

1 **Synthetic Siglec-9 agonists inhibit neutrophil activation associated with COVID-19**

2

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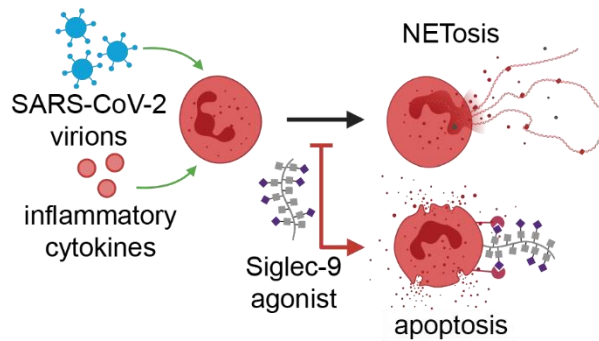
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20 **Abstract.**

21 Severe cases of coronavirus disease 2019 (COVID-19), caused by infection with SARS-CoV-2,
22 are characterized by a hyperinflammatory immune response that leads to numerous
23 complications. Production of proinflammatory neutrophil extracellular traps (NETs) has been
24 suggested to be a key factor in inducing a hyperinflammatory signaling cascade, allegedly
25 causing both pulmonary tissue damage and peripheral inflammation. Accordingly, therapeutic
26 blockage of neutrophil activation and NETosis, the cell death pathway accompanying NET

27 formation, could limit respiratory damage and death from severe COVID-19. Here, we
28 demonstrate that synthetic glycopolymers that activate signaling of the neutrophil checkpoint
29 receptor Siglec-9 suppress NETosis induced by agonists of viral toll-like receptors (TLRs) and
30 plasma from patients with severe COVID-19. Thus, Siglec-9 agonism is a promising therapeutic
31 strategy to curb neutrophilic hyperinflammation in COVID-19.

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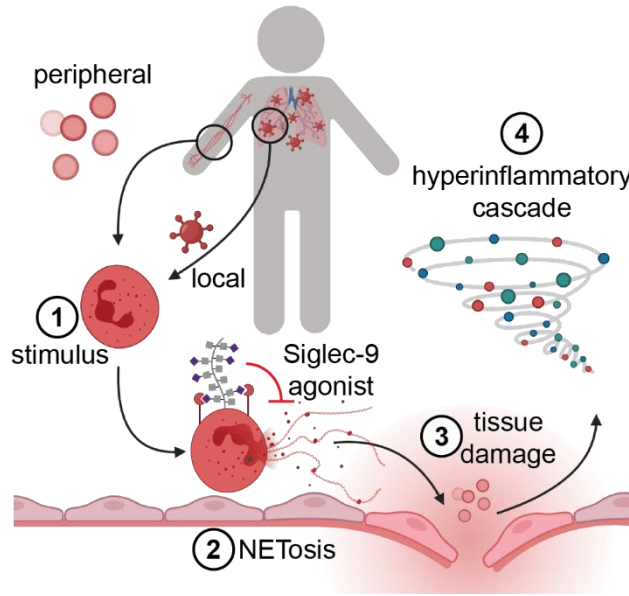
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34 **Abstract Figure.** In COVID-19, viral pathogen associated molecular patterns and viral-induced
35 cytokines can induce NETosis of neutrophils at the site of infection and in the periphery. Siglec-
36 9 agonists inhibit COVID-19 plasma-induced NETosis, potentially preventing deleterious
37 hyperinflammatory responses.

38 **Introduction.**

39 Runaway inflammation in coronavirus disease 2019 (COVID-19) is thought to lead to numerous
40 complications, including potentially fatal pneumonia and acute respiratory distress syndrome
41 (ARDS).¹⁻³ While the specific causal factors of inflammation in COVID-19-related ARDS are
42 unknown and likely multifarious, an emerging hypothesis posits that hyperactivation of
43 neutrophils initiates and drives this response (**Figure 1**).⁴⁻¹² Neutrophils are immune cells of the
44 myeloid lineage that are involved in numerous innate immune functions. It has been suggested
45 that neutrophils drive a hyperinflammatory response in COVID-19 through a death process
46 called NETosis, in which neutrophils rapidly decondense chromatin and spew out a neutrophil
47 extracellular trap (NET), an amalgam of genomic DNA, intracellular proteins (e.g. histones), and
48 tissue-damaging enzymes (e.g. neutrophil elastase, myeloperoxidase).^{13,14} Extracellular DNA
49 and tissue damage from NET-associated enzymes act as proinflammatory signals to other
50 immune cells¹⁵⁻¹⁷ and are proposed to initiate the hyperinflammatory cascade in COVID-19,
51 leading to ARDS and potentially death. Consistent with this hypothesis, NETs have been
52 extensively observed both at the site of infection (i.e., pulmonary tissue)¹⁸⁻²¹ and in the
53 periphery (i.e., sera and plasma).^{19,21}

54



55

56 **Figure 1.** Local and peripheral inflammatory stimuli induce NETosis and a subsequent
 57 hyperinflammatory cascade in COVID-19. Both local inflammatory stimuli at the site of SARS-
 58 CoV-2 infection (e.g. virions) and peripheral inflammatory stimuli (e.g. the proinflammatory
 59 cytokines IL-8 and G-CSF) associated with COVID-19 have been shown to induce NETosis *in*
 60 *vitro*. These factors are suspected to be causative agents of NETosis in those tissues, initiating
 61 a deleterious hyperinflammatory cascade leading to the symptoms of moderate and severe
 62 COVID-19. Agonists of the neutrophil-associated checkpoint receptor Siglec-9 could inhibit
 63 NETosis in COVID-19.

64

65 Both SARS-CoV-2 virions and serum/plasma from COVID-19 patients have been shown to
 66 induce NETosis of neutrophils isolated from healthy donors *in vitro*, consistent with both the
 67 local and peripheral inflammatory responses observed in COVID-19.^{19,21,22} However, the specific
 68 signals that induce NETosis in viral disease remain an open question; viral ligands for toll-like
 69 receptors (TLRs), host damage-associated molecular patterns, antiviral cytokines (e.g., IL-8 and
 70 IFN γ), and activated platelets have all been implicated, but which if any of these is sufficient to
 71 induce NETosis is still debated.^{21,23} Beyond viral disease, NETosis has been demonstrably

72 linked to numerous inflammatory pathologies, including thrombosis and sepsis, both of which
73 are observed in patients with COVID-19.⁴ During NETosis, inflammatory stimuli signal
74 neutrophils to import calcium ions, which activates protein arginine deiminase 4 (PADI4).^{24,25}
75 PADI4 mediates the conversion of arginine to the deiminated citrulline on histones.²⁵ The loss of
76 positive charges induces rapid unwinding of genomic DNA, which eventually ruptures the
77 nucleus and the cell.²⁵ When this happens, intracellular contents including genomic DNA, active
78 PADI4, tissue-damaging NET-associated enzymes, and citrullinated histones are emitted into
79 the extracellular space, all of which provoke an inflammatory response.^{24,25} Thus, strategies to
80 curb neutrophil-mediated inflammation could treat both COVID-19 as well as other neutrophilic
81 inflammatory pathologies.

82

83 Transcriptomic analyses of immune cells from severe COVID-19 patients show that neutrophils
84 upregulate the myeloid checkpoint receptor Siglec-9, a member of the sialic acid-binding
85 immunoglobulin-like lectin (Siglec) family that is also found on macrophages and activated T
86 cells.^{8,9,26–29} This sialoglycan-binding immunosuppressive receptor has an intracellular signaling
87 domain similar to the prominent lymphoid checkpoint molecule PD-1.^{30,31} And analogous to PD-
88 1, clustering of Siglec-9 by virtue of ligand engagement leads to inhibitory signaling that
89 quenches activation of the immune cells. Both erythrocytes and host-mimicking pathogens
90 have been shown to engage Siglec-9 to suppress neutrophil-mediated immunity.^{32–35}
91 Furthermore, engagement of Siglec-9 on primary neutrophils has been shown to induce
92 apoptotic pathways,²⁶ in a manner similar to the engagement of Siglec-8 on eosinophils that
93 recently led to an FDA-approved Siglec-8 agonist for eosinophilic inflammatory conditions.³⁶
94 Given that Siglec-9 is both anti-inflammatory and pro-apoptotic checkpoint molecule, we
95 hypothesized that engagement of Siglec-9 could simultaneously inhibit proinflammatory NETotic
96 cell death and induce quiet apoptotic cell death in COVID-19-related inflammation. Notably, an
97 agonist (CD24Fc, tradename SACCOVID) of the related myeloid checkpoint receptor Siglec-10

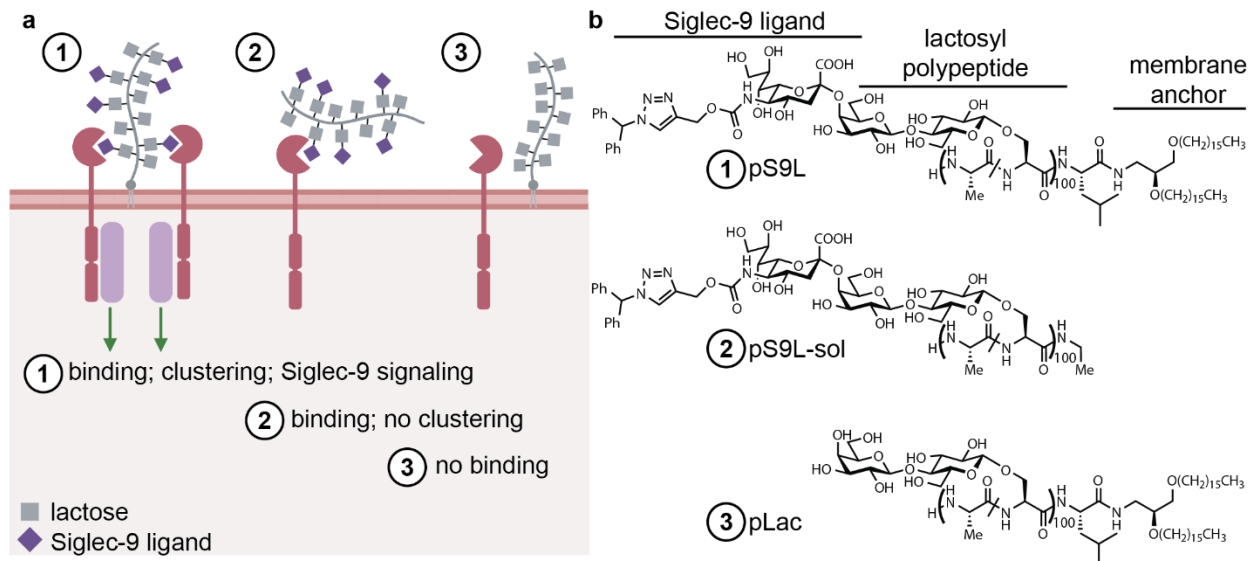
98 has recently shown great promise in suppressing viral hyperinflammation and is in a Phase III
99 clinical trial.^{37,38} However, unlike the CD24/Siglec-10,³⁹ a specific and high-affinity glycoprotein
100 ligand for Siglec-9 has not been described.⁴⁰

101

102 We recently reported⁴¹ the design and synthesis of a potent Siglec-9 agonist comprising a lipid-
103 conjugated glycopolypeptide bearing modified sialic acid residues that Paulson and coworkers
104 had previously found to confer high-affinity and specificity binding to Siglec-9 over other Siglec
105 family members (pS9L, **Figure 2**).⁴² The lipid group enabled passive insertion into cell
106 membranes, leading to engagement of Siglec-9 in cis on macrophage cell surfaces. This cell-
107 surface clustering, in turn, induced Siglec-9 signaling suppress macrophage activation.⁴¹ In this
108 recent study we also designed control glycopolypeptides lacking either Siglec-9 binding glycans
109 (i.e., the lactose-functionalized glycopolypeptide pLac) or a membrane anchoring lipid group
110 (i.e., the soluble glycopolypeptide pS9) (**Figure 2**). Notably, potent Siglec-9 agonism required
111 membrane anchoring and cis-engagement; the soluble congener pS9 was unable to stimulate
112 Siglec-9 signaling and suppress macrophage activity. We hypothesized that pSL9 might also be
113 able to suppress neutrophil activation and NETosis by clustering Siglec-9 on neutrophils.

114

115



116

117 **Figure 2.** Synthetic glycopolypeptides bearing high-affinity Siglec-9 ligands cluster and engage
 118 Siglec-9 signaling. (a) Membrane-anchored and cis binding glycopolypeptide 1 (pS9L) induces
 119 Siglec-9 signaling, while a non-cis binding control polypeptide 2 (pS9L-sol) or a non-binding but
 120 membrane-anchored control polypeptide 3 (pLac) do not. (b) Structures of the polypeptides
 121 **pS9L**, **pS9L-sol**, and **pLac**. Polypeptides are all based on an O-lactosyl poly-serine-co-alanine
 122 scaffold, and in some cases bear terminal Siglec-9 ligands and/or C-terminal membrane-
 123 anchoring lipids.

