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1 **Chemokines modulate glycan binding and the immunoregulatory activity of galectins**

2

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22 **Abstract**

23 Galectins are versatile glycan-binding proteins involved in immunomodulation. Evidence suggests  
24 that galectins can control the immunoregulatory function of cytokines and chemokines through  
25 direct binding. Here, we report on an inverse mechanism by which chemokines control the galectins  
26 immunomodulatory functions. We show the existence of several specific galectin-chemokine binding  
27 pairs, including galectin-1/CXCL4. NMR analyses show that CXCL4 binding induces changes in the  
28 galectin-1 carbohydrate binding site. Consequently, CXCL4 alters the glycan-binding affinity and  
29 specificity of galectin-1. Regarding immunomodulation, CXCL4 significantly increases the apoptotic  
30 activity of galectin-1 on activated CD8+ T cells, while no effect is observed in CD4+ T cells. The  
31 opposite is found for another galectin-chemokine pair, i.e., galectin-9/CCL5. This pair significantly  
32 reduces the galectin-9 induced apoptosis of CD4+ T cells and not of CD8+ T cells. Collectively, the  
33 current study describes an immunomodulatory mechanism in which specific galectin-chemokine  
34 interactions control the glycan-binding activity and immunoregulatory function of galectins.

## 35 **Introduction**

36       The human glycome is a complex and dynamic collection of cellular glycans and glycoconjugates  
37 that are involved in a plethora of cellular functions. Alterations in the glycan composition have been  
38 linked to different pathologies including genetic disorders, immune diseases and cancer<sup>1</sup>. In  
39 vertebrates, the information that is encoded by the glycome is deciphered by specific glycan binding  
40 proteins, called lectins. Interestingly, the complex repertoire of the glycome greatly exceeds the  
41 number of lectins<sup>1</sup>. This suggests that specific mechanisms exist to regulate and fine-tune lectin  
42 binding affinity in order to cover the broad spectrum of the glycome.

43       Recently, it was shown that galectin-3, a member of the galectin protein family, can form  
44 heterodimers with the chemokine CXCL12. While galectins are known to exert their function by  
45 binding to glycoconjugates<sup>2</sup>, the interaction was found to be glycan-independent and to hamper  
46 CXCL12 activity<sup>3</sup>. Apart from galectin-3/CXCL12, the authors found interactions of other chemokines  
47 with either galectin-3 or galectin-1<sup>3</sup>. These observations support our previous finding that  
48 chemokine-like peptides can directly bind to different members of the galectin family<sup>4,5</sup>. We  
49 described that the heterodimerization induces changes in the galectin protein structure which affects  
50 the glycan-binding affinity and specificity of galectin-1<sup>4,5</sup>. More recently, modulation of glycan-  
51 binding activity was also observed in response to the interaction of galectin-1 with a specific  
52 fragment of the pre-B-cell receptor<sup>6,7</sup>. Collectively, these findings suggest that heterodimerization of  
53 galectins with other proteins could represent a mechanism to regulate galectin-glycan interactions.  
54 Such a mechanism would extend the functionality of this glycome-decoding protein family. However,  
55 whether chemokines could represent a protein family that regulates galectin function is not yet fully  
56 understood.

57       In the present study, we present evidence that the galectin-chemokine heterodimerization can  
58 indeed serve as a mechanism to modulate galectin glycan-binding and function. We found specific  
59 interactions between different chemokines and two immunomodulatory galectins i.e., galectin-1 and  
60 galectin-9. Focusing on the interaction between platelet factor 4 (PF4/CXCL4) and galectin-1, we

61 show that the interaction modifies the glycan-binding properties of galectin-1 by inducing structural  
62 changes within its carbohydrate binding site. Moreover, we provide evidence that this interaction  
63 can modulate the immunomodulatory activity of galectin-1 as well as of galectin-9 on specific T cell  
64 subsets. Our findings provide evidence of galectin-chemokine cross-talk which can serve as a  
65 regulatory mechanism to guide and fine-tune glycoconjugate-galectin interactions leading to specific  
66 cellular responses.

67

68 **Results**

69 *Several specific and glycan-independent galectin-cytokine heterodimers exist.*

70 The current study aimed to explore whether galectin-chemokine interactions could serve as a  
71 mechanism to modulate galectin glycan-binding and function. First, we performed an interaction  
72 screen (**Supplementary figure 1**) and observed several specific interactions of galectin-1 and galectin-  
73 9 with different chemokines and cytokines. Based on our recent work showing complementary  
74 platelet responses induced by galectin-1 and platelet factor 4 (PF4 or CXCL4), we focused our  
75 investigation on this galectin-chemokine pair<sup>8</sup>. Spot blot analyses showed that CXCL4 binds galectin-1  
76 to a similar extent as the positive control peptide, i.e., anginex<sup>4</sup> (**Figure 1a**). To ensure that the  
77 interaction was not caused by changes in cytokine structure due to immobilization on the  
78 nitrocellulose filter, soluble galectin-1 with either CXCL4 or anginex were incubated in the presence  
79 of the crosslinking agent DSS. Subsequent gel-shift analyses confirmed the expected shift in protein  
80 size of galectin-1 monomers ( $\pm 14$  kD) corresponding to the size of either  $\pm 8$  kD (CXCL4) or  $\pm 4$  kD  
81 (anginex) (**Figure 1b**). The intensity of the shifted band was weak suggesting interference of the  
82 bound chemokine with antibody binding to galectin-1. Of note, crosslinking with CXCL4 also revealed  
83 a band just above the galectin-1 dimer which could either represent a single galectin-1 monomer  
84 bound to two CXCL4 molecules or a galectin-1 dimer bound to a single CXCL4 molecule. To confirm  
85 that the interaction involved CXCL4 and was also not caused by structural abnormalities due to the  
86 crosslinking agent, excess heparin was added prior to crosslinking. Heparin, a known high affinity  
87 ligand of CXCL4, serves as a CXCL4 scavenger and indeed completely neutralized the galectin-1/CXCL4  
88 interaction (**Figure 1c**). Heparin did not cause a shift in galectin-1 size suggesting no interaction of  
89 both molecules. At the same time, the addition of excess lactose, a carbohydrate ligand of galectin-1,  
90 did not prevent the interaction (**Figure 1d**). The latter indicated that CXCL4 does not directly compete  
91 for glycan binding in the carbohydrate recognition domain of galectin-1. Furthermore, the interaction  
92 was dose-dependent and extended beyond the galectin-1/CXCL4 pair (**Figure 1e + Supplementary**  
93 **Figure 1**).

