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2 **High mannose N-glycans on red blood cells as phagocytic ligands,**
3 **mediating both sickle cell anaemia and resistance to malaria**

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24 **Abstract**

25

26 In both sickle cell disease (SCD) and malaria, red blood cells (RBCs) are phagocytosed in the
27 spleen, but receptor-ligand pairs mediating uptake have not been identified. Here, we report that
28 patches of high mannose N-glycans (Man₅₋₉GlcNAc₂), expressed on diseased or oxidized RBC
29 surfaces, bind the mannose receptor (CD206) on phagocytes to mediate clearance. Extravascular
30 haemolysis in SCD correlates with high mannose glycan levels on RBCs. Infection of RBCs
31 with *Plasmodium falciparum* expose surface mannose N-glycans on healthy RBCs, which
32 occurred at significantly higher levels on RBCs from subjects with sickle cell trait compared to
33 those lacking haemoglobin S. The glycans were associated with high molecular weight
34 complexes and protease-resistant, lower molecular weight fragments containing spectrin.
35 Recognition of surface N-linked high mannose glycans, a novel response to cellular stress, is the
36 first molecular mechanism common to both the pathogenesis of SCD and resistance to severe
37 malaria in sickle cell trait.

38 Introduction

39
40 Sickle cell disease (SCD) comprises a group of disorders affecting over 20 million individuals
41 and is caused by a mutation causing an amino acid substitution (E6V) in the adult haemoglobin β
42 chain (1, 2), so that the physiological haemoglobin (Hb) A tetramer, $\alpha_2\beta_2$, is replaced by the HbS
43 tetramer $\alpha_2\beta^S_2$, which can form pathological polymers. The disease is variable, with modifiers
44 such as high levels of the fetal β haemoglobin chain, γ , resulting in $\alpha_2\gamma_2$ or $\alpha_2\beta^S\gamma$ tetramers that
45 terminate HbS polymers and ameliorate disease. Milder disease is also associated with
46 compound heterozygosity of the sickle cell allele with quantitative defects in α or β chains
47 (thalassaemias) or other haemoglobin chain variants like haemoglobin C.

48
49 SCD is characterized by a multi-system vasculopathy and haemolysis, which cause much
50 morbidity and mortality, especially in Africa. The anaemia has been ascribed to abnormal
51 physical properties of diseased red blood cells (RBCs), which interfere with their transit through
52 the splenic and hepatic vasculatures, so stimulating phagocytic uptake by tissue macrophages (3).
53 However, the observation that isolated macrophages take up SCD RBCs selectively *in vitro* (4)
54 indicates the presence of disease-specific ligands, which remain uncharacterized. Heterozygosity
55 for HbS, sickle cell trait (SCT), affects over 250 million individuals and is maintained in the
56 population by conferring protection against severe malaria. The mechanism underlying this
57 protection is not fully explained, but the mutation has long been known to prevent high levels of
58 parasitaemia (5, 6). Yet under most conditions *in vitro*, the parasites grow equally well in SCT
59 RBCs compared to those with normal haemoglobin (7, 8), implying that protection is due to

60 efficient immune clearance of infected SCT RBCs, and again raising questions as to the identity
61 of the ligands responsible for mediating phagocytosis.

62

63 **Results**

64

65 **Red blood cells from patients with SCD express high mannose N-glycans on their surfaces**

66

67 We postulated these putative uptake ligands might be N-linked glycans, given the prominence of
68 the glycocalyx on RBCs and the corresponding expression of lectins as key innate receptors on
69 macrophages (9). A survey for surface ligands using a panel of plant lectins identified two panel
70 members that bound preferentially to SCD RBC (Fig. 1a), *Galanthus nivalis* Agglutinin (GNA)
71 and *Narcissus pseudonarcissus* Lectin (NPL). Binding was specific (Extended Data Table 1,
72 Extended Data Figs. 1a-b, 2a) and both lectins were noted to have similar specificities for
73 terminal mannose residues (Extended Data Fig. 1a) (10, 11). Microscopy with fluorescent GNA
74 lectin revealed discrete patches on the surfaces of SCD (HbSS), but not healthy (HbAA), RBCs
75 (Fig. 1b, Extended Data Fig. 1c, d). Glycomic analysis using mass spectrometry showed that
76 SCD RBCs express N-linked high mannose glycans, hereafter high mannose glycans (Man₅-
77 ₉GlcNAc₂; Fig. 1c), which are known ligands for phagocytosis by macrophages (9, 12) and
78 therefore good candidates for mediating RBC uptake. High mannose glycans are also observed in
79 the N-glycome profiles from HbAA RBC ghosts (Fig. 1c, Extended Data Fig. 3, Extended Data
80 File 1). The proportions of high mannose glycans with respect to whole N-glycomes were not
81 significantly different between sickle and healthy RBC ghosts (Extended Data Fig. 1e). The

82 marked difference between GNA binding on the cell surface of HbAA compared to HbSS RBCs
83 is therefore not explained by the total high mannose glycan content of the ghosts.

84

85 **RBC surface mannose correlates with extravascular haemolysis in sickle cell disease**

86

87 To assess the relevance of high mannose N-glycan display for RBC uptake *in vivo*, we exploited
88 the heterogeneity of SCD arising from the interactions of HbS with other mutations in the globin
89 loci (such as HbC, α - and β -thalassaemias) that also protect against malaria (13). If mannoses
90 were phagocytic ligands in SCD, higher levels of mannose exposure should correlate with more
91 severe anaemia. Despite a similar glycomic profile, RBCs from patients who were homozygous
92 for HbS (HbSS) tended to exhibit higher binding of GNA lectin, compared to RBCs from healthy
93 individuals containing HbAA or those with SCT (HbAS) (Fig. 2a). Patients with SCD who were
94 compound heterozygotes for HbS and either HbC or β -thalassaemia, or who had HbSS but with
95 mitigating α -thalassaemia or high levels of HbF, tended to exhibit low to intermediate GNA
96 lectin binding (Fig. 2a). RBCs in other anaemias did not express high levels of exposed mannose
97 residues (Extended Data Fig. 2a). The classical apoptotic marker for phagocytosis,
98 phosphatidylserine, as measured by annexin V binding, was expressed at similar, low levels on
99 RBCs from each of the clinical groups (Extended Data Fig. 2b), although it was highly expressed
100 on positive control calcium ionophore treated, eryptotic RBCs (Extended Data Fig. 2c). Overall,
101 GNA lectin binding correlated significantly with more severe anaemia (Fig. 2b) and other
102 markers of haemolysis (Extended Data Fig. 2d, e), consistent with high mannose N-glycan
103 expression driving ‘extravascular’ uptake by hepatosplenic phagocytes, which is the major
104 mechanism of haemolysis in SCD (14). A minor, but significant, proportion of RBC loss in SCD

105 is also accounted for by intravascular haemolysis (14). However, plasma lactate dehydrogenase
106 (LDH) levels, a marker of intravascular haemolysis (14), did not correlate with RBC GNA lectin
107 binding (Fig. 2c, d, e) within HbSS patients. We postulated that mannose binding lectin might
108 bind and opsonize sickle cells, but no significant correlations between levels of this plasma
109 protein and haemolytic phenotypes were observed (Extended Data Fig. 4a-c). Furthermore, when
110 we added cells washed free of plasma to macrophages, SS, but not AA, RBCs were selectively
111 taken up and this could be inhibited by mannan (Fig. 2f), indicating the macrophages expressed a
112 receptor that interacted directly with surface mannoses.

113

114 **Surface mannoses can be induced on healthy RBC by oxidative stress and are recognized**
115 **by the mannose receptor (CD206)**

116

117 High mannose N-glycans (Man_{5,9}GlcNAc₂) were detected in glycomic analyses of healthy
118 (HbAA) RBCs (Fig 1c, Extended Data Fig. 3). Furthermore, permeabilization of healthy RBCs
119 allowed GNA lectin binding in patches that colocalized with the membrane skeleton (Extended
120 Data Fig. 5a). SCD is associated with intracellular oxidative stress (15, 16), so we determined
121 whether exposing healthy RBCs to an oxidizing agent (Extended Data Fig. 5b) would alter the
122 surface mannose exposure as assessed by GNA lectin binding (Fig. 3a). Under the experimental
123 conditions applied, oxidation of healthy RBCs indeed resulted in binding of GNA lectin, with
124 similar, but fewer, patches observed compared to unoxidized HbSS RBCs (Fig. 3b). Artefactual
125 GNA lectin-binding resulting from permeabilization of the cell by oxidative damage was ruled
126 out (Extended Data Fig. 5c), as was potential intracellular O-GlcNAc binding by GNA lectin
127 (Extended Data Fig. 5d). To identify the cognate receptor on macrophages, we measured the

128 binding of a panel of recombinant mammalian C-type lectin fusion proteins with different glycan
129 specificities to oxidized RBCs. This survey implicated the mannose receptor (17, 18) (MR,
130 CD206), in particular the mannose recognizing carbohydrate recognition domain (MR-CRD)
131 (Fig. 3c). We also observed that in cultures of oxidized RBCs with human monocyte derived
132 macrophages (HMDM) *in vitro*, uptake was restricted to MR positive cells (Fig. 3d).
133 Furthermore, siRNA knockdown of MR in macrophages (Extended Data Fig. 6c) specifically
134 reduced phagocytosis (Fig. 3e, Extended Data Fig. 6d); and phagocytosis was also inhibited by
135 the competing glycans mannan or chitin, each known to block MR-CRD (19), but not inhibited
136 by the control glycan laminarin (Fig. 3f, Extended Data Fig. 6e). Finally, MR-CRD-blocking
137 antibody also inhibited phagocytosis of both oxidized healthy and native SCD RBC (Fig. 3g, h),
138 which was also sensitive to mannan and chitin (Fig. 3i). Taken together, these results
139 demonstrate that phagocytosis of mannose-displaying RBC is dependent on MR, although
140 involvement of other receptors cannot be excluded.

141

142 **GNA lectin binding proteins comigrate with spectrin containing complexes**

143

144 The above data show that high mannose sugars occur in the glycomes of both HbAA and HbSS
145 RBC, and that these sugars can be detected on the surface of HbSS RBC and oxidized HbAA
146 RBC by GNA lectin binding. To identify the proteins carrying the high mannose sugars, extracts
147 of HbAA and HbSS RBC membranes were analysed by western blots probed with GNA lectin.
148 A GNA-binding doublet around 260kDa was identified in both HbAA and HbSS RBC (Fig. 4a),
149 which is similar in molecular weight to the abundant membrane skeleton proteins α - and β -
150 spectrin. Blots of HbSS ghosts showed additional GNA lectin-binding bands at ~160kDa,

151 ~100kDa, ~70kDa and ~50kDa, which were not seen in fresh HbAA and HbAS RBC (Fig. 4a-b).
152 When HbAA RBCs were stored for six weeks, to allow oxidative damage to membrane skeletal
153 proteins (20), lower molecular weight Endo-H (N-glycan specific glycosidase) sensitive GNA
154 lectin-binding bands corresponding in size to fragments seen in HbSS cells were seen on western
155 blotting, and GNA lectin precipitation enriched these fragments (Extended Data Fig. 7a, b). The
156 intensity of the 100kDa fragment was noted to correlate positively with RBC surface GNA lectin
157 binding assessed by flow cytometry (Fig. 4b, Extended Data Fig. 7c), suggesting a role in the
158 surface exposure of high mannose glycans. The specificity of the GNA lectin binding was
159 confirmed by treating RBC ghosts with N-glycan specific glycosidases (PNGase F and Endo-H)
160 prior to western blotting, which abolished GNA-binding to all of the above bands (Fig. 4c). We
161 next attempted to determine whether treatment of RBC ghosts with N-glycanases reduced the
162 sizes of GNA-binding bands. A molecular weight change was not observable for the high
163 molecular weight doublet around 260kDa, although the large sizes of these proteins made minor
164 shifts in difficult to observe. However, a ~70kDa band from HbSS ghosts did show an
165 appropriate reduction in molecular weight, consistent with cleavage of N-glycans after treatment
166 of RBC ghosts with PNGase F and Endo-H (Fig. 4d). Western blotting with antibodies to β -
167 spectrin, indicated the band contained an epitope derived from spectrin.

168
169 To investigate the association of spectrin with high mannose glycans species further, we carried
170 out mass spectrometric analysis of tryptic peptides from the 260kDa GNA-binding doublet from
171 both HbAA and HbSS RBC ghosts and found that both bands contained large quantities of α -
172 and β -spectrin (Extended Data Table 2). As has been previously reported in RBC proteomic
173 experiments (21), other abundant RBC proteins of lower molecular weight were also identified,

174 including integral membrane glycoproteins such as Band 3 and Glut-1 (Extended Data Table 2).
175 However, despite extensive mass spectrometric analyses from the 260 kDa doublet, no
176 conventionally glycosylated peptides were identified.

177

178 Further evidence indicating covalent linkages between high mannose glycan containing
179 glycoproteins/glycopeptides and spectrin derived peptides came from western blots of membrane
180 extracts from HbSS RBC or HbAA ghosts treated with trypsin, which exhibited anti-spectrin
181 binding lower molecular weight bands comigrating with GNA lectin signals (Extended Data Fig.
182 7c, d), particularly marked for the 50kDa GNA-binding band and α -spectrin antibodies. GNA
183 lectin precipitation of extracts from HbAA ghosts followed by western blotting with antibodies
184 to spectrin, detected a ~260 kDa protein (Fig. 4e). Finally, super resolution imaging of
185 permeabilized HbAA and HbSS RBC demonstrated GNA lectin co-localising with spectrin in
186 discrete patches scattered in the spectrin membrane skeletal network (Fig. 4f). Taken together
187 these data support the hypothesis that spectrin containing complexes in HbSS and oxidized
188 HbAA RBCs are N-glycosylated with high mannose glycans, although it was not possible to
189 detect specific N-glycosylated peptides through conventional glycoproteomic approaches.

190

191 **GNA lectin binds to low molecular weight complexes that include spectrin, are protease**
192 **resistant and derive from higher molecular weight aggregates**

193

194 In order to generate smaller fragments of high mannose-bearing fragments that would be more
195 amenable to characterization, HbSS ghosts were incubated with serial dilutions of trypsin,
196 spectrin was purified from them and then probed with GNA in western blots. This showed that

197 high concentrations of trypsin, sufficient to digest the 260kDa and 160kDa GNA lectin binding
198 proteins, failed to degrade the ~50kDa, ~70kDa and ~100kDa GNA lectin binding gel bands
199 (Fig. 4g and Extended Data Fig. 7e)). Indeed, the intensities of these bands, particularly that at
200 ~100kDa, increased with higher trypsin concentrations (Fig. 4g). Prolonged, high concentration
201 trypsin digestion eventually degraded the ~100kDa fragment, and, to some extent, the ~70kDa
202 GNA lectin binding bands, with concurrent appearance of a new GNA lectin binding band
203 around 40kDa, which we term F40 (Extended Data Fig. 7f). This same pattern of loss of the
204 ~100kDa and ~70kDa GNA lectin binding fragments with concurrent appearance of F40 was
205 also observed when HbSS erythrocytes were stored over five weeks (Extended Data Fig. 7g).
206 Hence, protease digestion results in formation of a 40kDa protease-resistant fragment that binds
207 GNA lectin and therefore carries high mannose glycans.