124

125 Here, we demonstrate that a synthetic cis-binding Siglec-9 agonist (pS9L, **Figure 2b**)⁴¹ inhibits
 126 NETosis in primary neutrophils in models of local (TLR-7/8 agonist) and peripheral (COVID-19
 127 plasma) COVID-19-associated inflammation. Using time-course live cell microscopy, we
 128 showed that TLR-7/8 activation by the nucleoside analog resimiquod (R848) is sufficient to
 129 induce specific NETosis in primary human neutrophils. R848 induces rapid citrullination of
 130 histone substrates, consistent with PADI4-mediated NETosis, and this process was blocked by
 131 Siglec-9 signaling induced by pS9L. Significantly, pS9L inhibited neutrophil NETosis induced by

132 treatment with plasma from severe COVID-19 patients. In light of these data, we propose that
133 Siglec-9 agonists could be therapeutic agents that inhibit COVID-19-associated inflammation.

134

135 **Results and Discussion.**

136 **TLR-7/8 agonist R848 induces NETosis of primary neutrophils *in vitro*.**

137 In COVID-19, evidence of extensive NETosis can be observed in infected lungs,¹⁸⁻²¹ and SARS-
138 CoV-2 virions have been shown to infect and induce NETosis of healthy neutrophils *in vitro*.²⁰
139 These reports implicate TLR-7 and/or TLR-8 in inducing NETosis of neutrophils at the site of
140 infection.^{20,43} Notably, TLR-7 and TLR-8 are ssRNA receptors with numerous substrates
141 identified in the SARS-CoV-2 genome.⁴⁴ Furthermore, consistent with the hypothesis that
142 SARS-CoV-2 induces TLR-7/8-mediated immunity, TLR-7 deficiency is associated with severe
143 COVID-19.⁴⁵ Thus, agonists of TLR-7/8 may provide a convenient means of modeling local
144 inflammation induced by viral infection *in vitro* without using live virus.

145

146 We assayed TLR agonists using the live-cell imaging techniques described by Gupta and
147 coworkers.⁴⁶ In this assay, freshly isolated neutrophils are cultured in low-serum media in the
148 presence of a fluorogenic and membrane impermeable DNA-intercalating dye (Cytotox Green).
149 Upon genomic DNA-externalization by NETosis, dye intercalates and fluorescence increases.
150 As previously demonstrated,⁴⁶ because NETs are much larger than the nuclei of apoptotic cells,
151 NETotic cells yield much larger areas of fluorescence than apoptotic cells, as observed by
152 microscopy. Thus, apoptotic cells can be filtered out by only counting large (i.e., >>100 μm^2)
153 fluorescent objects.

154

155 We found that a TLR-7/8 agonist, R848, was sufficient to induce NETosis of healthy neutrophils
156 *in vitro* (**Figure 3a-c, Figure S1**). We also assayed the citrullination status of the PADI4
157 substrate H3 by Western blot, and observed that R848 rapidly induced citrullination at R2, R8,

158 and R17 (**Figure S2**). Additionally, we performed quantitative phosphoproteomics⁴⁷ with lysates
159 of neutrophils treated with media, phorbol-12-myristate-13-acetate (PMA), or R848 (**Figure S3**,
160 **Table S1**). We observed similar results to previously published datasets using neutrophils
161 stimulated with either R848⁴⁸ or PMA⁴⁹. Furthermore, several phosphosites were found to be
162 differentially regulated in both datasets, including those involved in neutrophil degranulation and
163 calcium flux, consistent with the described mechanism of NETotic death.^{24,49} These results
164 indicate that the TLR-7/8 agonist R848 induces NETosis in primary neutrophils. Thus, this can
165 be used to model local inflammation associated with viral infection, including in COVID-19.

166

167 **A Siglec-9 agonist inhibits TLR-7/8-induced NETosis via SHP-1.**

168 Previous studies by von Guten and coworkers have shown that engagement of Siglec-9 leads to
169 apoptotic and nonapoptotic death pathways as well as immunosuppression in neutrophils.^{26,32}

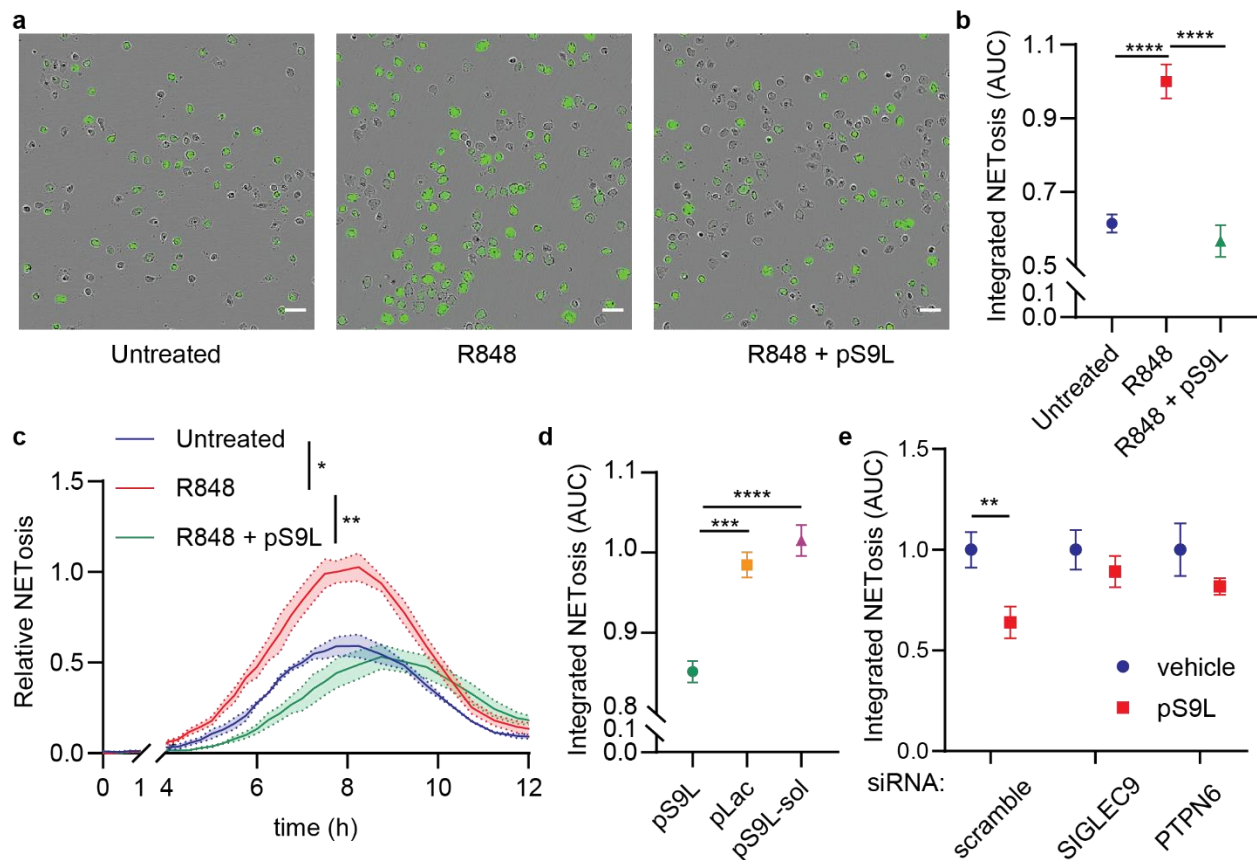
170 Thus, we hypothesized that Siglec-9 mediated immunosuppression and cell death could
171 override the NETotic effect of antiviral TLR signaling. To assay this, we employed the synthetic
172 Siglec-9 cis-binding agonist, pS9L, that we have previously described.⁴¹ pS9L is a lipid-tethered
173 glycopolypeptide, biomimetically inspired by native mucin proteins (**Figure 2**). When the lipid
174 inserts into the cell membrane, the polypeptide backbone can adopt extended conformations,⁵⁰
175 resulting in presentation of multivalent high-affinity Siglec-9 cis ligands.⁴¹ We have previously
176 observed that presentation in this manner results in binding and clustering with Siglec-9 in cis.⁴¹

177 We additionally used two control glycopolypeptides: pLac, a lipid-tethered glycopolypeptide
178 based on the same scaffold as pS9L, but lacking terminal Siglec-9 binding sialosides, and
179 pS9L-sol, a soluble lipid-free analogue of pS9L incapable of binding in cis (**Figure 2, S4**).

180

181 We assayed anti-NETotic activity by cotreatment of glycopolypeptide (500 nM) with R848 (10
182 μ M) in primary neutrophils in the live-cell assay described above (**Figure 3**). We observed that
183 pS9L was sufficient to inhibit NETosis induced by R848 treatment (**Figure 3a-c**). Moreover,

184 neither control polymer inhibited R848-induced NETosis (**Figure 3d**). We also confirmed that
 185 pS9L inhibits NETosis comparably to high concentrations of crosslinked anti-Siglec-9 antibody
 186 (clone 191240) (**Figure S5**).^{51,52} Additionally, we found that treatment with pS9L induced an
 187 oxidative burst in primary neutrophils (**Figure S6**), which von Guten and coworkers described as
 188 an important signaling step of Siglec-9-induced apoptotic signaling.²⁶ Furthermore, the oxidative
 189 burst was inhibited by the addition of the SHP-1/2 inhibitor NSC-87877, suggesting that SHP-1
 190 and/or SHP-2 mediate pS9L-induced oxidative burst in neutrophils, consistent with Siglec-9
 191 engagement (**Figure S6b**).



192
 193 **Figure 3.** A cis-binding Siglec-9 agonist (pS9L) inhibits R848-induced NETosis via Siglec-9 and
 194 SHP-1. (**a-c**) Primary neutrophils were cotreated with R848 (10 μ M) and glycopolypeptide (500
 195 nM) in IMDM supplemented 0.5% hiFBS containing the membrane impermeable DNA
 196 intercalators Cytotox Green or Red (250 nM). Images were acquired by fluorescence

197 microscopy every 15 min for 12 h. The area of all green fluorescent objects $>300 \mu\text{m}^2$ was
198 quantified and the total area was averaged across three images per well. Relative NETosis was
199 determined by normalizing to the maximal NET area from R848 treatment alone ($t = 8 \text{ h}$). **(a)**
200 Representative phase contrast and fluorescence images from $t = 8 \text{ h}$. Scale bars indicate 40
201 μm . **(b)** Quantitation of NETosis over time as area under the curve in **(c)**. Error bars represent
202 SD. **(c)** NET formation and degradation as a function of time. Error bands represent SEM. **(d)**
203 Treatment of R848-stimulated neutrophils with various glycopolypeptides. Error bars represent
204 SD. **(e)** pS9L is a mucin-like glycopolypeptide that bears high affinity and specific ligands for
205 Siglec-9 and is functionalized with a membrane-tethering lipid tail. **(f)** HL-60 cells were
206 transfected with siRNAs against SIGLEC9 (encoding Siglec-9), PTPN6 (encoding SHP-1), or a
207 scrambled control and then grown for two days. Cells were then cotreated with R848 ($10 \mu\text{M}$)
208 and vehicle or pS9L (500 nM). Relative NETosis is determined as in **(b)**, except all objects >200
209 μm^2 were quantified and the R848 maximum in dHL-60's was observed at 2.5 h post induction.
210 Error bars represent SD. Statistics were determined by two-way ANOVA **(b)** or one-way ANOVA
211 **(c,d,f)**. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

212

213 We performed quantitative phosphoproteomics using lysates of R848-stimulated primary
214 neutrophils cotreated with vehicle, pS9L, or pLac (**Figure S3, Table S2**). Notably, we found
215 increased phosphorylation of hyccin (HYCCI / FAM126A), a key component in phosphorylation
216 of phosphoinositides,⁵³ a class of signaling molecules implicated in mediating NETosis.⁵⁴
217 Additionally, we observed increased phosphorylation of RASAL3 (RASL3), a negative regulator
218 of the MAPK signaling pathway.⁵⁵ These data suggest that pS9L inhibits the calcium flux and
219 NADPH activity necessary for NETosis, as well as the MAPK-suppressive effects that have
220 been previously described for pS9L in macrophages.⁴¹