94 To characterize the binding kinetics of the galectin-1/CXCL4 heterodimer formation, surface  
95 plasmon resonance analysis was performed. Similar as previously described for the galectin-  
96 1:anginex interaction<sup>4</sup>, the galectin-1:CXCL4 interaction showed a 1:2 stoichiometry (single  
97 association followed by biphasic dissociation) with binding affinities in the high nanomolar and low  
98 micromolar range (**Supplementary Figure 2 + Table 1**). This also suggests that the high molecular  
99 weight band observed in the gel-shift assay most likely represents a galectin-1 monomer bound to a  
100 CXCL4 dimer. Collectively, all these data provide clear evidence of a direct interaction between  
101 galectin-1 and CXCL4.

102

103 *Structural characterization reveals that CXCL4 binds outside the galectin-1 carbohydrate binding site.*

104 To further characterize the galectin-1/CXCL4 interaction, chemical shift perturbation (CSP)  
105 mapping was performed using <sup>15</sup>N-labeled galectin-1 with or without CXCL4 protein. <sup>1</sup>H-<sup>15</sup>N HSQC  
106 spectra (**Figure 2a**) show that in the presence of CXCL4, several galectin-1 resonances experience  
107 CSPs (chemical shift deviations and peak intensity decrease), indicative of complex formation (**Figure**  
108 **2b + Supplementary figure 3**). Remarkably, perturbed resonances localize at two different sites on  
109 galectin-1 structure: (i) a surface at the edge of the galectin-1  $\beta$ -sandwich fold (opposite to the dimer  
110 interface) (**Figure 2c**), and (ii) a surface at the carbohydrate binding site (CBS) (**Figure 2c**). The first  
111 surface is defined by perturbed resonances belonging mainly to the  $\beta$ 6,  $\beta$ 8 and  $\beta$ 9 strands. Within the  
112 CBS, residues from strands  $\beta$ 3 to  $\beta$ 6 are perturbed upon CXCL4 interaction impacting not only the  
113 CBS core known to interact with the galactose unit but also the surrounding areas. However, our  
114 cross-linking data show that CXCL4 interaction does not compete with glycan binding activity of  
115 galectin-1, indicating that CXCL4 docks onto the perturbed surface of galectin-1 at the edge of its  $\beta$ -  
116 sheets and that this binding induces changes inside the CBS. To further investigate the structural  
117 basis of this interaction we performed NMR data-driven docking to generate a structural model of  
118 the galectin-1/CXCL4 complex. Residues from galectin-1 experiencing CSPs upon CXCL4 interaction  
119 and localized at the edge of the  $\beta$ -sandwich fold were defined as ambiguous restraints in the docking

120 calculations. On the CXCL4 side, residues from the  $\beta$ -sheet, homologous to the anginex peptide  
121 sequence, were also considered as ambiguous restraints. In the structural model showing the best  
122 HADDOCK score, the CXCL4  $\beta$ -sheet docks onto the galectin-1 surface involving mainly  $\beta$ 6 and  $\beta$ 9  
123 strands (**Figure 2d**). In addition, the CXCL4 C-terminal helix establishes contacts with strands  $\beta$ 8 and  
124  $\beta$ 9 strands. To further validate the proposed structural model of the galectin-1/CXCL4 complex  
125 formation, the CXCL4  $\beta$ -sheet region (CXCL4<sup>22-54</sup>; **Supplementary figure 4a+b**) was produced after  
126 which the interaction with <sup>15</sup>N-labeled galectin-1 was tested. The perturbations observed on galectin-  
127 1 spectra corresponded to residues from the CBS and from the  $\beta$ 6 and  $\beta$ 9 strands (**Supplementary**  
128 **Figure 4c**). When compared to CSPs induced by full-length CXCL4, the same regions of galectin-1  
129 were affected with CXCL4<sup>22-54</sup> except for a few residues belonging to the  $\beta$ 8 and  $\beta$ 9 strands, which  
130 belong to the galectin-1 residues that interact with the CXCL4 C-terminal helix that is absent from  
131 CXCL4<sup>22-54</sup> (**Supplementary Figure 4d+e**). Therefore, these data confirm the involvement of both the  
132 CXCL4  $\beta$ -sheet and its C-terminal helix in the interaction with galectin-1.

133

134 *Binding of CXCL4 to galectin-1 affects the glycan-binding affinity and specificity of galectin-1.*

135 Interestingly, while interacting at the edge of the galectin-1  $\beta$ -sheet, CXCL4 induced chemical  
136 shift perturbations within the galectin-1 CBS. Previously, we have shown that the direct protein-  
137 protein interactions can modulate the glycan binding affinity of galectin-1<sup>5,7</sup>. To explore whether  
138 binding of CXCL4 could also affect the carbohydrate binding affinity of galectin-1 we performed  
139 fluorescent anisotropy using a fluorescein-tagged high affinity tdga-probe<sup>9</sup>. Upon addition of 8  $\mu$ M  
140 CXCL4, the baseline signal slightly increased, indicating only minimal interaction between CXCL4 and  
141 the probe. Subsequent titration of increasing concentrations of galectin-1 showed that the binding  
142 affinity of galectin-1 for this probe increased almost 10-fold to a  $K_d$  of  $0.09 \pm 0.01$  as compared to  
143 galectin-1 alone ( $K_d$  of  $0.88 \pm 0.05$   $\mu$ M) (**Figure 3a**). In line with previous results<sup>5</sup>, the  $A_{max}$  (anisotropy  
144 of the galectin-probe complex) was also increased in the presence of CXCL4, which indicates a  
145 reduced mobility of the fluorescein tag. Similar as in the gel-shift experiments, the increased binding