208

209 The F40 fragment was concentrated through sequential 100kDa and 10kDa cut-off concentrators
210 (Extended Data Fig. 8a, b), and the resulting band cut out from a gel for glycoproteomic analysis.
211 Proteomic analysis of F40 identified peptides from α -spectrin, particularly the N-terminal 370
212 amino acids (Fig. 4h, Extended Data Table 3). PNGase-F treatment of the purified F40 fragment
213 released N-glycans consisting mainly of high mannoses (Man₆-Man₉) and complex structures
214 (Extended Data Fig. 8c). However, once again, no specific glycopeptides could be identified. We
215 postulated that the reason for this, and the relatively low identified protein sequence coverage,
216 could be unconventional peptide structures arising from oxidized and glycated aggregates.
217 Indeed, mass spectrometry confirmed the presence of lysine glycation in α -spectrin peptides
218 from F40 at amino acids K59, K270 and K281. Furthermore, although GNA lectin binding to
219 F40 was Endo-H sensitive, the enzyme required denaturing conditions to be effective (Fig. 4i),

220 consistent with the possibility of protein aggregates. Additionally, the N-terminal α -spectrin
221 antibody, B12, failed to bind to the full-length F40 band, but bound to smaller fragments after
222 treatment with a combination of proteases, indicating a cryptic epitope (Extended Data Fig. 8d, e,
223 f). Finally, when visualized in 3D-SIM, some HbSS cells show large aggregates of intracellular
224 spectrin, which correspond to dense GNA lectin surface staining (Fig. 4j). Overall, our data
225 demonstrate the existence of high mannose glycans in HbSS RBC extracts and suggest that the
226 main GNA lectin-binding molecules are spectrin-containing glycoprotein complexes with
227 atypical structures, including glycosylated forms, that make analysis by conventional
228 glycoproteomics challenging.

229

230 **Infection of RBCs with *P. falciparum* causes exposure of high mannose N-glycans,**
231 **especially those from donors with SCT**

232

233 As infection of RBC with malarial parasites is associated with oxidative stress (22), we
234 investigated whether exposure of high mannose N-glycans might be important in protection
235 against infection with *P. falciparum*, particularly in the context of SCT. First, we determined the
236 sensitivity of SCT RBCs to a given oxidative stress and found they bound more GNA lectin than
237 HbAA RBC (Fig. 5a). Interestingly, the proportion of HbS correlated well with the degree of
238 oxidative stress (Fig. 5b). These data suggested that exposure of high mannose N-glycans might
239 contribute to the resistance of individuals with SCT to severe malaria, by enhancing clearance of
240 infected cells. We therefore infected HbAA and HbAS RBC with *P. falciparum* and assessed
241 high mannose N-glycan exposure as the infection progressed through ring, trophozoite and
242 schizont stages. HbAA RBCs containing schizonts, but not trophozoites, expressed significantly

243 higher exposed high mannose N-glycans as indicated by increased GNA lectin binding (Fig. 5c).
244 Importantly, HbAS RBCs containing schizonts expressed even higher levels of exposed high
245 mannose N-glycans and this increased expression extended into the trophozoite stages (Fig. 5c).
246 *P. falciparum* infected RBCs cytoadhere to vascular endothelium, to avoid phagocytosis by
247 hepatosplenic macrophages (23, 24), and this adhesion is mediated by the expression of PfEMP1
248 on late stage infected RBC (25). Reduced display of PfEMP1 on the surface of HbAS-infected
249 RBCs is a potential mechanism of protection against malaria in SCT (26), and PfEMP1 levels
250 show considerable variation in different HbAS donors (27). We therefore determined PfEMP1
251 expression in addition to mannose display and noted a marked inverse correlation (Fig. 5d).

252

253

254 **Discussion**

255

256 This work has identified a novel receptor-ligand pair mediating RBC clearance that underlies
257 both the extravascular haemolysis of SCD and clearance of *P. falciparum* infected
258 RBCs. Immunologically, the latter can be regarded as protective immunity arising from
259 recognition of altered self, with the mannose receptor recognizing a pattern common to both
260 diseased and infected cells. Display of high mannose N-glycans on membrane proteins could
261 also be regarded as a new damage-associated molecular pattern (DAMP). The mannose receptor
262 is expressed in human spleens by Lyve-1+ cells lining venous sinuses, where they form a
263 physical barrier for blood cells to exit the red pulp and so are ideally located to perform a
264 filtering function (28). In infection with *P. falciparum*, the parasite evades passage through the
265 spleen by expressing adhesive proteins, notably PfEMP1, on the surface of infected RBCs, so
266 that they adhere to endothelial cells in the systemic circulation. The inverse correlation between

267 mannose exposure and surface PfEMP1 implies similar processes involving oxidation induced
268 membrane skeletal rearrangements underlie both phenomena (29, 30). Reduced PfEMP1 in
269 infected HbAS RBCs will lead to a failure of cytoadherence, and the exposed high mannose N-
270 glycans on circulating infected RBC will induce clearance by hepatosplenic phagocytes.
271 Together with the spleen's role in processing high mannose N-glycan bearing RBCs in SCD,
272 these data are consistent with spleen being the primary organ in removing RBCs infected with
273 malaria exposing high mannose N-glycans. Our work also potentially sheds light on the reasons
274 why those with SCD are so susceptible to infections with encapsulated bacteria, especially
275 *Streptococcus pneumoniae*, which is the commonest cause of death in children (1). Capsular
276 polysaccharides from pneumococcus are known to bind the carbohydrate binding domains of the
277 mannose receptor (31). It therefore seems likely that the high mannose N-glycans on the surfaces
278 of sickle cells would compete with bacteria for uptake by the mannose receptor.
279
280 Our work has also identified a new phenomenon whereby complexes of membrane skeletal
281 proteins, and fragments derived from them, are associated with high mannose N-glycans, which
282 act as an eat me signal. There are still only two accepted eat me signals in higher eukaryotes,
283 phosphatidylserine and calreticulin (32). This work therefore adds a third ligand to perform this
284 role. The expression of ligands for uptake attached to the membrane skeleton would allow
285 receptors on phagocytic cells to bind to molecules with high tensile strength, which may be
286 important for capturing cells as they transit through the spleen under conditions of high shear
287 stress.
288

289 Protein aggregates are thought to form after attacks by free radicals, reactive oxygen and
290 nitrogen species and glycation. These result in a wide variety of amino acid adducts (33), some
291 of which mediate the cross-links thought to underlie the formation of aggregates. Complexes of
292 damaged proteins, including spectrin, secondary to oxidative damage associated with haemolysis
293 have long been recognized in RBCs (34-37), partly arising from interaction with free radicals
294 generated by denatured haemoglobin species (hemichromes), including in SCD (38). Oxidative
295 degradation has been shown to be one of the main causes of membrane skeletal protein
296 alterations occurring in RBCs in storage, with proteolytic cleavage having a secondary role (20).
297 Interestingly, spectrin containing species were detected either as low molecular weight fragments
298 covering the N-terminus, as found in our proteomic analysis, or as high molecular aggregates
299 (20). Oxidation during the storage period of RBCs has also been shown to inactivate
300 glyceraldehyde 3-phosphate dehydrogenase, an important enzyme for ATP synthesis (20, 39). In
301 turn, this leads to dissociation of spectrin from the phosphatidylserine molecules of the RBCs
302 membrane, in an ATP dependent mechanism, resulting in increased spectrin-glycation products
303 (40).

304

305 Therefore, either by directly acting on the cytoskeletal proteins, or indirectly through ATP
306 dependent mechanisms, oxidative damage of RBCs is a mechanism that induces alterations in
307 membrane protein organization leading to aggregation of membrane glycoproteins. This is in
308 accordance with a recent report demonstrating that oxidative stress results in cluster-like
309 structures on the membrane of RBCs as a result of possible reorganization and aggregation (41).
310 RBCs contain various glycoproteins such as band 3, glycophorin, GLUT1, CD44 and CD47 (42).
311 Therefore, the enhanced phagocytosis we describe could potentially be driven by the aggregation

312 of RBC membrane glycoproteins increasing the local concentration of high mannose N-glycans,
313 thus favouring their recognition by the mannose receptor. This is in accordance with previous
314 reports showing that the binding affinity of the mannose receptor increases with the density of
315 mannose-containing glycoproteins (43). Taken together, we suggest that oxidative stress in RBCs
316 induces glycoprotein reorganization and aggregation, resulting in increased high mannose glycan
317 bearing densities that are recognized by the mannose receptor.

318

319 In summary, we describe a mechanism whereby oxidatively-damaged, membrane protein
320 complexes display high mannose N-glycans, which act as eat me signals important in the
321 haemolysis of sickle cell disease and resistance against severe malaria. It therefore represents the
322 first unified mechanism to explain both advantageous and deleterious consequences of the sickle
323 mutation.

324 **Methods**

325 *Donors*

326 Ethical approval was obtained for the study (North of Scotland REC Number 11/NS/0026).
327 Further samples, including donors with SCT, were obtained from the NHS Grampian
328 Biorepository scheme (application number TR000142). Discarded anonymized samples were
329 also obtained from patients with SCD from King's College Hospital. HbAA, HbAS and SCD
330 statuses were assessed by Hb HPLC (Tosoh G7). All samples from patients were collected into
331 EDTA tubes (Becton-Dickinson).

332 *RBC isolation*

333 Blood was collected into acid citrate dextrose solution tubes (ACD; 455055, Grenier) and RBC
334 isolated by sodium metrizoate density gradient centrifugation (1.077 g/ml, Lymphoprep;
335 1114547 Axis-Shield). Packed RBC were diluted with an equal volume of Dulbecco's modified
336 Eagle's medium (DMEM; 4.5 g/L glucose, L-glutamine; 41965, Gibco), stored in ACD (9 ml
337 RBC/DMEM per ACD tube) at 4°C and used within 3 days unless otherwise stated.

338 *Lectins*

339 Biotinylated lectins were all purchased from Vector Laboratories. They include: *Galanthus*
340 *nivalis* Agglutinin (GNA, B-1245, 4µg/ml), *Narcissus pseudonarcissus* Lectin (NPL, B-1375,
341 4µg/ml), *Griffonia simplicifolia* Lectin II (G.Simp, B-1215, 4µg/ml), *Solanum tuberosum* Lectin
342 (STL, B-1165, 20 ng/ml), *Aleuria aurantia* Lectin (AAL, B-1395, 33ng/ml), *Maackia amurensis*
343 Lectin II (MAL II, B-1265, 67ng/ml), *Sophora japonica* Lectin (Vector Laboratories, no longer
344 available, 1µg/ml). FITC conjugated Peanut agglutinin was purchased from Sigma-Aldrich
345 (PNA, L7381-2MG, 2µg/ml).

346 *Flow cytometry*

347 Whole blood flow cytometry assays were used for Fig. 1a, 2, Extended Data Fig. 1a, 2, 3b, d, e
348 and 8a. Whole blood was washed first in phosphate buffered saline (PBS). Purified RBC were
349 used for the other flow cytometry experiments. RBC gating was applied by forward and side
350 scatter gating of both whole blood and purified RBC flow cytometry experiments (Extended
351 Data Fig. 9a). Staining with anti-glycophorin A (GPA) confirmed gates contained >99% RBC
352 (data not shown).

353 For lectin flow cytometry, approximately 5×10^6 RBC were washed three times in PBS, and
354 incubated for 15 minutes at room temperature in calcium buffer (10mM HEPES, 150mM NaCl₂,
355 2.5mM CaCl₂, pH 7.4) containing 10% Carbo-Free Blocking Solution (SP5040, Vector
356 Laboratories) for whole blood flow cytometry or just buffer alone for purified RBC flow
357 cytometry. Biotinylated lectin staining was carried out at room temperature in the same buffer as
358 the initial blocking step. PNA-FITC and other antibody staining was carried out in PBS
359 throughout, without contact with calcium buffer or Carbo-Free Blocking Solution. Lectin and
360 antibody staining were carried out for 30 minutes at room temperature, protected from light.
361 Annexin V staining was carried out according to the manufacturer's instructions (640945,
362 Biologend). For biotinylated lectin staining, cells were then washed and incubated with
363 streptavidin PE-Cy7 (0.27 μ g/ml; 25431782, eBioscience) or PE (0.67 μ g/ml; 554061, BD
364 Pharmingen) for 30 minutes at room temperature. Humanized Fc fusions of murine C-type
365 lectins (17, 44) (5 μ g/ml) were incubated with RBC for 30 minutes at room temperature in
366 calcium buffer, then detected by Alexa Fluor 647 goat anti-human secondary antibody (2 μ g/ml;
367 109-605-098, Jackson ImmunoResearch Laboratories). In tests of their specificity for binding
368 RBC, lectin or Fc fusion proteins were first incubated with mannan (5 mg/ml, unless otherwise

369 stated) for 15 minutes at room temperature. Biotinylated BRIC 132 and BRIC 163 (10 µg/ml;
370 9458B and 9410B, International Blood Group Reference Laboratory) and anti-O-GlcNAc (1
371 µg/ml, RL2; 59624, Santa Cruz) binding to RBC were performed in PBS for 30 minutes at room
372 temperature, before incubation with streptavidin secondary (for BRIC 132/163) or anti-mouse PE
373 secondary (for anti-O-GlcNAc) for a further 30 minutes.
374 Prior to intracellular staining, RBC were fixed with glutaraldehyde (0.05%, 10 minutes, room
375 temperature), permeabilized with Triton X-100 (0.1% in PBS) for 5 minutes at room temperature
376 and then washed in PBS. Cells were washed before cytometric analysis. Data were acquired on a
377 FACSCalibur (BD) and analysed using FlowJo v10.0 (Treestar) software. Normalized geometric
378 mean fluorescences (gMFI) were calculated by subtracting the gMFI of secondary
379 antibody/streptavidin-only paired controls. For PNA-FITC and Annexin V analysis, unstained
380 controls were used for gMFI normalization.

381 *RBC ghost preparation*

382 Washed RBC were subjected to ice cold hypotonic lysis in 20 mM Tris, pH 7.6, with protease
383 inhibitor (05056489001, Roche) (45). Lysates were washed three times in hypotonic lysis buffer
384 (37000 g, 4°C, 30 minutes) before resuspension in minimal hypotonic lysis buffer. Protein
385 concentrations were determined by protein BCA assay (23227, Pierce). No trypsinization was
386 performed before any glycan analysis.

387 *Glycomic mass spectrometry (MS)*

388 N-linked glycan analysis from RBC ghosts were performed according to Jang-Lee *et al.* (46). MS
389 and MS/MS data from the permethylated purified glycan fractions were acquired on a 4800
390 MALDI-TOF/TOF mass spectrometer (Applied Biosystems). Data were processed using Data
391 Explorer 4.9 Software (Applied Biosystems). The processed spectra were subjected to manual

392 assignment and annotation with the aid of a glycoinformatics tool, GlycoWorkBench (47).
393 Proposed assignments for the selected peaks were based on ^{12}C isotopic composition together
394 with knowledge of the biosynthetic pathways, and structures were confirmed by data obtained
395 from MS/MS experiments.

