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Chemokines modulate glycan binding and the immunoregulatory activity of galectins

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Abstract

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

Introduction

The human glycome is a complex and dynamic collection of cellular glycans and glycoconjugates that are involved in a plethora of cellular functions. Alterations in the glycan composition have been linked to different pathologies including genetic disorders, immune diseases and cancer¹. In vertebrates, the information that is encoded by the glycome is deciphered by specific glycan binding proteins, called lectins. Interestingly, the complex repertoire of the glycome greatly exceeds the number of lectins¹. This suggests that specific mechanisms exist to regulate and fine-tune lectin binding affinity in order to cover the broad spectrum of the glycome.

Recently, it was shown that galectin-3, a member of the galectin protein family, can form heterodimers with the chemokine CXCL12. While galectins are known to exert their function by binding to glycoconjugates², the interaction was found to be glycan-independent and to hamper CXCL12 activity³. Apart from galectin-3/CXCL12, the authors found interactions of other chemokines with either galectin-3 or galectin-1³. These observations support our previous finding that chemokine-like peptides can directly bind to different members of the galectin family^{4,5}. We described that the heterodimerization induces changes in the galectin protein structure which affects the glycan-binding affinity and specificity of galectin-1^{4,5}. More recently, modulation of glycan-binding activity was also observed in response to the interaction of galectin-1 with a specific fragment of the pre-B-cell receptor^{6,7}. Collectively, these findings suggest that heterodimerization of galectins with other proteins could represent a mechanism to regulate galectin-glycan interactions. Such a mechanism would extend the functionality of this glycome-decoding protein family. However, whether chemokines could represent a protein family that regulates galectin function is not yet fully understood.

In the present study, we present evidence that the galectin-chemokine heterodimerization can indeed serve as a mechanism to modulate galectin glycan-binding and function. We found specific interactions between different chemokines and two immunomodulatory galectins i.e., galectin-1 and 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

Results

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

The current study aimed to explore whether galectin-chemokine interactions could serve as a mechanism to modulate galectin glycan-binding and function. First, we performed an interaction screen (**Supplementary figure 1**) and observed several specific interactions of galectin-1 and galectin-9 with different chemokines and cytokines. Based on our recent work showing complementary platelet responses induced by galectin-1 and platelet factor 4 (PF4 or CXCL4), we focused our investigation on this galectin-chemokine pair⁸. Spot blot analyses showed that CXCL4 binds galectin-1 to a similar extent as the positive control peptide, i.e., anginex⁴ (**Figure 1a**). To ensure that the interaction was not caused by changes in cytokine structure due to immobilization on the nitrocellulose filter, soluble galectin-1 with either CXCL4 or anginex were incubated in the presence of the crosslinking agent DSS. Subsequent gel-shift analyses confirmed the expected shift in protein size of galectin-1 monomers (± 14 kD) corresponding to the size of either ± 8 kD (CXCL4) or ± 4 kD (anginex) (**Figure 1b**). The intensity of the shifted band was weak suggesting interference of the bound chemokine with antibody binding to galectin-1. Of note, crosslinking with CXCL4 also revealed a band just above the galectin-1 dimer which could either represent a single galectin-1 monomer bound to two CXCL4 molecules or a galectin-1 dimer bound to a single CXCL4 molecule. To confirm that the interaction involved CXCL4 and was also not caused by structural abnormalities due to the crosslinking agent, excess heparin was added prior to crosslinking. Heparin, a known high affinity ligand of CXCL4, serves as a CXCL4 scavenger and indeed completely neutralized the galectin-1/CXCL4 interaction (**Figure 1c**). Heparin did not cause a shift in galectin-1 size suggesting no interaction of both molecules. At the same time, the addition of excess lactose, a carbohydrate ligand of galectin-1, did not prevent the interaction (**Figure 1d**). The latter indicated that CXCL4 does not directly compete for glycan binding in the carbohydrate recognition domain of galectin-1. Furthermore, the interaction was dose-dependent and extended beyond the galectin-1/CXCL4 pair (**Figure 1e + Supplementary Figure 1**).