146 was neutralized by the addition of heparin (**Figure 3a**). To further explore the modulation of glycan  
147 binding by CXCL4, comparable experiments were performed with a low affinity probe (lacto-N-  
148 tetraose; LNT-probe). While in the absence of CXCL4 no binding was observed due to very low  $A_{max}$ ,  
149 the addition of CXCL4 induced a measurable anisotropy and binding of the LNT-probe to galectin-1  
150 with a  $K_d$  in the range of the high-affinity tdga-probe, i.e.,  $0.48 \pm 0.18 \mu\text{M}$  (**Figure 3b**). At high galectin  
151 concentrations ( $>1.25 \mu\text{M}$ ) the anisotropy levels decreased again, due to competition of the free  
152 galectin for the probe. The altered carbohydrate binding affinity was further confirmed by a  
153 competition experiment in which increasing concentrations of an unlabeled inhibitor of the galectin-  
154 1-probe interaction, i.e., the glycoprotein asialofetuin (ASF), was added to a fixed concentration of  
155 galectin-1 (the  $EC_{50}$  of the corresponding slope in the absence of ASF). In the presence of CXCL4, the  
156 inhibitory potency of ASF was more than 600-fold increased (**Figure 3c**). Of note, the effects were  
157 galectin-1/CXCL4 specific as CXCL4 did not affect glycan binding of e.g. galectin-3 nor did CCL5 and IL-  
158 8 affect glycan binding affinity of galectin-1 (**Supplementary figure 5**). Altogether, these results show  
159 that heterodimerization with CXCL4 can alter the affinity and specificity of galectin-1 for glycan  
160 binding.

161

162 *Galectin-chemokine interactions can modify the pro-apoptotic activity of galectins in immune cells.*

163 To explore whether galectin-1/CXCL4 interactions represent an endogenous mechanism to  
164 control the function of galectin-1, we analyzed the effect of the heterodimer formation on immune  
165 cells. We focused on T cell apoptosis since galectins are known to induce apoptosis in activated T  
166 cells<sup>10-12</sup>. Indeed, in line with previous findings, we did see time- and concentration-dependent  
167 induction of T cell (Jurkat) apoptosis by galectin-1 (**Supplementary figure 6a**). Interestingly, apoptosis  
168 induction by galectin-1 was significantly increased by CXCL4 and not by CCL5 or CXCL8 (**Figure 4a**).  
169 The latter two were included since spot blot or gel shift assays indicated an interaction with galectin-  
170 1 as well (**Figure 1e** and **Supplementary figure 1**). The effects of CXCL4 were dose-dependent (**Figure**  
171 **4b**) and could be blocked by the addition of either lactose, which competes for galectin-glycan

172 binding, or heparin which scavenges CXCL4 (**Figure 4c**). CCL5 (RANTES) showed an interaction with  
173 both galectin-1 and galectin-9 (Supplementary figure 1), but did not affect galectin-1 function.  
174 Therefore, we explored whether CCL5 could affect galectin-9-induced T cell apoptosis. Similar as for  
175 galectin-1, treatment with galectin-9 alone resulted in a time- and concentration-dependent  
176 induction of Jurkat apoptosis (**Supplementary figure 6b**). Interestingly, the induction of apoptosis by  
177 galectin-9 was specifically inhibited by CCL5 and not by CXCL4 or IL-8 (**Figure 4d**). Again, the effect  
178 was dose-dependent (**Figure 4e**) and could be neutralized by lactose (**Figure 4f**). These results  
179 suggest that heterodimerization with specific chemokines can have opposite effects on T-cell  
180 apoptosis induction by specific galectins.

181

182 *Regulation of apoptotic activity by galectin-chemokine heterodimers depends on immune cell type*  
183 *and activation status.*

184 To further extend these findings, we next analyzed the effects of specific galectin-chemokine  
185 heterodimers on peripheral blood mononuclear cells (PBMCs) obtained from healthy donors. Firstly,  
186 we evaluated the effect of galectin-chemokine heterodimers on apoptosis in resting and activated  
187 PBMCs, the latter being more susceptible to galectin-induced apoptosis<sup>12</sup>. Cell activation was induced  
188 by PHA-L lectin treatment and confirmed by CD25 surface staining (**Supplementary figure 7**). In  
189 resting PBMCs, neither CXCL4 nor CCL5 affected the induction of apoptosis by galectin-1 and  
190 galectin-9, respectively (**Figure 5a**). However, in activated PBMCs (1 µg PHA-L/mL), the pro-apoptotic  
191 effect of galectin-1 was significantly induced by CXCL4 while the pro-apoptotic effect of galectin-9  
192 was significantly inhibited by CCL5 (**Figure 5b**). These observations corroborated our findings in  
193 Jurkat cells. Of note, in the absence of galectins, neither CXCL4 nor CCL5 induced apoptosis in resting  
194 or activated PBMCs (**Supplementary figure 8**).

195 To determine whether the effects could be linked to specific immune cell subsets, analyses were  
196 performed on different immune cell subpopulations, i.e., CD3<sup>+</sup> T cells, CD3<sup>+</sup>/CD8<sup>+</sup> cytotoxic T cells,  
197 CD3<sup>+</sup>/CD4<sup>+</sup> T helper cells, and CD14<sup>+</sup> monocytic cells (For gating strategy see **Supplementary figure**

198 9). Regarding the galectin-1/CXCL4 heterodimer, we observed a significant increment in the  
199 percentage of apoptotic cells in CD3<sup>+</sup> cells (**Figure 5c**) upon the addition of CXCL4 to galectin-1.  
200 Within the CD3<sup>+</sup> population, the increase of galectin-1-mediated apoptosis by CXCL4 could be mainly  
201 attributed to the CD8<sup>+</sup> cells while no effect was observed in CD4<sup>+</sup> cells (**Figure 5d**). This suggests that  
202 within the analyzed T cell subsets, CD3<sup>+</sup>/CD8<sup>+</sup> cells are particularly susceptible to galectin-1/CXCL4-  
203 induced apoptosis. The modulation of galectin-9 by CCL5 appears to display a different specificity  
204 regarding the modulation of immune cell survival. While CCL5 reduced the galectin-9-induced  
205 percentage of apoptotic CD3<sup>+</sup> cells (**Figure 5e**), this was only significant for CD3<sup>+</sup>/CD4<sup>+</sup> cells and not  
206 for CD3<sup>+</sup>/CD8<sup>+</sup> cells (**Figure 5f**). Comparable results were observed in CD14<sup>+</sup> cells in which CXCL4  
207 increased the effect of galectin-1 and CCL5 reduced the effect of galectin-9 (**Supplementary figure**  
208 **10**). Altogether, these results suggest that galectin-chemokine heterodimers can alter the apoptotic  
209 activity of specific galectins on specific immune cell subpopulations.