396 *Proteomics*

397 RBC ghost membranes were subjected to SDS-PAGE (10% Bis-Tris gel with MOPS running
398 buffer). SDS-gel bands a were excised, sliced into small pieces, and destained with 200 μL of 1:1
399 v:v acetonitrile:ammonium bicarbonate (50 mM, pH 8.4; AMBIC). Destained gel pieces were
400 then reduced by treatment with 10 mM DTT in AMBIC at 56 °C for 30 min, carboxymethylated
401 in 55 mM iodoacetic acid in AMBIC in the dark at room temperature, and then subjected to
402 overnight sequencing grade modified trypsin (Promega V5111) digestion in AMBIC at 37 °C.
403 After enzyme inactivation (100 °C water bath, 3 min), the digested peptides were extracted twice
404 from the gel pieces by incubating sequentially (15 min with vortexing) with 0.1% trifluoroacetic
405 acid and 100% acetonitrile. Finally, the volume was reduced with a Speed Vac. Eluted peptides
406 were analysed by LC-MS using a NanoAcquity UPLC™ system coupled to a Synapt™ G2-S
407 mass spectrometer (Waters MS Technologies, Manchester, UK) in positive ion mode. 5 μL of
408 sample was injected onto the analytical column (Waters, HSS T3, 75 μm \times 150 mm, 1.8 μm).
409 Peptides were eluted according to the following linear gradient program (A: 0.1% v/v formic
410 acid in water, B: 0.1% v/v formic acid in acetonitrile): 0-90 min, 3-50% of B. MS data were
411 acquired on the Synapt G2-S using a data-dependent acquisition program, calibrated using Leu-
412 Enkephalin peptide standard. The top 20 components were selected for MS/MS acquisition.
413 Identification of the eluted peptides was performed using ProteinLynx Global SERVER™ v3.03
414 (Waters) using human porcine trypsin database (Uniprot 1.0). The following were set as

415 workflow parameters on PLGS: fixed carboxymethyl modification for cysteine, variable
416 deamidation and oxidation modifications for glutamine and methionine respectively.

417 *Human monocyte-derived macrophage (HMDM) preparation and culture*

418 Mononuclear cells were isolated by density centrifugation from whole blood and seeded at 10^6
419 cells/ml in Roswell Park Memorial Institute medium (RPMI (21875-034, Gibco)), 100 U/ml
420 penicillin, 100 μ g/ml streptomycin, 292 μ g/ml L-glutamine (10378-016, Gibco) and 10% heat
421 inactivated autologous serum. Cultures were incubated at 37°C with 5% CO₂ for 14-21 days.
422 Cells were washed with RPMI three times prior to use.

423 *Phagocytosis Assay*

424 For identification of phagocytosis by microscopy, RBC were stained with Cell Trace Far Red
425 (CTFR) (C34564, Molecular Probes) according to the manufacturer's instructions with minor
426 alterations: CTFR was diluted at 1 in 500 (2 μ l/ml) in RPMI with penicillin and streptomycin
427 (RPMI/PS) media and incubated with 20 μ l packed RBC for 30 minutes at 37°C, after which
428 staining was inhibited by adding 10% FCS (10270-106, Gibco). Stained cells were washed in
429 RPMI/PS prior to counting and addition to macrophages. RBC were added to HMDM at 5×10^7
430 cells per well for 3 hours, before removal of cells, washing and fixation with 4%
431 paraformaldehyde (Extended Data Fig. 6a). RBC bound, but not ingested, by HMDM were then
432 stained with anti-glycophorin-FITC (HIR2 antibody; 306610, Biolegend). Cells were imaged
433 using an immunofluorescence microscope (Zeiss AxioObserver Z1). Phagocytic macrophages
434 were defined as containing at least one CTFR-positive GPA-FITC-negative RBC (determined by
435 bright field). Three examples of oxidized RBC phagocytosis are shown in Extended Data Fig.
436 6b, marked 'P'. RBC-binding macrophages were defined as associated with at least one
437 glycophorin-FITC/CTFR double positive RBC. Analysis of HMDM phagocytosis included only

438 the small, non-granular subset of macrophages, because of consistent association with
439 phagocytosis and binding of RBC. For quantification of phagocytosis, 200-500 such
440 macrophages were counted per treatment. To test specificity of HMDM recognition, the
441 polymers mannan (10 mg/ml; M-7504, Sigma-Aldrich), chitin (50 µg/ml; C9752, Sigma-
442 Aldrich) or laminarin (10 mg/ml; L9634, Sigma-Aldrich), or anti-CD206 blocking antibody (10
443 µg/ml clone 15.2 321102, BioLegend; isotype control mouse IgG1 kappa clone 107.3; 554721,
444 BD Biosciences) were added to cultures 60 minutes before phagocytosis assays. Coumarin-
445 stained 6 µm Fluoresbrite carboxylate microspheres, of similar size to RBC, were used to assess
446 RBC independent phagocytosis (Extended Data Fig. 6e).

447 *RBC oxidation and eryptosis*

448 Purified RBC were incubated for 60 or 30 minutes respectively with 0.2 mM copper sulphate and
449 5 mM ascorbic acid at 37°C in DMEM with 4.5 g/L glucose. Cells were washed in PBS 3 times
450 prior to use. To induce eryptosis, calcium ionophore (2µM, A23187, Sigma-Aldrich) was applied
451 at 37°C in DMEM, with 4.5 g/L glucose, to purified RBC for 3 hours.

452 *Reactive Oxygen Species (ROS) Production*

453 The rate of ROS production was determined by first loading purified oxidized or untreated RBC
454 with oxidation sensitive dye CM-H2DCFDA (10 µM; C6827, Molecular Probes) in PBS and
455 incubating for 60 minutes in the dark at 37°C. RBC were then washed three times, resuspended
456 in DMEM and fluorescence determined immediately by spectrofluorimeter (Fluostar Optima;
457 BMG Labtech) with excitation of 485 nm and emission 530 nm. The rate of ROS formation was
458 calculated for the linear portion of the fluorescence/time curve generated over six hours, which
459 typically lasted for three hours.

460 *Lectin/Immuno-blotting*

461 Ghost preparations were mixed in equal volumes with SDS sample buffer containing 8M urea
462 (45) and heated at 100°C for 10 minutes. Ghost protein samples were fractionated by gel
463 electrophoresis using NuPage 4-12% Bis-Tris gel (Invitrogen, NP0312BOX) and transferred by
464 western blotting (30V, 1 hour) to polyvinylidene fluoride membrane (P 0.45 µm; 10600023
465 Amersham Hybond, GE Healthcare). Blots were probed with biotinylated GNA lectin (40 µg/ml;
466 B1245, Vector Laboratories) and Streptavidin HRP (1:2500 dilution, 3999S, Cell Signalling) in
467 calcium binding buffer (10mM HEPES, 150mM NaCl₂, 2.5mM CaCl₂, pH 7.4) containing 1x
468 Carbo-Free Blocking Solution (Vector Laboratories, SP-5040) and protease inhibitor cocktail
469 (11836145001, Roche) before development in Amersham ECL Select substrate (RPN2235, GE
470 Healthcare). 0.1% Tween-20 was added in probing and washing steps. Loading of wells was
471 normalized by protein concentration (~6 µg per sample). Enzymes PNGase F (P0704S, New
472 England Biolabs) and Endo-Hf (P0703L, New England Biolabs) were used according to the
473 manufacturer's instructions to treat RBC ghost samples prior to electrophoresis.

474 *Lectin precipitation*

475 RBC ghosts were suspended in equal volumes of calcium binding buffer containing 2% Triton
476 X-100, pre-cleared with magnetic streptavidin beads (88816, Pierce) and incubated with
477 biotinylated GNA lectin (1 mg/ml; B1245, Vector Laboratories), biotinylated MAL-II lectin (1
478 mg/ml; B1265, Vector Laboratories) or buffer only overnight at 4°C. Precipitation with magnetic
479 streptavidin beads was performed in binding buffer and the beads washed with binding buffer
480 containing 0.1% Triton X-100. Washed precipitates were denatured at 100°C for 10 minutes and
481 supernatants loaded for gel electrophoresis and blotting. Blots were probed with anti-spectrin
482 antibody (1 in 20,000 dilution; S3396, Sigma-Aldrich) and anti-mouse-HRP secondary antibody

483 (1 in 10,000 dilution, 5887, Abcam). PBS/0.1% Tween 20 replaced calcium binding buffer for
484 lectin blotting.

485 *Spectrin purification*

486 Spectrin was purified following the method of Ungewickell *et al.* with slight modifications (48).
487 Briefly, the ghosts were washed twice and resuspended in 3 volumes of 37°C pre-warmed
488 sodium phosphate (0.3 mM, pH 7.2) (extraction buffer) and incubated for 20 min at 37°C. The
489 fragmented ghosts were pelleted by centrifugation at 40000g for 1 hour at 2°C. Supernatant was
490 used as spectrin preparation for analysis.

491 *Serial trypsin dilution treatment of spectrin*

492 Spectrin preparations or ghosts were analysed for protein content by BCA assay. Titrations of
493 trypsin at concentrations as a fraction of sample concentration were applied for one hour or
494 longer if indicated, at 37°C. No trypsin addition was applied to the untreated sample, which was
495 also incubated for the same duration. Samples were all heat inactivated at 100°C for 10 minutes,
496 after diluting with 8M urea sample buffer at a 1:1 ratio.

497 *Chymotrypsin and pepsin digestions*

498 Chymotrypsin and pepsin were applied at 1:5 dilution in sample. Combinatorial protease
499 treatment over 48 hours were performed with heat inactivation for 100°C, 10 minutes at 24
500 hours. During pepsin treatment, sample was pre-diluted 1:1 with HCl, pH2.0. Acid was
501 neutralized with NaOH after 24 hours, prior to addition of other proteases.

502 *Isolation of trypsin resistant sickle fragment (TRSF)(F40)*

503 Approximately 20ml of HbSS ghosts, having been washed with low cold salt extraction buffer
504 (0.3M sodium phosphate, pH 7.6), was treated with 1:5 trypsin: sample ratio overnight. Heat
505 inactivation at 100°C was carried out for 10 minutes. Supernatant was harvested and further

506 centrifuged to remove insoluble products. Clarified supernatant was subsequently concentrated
507 with a 100kDa cut-off concentrator (Pierce, Thermo Fisher, 88533) and supernatant applied to
508 and concentrated with a 10kDa cut-off concentrator to approximately 500 μ l.

509 *Mass spectrometry glycomic analysis of TRSF*

510 Urea containing sample buffer, as above, was applied to TRSF. Coomassie bands corresponding
511 to 40-44kD region were cut and analysed in three segments: 39-40kD, 41-42kD and 43-44kD.

512 *Dual colour western*

513 Amersham western blotting machines was used to detect GNA lectin and anti-spectrin binding
514 using Cy3 and Cy5 conjugated reagents. Data were acquired and analysed by Amersham's
515 inbuilt software.

516 *Immunofluorescence microscopy*

517 Cell surface GNA lectin binding for immunofluorescence was performed as for flow cytometry
518 with minor alterations (10^7 cells per test, 8 μ g/ml GNA lectin, 1 μ g/ml Streptavidin PE. For Fig.
519 1b and Extended Data Fig.1c and 1d, 8 μ g/ml GNA lectin and 1 μ g/ml Streptavidin PE were pre-
520 complexed overnight). Intracellular GNA lectin binding followed fixation (0.005%
521 glutaraldehyde/PBS, 10 minutes, room temperature) and permeabilization (0.1% Triton-X
522 100/PBS, 15 minutes, room temperature). Stained cells were pulse centrifuged ($\leq 300g$) for 30
523 seconds (including acceleration), in 24 well, flat bottom tissue culture plates (Greiner). To stain
524 CD206, cells were blocked for 15 minutes with 1% BSA/PBS at room temperature in the dark
525 and incubated with Alexa-488 conjugated anti-mannose receptor antibody (1.25 μ g/ml, Clone
526 19.2; 53-2069-47, eBiosciences). DAPI (as per manufacturer's instructions; D1306, Thermo
527 Fisher) staining was applied to cells post fixation/permeabilization for 30 minutes at room
528 temperature. Cells were washed in PBS and imaged at 32x magnification using an

529 immunofluorescence microscope (Zeiss AxioObserver Z1). Images were analysed by Zen (Black
530 and Blue versions, Zeiss).

531 *siRNA knockdown of mannose receptor*

532 Human mannose receptor (CD206) siRNA (UACUGUCGCAGGUAUCAUCCA) or a non-
533 targeting siRNA sequence control (4390843, Life Technologies) were transfected into HMDM
534 (RNAiMax, Life Technologies) (n = 4 donors for all siRNA experiments). Knockdown
535 efficiency was established by determining mannose receptor expression by microscopy using
536 CD206-Alexa-488 staining (described above) in the small non-granular macrophage sub-
537 population by merging bright field and mannose receptor fluorescence staining. Knockdown
538 efficiency was typically 65-85% (Extended Data Fig. 6c).

539 *Confocal microscopy for RBCs*

540 For spectrin-GNA lectin double staining experiments, permeabilized RBCs were stained with
541 anti-human spectrin antibody (1 in 50 dilution; S3396, Sigma Aldrich) concurrently with GNA
542 lectin (8 µg/ml) in calcium buffer. Alexa Fluor 647 anti-mouse antibody (10 µg/ml; A31571,
543 Thermo Fisher) was applied in conjunction with streptavidin PE (1 µg/ml; Beckman Dickinson)
544 following staining of primary reagents. RBCs were gravity sedimented (30 minutes at room
545 temperature, in the dark) onto poly-L-lysine (Sigma Aldrich) treated 8 well chamber slides
546 (LabTek). Images were acquired by a Zeiss LSM 710 microscope.

547 *3D-Structured Illumination Microscopy (3DSIM)*

548 GNA lectin/anti-spectrin stained RBCs were gravity sedimented onto poly-L-lysine treated
549 chamber slides (LabTek). 3DSIM images were acquired on a N-SIM (Nikon Instruments, UK)
550 using a 100x 1.49NA lens and refractive index matched immersion oil (Nikon Instruments).
551 Samples were imaged using a Nikon Plan Apo TIRF objective (NA 1.49, oil immersion) and an

552 Andor DU-897X-5254 camera using 561 and 640nm laser lines. Z-step size for Z stacks was set
553 to 0.120 μm , as required by the manufacturer's software. For each focal plane, 15 images (5
554 phases, 3 angles) were captured with the NIS-Elements software. SIM image processing,
555 reconstruction and analysis were carried out using the N-SIM module of the NIS-Element
556 Advanced Research software. Images were checked for artefacts using SIMcheck software
557 (<http://www.micron.ox.ac.uk/software/SIMCheck.php>). Images were reconstructed using NiS
558 Elements software v4.6 (Nikon Instruments, Japan) from a Z stack comprising $\geq 1\mu\text{m}$ of optical
559 sections. In all SIM image reconstructions, the Wiener and Apodization filter parameters were
560 kept constant. Reconstructed SIM images were rendered in 3 dimensions using Imaris (Bitplane).
561 Intracellular GNA lectin and anti-spectrin staining of healthy RBCs was performed following
562 fixation and permeabilization. In order to co-localize surface GNA lectin with intracellular
563 spectrin, SCD RBCs were stained with GNA lectin/streptavidin-PE staining before, and anti-
564 spectrin/donkey anti-mouse Alexa 647 after, fixation and permeabilization.