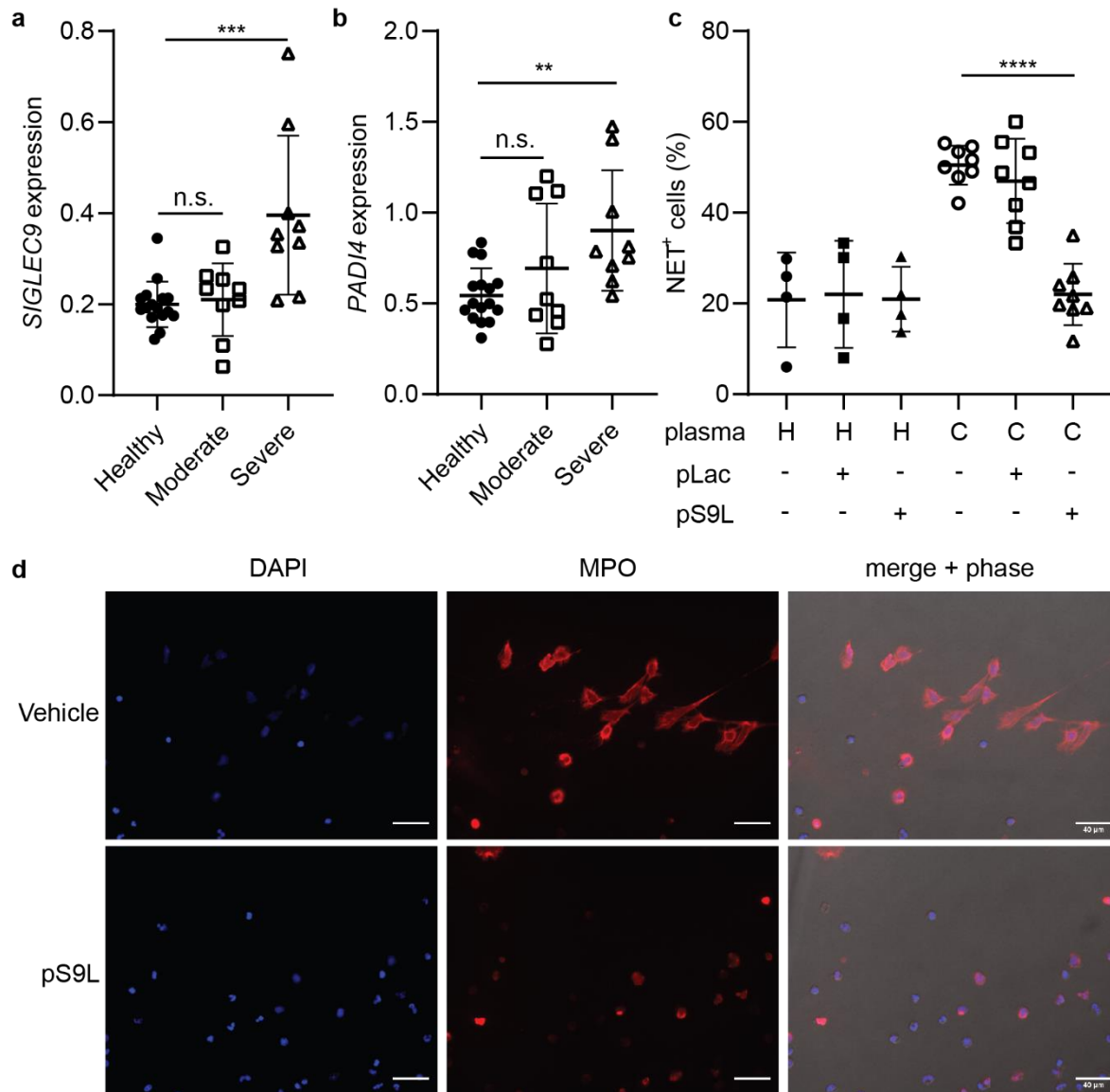
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222 To determine whether the anti-NETotic effect of pS9L is specifically mediated by Siglec-9
223 signaling, we recapitulated our results in the HL-60 cell line. The HL-60 line is a promyelocytic
224 leukemia that can be differentiated into a neutrophil-like cells (dHL-60) using all-trans retinoic
225 acid (ATRA, 100 nM) and dimethylsulfoxide (DMSO, 1.25% v/v), which have previously been
226 used to study NETosis *in vitro*.^{31,56} We observed that R848 induced NETosis in dHL-60 cell's
227 **(Figure S7)**. Furthermore, we observed that pS9L inhibited NETosis and that siRNA knockdown
228 of Siglec-9 (encoded by *SIGLEC9*) or SHP-1 (encoded by *PTPN6*) abrogated the effect of pS9L
229 **(Figure 3e, S8, S9)**. Therefore, the Siglec-9 agonist pS9L inhibits TLR7/8-induced NETosis via
230 Siglec-9 and SHP-1. This suggests that Siglec-9 agonists could inhibit NETosis at the site of
231 viral infection, thus preventing pulmonary inflammation in COVID-19.

232

233 **Siglec-9 is upregulated in severe COVID-19 and can inhibit NETosis induced by COVID-19**
234 **plasma.**

235 Sera and plasma from COVID-19 patients have been shown to be sufficient to induce NETosis
236 of neutrophils isolated from healthy donors *in vitro*.^{19,21} The causes of this are unclear, however
237 potential factors include viral TLR ligands, damage-associated molecular patterns that bind
238 TLRs, activated platelets, and (pro)inflammatory cytokines. Recent reports have described
239 increased levels of neutrophil-activating cytokines, predominantly IL-8 and G-CSF.⁵⁷ Consistent
240 with this observation, we also observed that the combination of IL-8 and G-CSF was sufficient to
241 induce NETosis *in vitro* **(Figure S10)**. Additionally, transcriptomic analyses of peripheral myeloid
242 cells⁸ and neutrophils⁹ in COVID-19 patients have observed increased *SIGLEC9* expression
243 **(Figure 4a, S11)** and *PADI4* expression **(Figure 4b, S11)**. We hypothesize that this is an
244 exhaustion-like phenotype of induced Siglec-9 expression of hyper-NETotic neutrophils, similar
245 to what has been observed with Siglec-9 on exhausted tumor-infiltrating T cells.⁵¹ This makes
246 Siglec-9 an attractive target for therapeutic blockade of hyperinflammatory NETosis in COVID-
247 19.



248

249 **Figure 4.** A Siglec-9 agonist inhibits NETosis of neutrophils induced by COVID-19 plasma. (a,b)

250 Analysis of publicly available single-cell transcriptomics data ⁸ for *SIGLEC9* expression (a) and

251 *PADI4* expression (b) on neutrophils in peripheral blood from healthy donors or COVID-19

252 patients. Error bars represent SD. Statistics were determined using mixed effects model. ** = p

253 < 0.01; *** = p < 0.001 (c,d) Primary neutrophils were cultured in undiluted and citrate

254 anticoagulated plasma from healthy donors or COVID-19 patients for 4 h. Cells were fixed,

255 stained for extracellular myeloperoxidase, and imaged in DAPI imaging media by fluorescence

256 microscopy. Cells were treated in technical triplicate and imaged across multiple fields of view.

257 (c) Proportion of NET-positive cells (%) across all fields of view. Each dot represents and
258 individual plasma sample. (d) Representative images from a COVID-19 patient plasma sample
259 with or without pS9L. Error bars represent SD. Statistics were determined using mixed effects
260 models to account for samples using repeat neutrophil donors. **** = $p < 0.0001$.

261

262 To test the hypothesis that pS9L can inhibit NETosis induced by COVID-19 plasma, we treated
263 neutrophils isolated from whole blood of healthy donors with citrate-anticoagulated heterologous
264 plasma from healthy donors or COVID-19 patients. Neutrophils in undiluted plasma were
265 cotreated with pS9L (500 nM), the non-binding analog pLac (500 nM), or vehicle. To satisfy
266 biosafety restrictions, cells were incubated in the presence of COVID-19 plasma for 4 h and
267 then fixed before assaying for extracellular complexes of myeloperoxidase (MPO) and DNA
268 (DAPI) (**Figure 4c,d**). The combination of these stains, which when observed extracellularly is
269 indicative of NETosis, has been previously used to identify NET⁺ cells in the context of
270 COVID-19.²⁰ We observed that COVID-19 plasma induced NETosis of neutrophils from healthy
271 donors, and that this effect was inhibited by pS9L (**Figure 4c,d**). This is indicated by the distinct
272 web-like morphology of NETs (**Figure 4d**). Furthermore, we observe that pLac does not inhibit
273 COVID-19-induced NETosis, and that neither polymer affects basal NETosis of *in vitro* cultured
274 neutrophils (**Figure 4c**). Additionally, we performed similar experiments staining neutrophils
275 treated with 10% plasma in IMDM (**Figure S12**) or undiluted plasma (**Figure S13**) for
276 extracellular H1/DNA complexes, another marker of NETs,⁵⁸⁻⁶⁰ and observed comparable
277 results.

278

279 Collectively, these data demonstrate that Siglec-9 agonists are sufficient to inhibit NETosis
280 induced by COVID-19 patient plasma, and thus could inhibit peripheral inflammation in patients
281 with COVID-19. Additionally, Siglec-9 agonists could resolve NET-associated pathologies
282 observed in COVID-19 and elsewhere such as immunothrombosis²¹ and sepsis.^{4,5}

283 **Conclusion.**

284 Here, we have demonstrated that Siglec-9 agonists can inhibit NETosis induced by COVID-19-
285 associated proinflammatory signals. Thus, if NETosis is a causal factor of COVID-19-related
286 hyperinflammation as has been hypothesized, Siglec-9 is a therapeutic target to inhibit
287 potentially fatal hyperinflammation associated with COVID-19 in an analogous fashion to the
288 highly effective therapeutics currently aimed at the Siglec-10/CD24 interaction. A CD24-Fc
289 fusion protein has been shown to both engage Siglec-10 as an immune checkpoint on
290 macrophages and sequester the nuclear protein HMGB1, which can act as a damage
291 associated molecular pattern by engaging TLR4.³⁹ The Siglec-9 agonists described here have
292 previously been shown to inhibit macrophage TLR4 signaling and engage macrophage Siglec-
293 9.⁴¹ Thus, Siglec-9 agonists may be multipurpose therapeutics, able to both inhibit the clinically
294 unaddressed problem of proinflammatory NETosis and also subsequent inflammatory signaling
295 from tissue damage that is currently being clinically investigated. Finally, Siglec-9 agonists have
296 the potential to expand beyond ARDS to other NET-related pathologies such as thrombosis,^{61,62}
297 atherosclerosis,⁶³ and cystic fibrosis.⁶⁴

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323

324 **Conflicts of Interest.**

325 C.S.D. and C.R.B. are coinventors on a patent application for cis-binding Siglec agonist
326 glycopolymers as immune suppressants (USPTO63046140). C.R.B. is a co-founder and
327 Scientific Advisory Board member of Lycia Therapeutics, Palleon Pharmaceuticals, Enable
328 Bioscience, Redwood Biosciences (a subsidiary of Catalent), and InterVenn Bio, and a member
329 of the Board of Directors of Eli Lilly & Company. C.A.B. is a Scientific Advisory Board member of
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331

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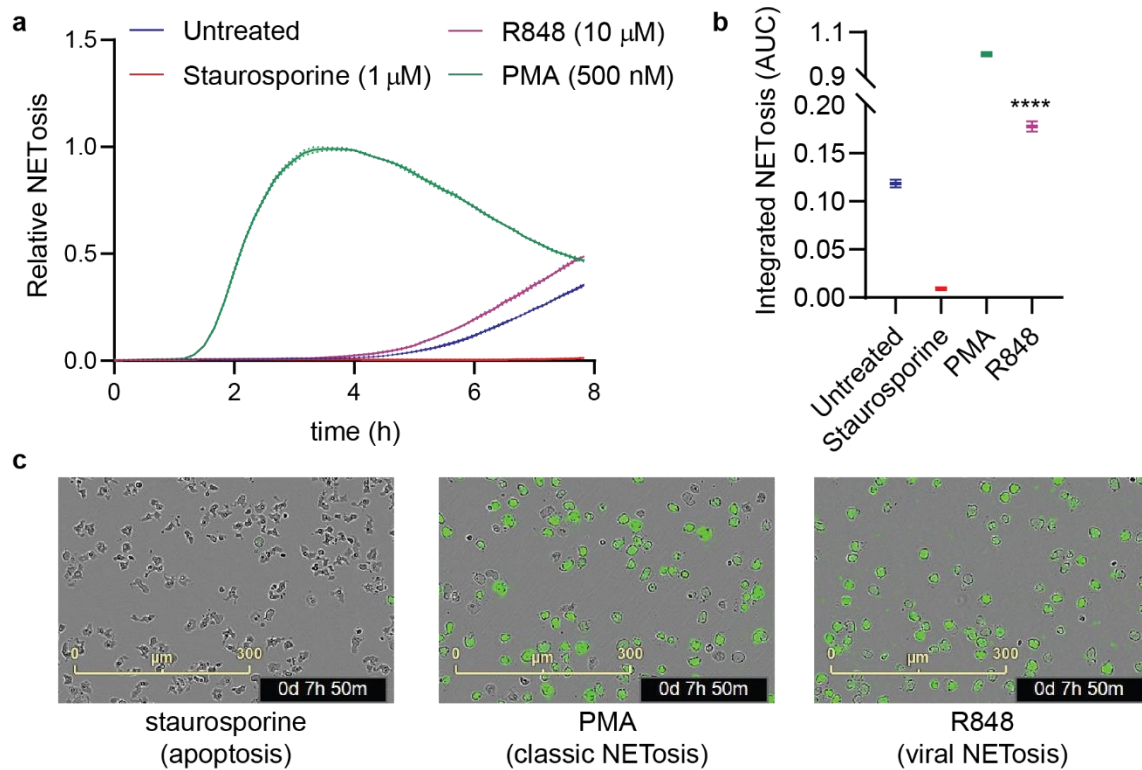
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596 **Supplementary Figures.**