To characterize the binding kinetics of the galectin-1/CXCL4 heterodimer formation, surface plasmon resonance analysis was performed. Similar as previously described for the galectin-1:anginex interaction⁴, the galectin-1:CXCL4 interaction showed a 1:2 stoichiometry (single association followed by biphasic dissociation) with binding affinities in the high nanomolar and low micromolar range (**Supplementary Figure 2 + Table 1**). This also suggests that the high molecular weight band observed in the gel-shift assay most likely represents a galectin-1 monomer bound to a CXCL4 dimer. Collectively, all these data provide clear evidence of a direct interaction between galectin-1 and CXCL4.

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

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

calculations. On the CXCL4 side, residues from the β -sheet, homologous to the anginex peptide sequence, were also considered as ambiguous restraints. In the structural model showing the best HADDOCK score, the CXCL4 β -sheet docks onto the galectin-1 surface involving mainly β 6 and β 9 strands (**Figure 2d**). In addition, the CXCL4 C-terminal helix establishes contacts with strands β 8 and β 9 strands. To further validate the proposed structural model of the galectin-1/CXCL4 complex formation, the CXCL4 β -sheet region (CXCL4²²⁻⁵⁴; **Supplementary figure 4a+b**) was produced after which the interaction with ¹⁵N-labeled galectin-1 was tested. The perturbations observed on galectin-1 spectra corresponded to residues from the CBS and from the β 6 and β 9 strands (**Supplementary Figure 4c**). When compared to CSPs induced by full-length CXCL4, the same regions of galectin-1 were affected with CXCL4²²⁻⁵⁴ except for a few residues belonging to the β 8 and β 9 strands, which belong to the galectin-1 residues that interact with the CXCL4 C-terminal helix that is absent from CXCL4²²⁻⁵⁴ (**Supplementary Figure 4d+e**). Therefore, these data confirm the involvement of both the CXCL4 β -sheet and its C-terminal helix in the interaction with galectin-1.

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

Interestingly, while interacting at the edge of the galectin-1 β -sheet, CXCL4 induced chemical shift perturbations within the galectin-1 CBS. Previously, we have shown that the direct protein-protein interactions can modulate the glycan binding affinity of galectin-1^{5,7}. To explore whether binding of CXCL4 could also affect the carbohydrate binding affinity of galectin-1 we performed fluorescent anisotropy using a fluorescein-tagged high affinity tdga-probe⁹. Upon addition of 8 μ M CXCL4, the baseline signal slightly increased, indicating only minimal interaction between CXCL4 and the probe. Subsequent titration of increasing concentrations of galectin-1 showed that the binding affinity of galectin-1 for this probe increased almost 10-fold to a K_d of 0.09 ± 0.01 as compared to galectin-1 alone (K_d of 0.88 ± 0.05 μ M) (**Figure 3a**). In line with previous results⁵, the A_{max} (anisotropy of the galectin-probe complex) was also increased in the presence of CXCL4, which indicates a reduced mobility of the fluorescein tag. Similar as in the gel-shift experiments, the increased binding

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

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

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

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

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

To further extend these findings, we next analyzed the effects of specific galectin-chemokine heterodimers on peripheral blood mononuclear cells (PBMCs) obtained from healthy donors. Firstly, we evaluated the effect of galectin-chemokine heterodimers on apoptosis in resting and activated PBMCs, the latter being more susceptible to galectin-induced apoptosis¹². Cell activation was induced by PHA-L lectin treatment and confirmed by CD25 surface staining (**Supplementary figure 7**). In resting PBMCs, neither CXCL4 nor CCL5 affected the induction of apoptosis by galectin-1 and galectin-9, respectively (**Figure 5a**). However, in activated PBMCs (1 µg PHA-L/mL), the pro-apoptotic effect of galectin-1 was significantly induced by CXCL4 while the pro-apoptotic effect of galectin-9 was significantly inhibited by CCL5 (**Figure 5b**). These observations corroborated our findings in Jurkat cells. Of note, in the absence of galectins, neither CXCL4 nor CCL5 induced apoptosis in resting or activated PBMCs (**Supplementary figure 8**).