210

211 **Discussion**

212 In this study, we present evidence that specific galectins and chemokines can form heterodimers  
213 that affect the function of galectins through modifications of their glycan binding activity. In  
214 particular, we show that galectin-1/CXCL4 and galectin-9/CCL5 heterodimers can exert opposing  
215 immunoregulatory activity with regard to immune cell apoptosis. This reveals a cross-talk mechanism  
216 by which galectins and chemokines can jointly control the immune response.

217 Both galectins and chemokines are well established (immuno)regulatory protein families that  
218 exert a plethora of biological functions, including regulation of (immune) cell recruitment, growth,  
219 differentiation, and survival<sup>13-15</sup>. Thus far, both galectins and chemokines have been considered as  
220 distinct immunomodulatory protein families. However, there is increasing evidence that suggests a  
221 link between both. For example, it has been recently shown in two studies that galectin-3 can directly  
222 interact with interferon-gamma<sup>16</sup> and CXCL12<sup>3</sup>, thereby affecting their activity. While these findings  
223 show that galectin heterodimers can govern cytokine/chemokine function, our results show that the  
224 interaction can also regulate the function of galectin-1 and galectin-9. This adds an additional level to  
225 the immunomodulatory activity of these two galectins. As shown in the current study, a potential  
226 mechanism underlying the observed effects might involve modulation of the galectin glycan-binding  
227 affinity and specificity by inducing structural changes in the carbohydrate binding site. Previously, it  
228 has been shown that introducing mutations distal from the carbohydrate binding site can also result  
229 in small modifications in the structure of galectin-1 that affect glycan binding affinity<sup>17</sup>. In addition,  
230 we and others have previously reported on a close relationship between the galectin CRD structure  
231 and ligand binding<sup>9,18-20</sup>. In line with this, we have previously described that non-endogenous  
232 chemokine-like peptides can directly bind to galectins and affect glycan binding affinity<sup>5</sup>. More  
233 recently, we provided the first evidence that galectin-1 directly interacts with an endogenous  
234 protein, i.e., the pre-B cell receptor. This interaction also induces structural changes in galectin-1  
235 which modulates glycan binding affinity, particularly with regard to LacNAc containing epitopes<sup>6,7</sup>.  
236 Our current findings extend these observations and suggest that the observed galectin-chemokine

237 heterodimerization could represent a mechanism that allows the modification of the glycan-binding  
238 affinity and even specificity of galectin-1 and galectin-9. At the same time, the exact mechanism  
239 underlying the glycan-binding effects of the galectin-cytokine interactions reported here is still  
240 unknown and might also involve, e.g., allosteric effects, as shown for other peptides/molecules<sup>21-24</sup>.  
241 Thus, while the exact reach and mechanisms of galectin-chemokine interactions remain to be  
242 explored, it is tempting to speculate that such interactions might be involved in adapting the glycan-  
243 binding repertoire of galectins. This is supported by our current results showing heterodimer  
244 formation of different chemokines with galectin-1 and/or galectin-9 as well as by a recent study by  
245 Eckardt et al. that identified several specific chemokine interactions with galectin-1 and/or galectin-  
246 3<sup>3</sup>. Of note, while some interactions were confirmed by both Eckhardt et al. and us, e.g., gal-1/CCL5,  
247 others were not, including gal-1/CXCL4. This is most likely related to differences in experimental  
248 setup and these findings warrant further studies to unravel the specific features that govern galectin-  
249 chemokine heterodimerization and to explore the functional consequences of these heterodimeric  
250 complexes.

251       Regarding the functional implications, we focused here on T cell apoptosis, instigated by the  
252 established role of galectin-1 and galectin-9 in this process<sup>10-12</sup>. For example, these two galectins, as  
253 well as others, can impair T cell function by inducing T cell apoptosis and skewing T cell  
254 differentiation towards a suppressive phenotype<sup>10,11,25,26</sup>. Our results confirm the previously  
255 described pro-apoptotic activity of galectin-1 and galectin-9 on T cells<sup>10,11</sup>. In addition, our current  
256 data show that this pro-apoptotic activity can be either increased or inhibited by specific galectin-  
257 chemokine heterodimers, i.e., gal-1/CXCL4 or gal-9/CCL5, respectively. The mechanism underlying  
258 this regulation should be further explored but it most likely involves an altered glycan-binding affinity  
259 of the specific galectin-chemokine heterodimers. For example, several T cell glycoproteins, e.g., CD3,  
260 CD7, CD43, CD45, have been linked to galectin-1-mediated T cell apoptosis<sup>12,27,28</sup>. In addition, it has  
261 been shown that the presence of specific glycan repertoires controls the ability of galectin-1 to  
262 induce apoptosis in specific T helper cells<sup>10</sup>. Thus, while speculative, it can be hypothesized that

263 (changes in) the immune cell glycan signature together with altered glycan-binding affinity of  
264 galectin-chemokine heterodimers will determine whether or not certain immune cell subset will  
265 undergo apoptosis. The observation that the effects were more potent in Jurkat cells as compared to  
266 primary PBMCs could reflect the diverse glycosylation of different immune cell subsets in the latter.  
267 In line with this, the regulatory effects in PBMCs were only observed after PHA activation, suggesting  
268 a role for altered glycosylation patterns in activated vs. non-activated cells. Thus, future studies  
269 should thus aim at deciphering which immune cell subsets are particularly sensitive to specific  
270 galectin-chemokine heterodimers and whether this is linked to the cell surface glycan signatures and  
271 activation status.