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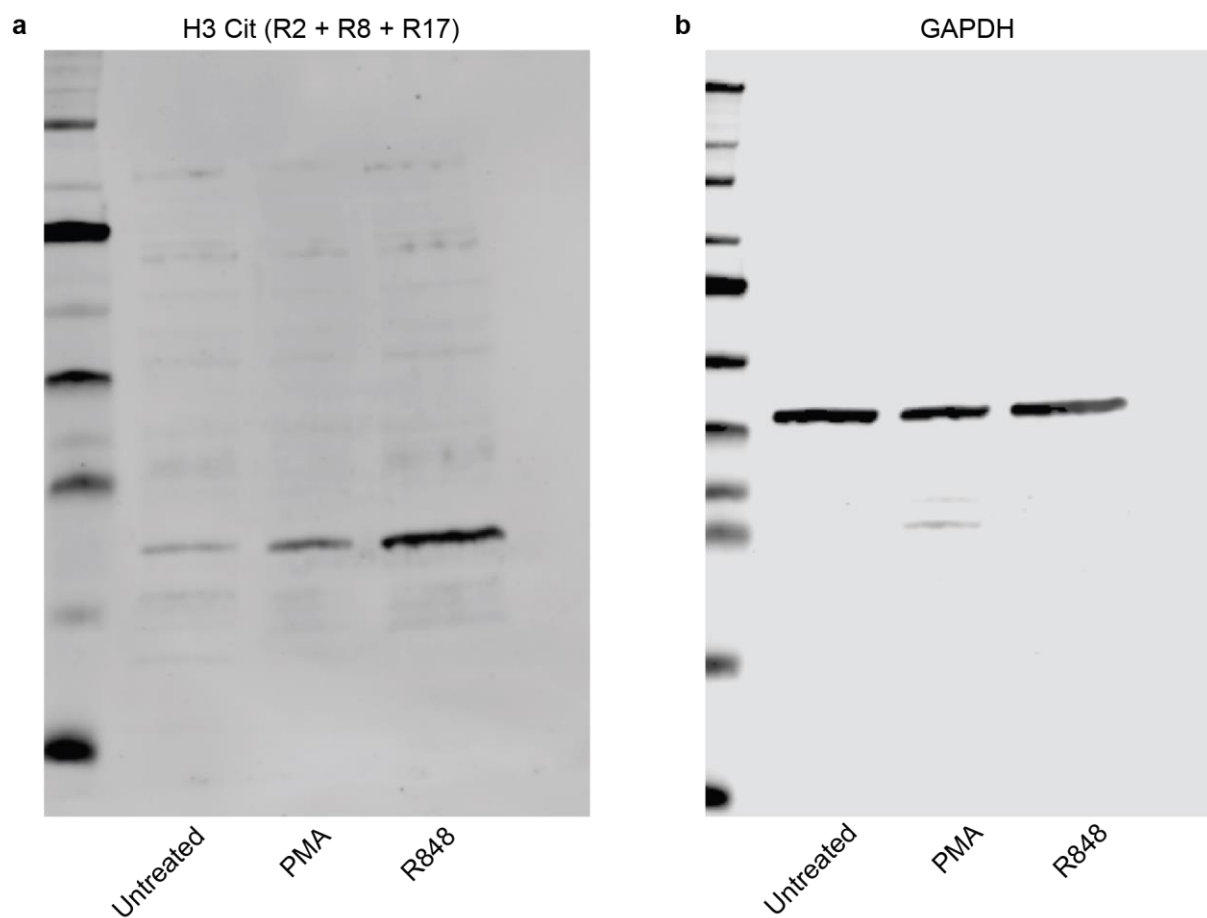


598

599 **Figure S1.**TLR-7/8 agonist R848 induces NETosis in primary neutrophils. **(a-c)** Primary
600 neutrophils were treated with compounds at the concentrations detailed in **(a)** in IMDM
601 supplemented 0.5% hiFBS containing the membrane impermeable DNA intercalator Cytotox
602 Green (250 nM). Images were acquired by fluorescence microscopy every 10 min for 8 h. The
603 area of all green fluorescent objects $>300 \mu\text{m}^2$ was quantified and averaged across three
604 images per well. Relative NETosis was determined by normalizing to the maximal NET area
605 from PMA treatment ($t = 3 \text{ h}$). **(a)** Time-course data for NET formation and degradation over
606 time. Error bands represent SEM. **(b)** Quantitation of **(a)** as area under the curve. Error bars
607 represent SD. All data are representative of multiple independent experiments using neutrophils
608 from different donors. **** $p < 0.0001$. **(c)** Representative images of neutrophils treated with
609 staurosporine, PMA, or R848 stained with Cytotox Green at $t = 7 \text{ h } 50 \text{ m}$.

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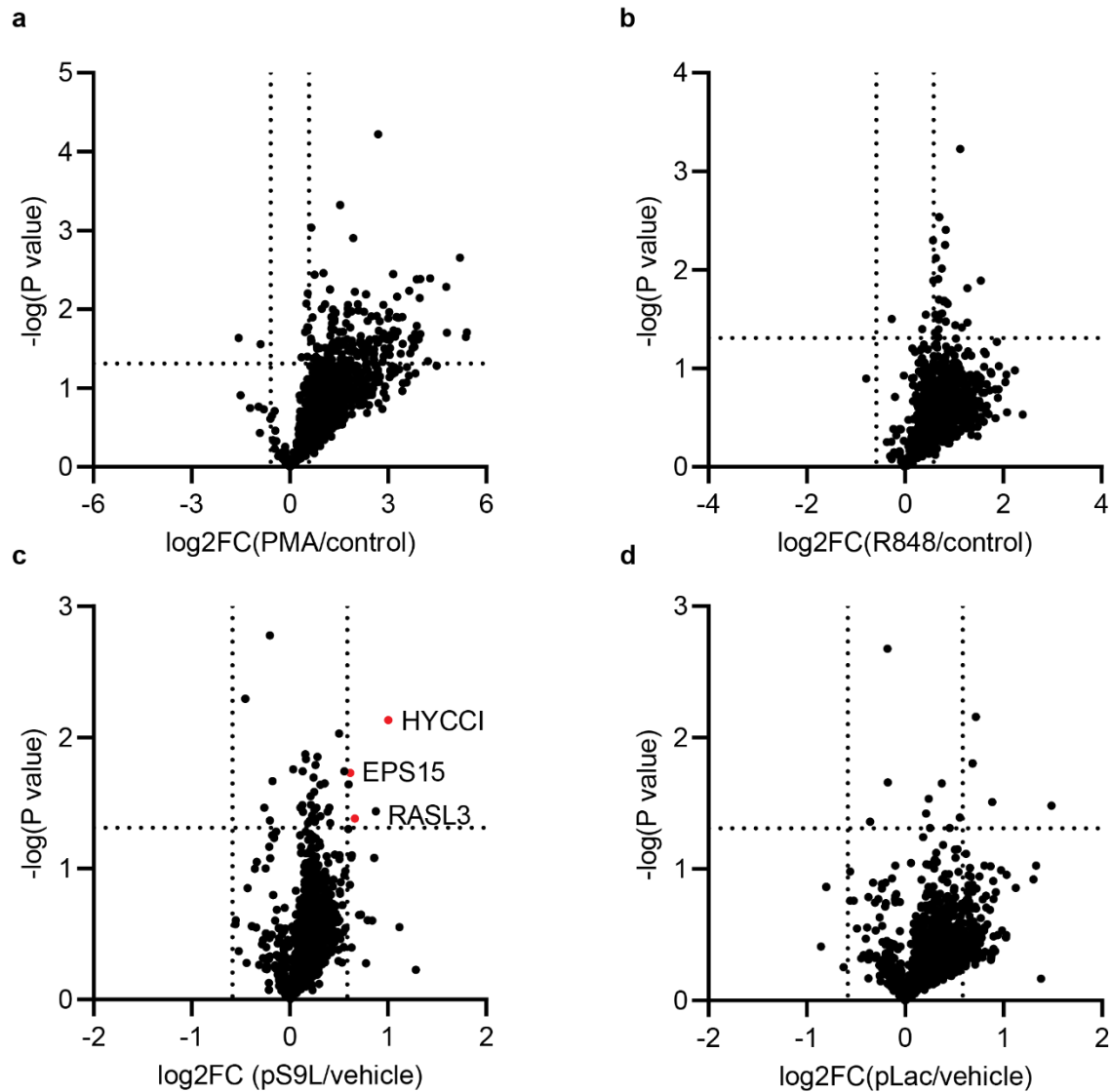
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613 **Figure S2.** R848 induces rapid citrullination of H3 in primary neutrophils. Primary neutrophils
614 were treated with compound in IMDM [+] 0.5% hiFBS for 30 min before being lysed and
615 analyzed by Western blot for citrullination of histone H3. (a) H3Cit (R2 + R8 + R17). (b) GAPDH.

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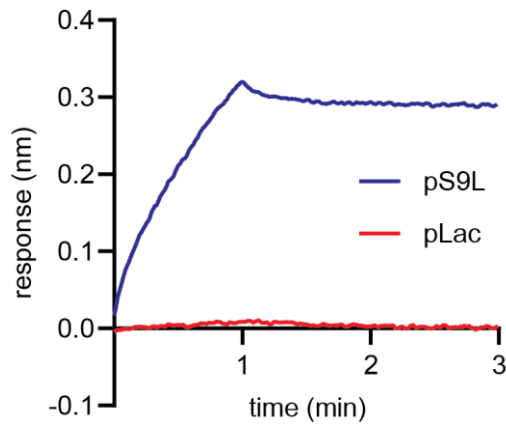


617

618 **Figure S3.** Volcano plots of significance vs. fold-change for phosphopeptides enriched from
 619 neutrophil lysates. Neutrophils isolated from healthy donor whole blood were treated with either
 620 PMA (500 nM) or R848 (10 μ M) or left untreated for 30 min (**a,b**) or with R848 (10 μ M) and one
 621 of pS9L (500 nM), pLac (500 nM) or vehicle for 15 min (**c,d**) in IMDM [+]
 622 0.5% hiFBS. The cells were lysed, digested, and phosphopeptides were enriched before being TMT-labeled and
 623 analyzed by LC-MS. Vertical lines represent $x = \pm 0.585$ (corresponding to 1.5-fold change)
 624 and horizontal lines represent $p = 0.05$ as determined by a paired t-test to account for variability
 625 between neutrophil donors. Each datapoint represents the average fold-change from three

626 different donors. **(a)** Fold-change of PMA compared to untreated neutrophils. **(b)** Fold-change of
627 R848 compared to untreated neutrophils. **(c)** Fold-change of pS9L-treated compared to vehicle-
628 treated R848-stimulated neutrophils. Red dots highlight significant hits unique to this dataset
629 compared to **(d)**. **(d)** Fold-change of pLac-treated compared to vehicle-treated R848-stimulated
630 neutrophils.

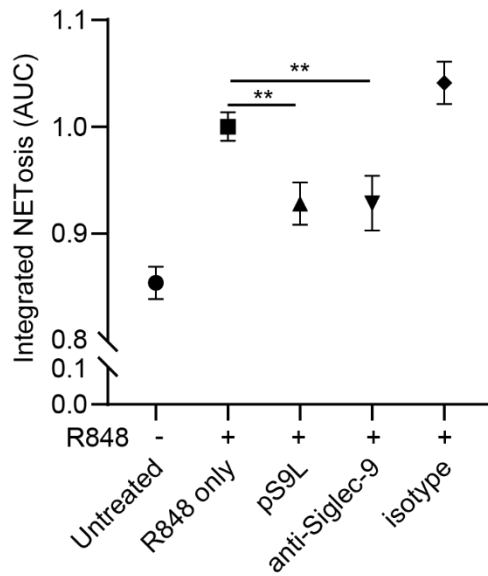
631



632

633 **Figure S4.** Siglec-9 binds pS9L but not pLac. Siglec-9-Fc was immobilized onto anti-hFc-coated
634 tips on an OctetRed96 to a threshold of 0.4 nm. Association of glycopolyptide (2.5 μ M) was
635 measured by dipping Siglec-9-Fc coated tips into a solution of glycopolyptide in PBS with
636 0.1% BSA to abrogate nonspecific binding. Tips were conditioned prior to the first assay and
637 regenerated between runs with three washes in 100 mM glycine buffer (pH 1.5).