565

566 *P. falciparum* culture in RBCs of different genotypes and flow cytometry analysis

567 *P. falciparum* IT/FCR3 parasites were cultured at 2% haematocrit in supplemented RPMI as
568 described (49). Mature trophozoite-infected erythrocytes were purified to >90% parasitaemia by
569 magnetic separation with a MACS CS column (Miltenyi Biotec, Germany) (50). The purified
570 infected erythrocytes were used to infect RBCs of different genotypes with a starting
571 parasitaemia of approximately 0.5%. RBCs were used within 10 days post-bleed, typically 3-5
572 days. Cultures were gassed with 1% oxygen, 3% carbon dioxide and 96% nitrogen, then
573 incubated at 37°C for 48-72 hours. After one cycle of invasion and growth, flow cytometry was
574 used to assess parasitaemia and parasite maturation (51), as well as GNA lectin -binding and

575 PfEMP1 antibody staining. This method uses internally controlled flow cytometry analysis to
576 separate uninfected red cells, ring-stage, trophozoite and schizont stage parasites within the same
577 culture by FACS gating, using a pair of DNA- and RNA-binding dyes (Extended Data Fig. 9b,
578 c). Hypoxia-induced reduction in parasite invasion and growth was observed in HbAS red cells,
579 as described by Archer *et al.* (8). However, a range of parasite stages was available within each
580 culture to allow investigation of high mannose exposure as parasites matured through the blood
581 stage cycle.

582 For flow cytometry, infected erythrocytes in binding buffer (10mM HEPES, 150mM NaCl,
583 2.5mM CaCl₂, pH 7.4) were stained with Vybrant Violet (Thermo Fisher, V35003, 2.5µM) and
584 ethidium bromide (Sigma-Aldrich, 46067-50ml-F, 1% in dH₂O). Biotinylated GNA lectin
585 staining was performed as described above. Streptavidin APC was used to detect biotinylated
586 GNA lectin. A BD LSRFortessa (BD Biosciences) was used for flow cytometry and
587 compensation between channels was carried out prior to the experiment. Relative GNA lectin
588 binding was calculated for ring, trophozoite and schizont gates by dividing the gMFI for each
589 gate by the value measured in the uninfected RBC gate. PfEMP1 expression was assessed using
590 a rabbit polyclonal antibody raised against the N-terminal region (DBLαCIDR didomain) of the
591 predominant PfEMP1 variant expressed in the culture (ITvar70, also known as AFBR6) (52), as
592 described previously (53). The staining with PfEMP1 antibody (purified total IgG at 10 µg/ml for
593 30 mins, followed by APC-conjugated goat anti-rabbit Alexa 647 (Invitrogen, A21244, 2 µg/ml))
594 was compared to rabbit IgG control antibody (10 µg/ml, total IgG from a non-immunized rabbit,
595 followed by secondary antibody as above). Normalized PfEMP1 gMFI was calculated by
596 subtracting gMFI for rabbit IgG control antibody staining from that of the PfEMP1 antibody
597 staining. Relative PfEMP1 expression for all samples is expressed as a percentage of the average

598 HbAA schizont PfeMP1 expression per experiment, which typically contained three HbAA
599 samples.

600 *Statistical analysis*

601 Most data are treated as non-parametrically distributed and presented with medians and
602 interquartile ranges, with the exception of Fig. 5c, where means and standard deviations are
603 shown. Statistical significance was assessed by either two-tailed Mann-Whitney (non-paired
604 data) or two-tailed Wilcoxon signed rank tests (paired data). Multiple comparisons between
605 stages of RBC infection by *P. falciparum* were analysed by ANOVA. All calculations were
606 implemented in Prism version 5.04 (GraphPad Software).

607

608 **Data Availability**

609 The authors declare that the data supporting the findings of this study are available within the
610 paper and its supplementary information files. Further data are available from the corresponding
611 author upon reasonable request.

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750

751

752 **Supplemental Information**

753 Extended Data Tables 1-3.

754 Extended Data Figs. 1-9.

755 Extended Data File 1.

756

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771

772 **Author contributions**

773 H.C. carried out experiments, analysed data and wrote the paper. S.H., A.M., B.P., J.S., H.W.,
774 M.A.F., E.B., S.L., G.K., B.M., M-L.W., A. Davie, D.T., M.M., L.H., C.L., W.P. carried out

775 experiments. J.B., supervised by D.C.R., and B.R. obtained blood samples. A.A. obtained and
776 analysed glycomic and proteomic data, supervised by S.M.H. and A. Dell. L.E., G.D.B. and
777 H.M.W. helped supervise the project. J.A.R. carried out experiments, analysed data and wrote
778 the paper. R.N.B. and M.A.V. conceived and supervised the project, and wrote the paper.

779

780 **Author information**

781 The authors declare the existence of a financial competing interest. The University of Aberdeen
782 has applied for patents covering diagnostic and therapeutic applications arising from the work
783 described in this paper. Correspondence and requests for materials should be addressed to
784 M.A.V. (m.a.vickers@abdn.ac.uk) or A.R. (Alex.Rowe@ed.ac.uk).

785

786 **Data Availability**

787 Some source data are provided in the online version of the paper. The other datasets generated
788 during and/or analysed during the current study are available from the corresponding authors on
789 reasonable request.

791 **Figure 1: HbSS RBCs are characterized by microdomain expression of surface mannoses.**

792 a) Whole blood flow cytometry analysis of HbAA and HbSS RBCs using fluorescently
793 labelled plant lectins, detailed in Methods. Vertical axes show normalized geometric
794 mean fluorescence (gMFI). Symbols of terminal carbohydrate detected by plant lectins
795 are indicated. Data shown as median +/- IQR, n=7 per group for significant differences, 2
796 tailed Mann-Whitney p values shown, distinct samples measured once each, 3 separate
797 experiments. Annotation uses conventional symbols for carbohydrates in accordance with
798 <http://www.functionalglycomics.org> guidelines: purple diamond, sialic acids; yellow
799 circle, galactose; blue square, N-acetyl glucosamine; green circle, mannose; red triangle,
800 fucose.

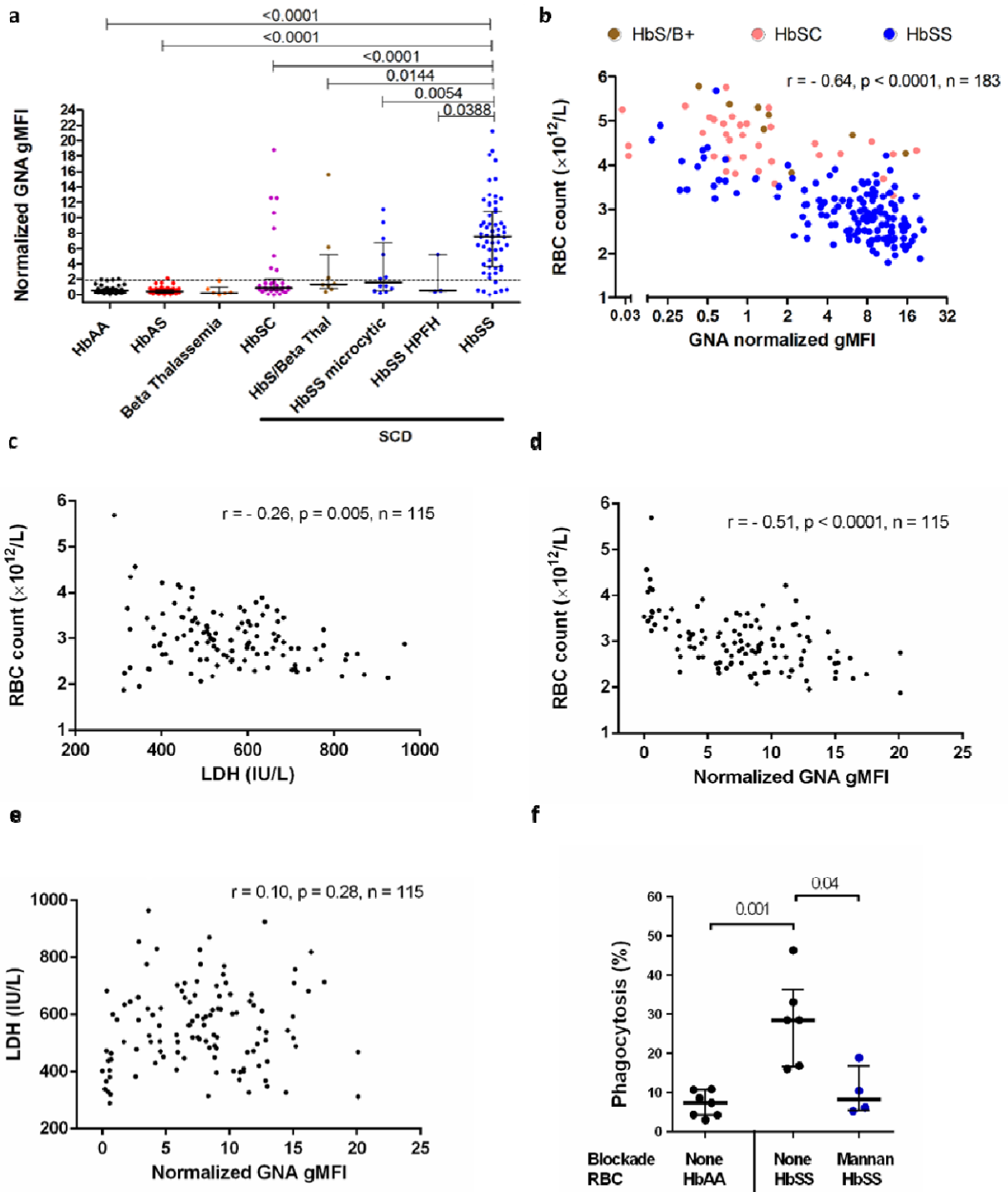
801 b) GNA lectin staining (red) of HbSS and HbAA RBCs, immunofluorescence, merged with
802 bright field.

803 c) MALDI-ToF mass spectra (m/z versus relative intensity) for glycomic analysis of N-
804 glycans from membrane ghosts from individual HbSS and HbAA donors. Red boxes
805 indicate high mannose structures. Annotation uses conventional symbols for
806 carbohydrates in accordance with <http://www.functionalglycomics.org> guidelines: purple
807 diamond, sialic acids; yellow circle, galactose; blue square, N-acetyl glucosamine; green
808 circle, mannose; red triangle, fucose. Only major structures are annotated for clarity. Full
809 spectra of both HbSS and HbAA donors are shown in Extended Data Fig. 9.

810

811

Figure 2



812

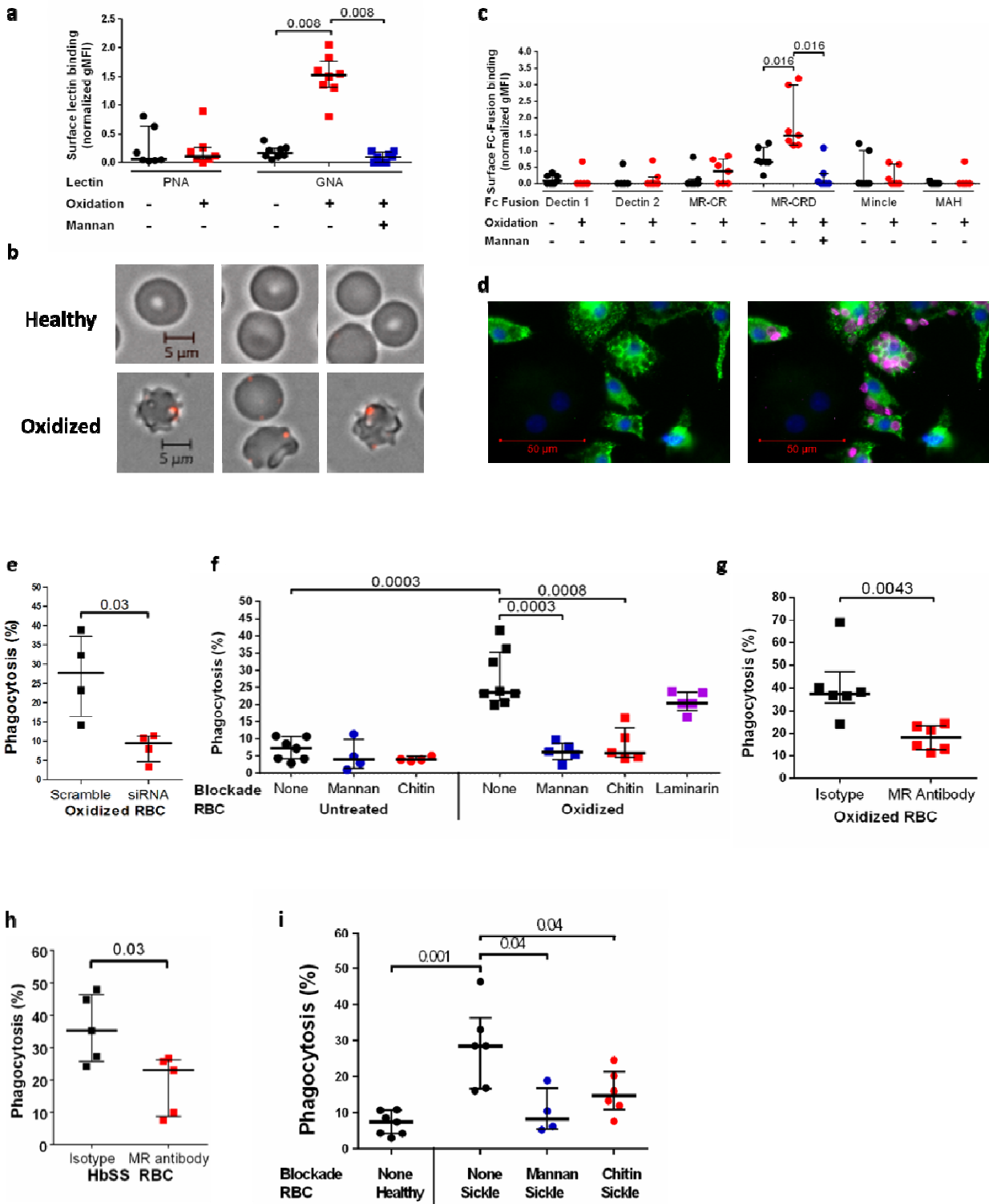
813

814 **Figure 2: Mannose expression correlate to clinical anaemia in SCD**

- 815 a) Normalized gMFI of GNA lectin staining of RBCs from peripheral blood samples,
816 comparing RBC from patients with sub-types of SCD (milder phenotypes: HbSC (n=34),
817 HbS/beta-thal (n=8), HbSS microcytic (n=12) and HbSS HPFH (n=3) indicate compound
818 heterozygosity for HbC, β -thalassaemia, α -thalassaemia and hereditary persistence of
819 fetal haemoglobin respectively) versus healthy donors (n=45), sickle cell trait (n=57) and
820 β -thalassaemia (n=6). Dotted line indicates 90th centile of GNA lectin binding within
821 healthy samples. Data shown as median +/- IQR, 2 tailed Mann-Whitney p values shown,
822 distinct samples measured once each, numerous experiments.
- 823 b) Plot of RBC count against normalized GNA gMFI for SCD: HbS/B+ and HbSC indicates
824 compound heterozygosity for HbS with β -thalassaemia and HbC respectively;
825 Spearman's rank correlation.
- 826 Plots of:
- 827 c) RBC count versus serum lactate dehydrogenase (LDH),
828 d) RBC count vs GNA binding,
829 e) LDH vs GNA lectin binding; HbSS RBCs for which corresponding serum LDH values
830 were available; Spearman's rank correlation,
831 f) Percentage phagocytosis of HbAA and HbSS RBCs by human monocyte derived
832 macrophages analysed by microscopy. Mannan inhibition as shown. Each data point
833 represents a different RBC donor. Mann-Whitney; pooled from two experiments.

