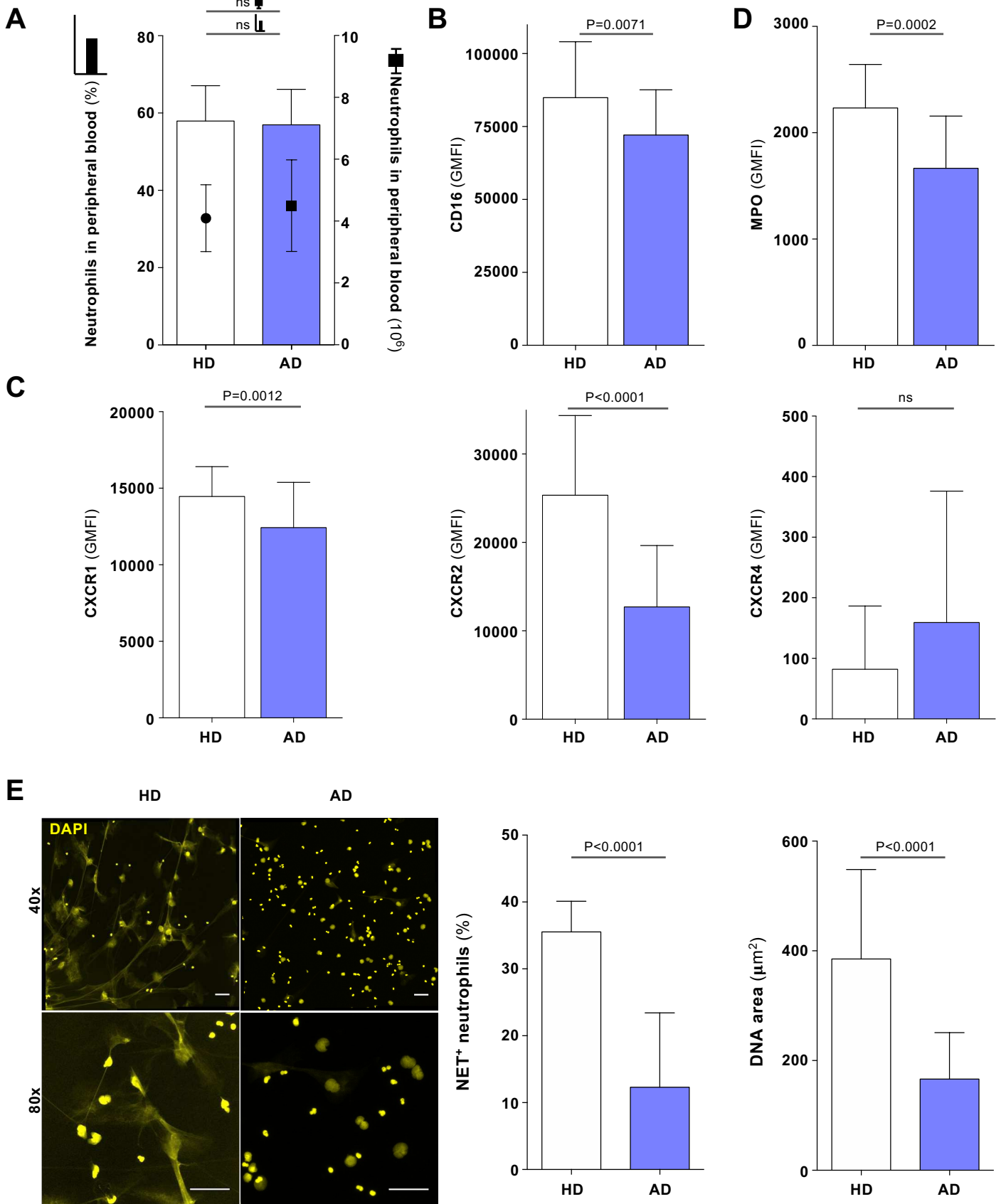
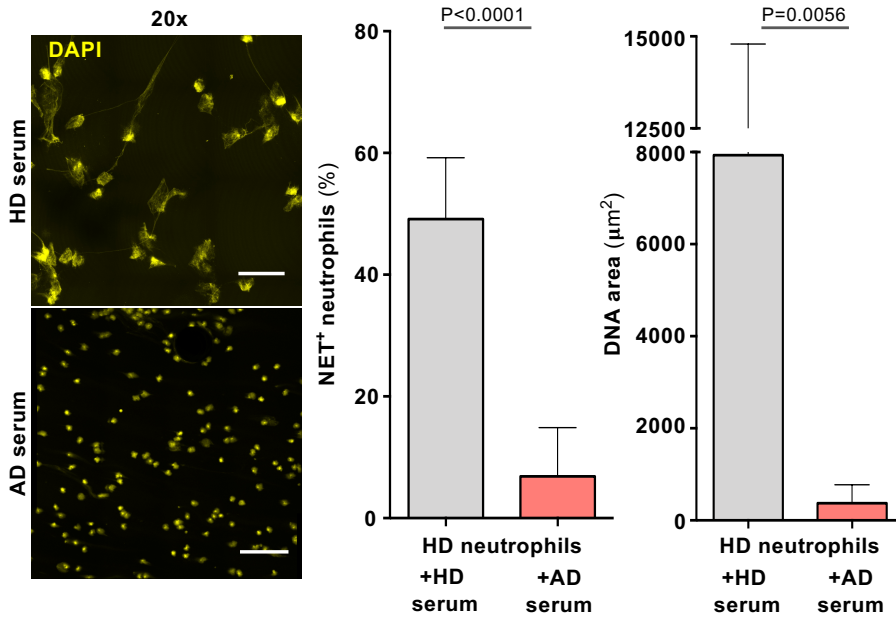