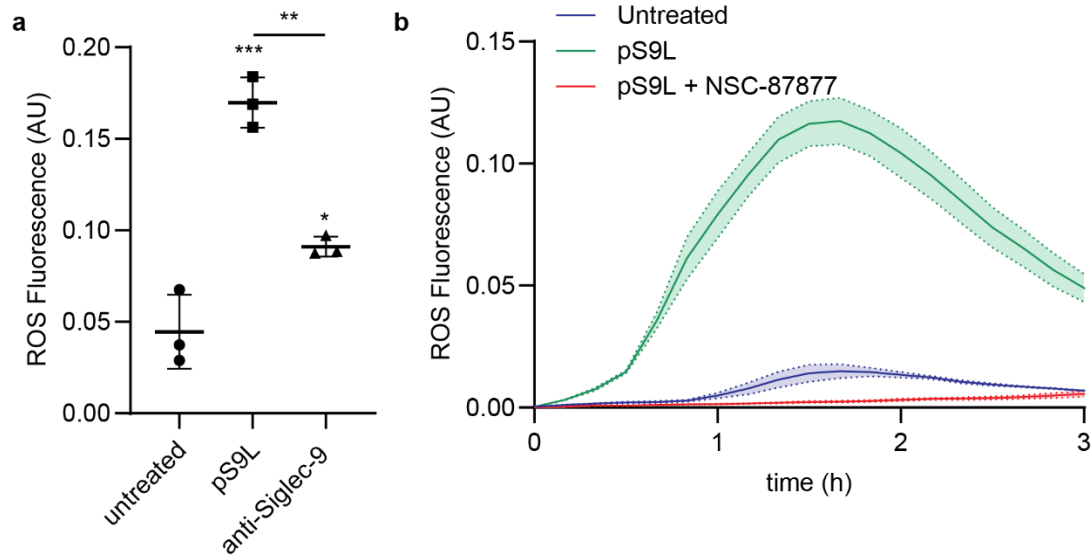
638



639

640 **Figure S5.** pS9L inhibits NETosis comparably to Siglec-9 agonist antibody. Primary neutrophils
 641 were cotreated with R848 (10 μ M) and either cis-binding Siglec-9 agonist pS9L (500 nM) or
 642 antibody (anti-Siglec-9 clone 191240 or an isotype control IgG) (35 μ g/mL) precomplexed with
 643 Protein A (5 μ g/mL) or vehicle in IMDM supplemented with 0.5% hiFBS and containing Cytotox
 644 Green (250 nM). Images were acquired by fluorescence microscopy on an Incucyte Zoom every
 645 1 h for 12 h. The area of all green fluorescent objects $>200 \mu\text{m}^2$ was quantified and averaged
 646 across three images per well. The area under the curve was quantified and normalized to the
 647 mean value for R848 treated neutrophils with no cotreatment.

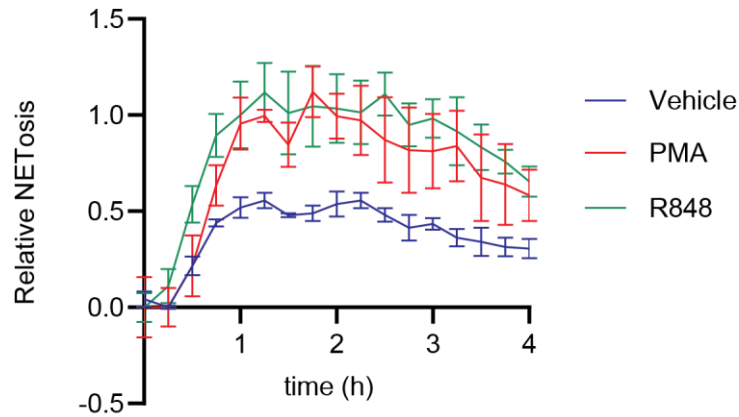
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650 **Figure S6.** Siglec-9 engagement induces a SHP-1/2 dependent oxidative burst. **(a)** Primary
 651 neutrophils were treated with either cis-binding Siglec-9 agonist pS9L (500 nM) or anti-Siglec-9
 652 (clone 191240) (35 $\mu\text{g}/\text{mL}$) precomplexed with Protein A (5 $\mu\text{g}/\text{mL}$) or vehicle in IMDM
 653 supplemented with 0.5% hiFBS and containing CellROX Deep Red (5 μM). Phase and red
 654 fluorescence images were acquired every 10 min using an Incucyte S3 in a 37 $^{\circ}\text{C}$ and 5% CO_2
 655 incubator. ROS fluorescence was quantitated using integrated intensity normalized to
 656 confluence. Statistics were determined by one-way ANOVA. * = $p < 0.05$; ** = $p < 0.01$; *** = $p <$
 657 0.001. **(b)** As in **(a)**, with or without the SHP-1/2 inhibitor NSC-87877 (50 μM), as has been
 658 previously used to study SHP-1/2-mediated Siglec activity.⁶⁵

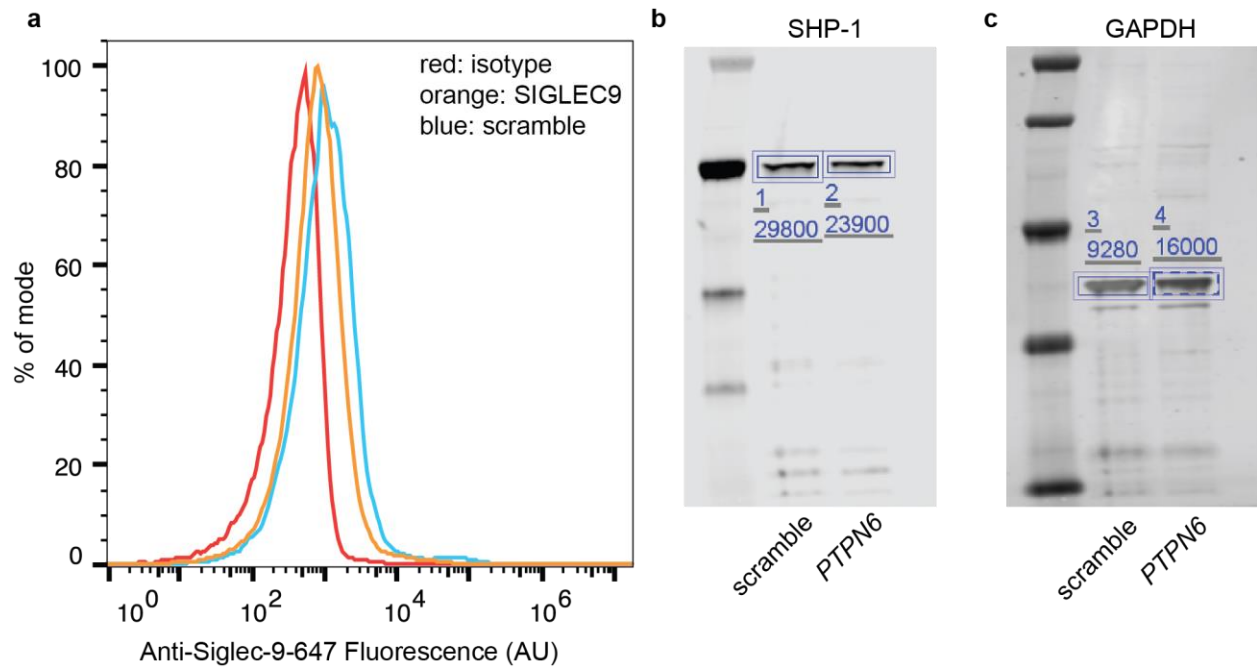
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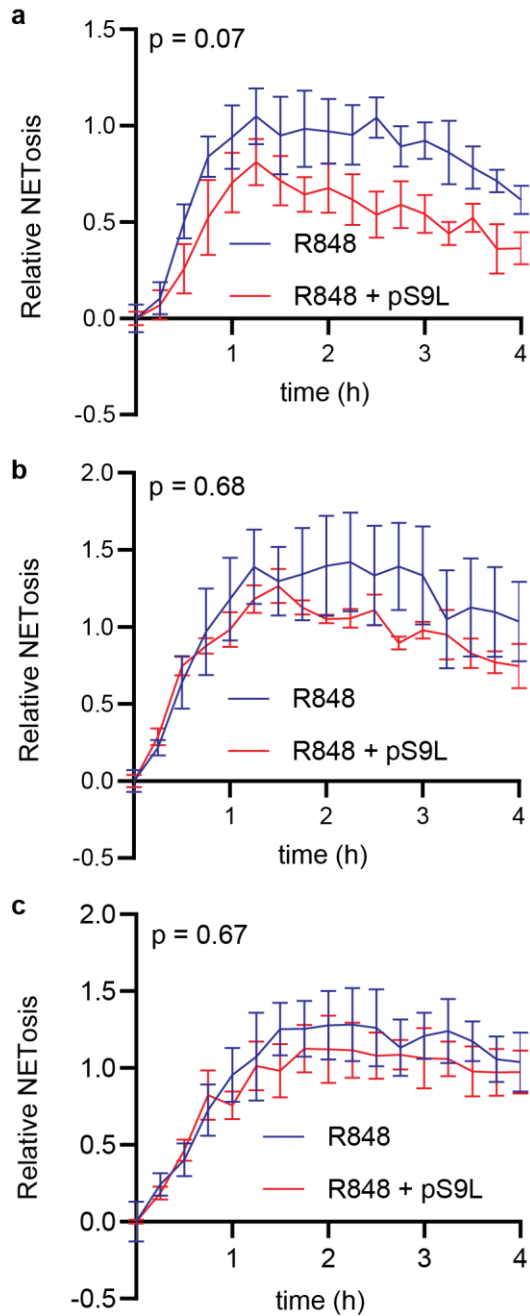
661 **Figure S7.** TLR-7/8 agonist R848 induces NETosis in dHL-60 cells. HL-60 cells were cultured in
 662 RPMI-1640 supplemented with 20% hiFBS in the presence of DMSO (1.25% v/v) and ATRA
 663 (100 nM) for 6 d. Cells were then seeded in serum-free RPMI-1640 containing Cytotox Green
 664 (250 nM) and NETosis was induced with PMA (100 nM), R848 (10 μ M), or vehicle. Phase and
 665 green fluorescence images were acquired every 15 min using an Incucyte S3. The area of all
 666 green fluorescent objects $>200 \mu\text{m}^2$ was quantified and averaged across three images per well.
 667 Relative NETosis was determined by normalizing to the maximal NET area from PMA treatment
 668 alone ($t = 2.5 \text{ h}$).

669



670

671 **Figure S8.** Siglec-9 and SHP-1 levels are reduced by siRNA knock-down of differentiated HL-
 672 60 neutrophil-like cells. HL-60 cells were cultured in RPMI-1640 supplemented with 20% hiFBS
 673 in the presence of DMSO (1.25% v/v) and ATRA (100 nM). After 4 d, the media was changed
 674 and cells were treated with siRNA's. On day 6, expression of target proteins was assayed. (a)
 675 dHL-60's treated with either SIGLEC9-targeting or scramble siRNA's were stained with an anti-
 676 Siglec-9 antibody (clone K8, AlexaFluor647 conjugate) or an isotype control and analyzed by
 677 flow cytometry. (b,c) Lysates from dHL-60's treated with siRNA's targeting PTPN6 (encoding
 678 SHP-1) or a scrambled negative control were analyzed by Western blot, staining for either SHP-
 679 1 (b) or GAPDH (c). Fluorescence signal was quantitated by LiCOR.

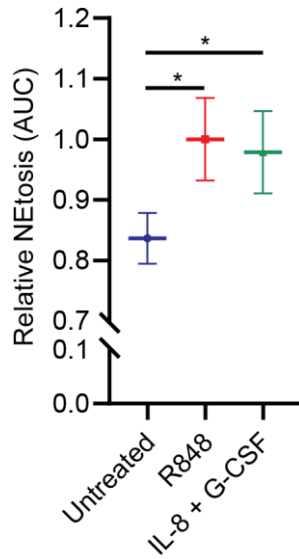


680

681 **Figure S9.** Cis Siglec-9 agonist pS9L inhibits NETosis via Siglec-9 and SHP-1. HL-60 cells
 682 were cultured in RPMI-1640 supplemented with 20% hiFBS in the presence of DMSO (1.25%
 683 v/v) and ATRA (100 nM). After 4 d, the media was changed and cells were treated with siRNA's.
 684 On day 6, cells were then seeded in serum-free RPMI-1640 containing Cytotox Green (250 nM)
 685 and NETosis was induced with R848 (10 μ M) with or without pS9L (500 nM). Phase and green