834

Figure 3



835

836

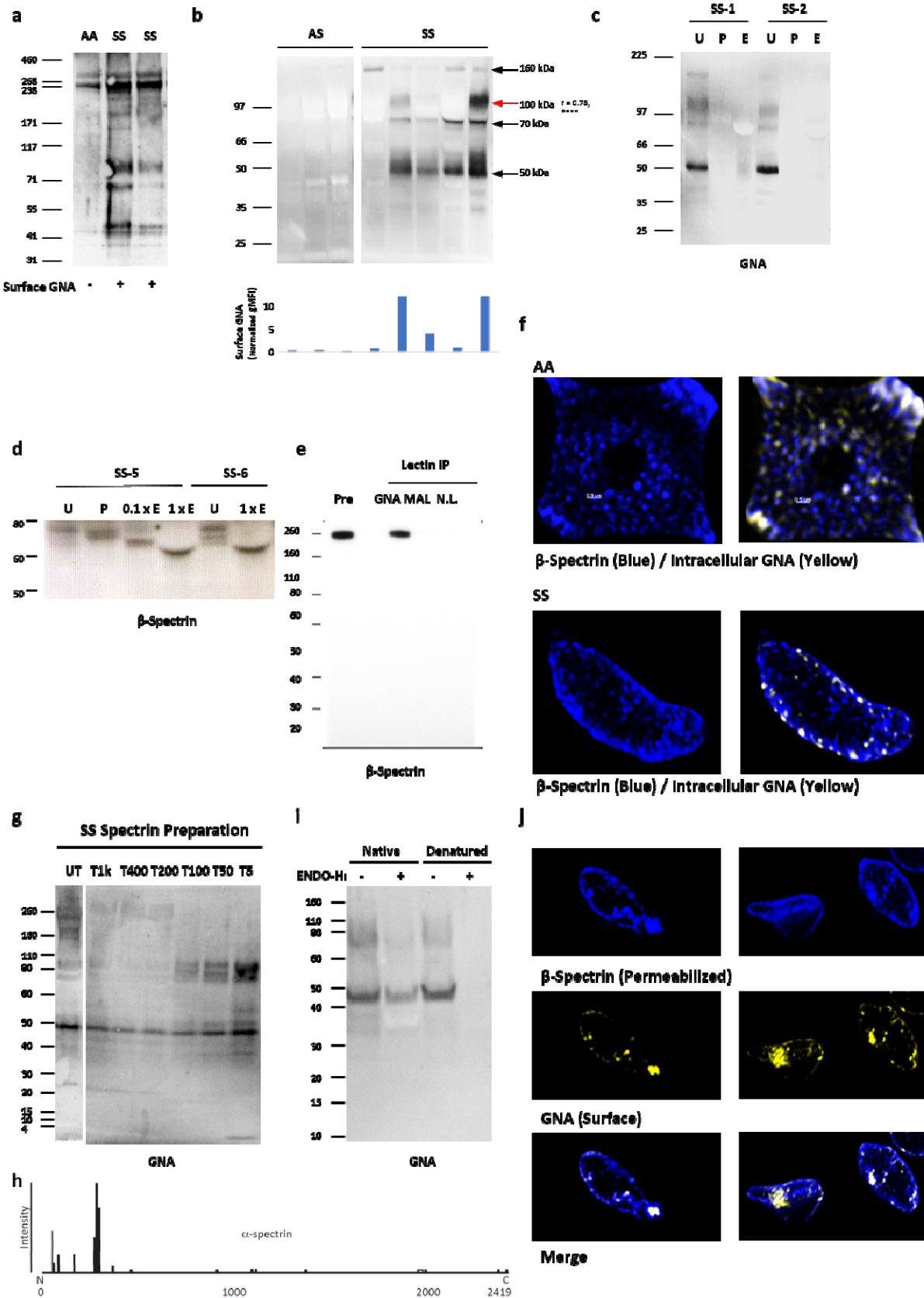
Figure 3: Display of membrane skeleton associated mannose patches is induced by

837 **oxidative stress and recognized by the mannose receptor on macrophages.**

- 838 a) PNA and GNA lectin binding to HbAA RBCs with or without oxidation. Mannan
839 blockade for GNA lectin binding shown in blue. Wilcoxon, paired data, pooled from two
840 independent experiments.
- 841 b) Immunofluorescence microscopy of GNA lectin/streptavidin (red) staining of healthy
842 HbAA RBCs (above) and after oxidative insult (below).
- 843 c) Normalized gMFI for binding analysed by flow cytometry of murine Fc fusions with C-
844 type lectins or sub-domains applied to oxidized versus undamaged RBCs. Mannan
845 blockade of MR-CRD binding is shown in blue. MAH, macrophage antigen H. CR,
846 cysteine rich. CRD, carbohydrate recognition domain. Wilcoxon, paired data, pooled
847 from two independent experiments.
- 848 d) Immunofluorescence microscopy image of human monocyte derived macrophages
849 (HMDM) stained with DAPI (blue) and for mannose receptor (green) after incubation
850 with oxidized HbAA RBCs, shown in magenta.
- 851 e) Percentage phagocytosis of oxidized RBCs by HMDM treated with human MR specific
852 or scrambled siRNA. Mann-Whitney, 2 experiments.
- 853 f) Percentage phagocytosis of healthy or oxidized HbAA RBCs by HMDM with or without
854 pre-blocking by mannan, chitin or laminarin
- 855 g) As (f) but oxidized RBCs are blocked by MR-CRD blocking antibody 15.2 as indicated.
856 Mann-Whitney.
- 857 h) Percentage phagocytosis of HbSS RBCs with or without pre-blocking by MR-CRD
858 blocking antibody 15.2. Mann-Whitney, 3 experiments.

- 859 i) HbAA unblocked and HbSS unblocked or mannan and chitin blockade phagocytosis
860 experiments as shown.

Figure 4

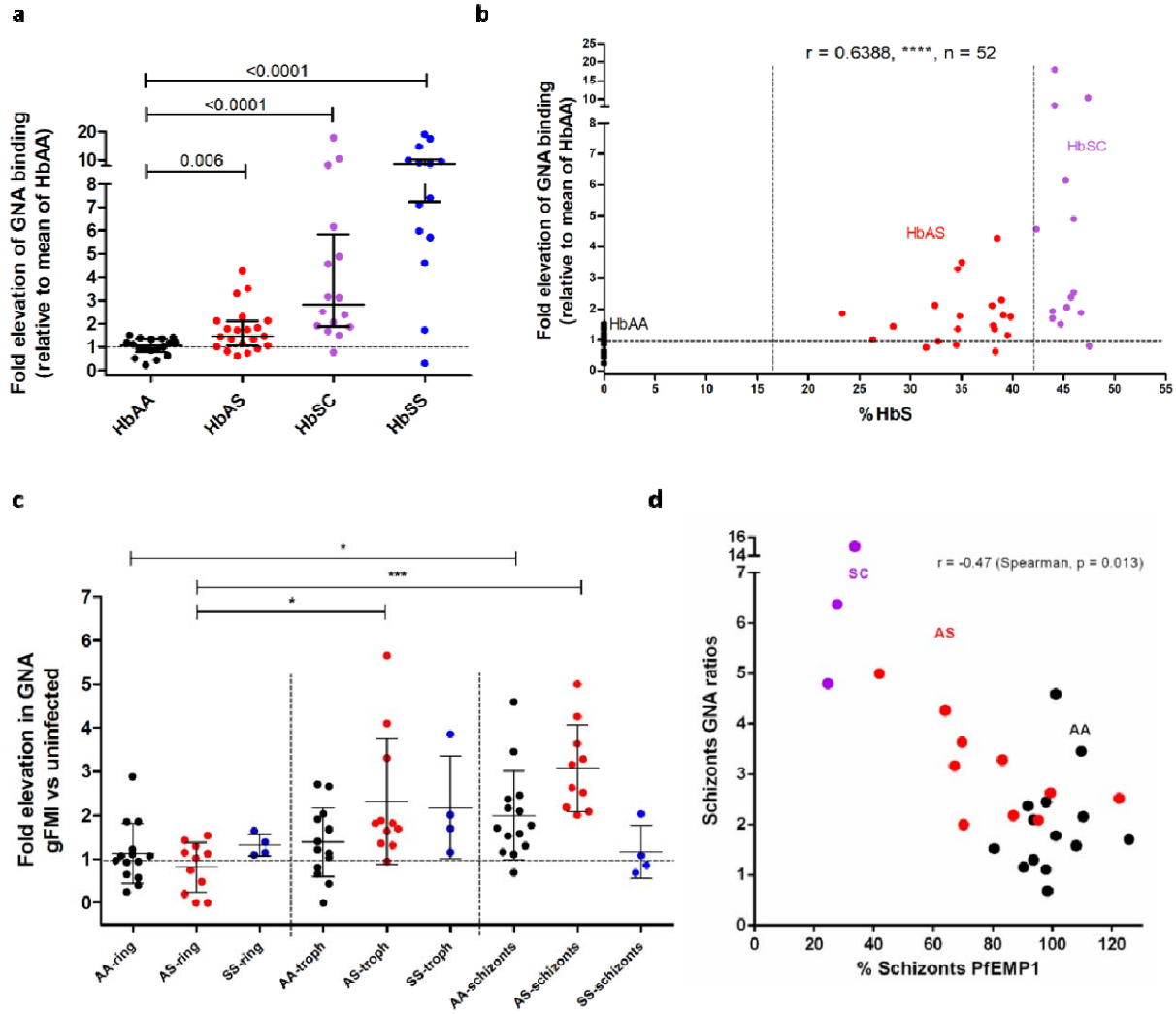


862 **Figure 4: High mannose decoration of spectrin containing fragments in sickle cell disease**

- 863 a) GNA lectin western blot from healthy (HbAA) and sickle (HbSS) ghosts.
- 864 b) Above are shown further GNA lectin western blots from HbAA and HBSS ghosts.
- 865 The histogram below the blot shows the flow cytometrically measured surface GNA
- 866 lectin staining values of the RBCs used to make the ghosts, with each bar
- 867 corresponding to the cells used to make the western lane above. The r value to the
- 868 right of the 100kDa size label is Spearman's rank correlation coefficient between
- 869 GNA lectin staining values and band intensities, both classified ordinally as high,
- 870 medium or low (n=27 measurements from 22 individuals). None of the other bands
- 871 yielded significant correlation coefficients.
- 872 c) GNA lectin blot from HbSS ghosts: untreated (U), treated by PNGase (P) or Endo-H
- 873 (E).
- 874 d) High exposure β -spectrin blot showing PNGase and partial/full (0.1X/1X) Endo-H
- 875 digestion of two HbSS ghosts.
- 876 e) Lectin precipitation of healthy ghosts with GNA or MAL-II lectins. No lectin control
- 877 is also shown. Immunoblot with β -spectrin specific antibody.
- 878 f) Super resolution microscopy image of spectrin membrane skeleton (blue) from
- 879 healthy (AA) and sickle cells (SS). Yellow clusters of GNA staining are overlaid. 3D
- 880 image is sliced to reveal single sheet of membrane skeleton network.
- 881 g) GNA lectin blot of spectrin released from HbSS ghosts after digestion with trypsin
- 882 for one hour. Untreated (UT), Tx indicates the dilution factor of trypsin relative to
- 883 spectrin material.

- 884 h) Peptide coverage and intensity map of α -spectrin from proteomic analysis of F40
885 following chymotrypsin treatment.
- 886 i) GNA lectin blots showing Endo-H treatment of the 10kDa concentrate from (e) under
887 native or denaturing conditions (urea/SDS/2-mercaptoethanol) for 24 hours.
- 888 j) 3D SIM super-resolution microscopy of surface GNA lectin binding and internal β -
889 spectrin in HbSS. HbSS RBC are first stained with GNA lectin (yellow), then
890 permeabilized, and stained with anti-spectrin antibody (blue).
- 891

Figure 5



893 **Figure 5: High levels of mannose are displayed by sickle cell trait RBCs in response to both**
894 **oxidative stress and infection with *P. falciparum*.**

895 a) GNA lectin binding to HbAA versus HbAS, HbSC, HbSS RBCs in response to oxidative
896 stress, expressed as ratio to mean of oxidized HbAA samples. >5 experiments, Mann-
897 Whitney.

898 b) HbS percentage is plotted against elevation of GNA lectin binding in response to
899 oxidative stress as in (a). HbAA, HbAS and HbSC donor samples are shown as indicated.
900 Spearman's rank statistics shown.

901 c) GNA binding to HbAA, HbAS and HbSS RBCs in response to infection with *P.*
902 *falciparum*. Values for rings, trophozoites and schizonts are expressed as ratios relative to
903 the uninfected gate. Linked ANOVA analysis for HbAA (*, $p < .05$), HbAS (***, $p < .001$),
904 HbSS (n.s.); Tukey's multiple comparison within each genotype, 5 experiments.

905 d) Plot of relative GNA lectin binding for RBCs infected by schizonts against relative
906 PfEMP1 expression. Haemoglobin phenotypes of donors as indicated, Spearman's rank.

907

908 **Extended Data Table 1: Exclusion of possible lectin binding artefacts for SCD RBCs.**

909

Potential artefact	Exclusion
Non-specific binding of GNA lectin	<ul style="list-style-type: none">• Wide lectin panel shows specificity (Fig.1a)• NPL lectin binding highly correlated with GNA lectin binding across all samples (Extended Data Fig. 1a)• Mannan blockade (Extended Data Fig. 1b)• No binding of GNA lectin to non-SCD haemolytic anaemia, HbAA and HbAS RBCs (Fig. 2a, Extended Data Fig. 2a)
Non-specific binding on sickle cells	Little binding with Annexin V (Extended Data Fig. 2b), in contrast to eryptotic RBC control (Extended Data Fig. 2c)
Sickle cells permeable to lectins	Lack of binding of other lectins or antibodies against intracellular antigens, BRIC-132/163 (Extended Data Fig. 4d)
O-GlcNAc detection instead of high mannose	No surface binding by antibody RL2, specific for O-GlcNAc (Extended Data Fig. 4e for oxidized RBCs and Extended Data Fig. 4f for HbSS RBCs)
Reticulocytosis	Lack of expression on non-SCD RBCs with high reticulocyte counts (Extended Data Fig. 3d, e).
Intravascular haemolysis	LDH independence (Fig. 2c, d, e)

910

911 **Extended Data Table 2.** Proteomic LC-MS analysis of 260kDa band cut from SDS-PAGE of
912 ghosts from healthy (HbAA) RBCs. Proteins included are with minimum: 99.5% probability, 3
913 peptides identified, and 5.0% sequence coverage. Common contaminants (e.g. keratins and
914 trypsin) have been removed. Identified proteins (UniProt accession, entry codes and description)
915 were sorted based on the number of sequence coverage (%Cov). MW, molecular weight in Da;
916 PLGS score, ProteinLynx Global Server score.