Fig. 7

A



B

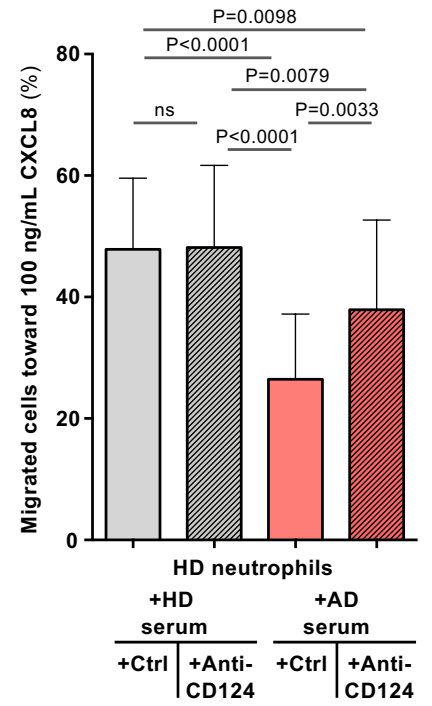
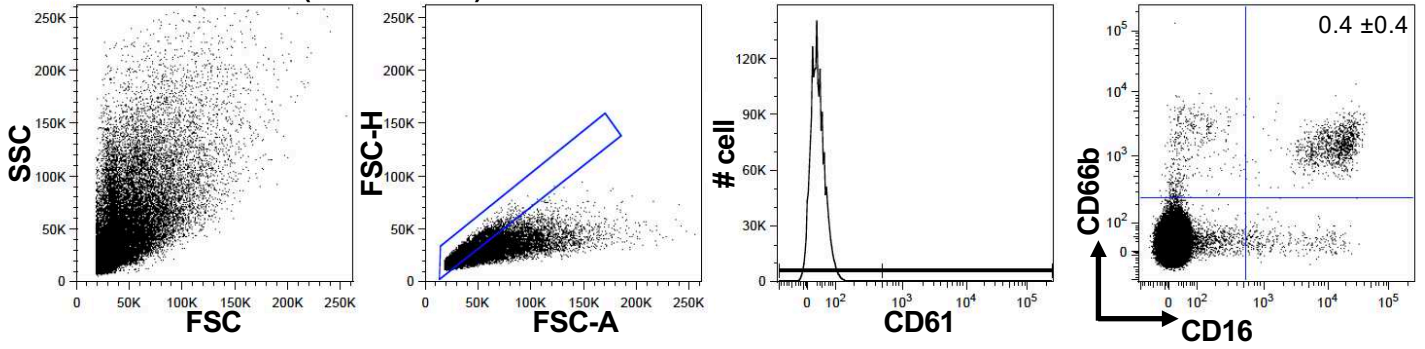