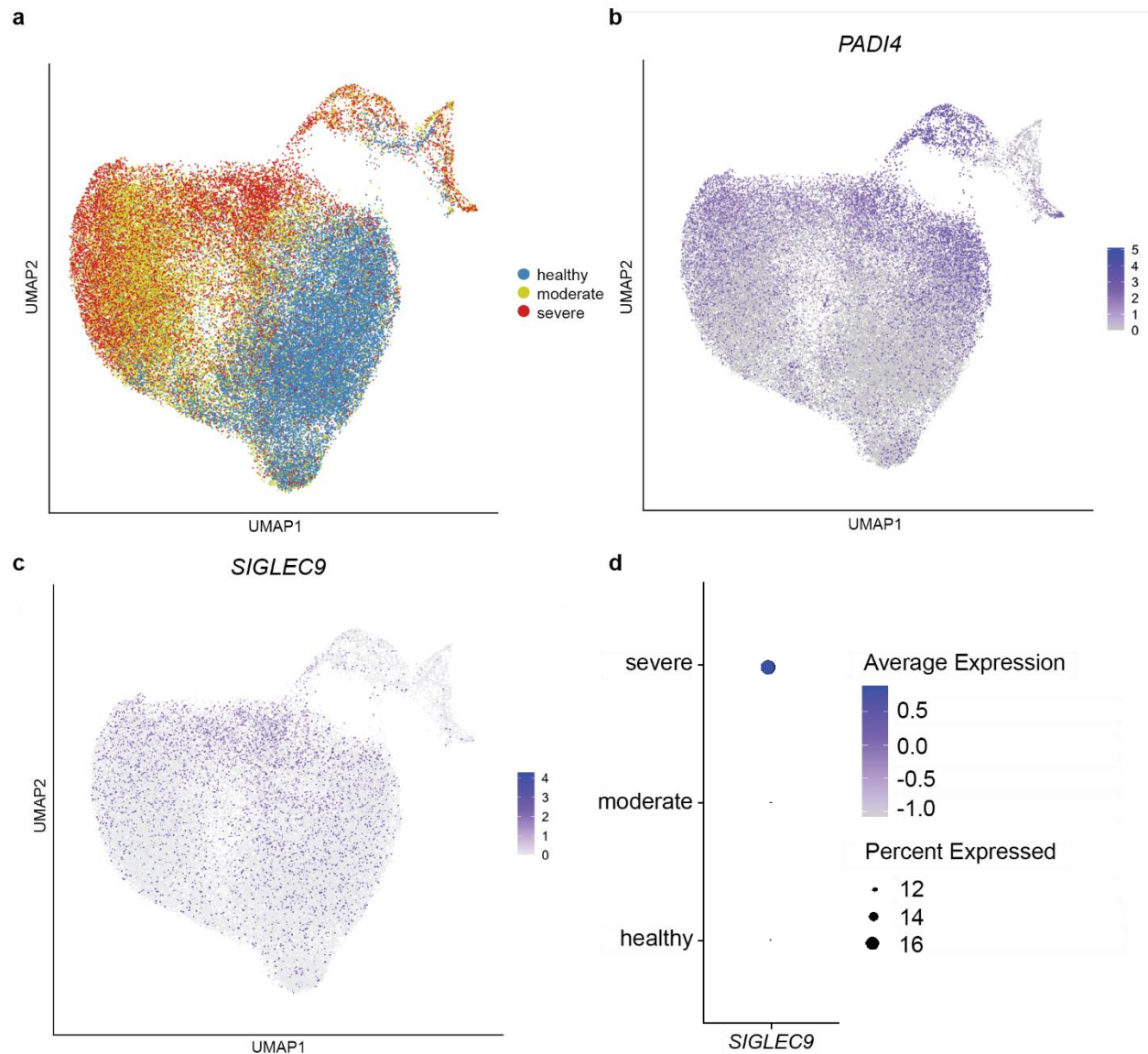
686 fluorescence images were acquired every 15 min using an Incucyte S3. The area of all green
687 fluorescent objects $>200 \mu\text{m}^2$ was quantified and averaged across three images per well.
688 Relative NETosis was determined by normalizing to the maximal NET area from PMA treatment
689 alone (t = 2.5 h). **(a)** Scramble siRNA control. **(b)** SIGLEC9 targeting siRNA cocktail. **(c)** PTPN6
690 siRNA. Statistics were calculated by two-way ANOVA.

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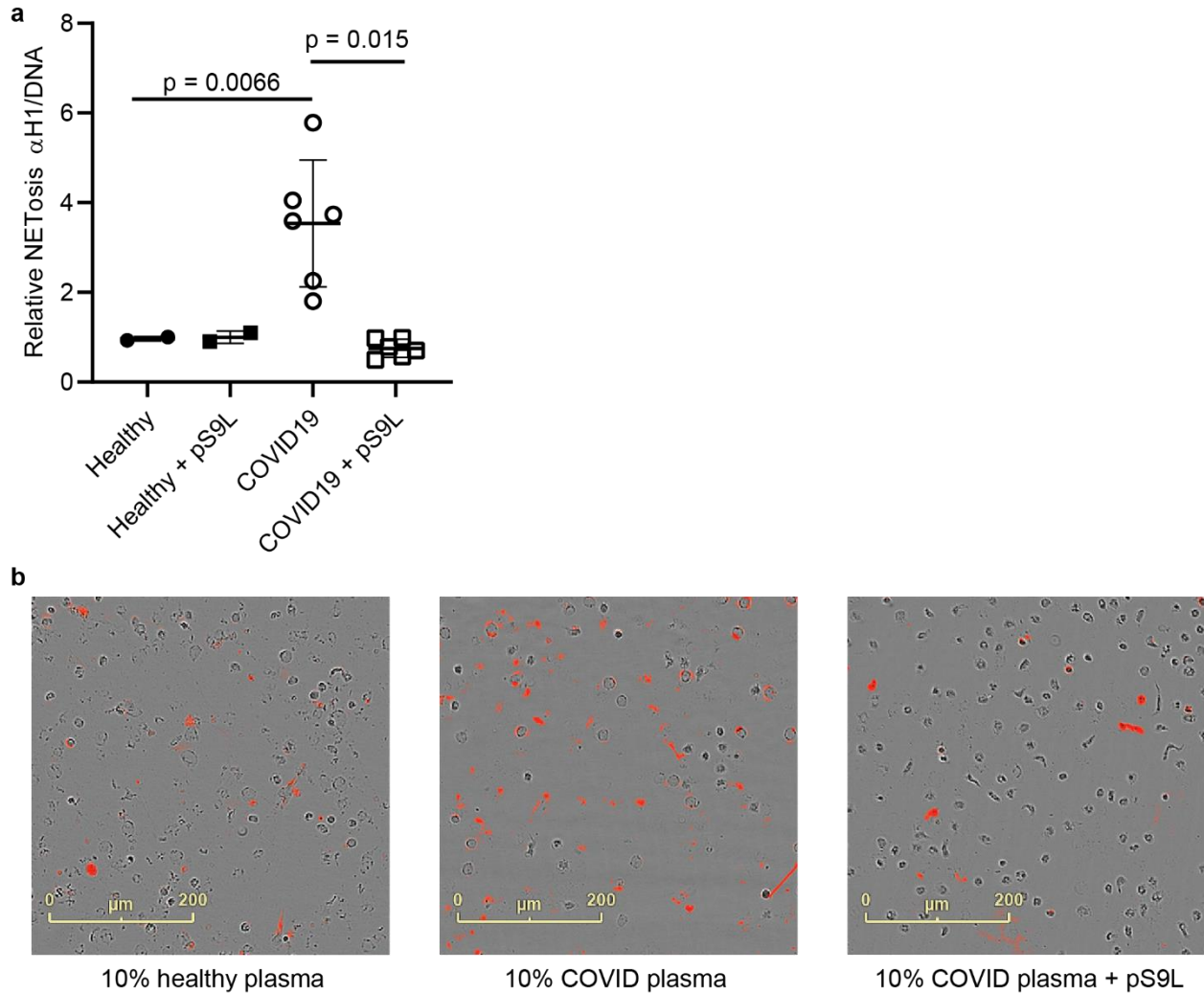
693 **Figure S10.** A combination of IL-8 and G-CSF induce NETosis in primary neutrophils. Primary
 694 neutrophils were stimulated with R848 (10 μ M), a combination of IL-8 (100 ng/mL) and G-CSF
 695 (100 ng/mL), or vehicle and cultured in IMDM supplemented 0.5% hiFBS containing the
 696 membrane impermeable DNA intercalator Cytotox Green (250 nM). Images were acquired by
 697 fluorescence microscopy every 1 h for 8 h. The area of all green fluorescent objects $>300 \mu\text{m}^2$
 698 was quantified and averaged across three images per well. Data were quantitated as area
 699 under the curve measurements. Error bars represent SD. Data are representative of multiple
 700 independent experiments using neutrophils from different donors. * $p < 0.05$.



701

702 **Figure S11.** *SIGLEC9* and *PADI4* are upregulated by neutrophils of patients with severe
 703 COVID-19. **(a-c)** Uniform Manifold Approximation and Projection (UMAP) plot of neutrophils
 704 from the single-cell transcriptomic dataset published by Schulte-Schrepping and coworkers.⁸ **(a)**
 705 Each cell is colored by the WHO severity score class (moderate, WHO score 4-5; severe, WHO
 706 score 6-8) of the patient at the time of sample collection, demonstrating strong severity-
 707 associated phenotypic reconfiguration of neutrophil transcriptome in COVID-19. **(b)** Each cell is
 708 colored by relative *PADI4* expression. **(c)** Each cell is colored by relative *SIGLEC9* expression.

709 (d) Dot plot depicting average and percent *SIGLEC9* expression by neutrophils in each WHO
710 severity score class, indicating upregulation of *SIGLEC9* in severe COVID-19.



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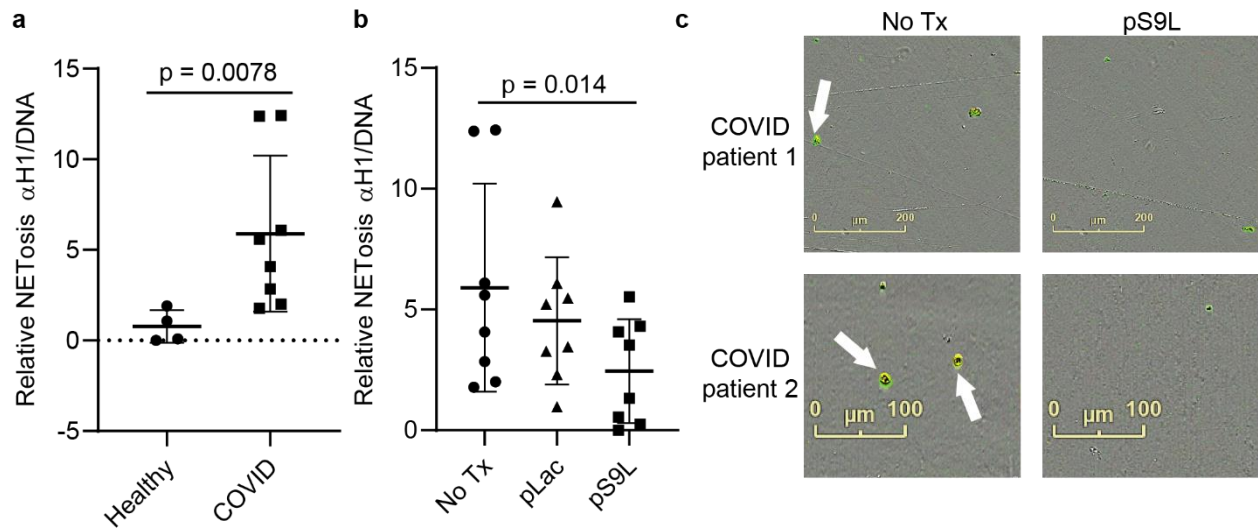
Figure S12. A Siglec-9 agonist inhibits NETosis of neutrophils induced by 10% COVID-19 plasma diluted in media. Neutrophils were isolated from healthy donors and cultured with pS9L (500 nM) in undiluted plasma for 4 h. Plasma was citrate anti-coagulated and from healthy donors or COVID-19 patients and diluted to 10% in IMDM. Cells were then fixed with 4% paraformaldehyde and blocked in 10% goat serum without permeabilization. (a) NETs were detected by immunocytochemistry with a mouse anti-H1/DNA complex primary antibody (MAB3864, 1:100) and a goat anti-mouse AlexaFluor594 (1:1000) secondary. Cells were counterstained with MemGlow488 prior to imaging but after immunostaining. The area of all red fluorescent objects $>25 \mu\text{m}^2$ and >2.0 RCU was quantified and averaged across four images per

721 well for three wells and normalized to cell count as determined by green fluorescent objects >50
722 μm^2 and >10.0 GCU. Each data point represents the mean value from technical replicates of an
723 individual donor/patient. Error bars represent SD. Statistics were determined using a mixed
724 effects model to account for differences in neutrophil donors and paired for matched patient
725 plasmas. **(b)** Representative images showing anti-H1/DNA staining.

726

727

728



729

730 **Figure S13.** A Siglec-9 agonist inhibits NETosis of neutrophils induced by undiluted COVID-19
 731 plasma. Neutrophils were isolated from healthy donors and cultured with pS9L (500 nM) or pLac
 732 (500 nM) in undiluted plasma for 4 h. Plasma was citrate anti-coagulated and from healthy
 733 donors or COVID-19 patients. Cells were then fixed with 4% paraformaldehyde and blocked in
 734 10% goat serum without permeabilization. (a) NETs were detected by immunocytochemistry
 735 with a mouse anti-H1/DNA complex primary antibody (MAB3864, 1:100) and a goat anti-mouse
 736 AlexaFluor594 (1:1000) secondary. Cells were counterstained with MemGlow488 prior to
 737 imaging but after immunostaining. The area of all red fluorescent objects $>25 \mu\text{m}^2$ and >2.0
 738 RCU was quantified and averaged across four images per well for three wells and normalized to
 739 cell count as determined by green fluorescent objects $>50 \mu\text{m}^2$ and >10.0 GCU. Each data point
 740 represents the mean value from technical replicates of an individual donor/patient. Error bars
 741 represent SD. Statistics were determined using a mixed effects model to account for differences
 742 in neutrophil donors and paired for matched patient plasmas. (b) Representative images
 743 showing anti-H1/DNA staining (red) overlaid with MemGlow488 (green).