917
918

Accession	Entry	Description	MW	PLGS score	Prob. (%)	Peptides	%Cov
P02549	SPTA1_HUMAN	Spectrin alpha chain, erythrocytic 1 OS=Homo sapiens	279840	9.9	100.0	125	53.5
P11277	SPTB1_HUMAN	Spectrin beta chain, erythrocytic OS=Homo sapiens	246313	9.9	100.0	46	26.6
P02730	B3AT_HUMAN	Band 3 anion transport protein OS=Homo sapiens	101727	9.9	100.0	14	19.0
P16157	ANK1_HUMAN	Ankyrin-1 OS=Homo sapiens	206136	9.9	100.0	25	17.0
P11166	GTR1_HUMAN	Solute carrier family 2, facilitated glucose transporter member 1 OS=Homo sapiens	54048	9.9	100.0	3	6.3

919

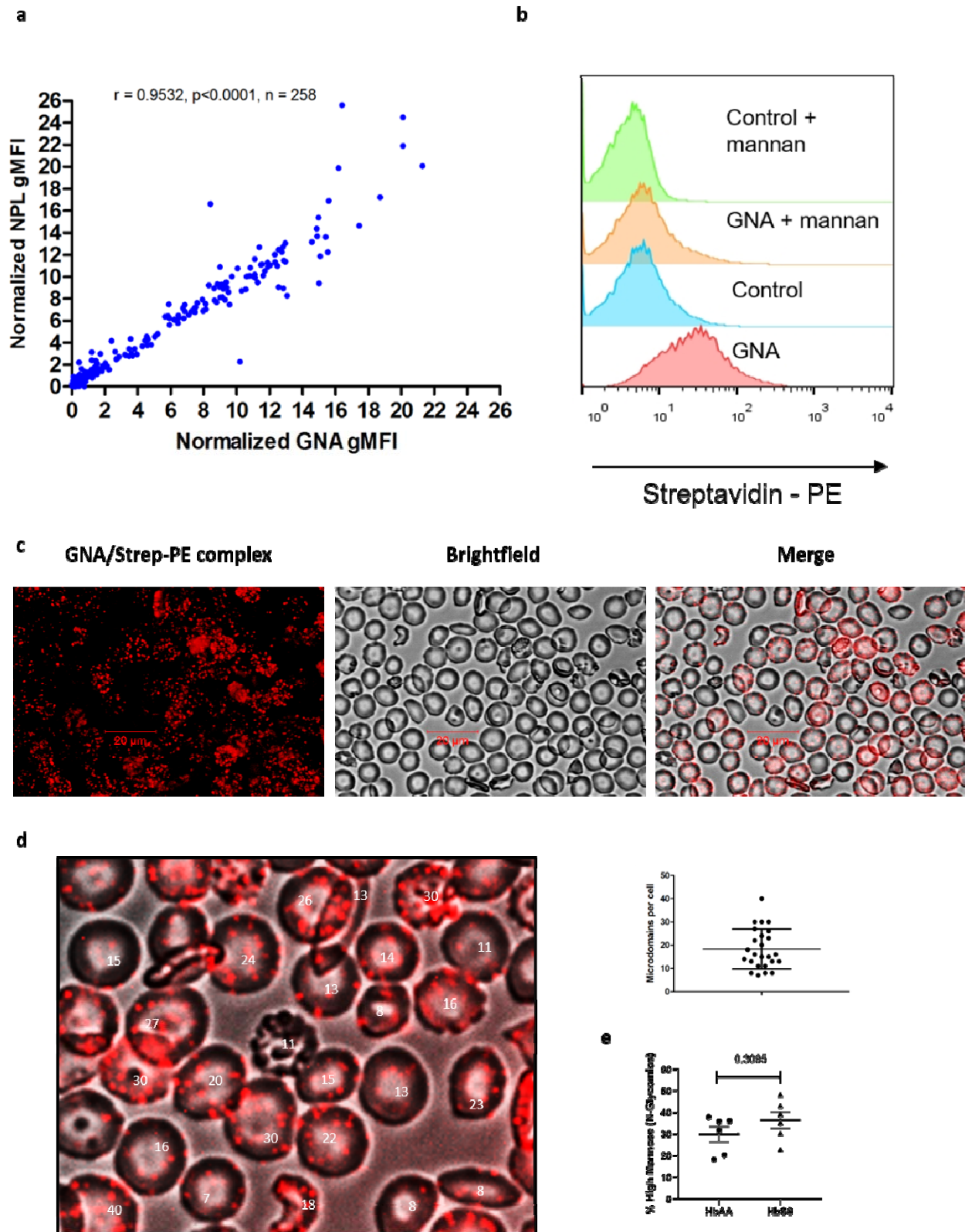
920 **Extended Data Table 3.** Proteomic analysis of F40 trypsin resistant gel band identified α -
 921 spectrin peptides (accession P02549, entry SPTA1_HUMAN), sorted on intensities. Peak MW,
 922 molecular weight of the protonated (MH+) peptide found in Da; peptide MW, theoretical
 923 molecular weight of the protonated (MH+) peptide in Da; delta, difference between peak MW
 924 and peptide MW in ppm; score, PLGS score. In peptide sequence, cysteine (C) amino acids in
 925 **bold** and underlined correspond to carboxymethylated cysteine amino acid.

926
 927

Peak MW (MH+)	Peptide MW (MH+)	Delta (ppm)	Score	Sequence		Sequence	Intensity
				Start	End		
1550.795	1550.795	0.08	6.8	280	292	IKEKEPVLTSEDY	317025
1135.598	1135.599	-1.18	6.6	293	303	GKDLVASEGLF	226142
643.359	643.356	4.36	6.3	54	58	HLQVF	146116
1245.657	1245.659	-1.14	6.1	270	279	KRDVTEAIQW	119871
1203.611	1203.612	-0.93	5.6	59	68	KRDADDLGKW	34163
1164.551	1164.553	-2.02	5.5	82	91	EDPTNIQGKY	63659
1263.555	1263.556	-0.65	5.4	164	173	VQEC <u>AD</u> ILEW	66792
852.448	852.446	1.73	5.2	364	370	EKLQATY	23647
966.491	966.489	2.03	5.1	2362	2370	QALAEGKSY	1717
5788.926	5788.981	-9.48	4.8	1869	1920	AVHETRVQNV <u>CA</u> QGEDILNKVLQEES QNKEISSKIEALNE KTPSLAKAIAAW	3774
802.330	802.337	-8.22	4.8	2413	2419	TNSYFGN	5889
1603.711	1603.739	-17.40	4.8	902	914	QQYLADLHEAET W	6290
2193.952	2193.977	-11.35	4.8	466	481	DERHRQYEQ <u>CL</u> DF HLF	1892
2065.934	2066.004	-33.71	4.7	1944	1963	IADKETSLKTNGN GADLGDF	6335
950.372	950.392	-21.38	4.6	1090	1097	EAGDMLEW	1078
3284.517	3284.539	-6.43	4.5	1385	1410	EKRKKILDQ <u>C</u> LEL QMFQGN <u>CD</u> QVES W	2553
2727.342	2727.442	-36.73	4.4	1124	1146	QKDLNTNEPRLRDI NKVADDLLF	1109

928
 929

Extended Data Fig. 1

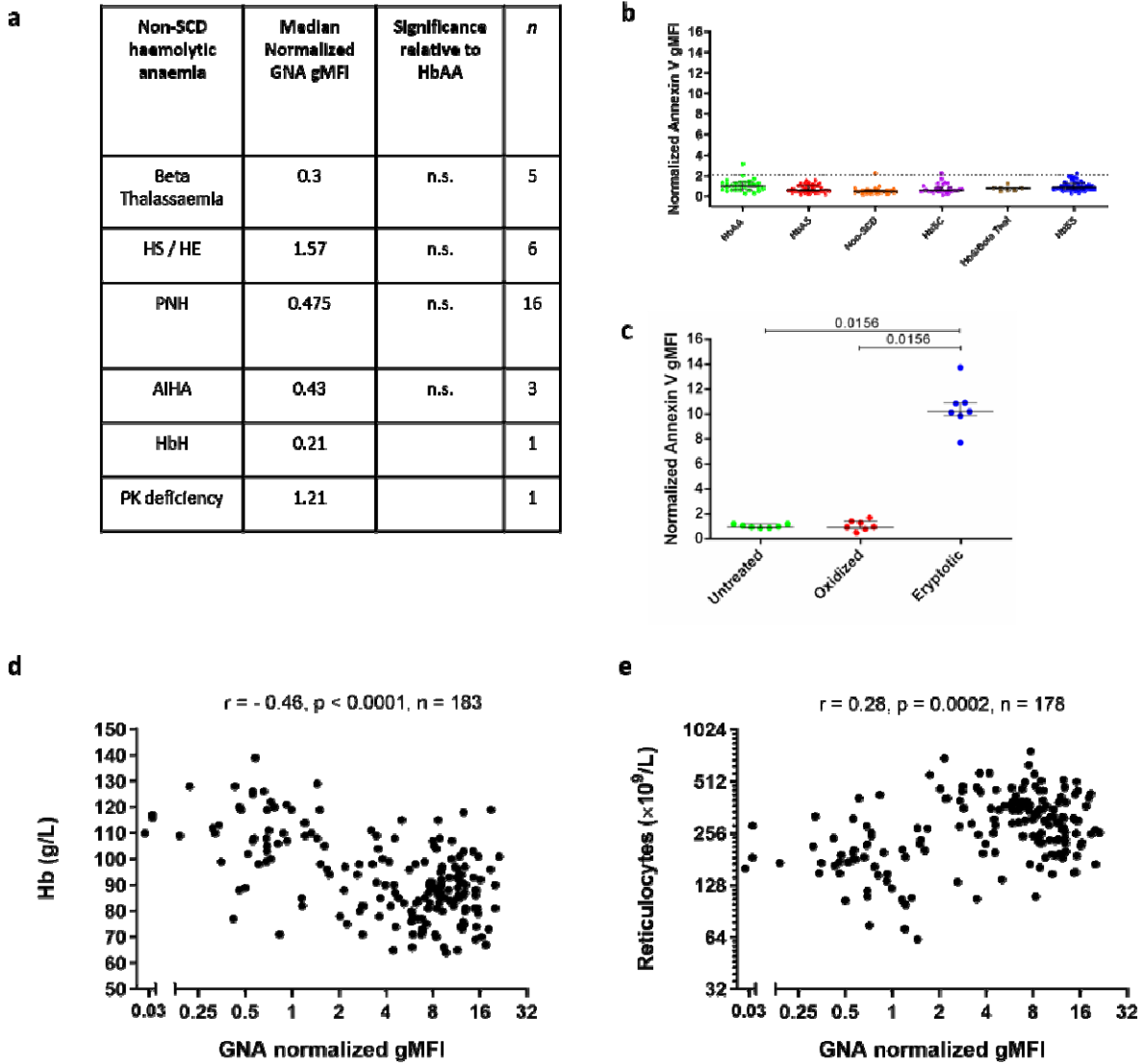


931 **Extended Data Figure 1: Specificity of binding of mannose binding lectins to RBCs.**

- 932 a) Correlation of NPL with GNA lectin surface binding; normalized gMFI, Spearman's rank
933 correlation.
- 934 b) Flow cytometric histograms of GNA lectin and streptavidin control binding for HbSS
935 RBCs with and without mannan blockade. Representative of 3 independent experiments
936 from different donors.
- 937 c) GNA lectin/Streptavidin PE staining of HbSS RBCs is visualized by fluorescence
938 microscopy (PE alone, Brightfield alone and merge).
- 939 d) HbSS RBCs from a section of image from c) are counted for the number of GNA lectin
940 binding patches visualized by fluorescence microscopy (left) and plotted on the right.
941 Mean 18.3, SD 8.6.
- 942 e) Percentage high mannose structures, with respect to total N-glycans. Untreated HbAA (n
943 = 2) and HbSS (n = 5) ghosts are analysed by N-glycome mass spectrometry. Results are
944 pooled from four independent experiments. High mannose and complex N-glycans total
945 100%. Mann Whitney statistical test.

946

Extended Data Fig. 2

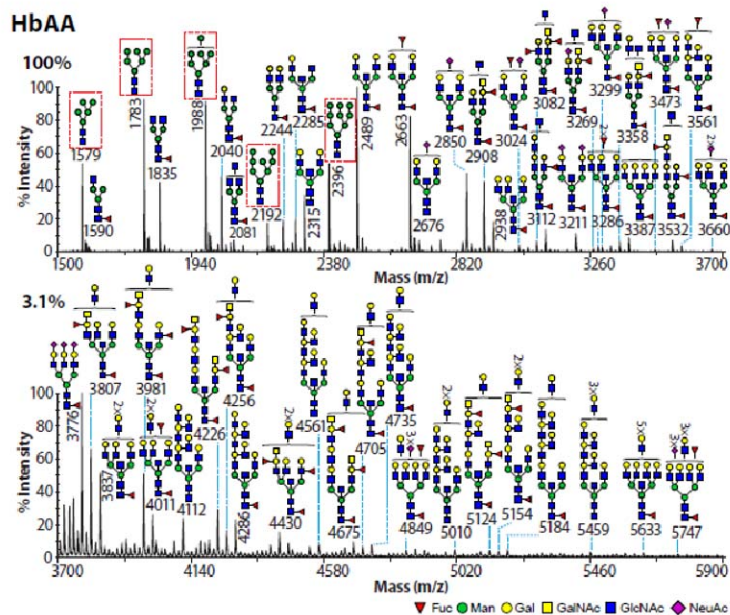


947

948 **Extended Data Figure 2: Binding of mannose binding lectins and annexin V to RBCs.**

- 949 a) Table of normalized gMFI values for GNA lectin binding to non-SCD haemolytic
950 anaemias, Mann-Whitney tests relative to HbAA.
- 951 b) Normalized gMFI of annexin V binding to RBCs in whole blood samples. Dotted line
952 shows 90th centile of annexin V staining within HbAA. Non-SCD include haemolytic
953 anaemias listed in Extended Data Fig. 2a. HbAA (n=29), HbAS (n=42), Non-SCD
954 (n=33), HbSC (n=30), HbS/Beta Thal (n=7), HbSS (n=52). No significant differences by
955 Mann-Whitney.
- 956 c) Normalized gMFI of annexin V binding to oxidized (copper sulphate/ascorbic acid),
957 eryptotic (calcium ionophore) or untreated purified HbAA RBCs; Wilcoxon.
- 958 d) Plots of haemoglobin concentrations,
- 959 e) and reticulocyte counts against normalized GNA lectin gMFI for SCD including HbS/B+,
960 HbSC (compound heterozygosity for HbS with β -thalassaemia and HbC respectively)
961 and HbSS patients. Spearman's rank correlation shown.

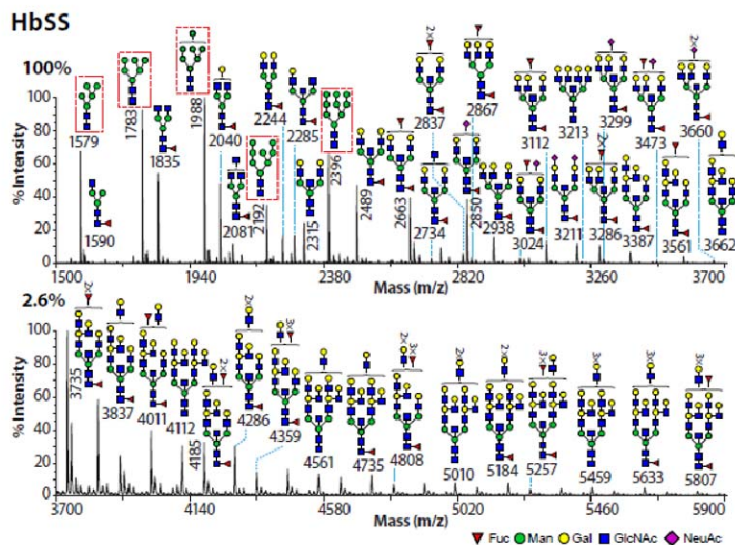
Extended Data Fig. 3



For calculation, upper panel of N-glycans has been taken into account.