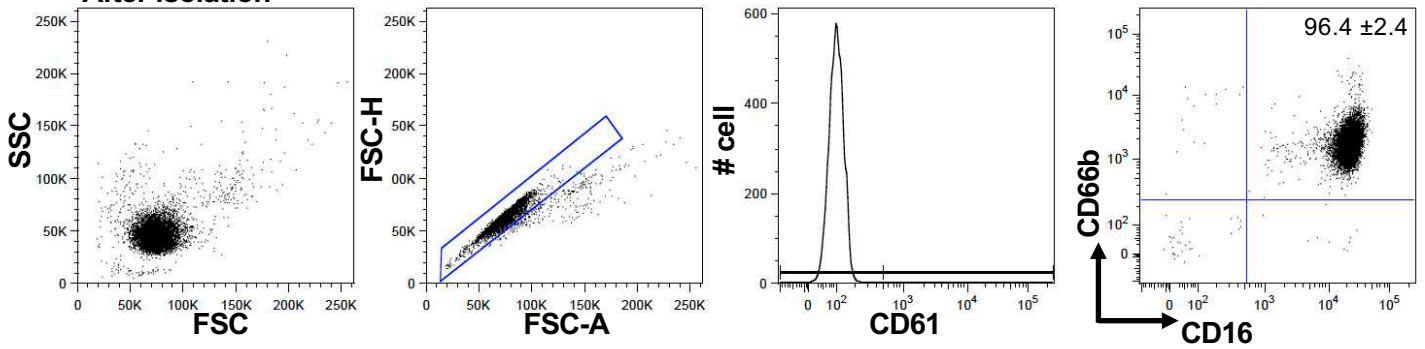
Fig. E1

A

Before isolation (whole blood)