744

745

747 **Materials and Methods.**

748 **Glycopolyptide synthesis.**

749 Glycopolyptides were synthesized as previously described.^{41,50} In brief, *N*-carboxyanhydride
750 monomers were polymerized either with a functionalized initiator that affords a membrane-
751 tethering moiety or with a Ni(0) initiator that affords a soluble polypeptide. Polypeptides were
752 deprotected with hydrazine monohydrate and purified by dialysis. In some cases, lactose-
753 bearing scaffolds were then chemoenzymatically functionalized to afford terminal Siglec-9
754 ligands.⁴¹ All glycopolyptides were endotoxin purified on Pierce endotoxin removal resin
755 (ThermoFisher, 88274) and sterile filtered (0.22 µm) before use.

756

757 **Primary cell isolation.**

758 Whole blood samples were obtained from the healthy donors and anti-coagulated with K2EDTA
759 at the Stanford Blood Center. Samples were de-identified by the Stanford Blood Center.
760 Neutrophils were purified on the same day as isolation, and the whole blood samples were kept
761 at room temperature between collection and neutrophil isolation. Neutrophils were isolated by
762 EasySep Direct Neutrophil Isolation Kit (StemCell Technologies, 19666) according to the
763 manufacturer's instructions in 5 mL aliquots on a magnetized rack (StemCell Technologies,
764 18103). Efficiency of isolation was determined by flow cytometry to identify CD45 and CD14 hi
765 cells. Neutrophils were routinely obtained in >96% purity via this method.

766

767 **COVID-19 patients and specimen collection.**

768 Peripheral blood was collected from patients enrolled in the IRB-approved Stanford University
769 Emergency Department COVID-19 Biobank beginning in April 2020 after written informed

770 consent from patients or their surrogates. Eligibility criteria included age ≥ 18 years and
771 presentation to the Stanford Hospital with a positive SARS-CoV-2 nasopharyngeal swab by RT-
772 PCR. Patients were phenotyped for COVID-19 severity according to the universal World Health
773 Organization (WHO) ordinal scale. Blood draws from patients occurred upon presentation to the
774 Stanford Hospital Emergency Department in concert with usual care to avoid unnecessary
775 personal protective equipment usage. Blood was collected into CPT vacutainers (Becton,
776 Dickinson, and Co.) and plasma isolated, aliquoted, and stored at -80°C after centrifugation at
777 $1800 \times g$ for 20 minutes at 25°C . All sample processing occurred under BSL2+ biosafety
778 precautions as approved by Stanford University APB.

779

780 **Cell culture.**

781 The acute promyelocytic leukemia cell line HL-60 (ATCC, CCL-240) were cultured in RPMI-
782 1640 supplemented with 20% hiFBS (ThermoFisher, 10-438-026). Cultures were thoroughly
783 washed with prewarmed complete media when bringing out of cryostorage to completely
784 remove DMSO, as residual DMSO can induce differentiation in HL-60s.⁶⁶ Cells were allowed to
785 recover for at least two weeks prior to any NETosis experiments. Cultures were not allowed to
786 exceed a density of 5×10^5 cells per mL of culture media, as high densities can also induce
787 differentiation.⁶⁶

788

789 To induce differentiation of HL-60's, 2×10^6 cells were harvested by centrifugation (300 rcf, 5 min)
790 and resuspended in complete media (RPMI-1640 +20% hiFBS) supplemented with 100 nM
791 ATRA (Sigma-Aldrich, R2625) and DMSO (Sigma-Aldrich, D8414). Cells were cultured for 5-6
792 days before use, with a complete media change 48 h prior to use.

793

794 **NETosis assays.**

795 For live cell NETosis assays were performed in 96 well plates (Corning, 3595) and monitored by
796 fluorescence microscopy with an Incucyte S3 (Essen Biosciences) or an Incucyte ZOOM (Essen
797 Biosciences). DNA staining was performed using membrane impermeable fluorogenic DNA
798 intercalators Cytotox Green (Essen Biosciences, 4633) or Cytotox Red (Essen Biosciences,
799 4632).

800

801 Plates were prepared containing 10X solutions of compounds (20 μ L) and a 2X solution of DNA
802 dye (100 μ L) in serum-free media (IMDM for primary neutrophils, RPMI-1640 for HL-60s)
803 lacking phenol red. Freshly harvested neutrophils in media (IMDM with 1.2% hiFBS for primary
804 neutrophils for 0.5% final concentration of hiFBS, serum free RPMI-1640 for HL-60s) were then
805 added to the plate (80 μ L, 2.5×10^5 per mL, 2×10^4 per well) from a cell suspension. The plates were
806 briefly centrifuged at 300 rcf for 1 min to settle the cell suspension, and the plates were
807 immediately transferred to the Incucyte for periodic monitoring (every 10 min for 8 h or every 15
808 min for 12 h).

809

810 For NETosis assays with diluted COVID-19 plasma, freshly isolate neutrophils were plated on
811 96 well plates (Corning, 3595) coated with 0.01% poly-L-lysine (Sigma, P4707) in IMDM
812 supplemented with 0.5% hiFBS. The neutrophils were allowed to settle and adhere for 20 min
813 before the media was removed and IMDM containing 10% healthy or COVID-19 plasma and
814 with or without glycopolypeptide (500 nM) was added. Alternatively, control wells were treated
815 with no inducer, PMA (500 nM) or R848 (10 μ M) in IMDM with 0.5% hiFBS. Plates were
816 incubated for 4 h at 37 $^{\circ}$ C and the media was gently removed. The cells were then fixed for 15
817 min at room temperature in a solution of 4% PFA in PBS (ThermoFisher, FB002). The fixed

818 cells were then blocked in 10% goat serum in PBS, stained with anti-H1/DNA (EMD Millipore,
819 MAB3864), and visualized with anti-mouse AlexaFluor 594 (Jackson ImmunoResearch, 115-
820 585-174). Plates were imaged using an Incucyte S3.

821

822 For NETosis assays with undiluted COVID-19 plasma, freshly isolate neutrophils were plated on
823 black-walled half-area 96 well plates (Greiner, 675090) coated with 0.01% poly-L-lysine (Sigma,
824 P4707) in IMDM supplemented with 0.5% hiFBS. The neutrophils were allowed to settle and
825 adhere for 20 min before the media was removed and either healthy or COVID-19 plasma and
826 with or without glycopolypeptide (500 nM) was added. Alternatively, control wells were treated
827 with no inducer or R848 (10 μ M) in IMDM with 0.5% hiFBS. Plates were incubated for 4 h at 37
828 $^{\circ}$ C and the media was gently removed. The cells were then fixed for 15 min at room
829 temperature in a solution of 4% PFA in PBS (ThermoFisher, FB002). The fixed cells were
830 washed. Then cells were then blocked in 10% goat serum. For plates imaged by Incucyte S3,
831 cells were stained with anti-H1/DNA (EMD Millipore, MAB3864) and visualized with a
832 combination of anti-mouse AlexaFluor 594 (Jackson ImmunoResearch, 115-585-174) and
833 MemGlow488 (Cytoskeleton, MG01-02). For plates imaged by immunofluorescence using a
834 Keyence BZ-710, cells were stained with anti-MPO (Thermo Scientific, MA516383) and
835 visualized with anti-rabbit AlexaFluor 555 (Thermo Scientific, A27039). Cells were imaged in
836 HBSS containing DAPI imaging solution (Thermo Scientific, R37606). Images were collected
837 using a Keyence BZ-X710 and images were analyzed using ImageJ.

838

839 Incucyte images were analyzed using the onboard Incucyte analysis software. For Incucyte S3
840 analyses: phase images were analyzed to identify cells using the following settings:
841 segmentation adjustment = 0.8; minimum area filter = 25 μ m². For primary neutrophils,

842 fluorescence areas were determined using the following settings: top hat segmentation; radius
843 (μm) = 10.0; threshold = 10.0 GCU or 0.1 RCU; area filter = $300 \mu\text{m}^2$. For dHL-60s,
844 fluorescence areas were determined as for primary neutrophils with the following variation: area
845 filter = $200 \mu\text{m}^2$. For Incucyte ZOOM analyses: phase images were analyzed to identify cells
846 using the following settings: segmentation adjustment = 0.8; minimum area filter = $25 \mu\text{m}^2$. For
847 primary neutrophils, fluorescence areas were determined using the following settings: top hat
848 segmentation; radius (μm) = 10.0; threshold = 5.0 GCU; area filter = $200 \mu\text{m}^2$

849

850 **Reactive oxygen species measurements.**

851 Induction of reactive oxygen species was measured by fluorescence microscopy using an
852 Incucyte S3 (Essen Biosciences). Cells were prepared as for NETosis assays, with the
853 difference that rather than the inclusion of Cytotox dye in the media, CellROX Deep Red
854 (Thermo Fisher, C10422) was included at a final concentration of $5 \mu\text{M}$. Data were analyzed as
855 in NETosis assays, with the following changes in settings for quantifying red fluorescence: top
856 hat segmentation; radius (μm) = 10.0; threshold = 1.6 RCU; area filter = $25 \mu\text{m}^2$.

857

858 **siRNA knock downs.**

859 For siRNA knock down experiments, HL-60's were differentiated as indicated above. On day 4,
860 the media was changed and 3 mL of culture was transfected with 30 pmol siRNA's (IDT) using
861 Lipofectamine RNAiMAX (Thermo Fisher, 13778100) according to the manufacturer's
862 instructions. Transfectants were cultured for 48 h prior to use in NETosis assays, with a
863 complete media change at 24 h post transfection. Protein expression was validated by Western
864 blot or flow cytometry, as applicable.

865

866 For siRNA KD of SIGLEC9, the top two suggested predesigned dicer substrate siRNA's from
867 IDT (hs.Ri.SIGLEC9.13.1 and hs.Ri.SIGLEC9.13.2) were combined in a 1:1 molar ratio. For
868 siRNA KD of PTPN6, the top suggested predesigned dicer substrate siRNA (hs.Ri.PTPN6.13.1)
869 was used. As a negative control, scramble siRNA's were obtained from IDT.

870

871 **Flow cytometry.**

872 All flow cytometry experiments were performed using a BD Accuri 6 flow cytometer and
873 analyzed using FlowJo 10. For all flow cytometry experiments, live cells were isolated from
874 culture and resuspended in cold PBS supplemented with 1% BSA at a concentration of 1e7
875 cells per mL. Aliquots of 5e5 cells were then stained at the concentrations indicated in the
876 Reagents Table and Usage for 30 min on ice in the dark. Cells were then washed three times
877 (500 rcf, 5 min) with in cold PBS supplemented with 1% BSA before being analyzed by flow
878 cytometry.

879

880 **SDS-PAGE and Western blotting.**

881 Cell pellets were washed twice with cold PBS supplemented with 1 mM EDTA and were lysed in
882 RIPA buffer (50 μ L for each 1e6 cells) (Thermo Fisher, PI89900) supplemented with Benzonase
883 (1:1000) (Sigma Aldrich, E1014), HALT phosphatase inhibitor (1:100) (Thermo Fisher, 78420),
884 and HALT protease inhibitor (1:100) (Thermo Fisher, 87786) for 30 min at 4 °C. Lysates were
885 clarified by centrifugation at 4 °C for 15 min at 16000 rcf. Protein concentration was quantified
886 by Rapid Gold BCA (Thermo Fisher, A53226).

887

888 Lysates were diluted into SDS-buffer with beta-mercaptoethanol and were separated by SDS-
889 PAGE on 4-12% bisacrylamide gels loading 10 µg per lane. For analysis of histone H3
890 citrullination, lysates were separated in XT-MES at 200 V for 35 min. For analysis of SHP-1
891 levels after siRNA KD, lysates were separated in XT-MOPS at 200 V for 1 h. Blots were
892 transferred to nitrocellulose and blocked with 5% BSA in TBS before being stained with primary
893 antibodies and IR-dye conjugated secondary antibodies for analysis by LiCOR.

894

895 **Protein expression and purification.**

896 All proteins for enzymatic synthesis of glycopolypeptides were expressed and purified as
897 previously described.⁴¹

898 His-tagged proteins cultures were grown at 37 °C to an OD600 of 0.8-1.0 in 1 L of LB containing
899 the appropriate antibiotic selection marker, at which point expression was induced with IPTG
900 (0.1 mM) and cultures were grown overnight at 20 °C with shaking at 220 rpm. After 24h, cells
901 were pelleted by centrifugation and lysed in buffer (250 mM TrisHCl, 0.5 M NaCl, 20 mM
902 imidazole, 0.1% TritonX100) supplemented with protease inhibitor cocktail (Sigma Aldrich,
903 04693132001) (one tablet per 40 mL) and DNaseI (Thermo Fisher, 90083) (10 µL per 40 mL).
904 Cells were lysed using a dounce homogenizer followed by French press. Lysates were clarified
905 by centrifugation and purified on HisTRAP columns (GE Life Sciences, 17-5247-01) using a
906 gradient of 20 mM to 200 mM imidazole on an AKTA FPLC. Fraction purity was determined by
907 SDS-PAGE and pure fractions were combined, purified by dialysis against storage buffer (50
908 mM Tris HCl, 250 mM NaCl, 10% glycerol), aliquoted, and flash-frozen in liquid nitrogen for
909 storage in a -80 °C freezer.