High mannose N-glycans: 35.9%

Complex N-glycans: 64.1%



For calculation, upper panel of N-glycans has been taken into account.

High mannose N-glycans: 48.3%

Complex N-glycans: 51.7%

963 **Extended Data Figure 3. Full N-glycome spectra from HbAA and HbSS ghosts.**

964 Spectra from individual HbAA (upper panel) and HbSS (lower panel) ghosts are shown. Zoom

965 factors are indicated by the percentages on top of the intensity axis. For calculations of

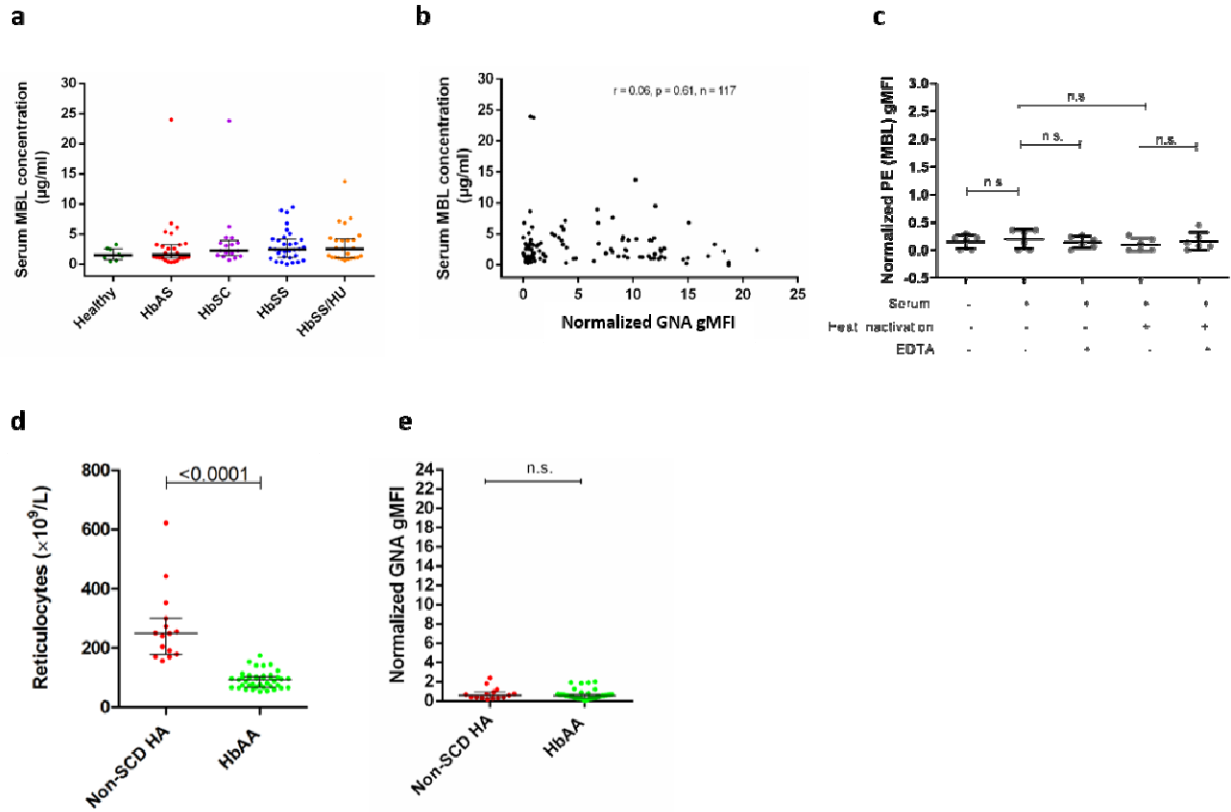
966 percentages high mannose and complex N-glycans, the upper panel of each N-glycan profile was

967 used.

968

969

Extended Data Fig. 4



970

971 **Extended Data Figure 4: Mannose binding lectin correlations and stratification of mannose**
972 **relationship to anaemia.**

973 Concentrations of serum mannose binding lectin (MBL): (a) split by clinical phenotype (HbAA
974 (n=8); HbAS (n=27); HbSC (n=17); HbSS (not receive hydroxycarbamide treatment, n=32);
975 HbSS/HU (receiving hydroxycarbamide treatment, n=25), (b) correlation with surface GNA
976 lectin binding and (c) lack of direct binding to HbSS RBCs (n=6). Comparison of reticulocyte
977 counts (d) or normalized GNA lectin binding gMFI (e) between HbAA and non-SCD haemolytic
978 anaemias (Non-SCD HA). For d), HbAA (n=15), non-SCD (n=37). For e), HbAA (n=15), non-
979 SCD (n=41).

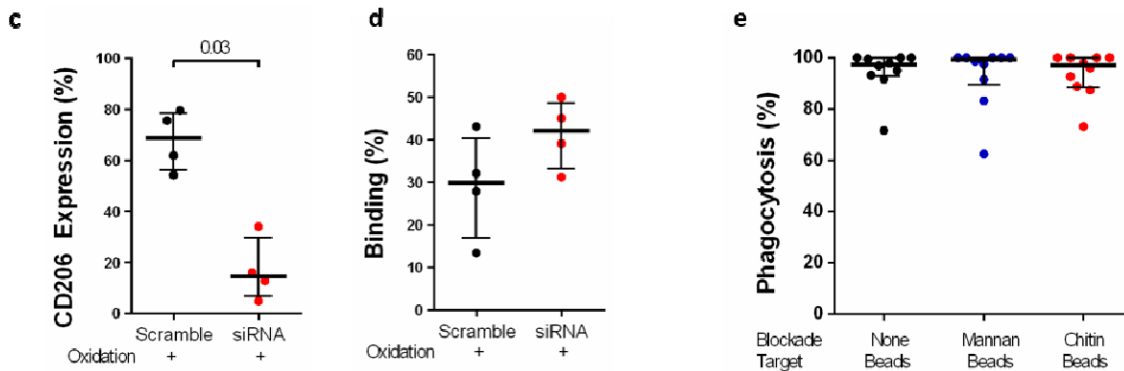
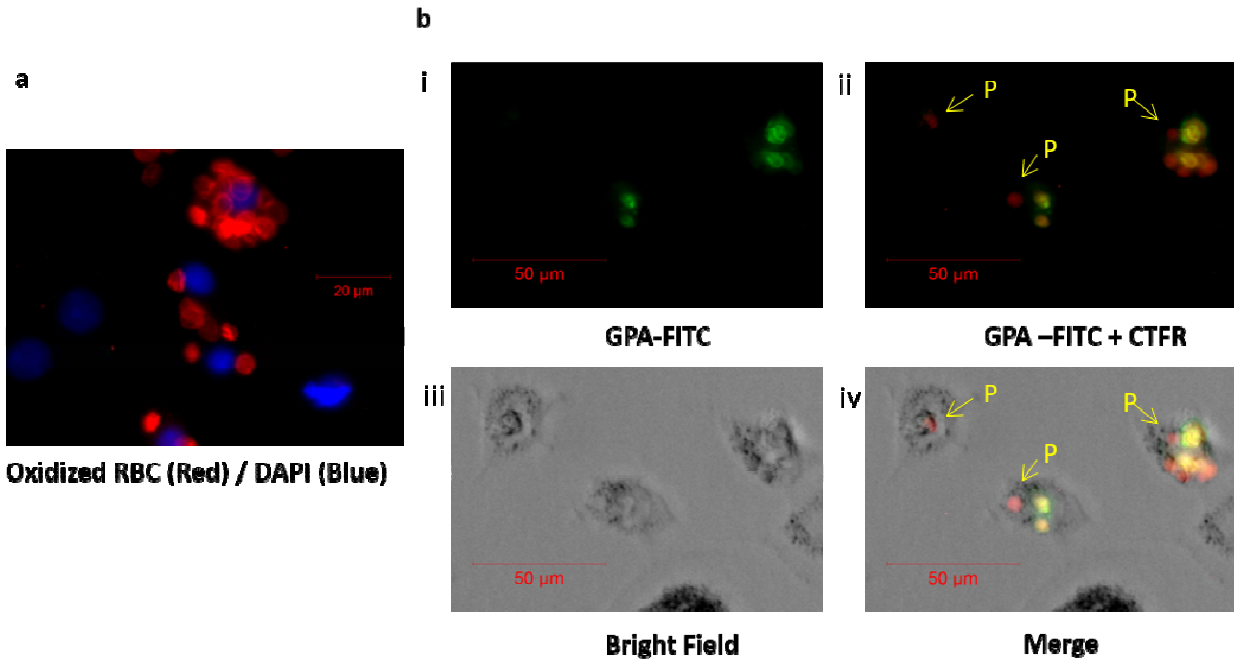
981 **Extended Data Figure 5: Oxidation exposes mannose on the surface of RBCs**

- 982 a) Confocal microscopy of HbAA RBCs permeabilized before staining with biotinylated
983 GNA lectin, streptavidin (yellow) and anti-spectrin (blue).
- 984 b) Rate of radical formation calculated from six-hour time course measurement for healthy
985 RBCs with or without oxidation by CuSO₄/ascorbic acid during time course.
- 986 c) Flow cytometric analysis of healthy, oxidized and HbSS RBCs using BRIC 132 (anti-
987 cytoplasmic band 3) or BRIC 163 (anti-cytoplasmic glycophorin A). Left hand panels: i)
988 histograms for permeabilized versus non-permeabilized binding of HbAA RBCs to BRIC
989 antibodies. ii) BRIC antibody binding to surfaces of oxidized and undamaged HbAA
990 RBCs. Differences not significant by Mann-Whitney. Right hand panel (iii): BRIC
991 antibody and GNA lectin binding to RBCs from two HbSS donors, without
992 permeabilization.
- 993 d) Flow cytometric analysis of healthy, oxidized and HbSS RBC using O-GlcNAc specific
994 antibody, RL2. i) Normalized GNA lectin and RL2 binding for undamaged and oxidized
995 RBCs, without permeabilization. ii) Histograms for RL2 binding to permeabilized
996 nucleated cells shown. Right hand panel (iii): RL2 antibody and GNA lectin binding to
997 RBCs from two HbSS donors, without permeabilization.

998

Extended Data Fig. 6

Two-toned fluorescence phagocytosis (TTFP) assay



999

1000

1001 **Extended Data Figure 6: Two-toned fluorescence phagocytosis (TTFP) assay**

1002 a) Oxidized RBCs stained with Cell Trace Far Red (CTFR, red) are incubated with HMDM
1003 for 3 hours, washed with PBS, permeabilized and stained for nucleus (DAPI, blue).
1004 Immunofluorescence. Scale bar shown.

1005 b) Oxidized RBCs stained with Cell Trace Far Red (CTFR, red) are incubated with HMDM
1006 for 3 hours, washed with PBS, and counter-stained prior to immunofluorescence
1007 microscopy with anti-GPA-FITC antibody (green) to identify RBCs that are not
1008 sequestered inside macrophages: GPA-FITC only (i), GPA-FITC and CTFR (ii), bright
1009 field only (iii) and merged (iv). CTFR (red) single positive cells are counted as having
1010 been phagocytosed (letter E). Double positive (CTFR and GPA) cells are not counted as
1011 having been phagocytosed, but bound to the macrophage cell surface. Scale bar 50 μ m
1012 scale as shown.

1013 c) Mannose receptor (CD206) expression assessed by microscopy for human monocyte
1014 derived macrophages treated with siRNA or scramble control. Mann-Whitney.

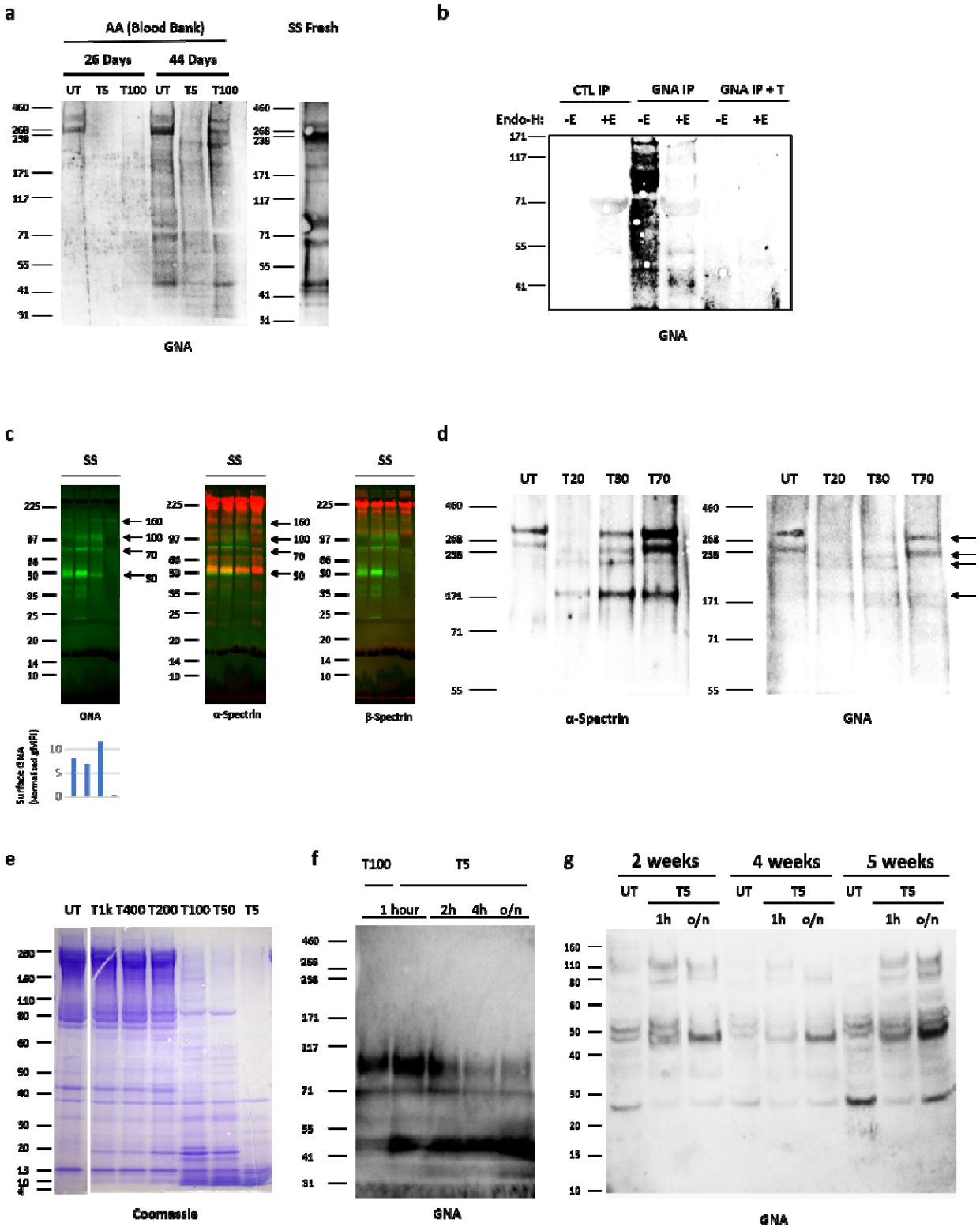
1015 d) Percentage surface binding of HbSS RBCs assessed by microscopy for human monocyte
1016 derived macrophages treated with siRNA or scramble control.