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

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

Discussion

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

Both galectins and chemokines are well established (immuno)regulatory protein families that exert a plethora of biological functions, including regulation of (immune) cell recruitment, growth, differentiation, and survival^{13–15}. Thus far, both galectins and chemokines have been considered as distinct immunomodulatory protein families. However, there is increasing evidence that suggests a link between both. For example, it has been recently shown in two studies that galectin-3 can directly interact with interferon-gamma¹⁶ and CXCL12³, thereby affecting their activity. While these findings show that galectin heterodimers can govern cytokine/chemokine function, our results show that the interaction can also regulate the function of galectin-1 and galectin-9. This adds an additional level to the immunomodulatory activity of these two galectins. As shown in the current study, a potential mechanism underlying the observed effects might involve modulation of the galectin glycan-binding affinity and specificity by inducing structural changes in the carbohydrate binding site. Previously, it has been shown that introducing mutations distal from the carbohydrate binding site can also result in small modifications in the structure of galectin-1 that affect glycan binding affinity¹⁷. In addition, we and others have previously reported on a close relationship between the galectin CRD structure and ligand binding^{9,18–20}. In line with this, we have previously described that non-endogenous chemokine-like peptides can directly bind to galectins and affect glycan binding affinity⁵. More recently, we provided the first evidence that galectin-1 directly interacts with an endogenous protein, i.e., the pre-B cell receptor. This interaction also induces structural changes in galectin-1 which modulates glycan binding affinity, particularly with regard to LacNAc containing epitopes^{6,7}. Our current findings extend these observations and suggest that the observed galectin-chemokine

heterodimerization could represent a mechanism that allows the modification of the glycan-binding affinity and even specificity of galectin-1 and galectin-9. At the same time, the exact mechanism underlying the glycan-binding effects of the galectin-cytokine interactions reported here is still unknown and might also involve, e.g., allosteric effects, as shown for other peptides/molecules²¹⁻²⁴. Thus, while the exact reach and mechanisms of galectin-chemokine interactions remain to be explored, it is tempting to speculate that such interactions might be involved in adapting the glycan-binding repertoire of galectins. This is supported by our current results showing heterodimer formation of different chemokines with galectin-1 and/or galectin-9 as well as by a recent study by Eckardt et al. that identified several specific chemokine interactions with galectin-1 and/or galectin-3³. Of note, while some interactions were confirmed by both Eckhardt et al. and us, e.g., gal-1/CCL5, others were not, including gal-1/CXCL4. This is most likely related to differences in experimental setup and these findings warrant further studies to unravel the specific features that govern galectin-chemokine heterodimerization and to explore the functional consequences of these heterodimeric complexes.

Regarding the functional implications, we focused here on T cell apoptosis, instigated by the established role of galectin-1 and galectin-9 in this process¹⁰⁻¹². For example, these two galectins, as well as others, can impair T cell function by inducing T cell apoptosis and skewing T cell differentiation towards a suppressive phenotype^{10,11,25,26}. Our results confirm the previously described pro-apoptotic activity of galectin-1 and galectin-9 on T cells^{10,11}. In addition, our current data show that this pro-apoptotic activity can be either increased or inhibited by specific galectin-chemokine heterodimers, i.e., gal-1/CXCL4 or gal-9/CCL5, respectively. The mechanism underlying this regulation should be further explored but it most likely involves an altered glycan-binding affinity of the specific galectin-chemokine heterodimers. For example, several T cell glycoproteins, e.g., CD3, CD7, CD43, CD45, have been linked to galectin-1-mediated T cell apoptosis^{12,27,28}. In addition, it has been shown that the presence of specific glycan repertoires controls the ability of galectin-1 to induce apoptosis in specific T helper cells¹⁰. Thus, while speculative, it can be hypothesized that

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

Apart from further exploring the role of galectin-chemokine interactions in the regulation of immune cell apoptosis, our findings raise additional questions that should be addressed. For example, it is currently unknown to what extent specific galectin-chemokine heterodimerization affects other processes of the immune response, e.g., cell growth or cell differentiation/maturation. It has been shown that both galectin-1 and CXCL4 are involved in Th17 differentiation^{29–31}. Whether the galectin-1/CXCL4 heterodimers stimulate or hamper this effect is unknown. In addition, next to immune cells, both galectin-1 and CXCL4 have been shown to regulate other cell types as well, e.g., endothelial cells during angiogenesis^{4,32–34} and platelets during hemostasis^{35,36}. Regarding the latter, we have recently shown that galectin-1 and CXCL4 colocalize on the platelet surface⁸. Since co-incubation did not affect binding of galectin-1 to platelets and the observed effects on platelet function appeared to be additive rather than synergistic, it appears that this specific heterodimer does not play a major effect in platelet homeostasis. Whether this is true for other cell types and other heterodimers remains to be studied. However, given our current observations, it can be assumed that the effects of certain galectin-chemokine heterodimerization extend beyond the immune cell compartment.