910

911 **Phosphoproteomics.**

912 Primary neutrophils were treated in media containing the indicated compounds in IMDM [+]
913 0.5% hiFBS for the indicated time period, or in plasma from either healthy donors or COVID-19
914 patients, with or without pS9L (500 nM) for 15 min. Cell pellets were washed twice with cold
915 PBS supplemented with 1 mM EDTA and were lysed in RIPA buffer (50 μ L for each 1e6 cells)
916 (Thermo Fisher, PI89900) supplemented with Benzonase (1:1000) (Sigma Aldrich, E1014),
917 HALT phosphatase inhibitor (1:100) (Thermo Fisher, 78420), and HALT protease inhibitor
918 (1:100) (Thermo Fisher, 87786) for 30 min at 4 °C. Lysates were clarified by centrifugation at 4
919 °C for 15 min at 16000 rcf. Protein concentration was quantified by Rapid Gold BCA (Thermo
920 Fisher, A53226).

921 Digestion was performed on 100 μ g protein using a mini S-trap protocol provided by the
922 manufacturer (Protifi).⁶⁷ Here, proteins brought to 5% SDS and reduced with 5 mM DTT for 10
923 minutes at 95 C. Cysteines were alkylated using 30 mM iodoacetamide for 45 minutes each at
924 room temperature in the dark. The lysate was then acidified with phosphoric acid, brought to
925 approximately 80-90% methanol with 100 mM TEAB in 90% methanol, and loaded onto the S-
926 trap column. Following washing with 100 mM TEAB in 90% methanol, trypsin (Promega) was
927 added to the S-trap at a 20:1 protein:protease ratio for 90 minutes at 47 °C. Peptides from each
928 lysate were labeled with 11-plex TMT (Tandem Mass Tags, Thermo Fisher Scientific) for 2
929 hours at room temperature using recently published protocols.^{47,68} Labeling schemes for the
930 stimulated study comparing R848 and PMA to no treatment (NT) were: NT replicates in
931 channels 126C (Donor 1), 127N (Donor 2), and 130C (Donor 3); PMA replicates in 127C (Donor
932 1), 128N (Donor 2) and 131N (Donor 3); R848 replicates in 128C (Donor 1), 129N (Donor 2)
933 and 131C (Donor 3). For the polymer experiment, the labeling scheme was: vehicle replicates in
934 channels 126C (Donor 1), 127N (Donor 2), and 130C (Donor 3); pS9L replicates in 127C (Donor
935 1), 128N (Donor 2) and 131N (Donor 3); pLac replicates in 128C (Donor 1), 129N (Donor 2) and
936 131C (Donor 3). A test mix was run to confirm >99% labeling efficiency and even distribution of

937 signal across all channels prior to quenching of the TMT labeling reaction (0.5 μ L 50%
938 hydroxylamine reacted for 15 min). Peptides from each channel were then combined prior to
939 phosphopeptide enrichment, which was performed as previously described.⁶⁹ Briefly, 100 μ L
940 magnetic titanium(IV) immobilized metal ion affinity chromatography (Ti(IV)-IMAC, ReSyn
941 Biosciences) beads were washed three times with 1 mL 80% acetonitrile/6% TFA (all washes
942 were 1 mL).⁷⁰ Peptides were dissolved in 1 mL 80% acetonitrile/6% TFA and gently vortexed
943 with the Ti(IV)-IMAC beads for 45 minutes. Unbound peptides were kept as flow through for
944 total protein analysis, followed by three 80% acetonitrile/6% TFA, one 80% acetonitrile, one 0.5
945 M glycolic acid/80% acetonitrile, and two 80% acetonitrile washes. Peptides were eluted with
946 500 μ L 50% acetonitrile, 1% ammonium hydroxide. Both eluate and flow through were dried
947 down in a speed vac and further cleaned up on Strata-X SPE cartridges (Phenomenex) by
948 conditioning the cartridge with 1 mL ACN followed by 1 mL 0.2% formic acid (FA) in water.
949 Peptides were resuspended in 0.2% FA in water and then loaded on to the cartridge, followed
950 by a 1 mL wash with 0.2% FA in water. Peptides were eluted with 400 μ L of 0.2% FA in 80%
951 ACN, were dried via lyophilization.

952

953 All samples were resuspended in 0.2% formic acid in water prior to LC-MS/MS analysis. Total
954 protein samples were resuspended in 500 μ L with 1 μ L injected on column, while enriched
955 phosphopeptides were resuspended in 15 μ L total with 4 μ L injected per analysis. Triplicate
956 injections were collected for all samples. All (phospho)peptide mixtures were separated over a
957 25 cm EasySpray reversed phase LC column (75 μ m inner diameter packed with 2 μ m, 100 \AA ,
958 PepMap C18 particles, Thermo Fisher Scientific). The mobile phases (A: water with 0.2% formic
959 acid and B: acetonitrile with 0.2% formic acid) were driven and controlled by a Dionex Ultimate
960 3000 RPLC nano system (Thermo Fisher Scientific). An integrated loading pump was used to
961 load peptides onto a trap column (Acclaim PepMap 100 C18, 5 μ m particles, 20 mm length,

962 Thermo Fisher Scientific) at 8 μ L/min, which was put in line with the analytical column 4 minutes
963 into the gradient for the total protein samples. The gradient increased from 0% to 5% B over the
964 first 4 minutes of the analysis, followed by an increase from 5% to 25% B from 4 to 158 minutes,
965 an increase from 25% to 90% B from 158 to 162 minutes, isocratic flow at 90% B from 162 to
966 168 minutes, and a re-equilibration at 0% for 12 minutes for a total analysis time of 180 minutes.
967 Eluted (phospho)peptides were analyzed on an Orbitrap Fusion Tribrid MS system (Thermo
968 Fisher Scientific). Precursors were ionized using an EASY-Spray ionization source (Thermo
969 Fisher Scientific) source held at +2.2 kV compared to ground, and the column was held at 40
970 $^{\circ}$ C. The inlet capillary temperature was held at 275 $^{\circ}$ C. Survey scans of peptide precursors were
971 collected in the Orbitrap from 350-1350 Th with an AGC target of 1,000,000, a maximum
972 injection time of 50 ms, and a resolution of 60,000 at 200 m/z. Monoisotopic precursor selection
973 was enabled for peptide isotopic distributions, precursors of $z = 2-5$ were selected for data-
974 dependent MS/MS scans for 2 second of cycle time, and dynamic exclusion was set to 30
975 seconds with a ± 10 ppm window set around the precursor monoisotope. An isolation window of
976 1 Th was used to select precursor ions with the quadrupole. MS/MS scans were collected using
977 HCD at 30 normalized collision energy (nce) with an AGC target of 100,000 and a maximum
978 injection time of 118 ms. Mass analysis was performed in the Orbitrap with a resolution of
979 60,000 with a first mass set at 100 Th.

980

981 **Phosphoproteomic data analysis.**

982 All data were searched with the Andromeda search engine⁷¹ in MaxQuant⁷² using the entire
983 human proteome downloaded from Uniprot⁷³ (reviewed, 20428 entries). Each separate TMT
984 experiment (resting, activated, and pLac control) was searched separately, with the flow
985 through/total protein triplicate injections labeled as Group0 and False under "PTM" and
986 phosphopeptide enriched triplicate injections labeled as Group1 and True under "PTM". Group0

987 had cleavage specificity set to Trypsin/P with 2 missed cleavage allowed and variable
988 modifications of oxidation of methionine and acetylation of the protein N-terminus with 4
989 maximum modifications per peptide. Group1 had cleavage specificity set to Trypsin/P with 3
990 missed cleavage allowed and variable modifications of phosphorylation on
991 serine/threonine/tyrosine, oxidation of methionine, and acetylation of the protein N-terminus with
992 4 maximum modifications per peptide. The experiment type for both Group0 and Group1 was
993 set to Reporter ion MS2 and only TMT channels used (as described above) were selected to be
994 included. The reporter ion mass tolerance was set to 0.3 Da and the minimum reporter PIF
995 score was set to 0.75. Defaults were used for the remaining settings, including PSM and protein
996 FDR thresholds of 0.01 and 20 ppm, 4.5 ppm, and 20 ppm for first search MS1 tolerance, main
997 search MS1 tolerance, and MS2 product ion tolerance, respectively. Match between runs was
998 not enabled. Quantified phosphosites were then processed in Perseus.⁷⁴ Contaminants and
999 reverse hits were removed, results were filtered for phosphosites that had localization
1000 probabilities > 0.75, and signal in all relevant TMT channels was required. Significance testing
1001 was performed using a two-tailed pair-ed sample t-test calculated in Microsoft Excel, using one
1002 condition versus control (NT or vehicle for stimulated and polymer experiments, respectively) for
1003 pairwise comparisons. Data have been deposited to the ProteomeXchange Consortium via the
1004 PRIDE partner repository with the dataset identifier PXD022990.⁷⁵

1005

1006 **Analysis of publicly-available single-cell RNA-sequencing (scRNA-seq) data.**

1007 The open source statistical software R (www.r-project.org; v3.6.1) and the R package Seurat (v
1008 3.2.2) was used for scRNA-seq data analysis.⁷⁶ A pre-processed Seurat object containing
1009 scRNA-seq data and metadata of neutrophils profiled using the BD Rhapsody platform was
1010 retrieved from www.fastgenomics.org as outlined in the data availability statement of Schulte-
1011 Schrepping, et al.⁸ The average expression of *SIGLEC9* or *PADI4* was defined as the mean of

1012 log-normalized transcript counts, calculated by NormalizeData() function, in a given sample.

1013 DotPlot() was used to visualize average and percent expression of *SIGLEC9* or *PADI4*.

1014

1015 Reagent Table and Usage.

1016 IN – Incucyte S3 (microscopy); OC – Octet (*in vitro* protein binding); FC – flow cytometry; WB –

1017 western blot; KD – siRNA knock down; IF – immunofluorescence

Reagent	Source (#)	Usage, dilution/concentration
Cytotox Green	Essen Biosciences (4633)	IN, 1:4,000
Cytotox Red	Essen Biosciences (4632)	IN, 1:4,000
CellROX Deep Red	ThermoFisher (C10422)	IN, 1:500
Anti-Siglec-9 clone K8 / AlexaFluor 647	BioLegend (351509)	FC, 1:50
Anti-human CD45 clone HI30 / APC	Stemcell Technologies (60018AZ.1)	FC, 1:50
Anti-human CD16 clone 3G8 / AlexaFluor 488	Stemcell Technologies (60041AD.1)	FC, 1:50
Mouse IgG1 isotype clone MOPC-21 / FITC	BD Biosciences (551954)	FC, 1:50
Mouse IgG1 isotype clone MOPC-21 / APC	BD Biosciences (550854)	FC, 1:50
Siglec-9-Fc	R&D Systems (1139-SL-050)	OC, 400 nM
DsiRNA (SIGLEC9)	IDT (hs.Ri.SIGLEC9.13.1)	KD, 30 nM
DsiRNA (SIGLEC9)	IDT (hs.Ri.SIGLEC9.13.2)	KD, 30 nM
DsiRNA (PTPN6)	IDT (hs.Ri.PTPN6.13.1)	KD, 30 nM
siRNA negative control	IDT (51-01-19-08)	KD, 30 nM
Rabbit anti-CitH3 (R2/R8/R17)	Abcam (ab5103)	WB, 1:1,000
Mouse anti-GAPDH	Sigma-Aldrich (G8795-100UL)	WB, 1:10,000
NSC-87877	Sigma-Aldrich (565851-50MG)	IN, 50 μ M

Rabbit anti-SHP1 (clone Y476)	Abcam (ab32559)	WB, 1:1,000
goat anti-mouse 680RD	LiCOR (926-68070)	WB, 1:10,000
goat anti-rabbit 800CW	LiCOR (926-32211)	WB, 1:10,000
anti-H1/DNA	EMD Millipore (MAB3864)	IF, 1:100
goat anti-mouse AlexaFluor 594	Jackson ImmunoResearch (115-585-174)	IF, 1:1000
MemGlow488	Cytoskeleton (MG01-02)	IF, 1:200
anti-myeloperoxidase clone SP72	Thermo Scientific (MA516383)	IF, 1:100
goat anti-rabbit AlexaFluor 555	Thermo Scientific (A27039)	IF, 1:1000
DAPI solution	Thermo Scientific (R37606)	IF, 2 drops per mL

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1019