1017 e) Quantified phagocytosis beads by HMDM with and without glycan polymer blockade,
1018 applied in the same way and the same concentration as for RBC phagocytosis
1019 experiments.

1020

1021

Extended Data Fig. 7



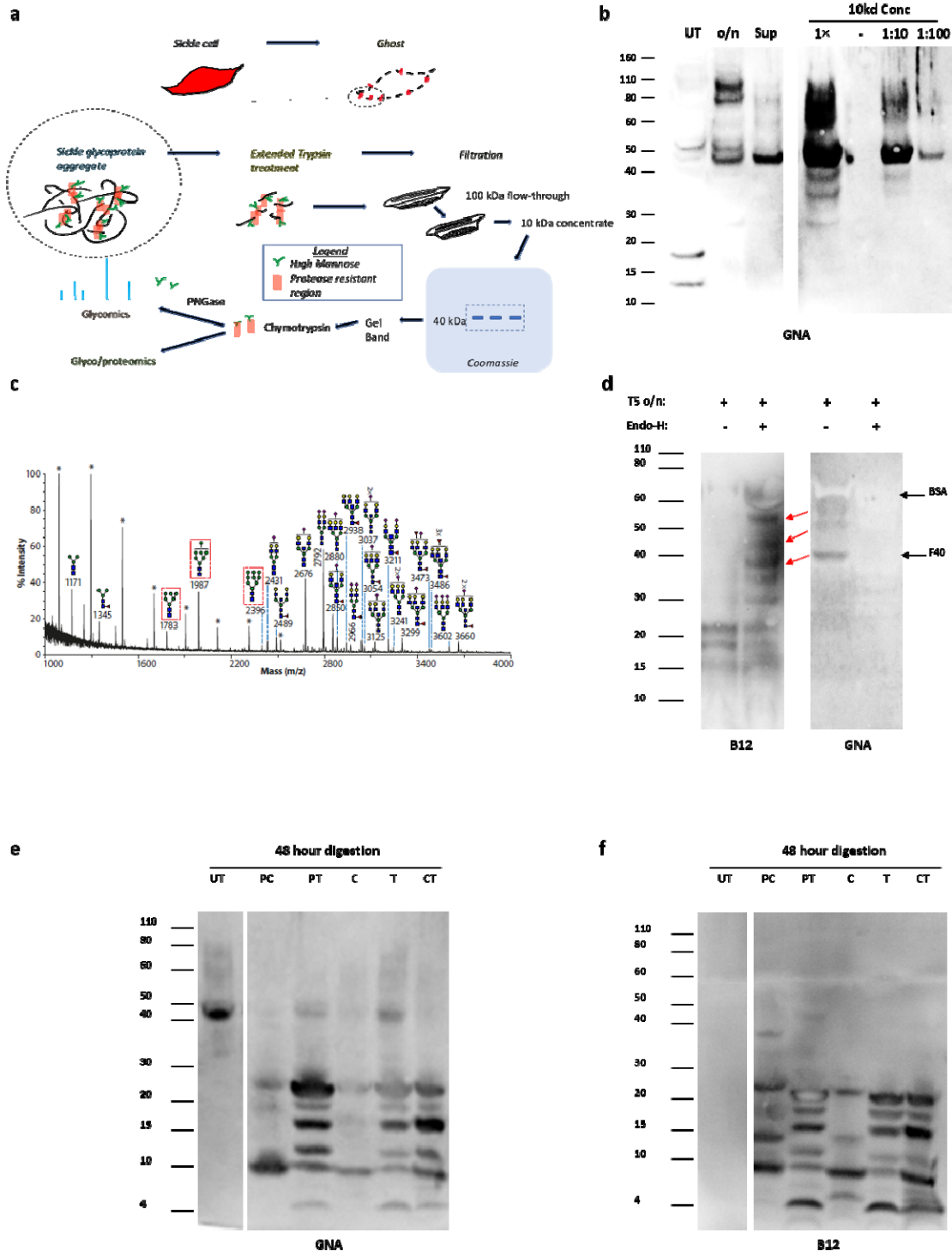
1022

1023 **Extended Data Figure 7: High mannose positive fragments derived from aging and**
1024 **proteolysis**

- 1025 a) GNA lectin western from HbAA ghosts isolated from erythrocytes stored from 26 to 44
1026 days. T5 and T100 indicate trypsin digestion of ghosts, where the number indicates the
1027 dilution factor of trypsin. Comparison HbSS GNA lectin blot is shown, demonstrating
1028 correspondence in the positions of the ~160kDa, ~100kDa, ~70kDa and ~50kDa
1029 fragments.
- 1030 b) Stored RBC (>40 days) ghosts are Triton treated and subjected to GNA lectin
1031 precipitation or control (no lectin) precipitation. Endo-H (+E) or control digestion (-E) is
1032 applied to the eluates. Overnight trypsin treatment is also applied to a subset of GNA IP
1033 elution (GNA IP + T), prior to Endo-H or control digestions.
- 1034 c) Dual colour western blot from HbSS ghosts using GNA lectin (green) with either α -
1035 spectrin or β -spectrin antibodies (both red). Below are corresponding surface FACS GNA
1036 lectin staining (normalized gMFI). N.B. The 8-18% gradient gel used here does not yield
1037 bright 260kDa GNA lectin binding bands.
- 1038 d) Partial trypsin digestion of HbAA ghosts (T70 indicates 1:70 ratio of trypsin to sample,
1039 etc) then blotted with polyclonal α -spectrin antibody or GNA lectin.
- 1040 e) Coomassie stain of spectrin released from HbSS ghosts after digestion with trypsin for
1041 one hour. Untreated (UT), Tx indicates the dilution factor of trypsin relative to spectrin
1042 material.
- 1043 f) GNA lectin blot of HbSS ghost after prolonged trypsin treatment from 1 hour to
1044 overnight (20hours).

- 1045 g) GNA lectin blot of ghosts made from HbSS erythrocytes aged from 2 to 5 weeks.
- 1046 Untreated (UT), one hour (1h) and overnight (o/n) high concentration trypsin digestions
- 1047 (T5).

Extended Data Fig. 8



1048

1049 Extended Data Figure 8: Purification and analysis of protease resistant F40 from sickle

1050 cells.

- 1051 a) Cartoon outlining purification process for peptide F40. Sickle cells (top right) are used to
1052 make RBC ghosts, which are then subjected to prolonged incubation with trypsin before
1053 removing the ghosts by centrifugation. The supernatant is then passed through a 100 kDa
1054 filter and concentrated on a 10 kDa filter before running on a polyacrylamide gel. The
1055 ~40 kDa band is cut out and digested with chymotrypsin (then PNGase) before analysis
1056 by mass spectroscopy for glycopeptides (and glycans).
- 1057 b) GNA lectin blot of enrichment process of F40 from aged HbSS erythrocyte ghosts.
1058 Untreated (UT); overnight T5 (o/n); supernatant from heat inactivated o/n sample (Sup).
1059 Final product from sequential concentration with 100kDa and 10kDa concentrators is
1060 shown as 1×, 1:10 and 1:100 dilution series. See explanatory notes below.
- 1061 c) Glycomic analysis following PNGase release of N-linked glycans from F40. Red boxes
1062 indicate high mannoses. Putative structures based on composition, tandem MS and
1063 biosynthetic pathways. All ions are $[M+Na]^+$. Peaks annotated with an asterisk (*) do not
1064 correspond to glycan structures. Major structures are annotated for clarity.
- 1065 d) HbSS 10kDa concentrate (from a) was digested with trypsin (T5) overnight, heat
1066 inactivated, then digested with Endo-H or control for 24 hours. GNA lectin and B12
1067 western blots. Red arrows show likely migration of GNA lectin and B12 reactive bands.
1068 Black arrows indicate contaminating BSA and position of F40.
- 1069 e) HbSS 10kDa concentrate (from a) subjected to combinatorial protease digestion in mild
1070 denaturing conditions over 48 hours. Western blot with GNA lectin. (UT) untreated,
1071 (PC) pepsin 24 hours then chymotrypsin 24 hours, (PT) pepsin 24 hours then trypsin 24
1072 hours, (C) chymotrypsin 48 hours, (T) trypsin 48 hours, (CT) chymotrypsin 24 hours then
1073 trypsin 24 hours, with heat inactivation at 24 hours.

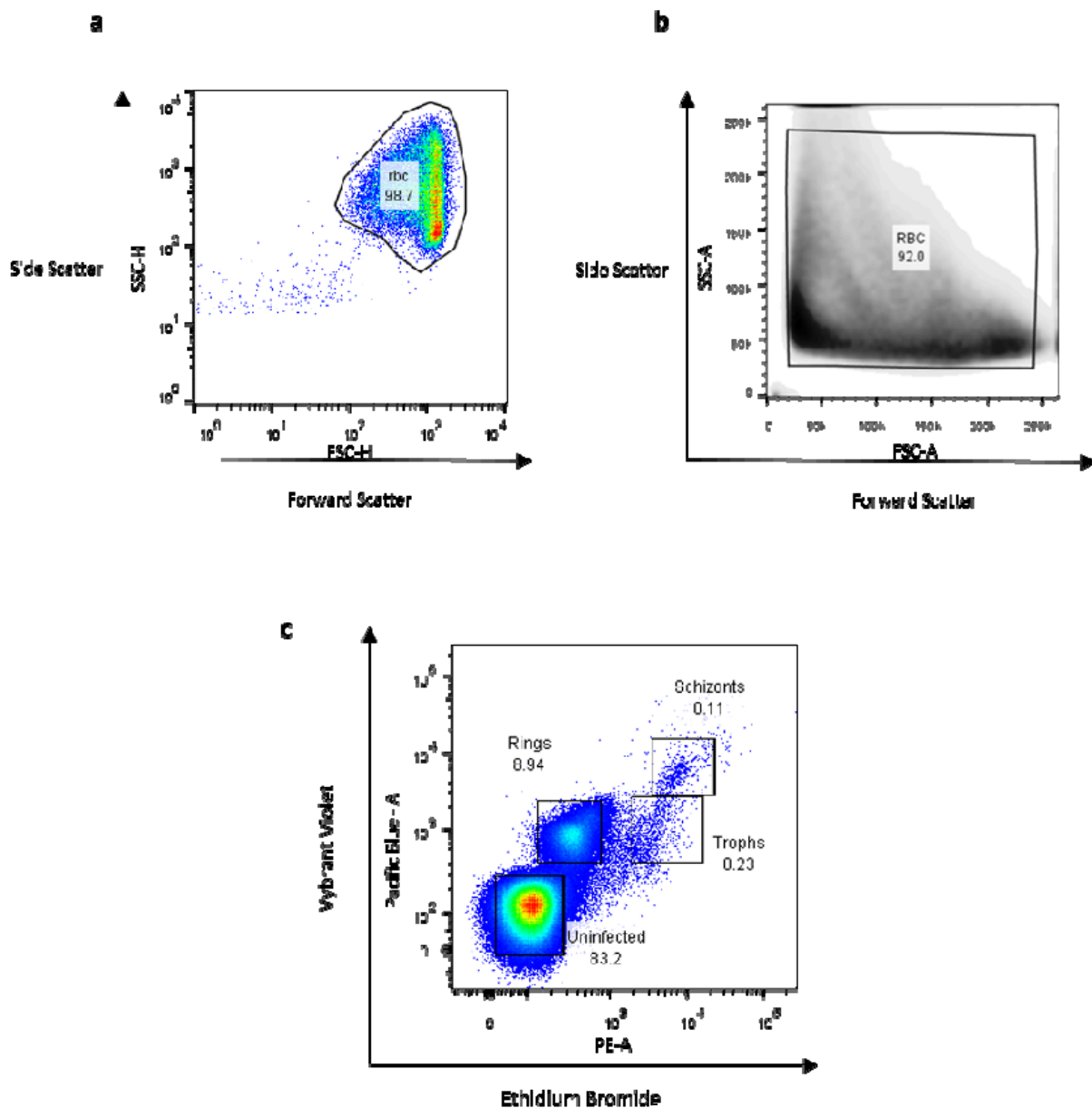
1074 f) As d) but with western blotting using antibody B12.

1075 **Purification and properties of F40:** F40 from overnight trypsin digestion of HbSS RBC
1076 ghosts partitions to the supernatant following heat inactivation (a). We thereby concentrated
1077 F40, through sequential 100kDa and 10kDa cut off concentrators, by approximately 50-fold
1078 (a). This preparation exhibited remarkably high GNA lectin binding, approximately 100-fold
1079 greater than full length spectrin. Sufficient F40 was purified for glycoproteomic analysis.
1080 PNGase released glycans including three high mannoses, a tri-mannose structure and other
1081 complex glycans (b). The Endo-H sensitive nature of F40 GNA lectin binding restricts the
1082 GNA lectin ligands within this pool of structures to the high mannoses: $\text{Man}_6\text{GlcNAc}_2$,
1083 $\text{Man}_8\text{GlcNAc}_2$ and $\text{Man}_9\text{GlcNAc}_2$. GNA lectin reactivity to F40 shows partial sensitivity to
1084 digestion with chymotrypsin and proteomic analysis of this digest showed the main protein
1085 represented was α -spectrin (Fig. 4i). The most abundant peptides came from the N-terminal
1086 370 amino acids, spanning spectrin repeats 1 to 3, with only scattered, low abundance
1087 peptides identified from the rest of the molecule and none from β -spectrin. Excluding mass
1088 spectrometry incompatible regions from this region yielded 34% coverage across spectrin
1089 repeats 1 to 3, with 64% coverage within repeat 3.

1090 Identification of the N-terminal portion of α -spectrin as a major constituent of F40 from mass
1091 spectrometry analysis was also supported by western blotting using B12, an N-terminus
1092 specific α -spectrin antibody. The antibody does not bind GNA lectin reactive F40 or two
1093 higher molecular weight bands directly, but epitopes are revealed by removal of high
1094 mannoses using Endo-H (c). Furthermore, three new B12-binding bands migrated at a
1095 slightly lower positions relative to the three GNA lectin binding bands, consistent with
1096 glycosidase induced cleavages (c). 48 hour-long digestions with combinations of proteases

1097 were able to cleave F40 and, even without Endo-H treatment, unmask B12-binding epitopes
1098 (e). The GNA lectin and B12-binding fragments align remarkably well under all treatment
1099 combinations (d, e). These data suggest the high mannose decoration and unusual protein
1100 structure of F40 limits antibody access and may account for its resistance to proteases.
1101

Extended Data Fig. 9



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1103 **Extended Data Figure 9. Gating strategy for flow cytometric analysis.**

1104 a) Example of gating strategy for selecting whole red blood cells used in both whole blood
1105 and purified RBC flow cytometry (with the exception of *P. falciparum* infected RBC
1106 flow cytometry).

1107 b) Example of first gating of whole red blood cells used in analysis of *P. falciparum*
1108 infected RBC flow cytometry.

1109 c) Example of second gating of *P. falciparum* infected RBCs by maturation stages as
1110 defined by ethidium bromide (PE) and Vybrant Violet (Pacific Blue) staining.

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