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

galectins³⁷ and the growing arsenal of drugs that unleash the immune system against tumors^{38,39}. It is essential to unravel the mechanisms by which the local tumor microenvironment (TME) controls the immune response and how these mechanisms interact^{10,40}. Especially since tumor-controlled immunomodulation frequently involves impaired recruitment and/or function of immune cells due to the presence or absence of soluble factors like galectins and chemokines^{41–43}. In that regard, the local balance between galectins, chemokines and their heterodimers could underlie the opposing effects seen in different experimental settings and biological contexts, e.g., the pro- or antiapoptotic activity of CXCL4^{44–48}. This also links to the recent findings that galectins can serve as scavenger molecules for cytokines/chemokines, thereby hampering immune cell recruitment^{3,16}. An additional intriguing but unexplored concept is whether galectins not only scavenge but also serve as chaperones for cytokines and chemokines by directing them, in a cytokine/chemokine-specific manner, to sites displaying a specific glycosylation pattern, e.g., glycoprotein cell-surface receptors. Further insight in the reciprocal relationship between galectins and cytokines/chemokines will therefore likely benefit the development of more effective treatment strategies.

A limitation of the current study is that we only explored the effects of specific galectin-chemokine combinations *in vitro*. To better understand the contribution of these interactions in different biological processes, *in vivo* experiments should be performed. However, to our opinion, this first requires further insight in the complex interactions between galectins and cytokines that might occur *in vitro*. For example, it has been suggested that heterodimers can also form between different members of the galectin family⁴⁹ or the chemokine family^{50,51}. This suggests the existence of a complex regulatory network that links the local expression levels of galectins and chemokines to the complex glycome in order to regulate cellular functions *in vivo*. The width of such a network is still unknown and while the present *in vitro* study adds to the identification of this complex network, a full understanding of the extent of specific galectin-chemokine interactions and how they 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

Materials and Methods

Materials

Recombinant galectin-1 and galectin-9 were in-house produced or purchased from R&D systems. For fluorescent anisotropy and apoptosis assays, the oxidation resistant mutant galectin-1⁵², i.e., galectin-1 C3S (in-house produced) was used.

The synthetic high-affinity tdga-probe (0.1 μ M, 3'-[4-(fluorescein-5-amidomethyl)-1*H*-1,2,3-triazol-1-yl]-3'-deoxy- β -D-galactopyranosyl 3-(3,5-dimethoxybenzamido)-3-deoxy-1-thio- β -D-galactopyranoside)⁹ as well as the low affinity probe (lacto-N-tetraose; LNT-probe)⁵³ were in-house produced.

Chemokines CXCL4 and CCL5 were either in-house produced⁵¹ or purchased from R&D systems. All other chemokines and cytokines were purchased from R&D systems. The beta-sheet region, CXCL4²²⁻⁵⁴, was chemically synthesized and purchased from Schafer-N. Anginex was a kind gift from prof. K. Mayo (University of Minnesota, Minneapolis, USA). Antibodies were purchased from BD Biosciences unless indicated otherwise.

Primary cells and cell lines

The human leukemia cell line Jurkat (originally purchased from and STR authenticated by ATCC) was maintained in RPMI 1640, 2 mM glutamine medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 100 U/mL penicillin and 100 μ g/mL streptomycin (Invitrogen). Cells were cultured in a humidified incubator at 37°C and 5% CO₂.

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

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

Spot blot

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

Gel-shift analysis

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

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

Surface plasmon resonance

Binding kinetics between galectin-1 and CXCL4 were studied using the BIAcore 1000 biosensor system (BiaCore) as described previously⁴. In short, galectin-1 was covalently attached to CM5 Sensor Chips (BiaCore) using amine-coupling chemistry according to the manufacturer's instructions. Next, binding measurements were performed at 25°C using HBS-EP buffer containing 10 mM HEPES, 150 mM NaCl, 3 mM EDTA and 0.005 % surfactant P20 adjusted to pH 7.4. Interactions between galectin-1 and CXCL4 were analyzed by injection of different CXCL4 concentrations (20 µL at a flow rate of 30 µL/minute). Flow cells were regenerated by injection of 20 µL regeneration buffer (10 mM glycine-HCl, pH 2.0). Association-rate (K_a) and dissociation-rate (K_d) constants were obtained by analysis of the sensorgrams using the Biaevaluation software, version 3.2. All measurements were performed at least in duplicate at all concentrations and the experiment was performed in duplo.