272         Apart from further exploring the role of galectin-chemokine interactions in the regulation of  
273 immune cell apoptosis, our findings raise additional questions that should be addressed. For  
274 example, it is currently unknown to what extent specific galectin-chemokine heterodimerization  
275 affects other processes of the immune response, e.g., cell growth or cell differentiation/maturation.  
276 It has been shown that both galectin-1 and CXCL4 are involved in Th17 differentiation<sup>29-31</sup>. Whether  
277 the galectin-1/CXCL4 heterodimers stimulate or hamper this effect is unknown. In addition, next to  
278 immune cells, both galectin-1 and CXCL4 have been shown to regulate other cell types as well, e.g.,  
279 endothelial cells during angiogenesis<sup>4,32-34</sup> and platelets during hemostasis<sup>35,36</sup>. Regarding the latter,  
280 we have recently shown that galectin-1 and CXCL4 colocalize on the platelet surface<sup>8</sup>. Since co-  
281 incubation did not affect binding of galectin-1 to platelets and the observed effects on platelet  
282 function appeared to be additive rather than synergistic, it appears that this specific heterodimer  
283 does not play a major effect in platelet homeostasis. Whether this is true for other cell types and  
284 other heterodimers remains to be studied. However, given our current observations, it can be  
285 assumed that the effects of certain galectin-chemokine heterodimerization extend beyond the  
286 immune cell compartment.

287         Our finding that galectin-chemokine interactions modulate glycan binding affinity and specificity  
288 could also have implications for the efficacy of glycan-based small molecule inhibitors that target

289 galectins<sup>37</sup> and the growing arsenal of drugs that unleash the immune system against tumors<sup>38,39</sup>. It is  
290 essential to unravel the mechanisms by which the local tumor microenvironment (TME) controls the  
291 immune response and how these mechanisms interact<sup>10,40</sup>. Especially since tumor-controlled  
292 immunomodulation frequently involves impaired recruitment and/or function of immune cells due to  
293 the presence or absence of soluble factors like galectins and chemokines<sup>41-43</sup>. In that regard, the local  
294 balance between galectins, chemokines and their heterodimers could underlie the opposing effects  
295 seen in different experimental settings and biological contexts, e.g., the pro- or antiapoptotic activity  
296 of CXCL4<sup>44-48</sup>. This also links to the recent findings that galectins can serve as scavenger molecules  
297 for cytokines/chemokines, thereby hampering immune cell recruitment<sup>3,16</sup>. An additional intriguing  
298 but unexplored concept is whether galectins not only scavenge but also serve as chaperones for  
299 cytokines and chemokines by directing them, in a cytokine/chemokine-specific manner, to sites  
300 displaying a specific glycosylation pattern, e.g., glycoprotein cell-surface receptors. Further insight in  
301 the reciprocal relationship between galectins and cytokines/chemokines will therefore likely benefit  
302 the development of more effective treatment strategies.

303         A limitation of the current study is that we only explored the effects of specific galectin-  
304 chemokine combinations *in vitro*. To better understand the contribution of these interactions in  
305 different biological processes, *in vivo* experiments should be performed. However, to our opinion,  
306 this first requires further insight in the complex interactions between galectins and cytokines that  
307 might occur *in vitro*. For example, it has been suggested that heterodimers can also form between  
308 different members of the galectin family<sup>49</sup> or the chemokine family<sup>50,51</sup>. This suggests the existence of  
309 a complex regulatory network that links the local expression levels of galectins and chemokines to  
310 the complex glycome in order to regulate cellular functions *in vivo*. The width of such a network is  
311 still unknown and while the present *in vitro* study adds to the identification of this complex network,  
312 a full understanding of the extent of specific galectin-chemokine interactions and how they  
313 contribute to *in vivo* biology requires further research.

314 In summary, we present evidence that galectins and chemokines can directly interact and that  
315 this heterodimerization can affect the glycan-binding properties and immunoregulatory activity of  
316 galectins. These findings suggest the existence of a reciprocal mechanism to fine-tune the activity of  
317 galectins by chemokines. A better understanding of this mechanism will provide insight in the  
318 complex process of immunomodulation and will help the development of future  
319 (immuno)therapeutic strategies.  
320



321 **Materials and Methods**

322 *Materials*

323 Recombinant galectin-1 and galectin-9 were in-house produced or purchased from R&D systems. For  
324 fluorescent anisotropy and apoptosis assays, the oxidation resistant mutant galectin-1<sup>52</sup>, i.e.,  
325 galectin-1 C3S (in-house produced) was used.

326 The synthetic high-affinity tdga-probe (0.1  $\mu$ M, 3'-[4-(fluorescein-5-amidomethyl)-1H-1,2,3-triazol-1-  
327 yl]-3'-deoxy- $\beta$ -D-galactopyranosyl 3-(3,5-dimethoxybenzamido)-3-deoxy-1-thio- $\beta$ -D-  
328 galactopyranoside)<sup>9</sup> as well as the low affinity probe (lacto-N-tetraose; LNT-probe)<sup>53</sup> were in-house  
329 produced.

330 Chemokines CXCL4 and CCL5 were either in-house produced<sup>51</sup> or purchased from R&D systems. All  
331 other chemokines and cytokines were purchased from R&D systems. The beta-sheet region, CXCL4<sup>22-</sup>  
332 <sup>54</sup>, was chemically synthesized and purchased from Schafer-N. Anginex was a kind gift from prof. K.  
333 Mayo (University of Minnesota, Minneapolis, USA). Antibodies were purchased from BD Biosciences  
334 unless indicated otherwise.

335

336 *Primary cells and cell lines*

337 The human leukemia cell line Jurkat (originally purchased from and STR authenticated by ATCC) was  
338 maintained in RPMI 1640, 2 mM glutamine medium (Gibco) supplemented with 10% heat-inactivated  
339 fetal bovine serum (Invitrogen), 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin (Invitrogen). Cells  
340 were cultured in a humidified incubator at 37°C and 5% CO<sub>2</sub>.