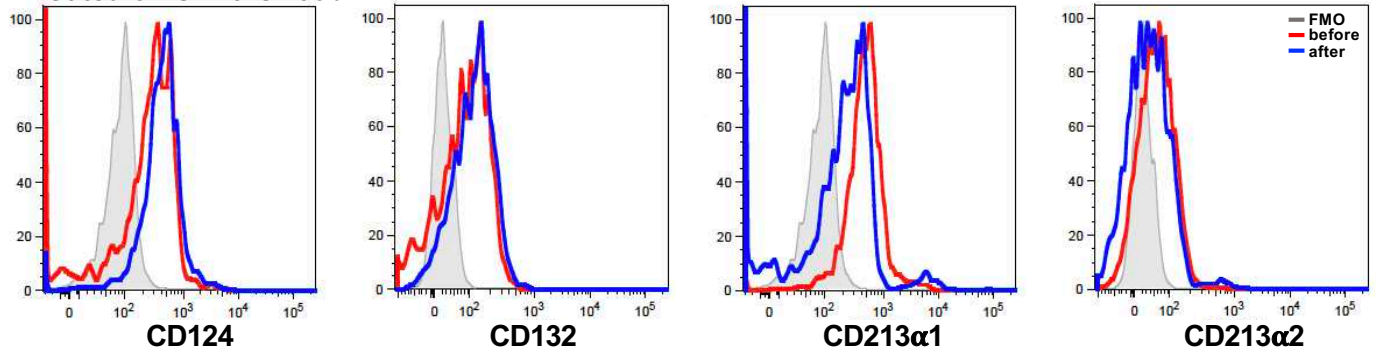


After isolation



B

Gated on CD16⁺CD66b⁺



C

Gated on CD16⁺CD66b⁺

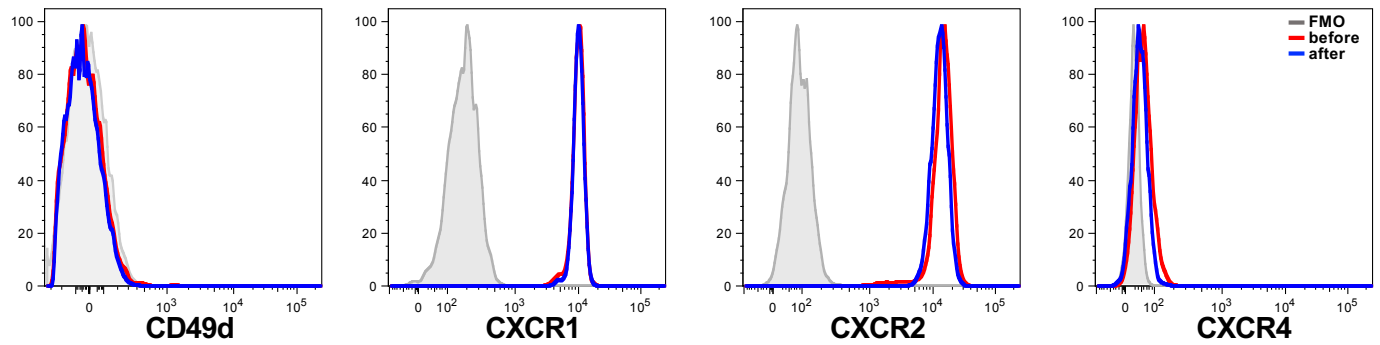


Fig. E2

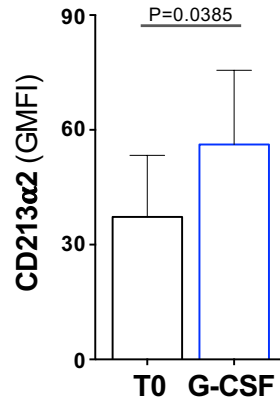
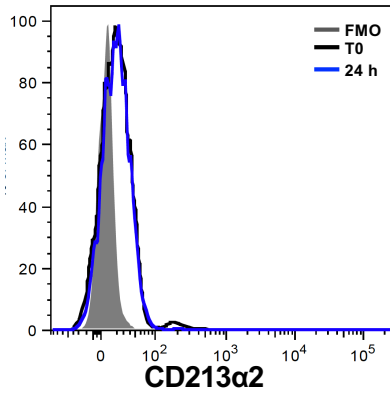


Fig. E3

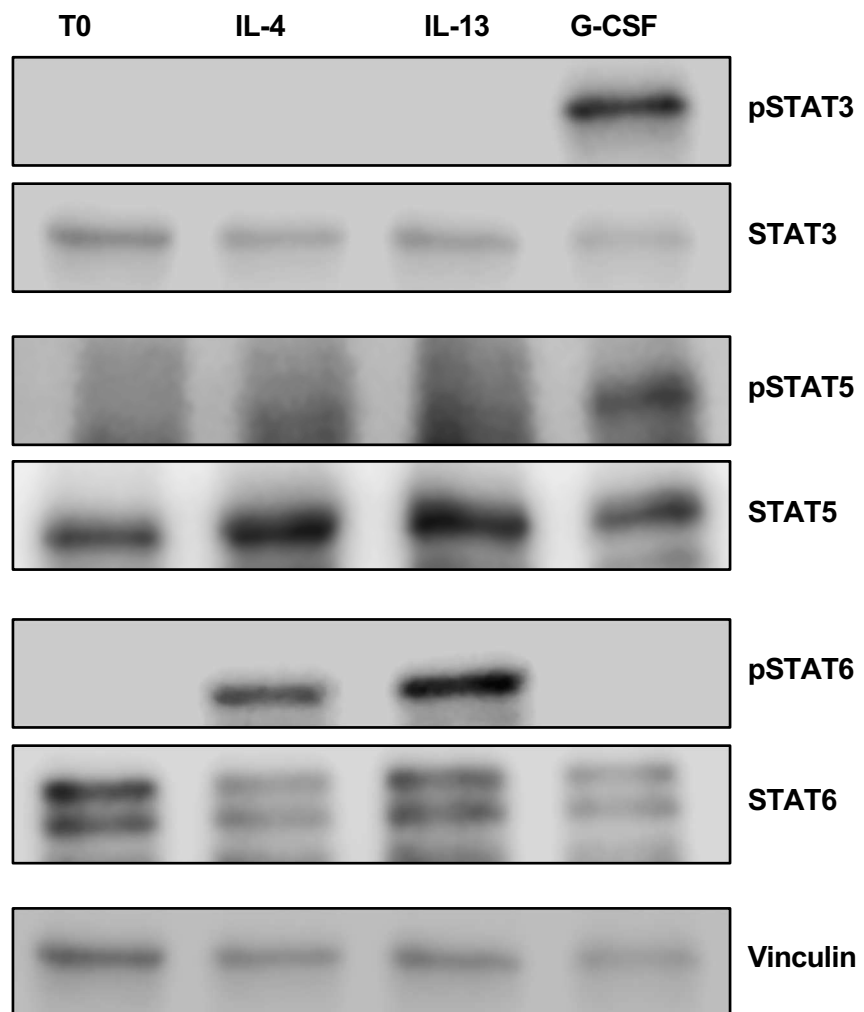


Fig. E4