Fluorescence anisotropy

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

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

Nuclear magnetic resonance

For NMR experiments, galectin-1 has been produced as 15 N-labeled protein as previously described⁶ 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 Bruker AVANCEIII spectrometer operating at 600 MHz and equipped with cold probe. NMR data were processed with TopSpin and analyzed with Ccpnmr Analysis⁵⁶. For interaction experiments with CXCL4, 1H - ^{15}N -HSQC spectra have been recorded on ^{15}N -labeled galectin-1 (40 μ M) in the absence and in the presence 80 μ M of CXCL4. The same experiments have been performed with ^{15}N -labeled galectin-1 (350 μ M) in the presence of CXCL4²²⁻⁵⁴ (700 μ M). The following formula was used for extracting chemical shift perturbations (CSPs), which takes into consideration the 1H and ^{15}N 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, respectively, the proton and nitrogen chemical shift variations of each residue.

NMR-Data Driven Docking

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

425 residues homologous to the angonex⁵⁸ 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 1×10^5 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*

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

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Data availability

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

Competing interests

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

Author contributions

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

References

1. Cummings, R. D. The repertoire of glycan determinants in the human glycome. *Mol Biosyst* **5**, 1087-1104 (2009). 10.1039/b907931a
2. Thijssen, V. L., Heusschen, R., Caers, J. & Griffioen, A. W. Galectin expression in cancer diagnosis and prognosis: A systematic review. *Biochim Biophys Acta* **1855**, 235-247 (2015). 10.1016/j.bbcan.2015.03.003
3. Eckardt, V. et al. Chemokines and galectins form heterodimers to modulate inflammation. *EMBO Rep* e47852 (2020). 10.15252/embr.201947852
4. Thijssen, V. L. et al. Galectin-1 is essential in tumor angiogenesis and is a target for antiangiogenesis therapy. *Proc Natl Acad Sci U S A* **103**, 15975-15980 (2006). 10.1073/pnas.0603883103
5. Salomonsson, E., Thijssen, V. L., Griffioen, A. W., Nilsson, U. J. & Leffler, H. The anti-angiogenic peptide anginex greatly enhances galectin-1 binding affinity for glycoproteins. *J Biol Chem* **286**, 13801-13804 (2011). 10.1074/jbc.C111.229096
6. Elantak, L. et al. Structural basis for galectin-1-dependent pre-B cell receptor (pre-BCR) activation. *J Biol Chem* **287**, 44703-44713 (2012). 10.1074/jbc.M112.395152
7. Bonzi, J. et al. Pre-B cell receptor binding to galectin-1 modifies galectin-1/carbohydrate affinity to modulate specific galectin-1/glycan lattice interactions. *Nat Commun* **6**, 6194 (2015). 10.1038/ncomms7194
8. Dickhout, A. et al. Galectin-1 and platelet factor 4 (CXCL4) induce complementary platelet responses in vitro. *PLoS One* **16**, e0244736 (2021). 10.1371/journal.pone.0244736
9. Salomonsson, E. et al. Monovalent interactions of galectin-1. *Biochemistry* **49**, 9518-9532 (2010). 10.1021/bi1009584
10. Toscano, M. A. et al. Differential glycosylation of TH1, TH2 and TH-17 effector cells selectively regulates susceptibility to cell death. *Nat Immunol* **8**, 825-834 (2007).

501 11. Bi, S., Earl, L. A., Jacobs, L. & Baum, L. G. Structural features of galectin-9 and galectin-1 that
502 determine distinct T cell death pathways. *J Biol Chem* **283**, 12248-12258 (2008).

503 12. Perillo, N. L., Pace, K. E., Seilhamer, J. J. & Baum, L. G. Apoptosis of T cells mediated by galectin-
504 1. *Nature* **378**, 736-739 (1995).