341 Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from healthy donors  
342 (Purchased from Sanquin, no approval from ethical committee required) by density gradient  
343 centrifugation using Lymphoprep (Nycomed Pharma). Recovered cells were washed twice in sterile  
344 PBS with 0.1% bovine serum albumin and resuspended in RPMI-1640 2 mM glutamine (Gibco),  
345 containing 10% heat-inactivated fetal bovine serum (Invitrogen), 100 U/mL penicillin and 100  $\mu$ g/mL  
346 streptomycin (Invitrogen). When indicated, PBMCs were treated with 1  $\mu$ g/mL phytohemagglutinin-L

347 (PHA-L, Invitrogen) for 24 hours prior to stimulation with galectins/cytokines. All studies involving  
348 human samples were conducted following the Declaration of Helsinki principles and current  
349 legislation on the confidentiality of personal data.

350

#### 351 *Spot blot*

352 Spot blot analysis was performed with 10  $\mu$ L drops containing 3  $\mu$ g of the specific proteins that were  
353 spotted onto a nitrocellulose membrane. The membrane was blocked with Odyssey blocking buffer  
354 (LI-COR Biosciences) for 1 hour, washed with PBS/0.1% Tween, and incubated with 1  $\mu$ g/ml  
355 recombinant galectin-1 or galectin-9 in PBS/0.1% Tween for 1 hour. As control, the incubation step  
356 with recombinant galectin-1 was omitted. After washing with PBS/0.1% Tween, the membrane was  
357 incubated with rabbit anti-galectin-1 antibody (1:1000, Peprotech) or goat anti-galectin-9 antibody  
358 (1:250, R&D Systems) for 1 hour. Following three washing steps, the appropriate IRDye-labeled  
359 secondary antibody (1:10000, LI-COR Biosciences) was applied for 1 hour. Finally, the membrane was  
360 washed twice with PBS/0.1% Tween and rinsed in PBS. Images were obtained by scanning the  
361 membrane with the Odyssey infrared imaging system (LI-COR Biosciences).

362

#### 363 *Gel-shift analysis*

364 For gel-shift analysis, 2  $\mu$ g of the specific proteins were combined in a total volume of 10  $\mu$ L in PBS  
365 and incubated together with 2  $\mu$ L DSS (Disuccinimidyl suberate, Thermo Scientific) for 2 hours on ice  
366 to fix existing protein interactions. Where indicated, 25 mg/mL heparin or 5 mM lactose (Sigma  
367 Aldrich) was added. The reaction was quenched with 1  $\mu$ L Tris buffer pH 7.5 for 15 minutes at room  
368 temperature. Subsequently, the protein mixture was suspended in Laemmli sample buffer (Biorad)  
369 supplemented with 1:20  $\beta$ -mercapto-ethanol, boiled for 5 minutes and subjected to SDS-PAGE  
370 followed by Western immunoblotting using anti-galectin-1 antibody (1:1000, Peprotech). Following  
371 three washing steps, the appropriate IRDye-labeled secondary antibody (1:10000, LI-COR  
372 Biosciences) was applied for 1 hour. Finally, the membrane was washed twice with PBS/0.1% Tween

373 and rinsed in PBS. Images were obtained by scanning the membrane with the Odyssey infrared  
374 imaging system (LI-COR Biosciences).

375

#### 376 *Surface plasmon resonance*

377 Binding kinetics between galectin-1 and CXCL4 were studied using the BIAcore 1000 biosensor  
378 system (BiaCore) as described previously<sup>4</sup>. In short, galectin-1 was covalently attached to CM5 Sensor  
379 Chips (BiaCore) using amine-coupling chemistry according to the manufacturer's instructions. Next,  
380 binding measurements were performed at 25°C using HBS-EP buffer containing 10 mM HEPES, 150  
381 mM NaCl, 3 mM EDTA and 0.005 % surfactant P20 adjusted to pH 7.4. Interactions between galectin-  
382 1 and CXCL4 were analyzed by injection of different CXCL4 concentrations (20 µL at a flow rate of 30  
383 µL/minute). Flow cells were regenerated by injection of 20 µL regeneration buffer (10 mM glycine-  
384 HCl, pH 2.0). Association-rate ( $K_a$ ) and dissociation-rate ( $K_d$ ) constants were obtained by analysis of  
385 the sensorgrams using the Biaevaluation software, version 3.2. All measurements were performed at  
386 least in duplicate at all concentrations and the experiment was performed in duplo.

387

#### 388 *Fluorescence anisotropy*

389 Fluorescence anisotropy analysis was performed as described previously<sup>5,54</sup>. For these experiments,  
390 the oxidation-resistant mutant galectin-1, i.e., galectin-1 C3S, was used to avoid the necessity to add  
391 β-mercaptoethanol, which may disturb interacting glycoprotein ligands<sup>52,55</sup>. In brief, 40 µL reaction  
392 mixture containing different concentrations of galectin-1 C3S and 0.1 µM of fluorescein labeled  
393 saccharide probes either with or without CXCL4 at the indicated concentrations was incubated under  
394 slow rotary shaking for 5 minutes in black 96-well half-area polystyrene microplates. All dilutions  
395 were prepared in HEPES buffer with pH 7.4 and in the absence of salt to prevent precipitation of  
396 CXCL4. Fluorescence anisotropy of fluorescein-tagged saccharide probes was measured with  
397 excitation at 485 nm and emission at 520 nm using a using a PheraStarFS plate reader with software  
398 PHERAstar Mars version 2.10 R3 (BMG). Binding curves were generated by plotting anisotropy

399 against increasing concentrations of galectin-1. Anisotropy was measured without galectin-1 ( $A_0$   
400 representing free probe), at experimental conditions (A) and at saturating concentrations of galectin-  
401 1 ( $A_{max}$ ). The experiments were performed at least in duplicate. For the inhibition assay, increasing  
402 concentrations of the glycoprotein asialofetuin (ASF), an inhibitor of the galectin-1-probe interaction,  
403 were added to fixed low concentrations of galectin-1C3S (0.1  $\mu$ M) and tdga-probe in the presence or  
404 absence of 8  $\mu$ M CXCL4. Data plotting, non-linear regression analysis and curve construction was  
405 performed with Prism 9 software (Graphpad).

406

#### 407 *Nuclear magnetic resonance*

408 For NMR experiments, galectin-1 has been produced as  $^{15}$ N-labeled protein as previously described<sup>6</sup>  
409 and prepared in 20 mM  $KPO_4$  pH 5.6 buffer with 8%  $D_2O$ . All NMR spectra were acquired at 30°C on a  
410 Bruker AVANCEIII spectrometer operating at 600 MHz and equipped with cold probe. NMR data were  
411 processed with TopSpin and analyzed with Ccpnmr Analysis<sup>56</sup>. For interaction experiments with  
412 CXCL4,  $^1H$ - $^{15}N$ -HSQC spectra have been recorded on  $^{15}N$ -labeled galectin-1 (40  $\mu$ M) in the absence  
413 and in the presence 80  $\mu$ M of CXCL4. The same experiments have been performed with  $^{15}N$ -labeled  
414 galectin-1 (350  $\mu$ M) in the presence of CXCL4<sup>22-54</sup> (700  $\mu$ M). The following formula was used for  
415 extracting chemical shift perturbations (CSPs), which takes into consideration the  $^1H$  and  $^{15}N$   
416 chemical shift range of NMR signals:  $CSP (ppm) = [\Delta\delta_{HN}^2 + (\Delta\delta_N^2/25)]^{1/2}$  where  $\Delta\delta_{HN}$  and  $\Delta\delta_N$  are,  
417 respectively, the proton and nitrogen chemical shift variations of each residue.

418

#### 419 *NMR-Data Driven Docking*

420 Models of galectin-1 in complex with CXCL4 were built with the version HADDOCK2.4 of “The  
421 HADDOCK web server for data-driven biomolecular docking”<sup>57</sup>. As starting structures, we used the  
422 structures of galectin-1 (PDB ID 2KM2) and CXCL4 (PDB ID 1F9Q). The galectin-1 and CXCL4 structures  
423 were then docked within HADDOCK. Residues of galectin-1 that showed CSPs above the  
424 threshold were considered as active Ambiguous Restraints (AIR). On the CXCL4 side, the beta-sheet

425 residues homologous to the angix<sup>58</sup> sequence were considered as AIR. Calculations were  
426 performed with 2,000 structures during the HADDOCK rigid body energy minimization, 400  
427 structures during the refinement, and 200 structures during the refinement in explicit water.

428

#### 429 *Apoptosis assays*

430 For analysis of apoptosis, either  $1 \times 10^5$  Jurkat cells or PBMCs per well were plated in 24-well plates  
431 and incubated for the indicated time and concentration with galectin-1 or galectin-9 alone or in  
432 combination with the indicated concentration of chemokines.

433 Following treatment, Jurkat cells were washed twice in ice-cold PBS and stained with (FITC)-  
434 conjugated annexin A5 (Immunotools) and propidium iodide, following the manufacturer's  
435 instructions. Cells were subsequently analyzed using a FACSCalibur instrument (BD Biosciences).

436 When indicated, 0.1 M lactose (Sigma-Aldrich) or 0.1 M heparin (Leo Pharma) were added to the cell  
437 culture just prior to galectin/chemokine treatment. Experiments were performed at least in  
438 triplicate.

439 Following PBMC treatment, PBMCs were blocked during 20 minutes with 1% BSA/PBS at 4°C, washed  
440 in ice-cold PBS and incubated during 30 minutes at 4°C with a combination of fluorescently  
441 conjugated monoclonal antibodies against CD3, CD8, CD4 and CD14: Anti CD3-APC (BD Clone SK7),  
442 anti-CD8-V500 (BD Clone SK1), anti-CD4-PerCP Cy5.5 (BD Clone SK73), anti-CD14-PE BD Clone MφP9).

443 Subsequently, cells were washed twice with 0.1% BSA/PBS and stained with (FITC)-conjugated  
444 annexin A5 (Immunotools) following the manufacturer's instructions. Data acquisition was  
445 performed using a FACSCanto II instrument (BD Biosciences). Experiments were performed using  
446 PBMCs from at least six different donors. All data were analyzed using Kaluza analysis software  
447 (Beckman Coulter).

448

#### 449 *Statistics and reproducibility*

450 Of all experiments, the individual data are presented together with the mean of at least three  
451 independent experiments unless indicated otherwise. To calculate statistically significant differences  
452 of the Jurkat and PBMC apoptosis data, the paired student's t-test and the one-way ANOVA with  
453 Tukey's multiple comparison tests were used. Statistical computations were performed using  
454 Graphpad Prism software (v8 or v9) and p-values < 0.05 were considered as statistically significant.

455

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460

#### 461 **Data availability**

462 The data that support the findings of this study are available in the supplementary material of this  
463 article (Supplementary data). This includes the original Western blot scans from Figure 1b-e and the  
464 data presented in Figures 3-5.

465

#### 466 **Competing interests**

467 The authors declare the following competing interests: H.L. and U.J.N are shareholders in Galecto  
468 Biotech AB, a company developing galectin inhibitors. The remaining authors declare no no  
469 competing interests.

470

#### 471 **Author contributions**

472 LS, IS, PT, RH, EA, KC, VT conducted experiments and generated data. LS, IS, PT, RH, HK, AWG, LE, RK,  
473 VT analyzed and interpreted data. TdG, UN, HK, AWG, LE, RK, VT conceived, designed and/or  
474 supervised experiments. LS, IS, VT wrote the main manuscript. All authors approved the final  
475 manuscript.

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620 233-242 (2001).
- 621
- 622

623 **Table 1.** Rate and affinity constants for galectin-1 binding

Analyte	$k_a$ (Ms <sup>-1</sup> )	$k_{d1}$ (s <sup>-1</sup> )	$k_{d2}$ (s <sup>-1</sup> )	$K_1$	$K_2$
Anginex	$6.5 \times 10^3 (\pm 1.7)$	$4.2 \times 10^{-2} (\pm 0.4)$	$5.9 \times 10^{-4} (\pm 1.9)$	$6.4 \pm 1.7 \mu\text{M}$	$90.0 \pm 6.7 \text{ nM}$
CXCL4	$1.02 \times 10^4 (\pm 0.05)$	$9.2 \times 10^{-2} (\pm 1.2)$	$5.0 \times 10^{-3} (\pm 0.5)$	$9.0 \pm 0.8 \mu\text{M}$	$483 \pm 28 \text{ nM}$
BSA	-	-	-	-	-

624 The association rate constant ( $k_a$ ) was obtained by plotting the  $k_{\text{obs}}$  as a function of analyte  
 625 concentration.  $K_1$  is defined as  $k_{d1}/k_a$  and  $K_2$  is defined as  $k_{d2}/k_a$ . BSA = bovine serum albumin. - = No  
 626 interaction detected.