505 13. Nagarsheth, N., Wicha, M. S. & Zou, W. Chemokines in the cancer microenvironment and their
506 relevance in cancer immunotherapy. *Nat Rev Immunol* **17**, 559-572 (2017).
507 10.1038/nri.2017.49

508 14. Rabinovich, G. A. & Croci, D. O. Regulatory circuits mediated by lectin-glycan interactions in
509 autoimmunity and cancer. *Immunity* **36**, 322-335 (2012). 10.1016/j.immuni.2012.03.004

510 15. Zlotnik, A. & Yoshie, O. The chemokine superfamily revisited. *Immunity* **36**, 705-716 (2012).
511 10.1016/j.immuni.2012.05.008

512 16. Gordon-Alonso, M., Hirsch, T., Wildmann, C. & van der Bruggen, P. Galectin-3 captures
513 interferon-gamma in the tumor matrix reducing chemokine gradient production and T-cell
514 tumor infiltration. *Nat Commun* **8**, 793 (2017). 10.1038/s41467-017-00925-6

515 17. López-Lucendo, M. F. et al. Growth-regulatory human galectin-1: crystallographic
516 characterisation of the structural changes induced by single-site mutations and their impact on
517 the thermodynamics of ligand binding. *J Mol Biol* **343**, 957-970 (2004).
518 10.1016/j.jmb.2004.08.078

519 18. Nesmelova, I. V. et al. Lactose binding to galectin-1 modulates structural dynamics, increases
520 conformational entropy, and occurs with apparent negative cooperativity. *J Mol Biol* **397**, 1209-
521 1230 (2010).

522 19. Chien, C. H., Ho, M. R., Lin, C. H. & Hsu, S. D. Lactose Binding Induces Opposing Dynamics
523 Changes in Human Galectins Revealed by NMR-Based Hydrogen-Deuterium Exchange.
524 *Molecules* **22**, E1357 (2017). 10.3390/molecules22081357

- 525 20. Romero, J. M., Trujillo, M., Estrin, D. A., Rabinovich, G. A. & Di Lella, S. Impact of human
526 galectin-1 binding to saccharide ligands on dimer dissociation kinetics and structure.
527 *Glycobiology* **26**, 1317-1327 (2016). 10.1093/glycob/cww052
- 528 21. Dings, R. P. M. et al. Antitumor agent calixarene 0118 targets human galectin-1 as an allosteric
529 inhibitor of carbohydrate binding. *J Med Chem* **55**, 5121-5129 (2012). 10.1021/jm300014q
- 530 22. Dings, R. P. M. et al. Structure-Based Optimization of Angiostatic Agent 6DBF7, an Allosteric
531 Antagonist of Galectin-1. *J Pharmacol Exp Ther* **344**, 589-599 (2013). 10.1124/jpet.112.199646
- 532 23. Ermakova, E. et al. Lactose binding to human galectin-7 (p53-induced gene 1) induces long-
533 range effects through the protein resulting in increased dimer stability and evidence for
534 positive cooperativity. *Glycobiology* **23**, 508-523 (2013). 10.1093/glycob/cwt005
- 535 24. Miller, M. C. et al. Targeting the CRD F-face of Human Galectin-3 and Allosterically Modulating
536 Glycan Binding by Angiostatic PTX008 and a Structurally Optimized Derivative. *ChemMedChem*
537 **16**, 713-723 (2021). 10.1002/cmdc.202000742
- 538 25. Stillman, B. N. et al. Galectin-3 and galectin-1 bind distinct cell surface glycoprotein receptors
539 to induce T cell death. *J Immunol* **176**, 778-789 (2006).
- 540 26. Wada, J., Ota, K., Kumar, A., Wallner, E. I. & Kanwar, Y. S. Developmental regulation,
541 expression, and apoptotic potential of galectin-9, a beta-galactoside binding lectin. *J Clin Invest*
542 **99**, 2452-2461 (1997).
- 543 27. Pace, K. E., Hahn, H. P., Pang, M., Nguyen, J. T. & Baum, L. G. CD7 delivers a pro-apoptotic
544 signal during galectin-1-induced T cell death. *J Immunol* **165**, 2331-2334 (2000).
545 10.4049/jimmunol.165.5.2331
- 546 28. Pace, K. E., Lee, C., Stewart, P. L. & Baum, L. G. Restricted receptor segregation into membrane
547 microdomains occurs on human T cells during apoptosis induced by galectin-1. *J Immunol* **163**,
548 3801-3811 (1999).