627

628 **Figure legends**

629

630 **Figure 1. Specific protein-protein interactions occur between galectin-1 and different chemokines.**

631 **a** Representative spot blot analysis. Indicated proteins were spotted onto nitrocellulose filter and  
632 following incubation with galectin-1 protein-protein interaction was revealed by staining with anti-  
633 galectin-1 antibody. Anginex (Ax) and anti-galectin-1 monoclonal antibody served as positive  
634 controls, bovine serum albumin (BSA) and anti-ICAM monoclonal antibody served as negative  
635 control. **b** Representative gel-shift analysis. Proteins (0.2 mg/mL) were incubated for 2 hours in the  
636 presence or absence of cross-linking agent disuccinimidyl suberate (DSS). Standard Western blot with  
637 anti-galectin-1 polyclonal antibody. The arrowheads point towards a shift of galectin-1. **c** Similar as in  
638 (b) but in the presence or absence of excess heparin. **d** Similar as in (b) but in the presence or  
639 absence of excess  $\beta$ -lactose. **e** Similar as in (b) but in the presence of different cytokines or increasing  
640 concentrations of CXCL4.

641

642 **Figure 2. Structural characterization of the galectin-1/CXCL4 complex.**

643 **a** Overlay of  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of  $^{15}\text{N}$ -labeled galectin-1 free (black trace) and bound to CXCL4  
644 (light orange trace). **b** Histogram plot of CSPs observed for each galectin-1 resonance upon  
645 interaction to CXCL4. The dashed line represents  $1\sigma$  from the average CSP, thereby defining the  
646 threshold selection for the most affected residues. Light orange bars correspond to CSP above the  
647 threshold. Arrows are pointing toward residue showing intensity decrease upon interaction to CXCL4  
648 (**Supplementary figure 3**). Galectin-1 secondary structures are depicted above the plot. **c** Chemical  
649 shift perturbation mapping onto galectin-1 monomer structure which is shown as  
650 a semitransparent solvent-accessible surface with a ribbon model displayed below the surface.  
651 Residues presenting CSPs above the threshold (defined in **b**) are colored in light orange. Main  $\beta$ -  
652 strands affected are labeled. Two galectin-1 orientations are presented: (i) the galectin-1 edge view,  
653 opposite to the dimer interface, and (ii) the CBS view with the CBS boundaries plotted in dotted lines.

654 **d** The structural model of the galectin-1/CXCL4 complex. Galectin-1 is shown as grey ribbon whereas  
655 CXCL4 is colored yellow. Galectin-1 residues found at the interface of the complex and presenting  
656 CSPs above the threshold (defined in **b**) are colored in light orange.

657

658 **Figure 3. Interactions between galectin-1 and CXCL4 affect glycan-binding affinity and specificity of**  
659 **galectin-1.**

660 **a** Anisotropy analyses using a fluorescently labeled high affinity thiodigalactoside amide (tdga)-probe  
661 with increasing galectin-1 concentrations in the absence (blue line, N=7) or presence (green line,  
662 N=4) of CXCL4 and excess heparin (red line, N=1). **b** Anisotropy analyses using a fluorescently labeled  
663 low affinity lacto-N-triose (LNT)-probe with increasing galectin-1 concentrations in the absence (blue  
664 line, N=2) or presence (green line, N=2) of CXCL4. **c** Anisotropy analyses using a fluorescently labeled  
665 high affinity tdga-probe with fixed galectin-1 concentrations and increasing concentrations of an  
666 unlabeled high affinity asialofetuin (ASF)-probe in the presence (green line, N=2) and absence (blue  
667 line, N=2) of CXCL4.

668

669 **Figure 4. Specific galectin-chemokine interactions modify the pro-apoptotic function of galectins on**  
670 **Jurkat cells.**

671 **a** Apoptosis as determined by FACS analysis (Annexin A5<sup>+</sup> and PI<sup>+</sup> staining). Jurkat cells were  
672 incubated for 1 hour with 6 μM galectin-1 in the presence or absence of the indicated chemokines at  
673 equimolar concentrations. N=4 independent experiments. **b** Similar as in (a) but with increasing  
674 concentrations of CXCL4 (0, 1.5, 3, 6 and 12 μM). N=4 independent experiments. **c** Similar as in (a)  
675 but with the addition of either excess β-lactose or heparin. N=4 independent experiments. **d**  
676 Apoptosis as determined by FACS analysis (Annexin A5<sup>+</sup> and PI<sup>+</sup> staining). Jurkat cells were incubated  
677 for 4 hours with 36 nM galectin-9 in the presence or absence of the indicated chemokines at  
678 equimolar concentrations. N=6 independent experiments. **e** Similar as in (d) but with increasing

679 concentrations of CCL5 (0, 9, 18, 36, 72 nM). **f** Similar as in (d) but with the addition of excess lactose.

680 N=3 independent experiments.

681

682 **Figure 5. Specific galectin-chemokine interactions modify the pro-apoptotic function of galectins on**  
683 **specific immune cell subsets.**

684 **a** Apoptosis as determined by FACS analysis (Annexin A5<sup>+</sup>). Peripheral blood mononuclear cells

685 (PBMCs) were incubated for 24 hours with 12 μM galectin-1 or 36 nM galectin-9 in the presence or

686 absence of the indicated chemokines at equimolar concentrations. N=11 different donors. **b** Similar

687 as in (a) but PBMCs were activated for 24 hours with 1 μg/mL phytohemagglutinin-L (PHA-L) prior to

688 treatment with galectins and chemokines. N=11 different donors. **c-f** Treatment similar as in (b)

689 followed by FACS analysis with gating for specific subpopulations, i.e., CD3<sup>+</sup>, CD3<sup>+</sup>/CD4<sup>+</sup>, CD3<sup>+</sup>/CD8<sup>+</sup>.

690 Graphs show percentage of Annexin A5<sup>+</sup> cells within the indicated populations. N≥6.