549 29. Cedeno-Laurent, F., Opperman, M., Barthel, S. R., Kuchroo, V. K. & Dimitroff, C. J. Galectin-1
550 triggers an immunoregulatory signature in Th cells functionally defined by IL-10 expression. *J*
551 *Immunol* **188**, 3127-3137 (2012). 10.4049/jimmunol.1103433

552 30. de la Fuente, H. et al. The leukocyte activation receptor CD69 controls T cell differentiation
553 through its interaction with galectin-1. *Mol Cell Biol* **34**, 2479-2487 (2014).
554 10.1128/MCB.00348-14

555 31. Affandi, A. J. et al. CXCL4 is a novel inducer of human Th17 cells and correlates with IL-17 and
556 IL-22 in psoriatic arthritis. *Eur J Immunol* **48**, 522-531 (2018). 10.1002/eji.201747195

557 32. Croci, D. O. et al. Glycosylation-Dependent Lectin-Receptor Interactions Preserve Angiogenesis
558 in Anti-VEGF Refractory Tumors. *Cell* **156**, 744-758 (2014). 10.1016/j.cell.2014.01.043

559 33. Maione, T. E. et al. Inhibition of angiogenesis by recombinant human platelet factor-4 and
560 related peptides. *Science* **247**, 77-79 (1990).

561 34. Sharpe, R. J., Byers, H. R., Scott, C. F., Bauer, S. I. & Maione, T. E. Growth inhibition of murine
562 melanoma and human colon carcinoma by recombinant human platelet factor 4. *J Natl Cancer*
563 *Inst* **82**, 848-853 (1990).

564 35. Pacienza, N. et al. The immunoregulatory glycan-binding protein galectin-1 triggers human
565 platelet activation. *Faseb J* **22**, 1113-1123 (2008).

566 36. Eslin, D. E. et al. Transgenic mice studies demonstrate a role for platelet factor 4 in thrombosis:
567 dissociation between anticoagulant and antithrombotic effect of heparin. *Blood* **104**, 3173-
568 3180 (2004).

569 37. Hirani, N. et al. Target-inhibition of Galectin-3 by Inhaled TD139 in Patients with Idiopathic
570 Pulmonary Fibrosis. *Eur Respir J* (2020). 10.1183/13993003.02559-2020

571 38. Berraondo, P. et al. Cytokines in clinical cancer immunotherapy. *Br J Cancer* **120**, 6-15 (2019).
572 10.1038/s41416-018-0328-y

573 39. Mahoney, K. M., Rennert, P. D. & Freeman, G. J. Combination cancer immunotherapy and new
574 immunomodulatory targets. *Nat Rev Drug Discov* **14**, 561-584 (2015). 10.1038/nrd4591

575 40. Munn, D. H. & Bronte, V. Immune suppressive mechanisms in the tumor microenvironment.
576 *Curr Opin Immunol* **39**, 1-6 (2016). 10.1016/j.coi.2015.10.009

577 41. Sharma, P., Hu-Lieskovan, S., Wargo, J. A. & Ribas, A. Primary, Adaptive, and Acquired
578 Resistance to Cancer Immunotherapy. *Cell* **168**, 707-723 (2017). 10.1016/j.cell.2017.01.017

579 42. Crome, S. Q. et al. A distinct innate lymphoid cell population regulates tumor-associated T cells.
580 *Nat Med* **23**, 368-375 (2017). 10.1038/nm.4278

581 43. De Henau, O. et al. Overcoming resistance to checkpoint blockade therapy by targeting PI3K γ in
582 myeloid cells. *Nature* **539**, 443-447 (2016). 10.1038/nature20554

583 44. Deng, S. et al. Non-platelet-derived CXCL4 differentially regulates cytotoxic and regulatory T
584 cells through CXCR3 to suppress the immune response to colon cancer. *Cancer Lett* **443**, 1-12
585 (2019). 10.1016/j.canlet.2018.11.017

586 45. Gao, J. et al. Activation of p38-MAPK by CXCL4/CXCR3 axis contributes to p53-dependent
587 intestinal apoptosis initiated by 5-fluorouracil. *Cancer Biol Ther* **15**, 982-991 (2014).
588 10.4161/cbt.29114

589 46. Liang, P. et al. Platelet factor 4 induces cell apoptosis by inhibition of STAT3 via up-regulation of
590 SOCS3 expression in multiple myeloma. *Haematologica* **98**, 288-295 (2013).
591 10.3324/haematol.2012.065607

592 47. Kasper, B. et al. CXCL4-induced monocyte survival, cytokine expression, and oxygen radical
593 formation is regulated by sphingosine kinase 1. *Eur J Immunol* **40**, 1162-1173 (2010).
594 10.1002/eji.200939703

595 48. Kasper, B., Brandt, E., Brandau, S. & Petersen, F. Platelet factor 4 (CXC chemokine ligand 4)
596 differentially regulates respiratory burst, survival, and cytokine expression of human
597 monocytes by using distinct signaling pathways. *J Immunol* **179**, 2584-2591 (2007).
598 10.4049/jimmunol.179.4.2584

599 49. Miller, M. C. et al. Adhesion/growth-regulatory galectins tested in combination: evidence for
600 formation of hybrids as heterodimers. *Biochem J* **475**, 1003-1018 (2018). 10.1042/BCJ20170658

50. von Hundelshausen, P. et al. Heterophilic interactions of platelet factor 4 and RANTES promote monocyte arrest on endothelium. *Blood* **105**, 924-930 (2005).
51. Koenen, R. R. et al. Disrupting functional interactions between platelet chemokines inhibits atherosclerosis in hyperlipidemic mice. *Nat Med* **15**, 97-103 (2009). 10.1038/nm.1898
52. Hirabayashi, J. & Kasai, K. Effect of amino acid substitution by sited-directed mutagenesis on the carbohydrate recognition and stability of human 14-kDa beta-galactoside-binding lectin. *J Biol Chem* **266**, 23648-23653 (1991).
53. Carlsson, S. et al. Affinity of galectin-8 and its carbohydrate recognition domains for ligands in solution and at the cell surface. *Glycobiology* **17**, 663-676 (2007).
54. Sörme, P., Kahl-Knutsson, B., Huflejt, M., Nilsson, U. J. & Leffler, H. Fluorescence polarization as an analytical tool to evaluate galectin-ligand interactions. *Anal Biochem* **334**, 36-47 (2004). 10.1016/j.ab.2004.06.042
55. Nishi, N. et al. Functional and structural bases of a cysteine-less mutant as a long-lasting substitute for galectin-1. *Glycobiology* **18**, 1065-1073 (2008). 10.1093/glycob/cwn089
56. Vranken, W. F. et al. The CCPN data model for NMR spectroscopy: development of a software pipeline. *Proteins* **59**, 687-696 (2005). 10.1002/prot.20449
57. van Zundert, G. C. P. et al. The HADDOCK2.2 Web Server: User-Friendly Integrative Modeling of Biomolecular Complexes. *J Mol Biol* **428**, 720-725 (2016). 10.1016/j.jmb.2015.09.014
58. Griffioen, A. W. et al. Anginex, a designed peptide that inhibits angiogenesis. *Biochem J* **354**, 233-242 (2001).

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

Analyte	k_a (Ms ⁻¹)	k_{d1} (s ⁻¹)	k_{d2} (s ⁻¹)	K_1	K_2
Anginex	6.5×10^3 (± 1.7)	4.2×10^{-2} (± 0.4)	5.9×10^{-4} (± 1.9)	6.4 ± 1.7 μ M	90.0 ± 6.7 nM
CXCL4	1.02×10^4 (± 0.05)	9.2×10^{-2} (± 1.2)	5.0×10^{-3} (± 0.5)	9.0 ± 0.8 μ M	483 ± 28 nM
BSA	-	-	-	-	-

624 The association rate constant (k_a) was obtained by plotting the k_{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

Figure legends

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

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

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

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

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

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

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

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

a Apoptosis as determined by FACS analysis (Annexin A5⁺ and PI⁺ staining). Jurkat cells were incubated for 1 hour with 6 μ M galectin-1 in the presence or absence of the indicated chemokines at equimolar concentrations. N=4 independent experiments. **b** Similar as in (a) but with increasing concentrations of CXCL4 (0, 1.5, 3, 6 and 12 μ M). N=4 independent experiments. **c** Similar as in (a) but with the addition of either excess β -lactose or heparin. N=4 independent experiments. **d** Apoptosis as determined by FACS analysis (Annexin A5⁺ and PI⁺ staining). Jurkat cells were incubated for 4 hours with 36 nM galectin-9 in the presence or absence of the indicated chemokines at 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⁺). Peripheral blood mononuclear cells
685 (PBMCs) were incubated for 24 hours with 12 μ M 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 μ g/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⁺, CD3⁺/CD4⁺, CD3⁺/CD8⁺.
690 Graphs show percentage of Annexin A5⁺ cells within the indicated populations. N $\geq$ 6.