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1 **Galectin-3 modulation of T-cell activation: mechanisms of membrane remodelling.**

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10

11 **Abstract**

12 Galectin-3 (Gal3) is a multifaceted protein which belongs to a family of lectins and binds  $\beta$ -  
13 galactosides. Gal3 expression is altered in many types of cancer, with increased expression  
14 generally associated with poor prognosis. Although the mechanisms remain unknown, Gal3  
15 has been implicated in several biological processes involved in cancer progression, including  
16 suppression of T cell-mediated immune responses. Extracellular Gal3 binding to the plasma  
17 membrane of T cells alters membrane organization and the formation of an immunological  
18 synapse. Its multivalent capacity allows Gal3 to interact specifically with different membrane  
19 proteins and lipids, influencing endocytosis, trafficking and T cell receptor signalling. The  
20 ability of Gal3 to inhibit T cell responses may provide a mechanism by which Gal3 aids in  
21 cancer progression. In this review, we seek to give an overview of the mechanisms by which  
22 Gal3 alters the spatial organization of cell membranes and how these processes impact on T  
23 cell activation.

24

25 **Keywords:** Galectin-3, glycolipids, T cell activation, immunological synapse, T cell  
26 signalling, immunotherapy.

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## 29 **1 Introduction: Galectin-3.**

30 There is an urgent need for new cancer therapies that alleviate the immunosuppressive nature  
31 of tumours. Among them, adoptive T cell transfer, genetically engineered chimeric antigen  
32 receptors (CAR) and immune checkpoint inhibitors have shown great promise in clinical  
33 trials. Due to their complexity, the mechanisms by which tumours create an  
34 immunosuppressive environment remain poorly understood. One emerging mechanism is  
35 thought to be the expression and function of galectin-3 (Gal3), a lectin that is often up- or  
36 down-regulated in many types of cancer[1–5]. Both tumour and non-cancerous cells secrete  
37 Gal3 into the extracellular environment, from where it can bind to the extracellular matrix  
38 and the surface of circulating cells including T cells. Enhanced levels of Gal3 in tumours  
39 have been associated with apoptosis, inflammatory responses[6], reduced numbers of tumour  
40 infiltrating lymphocytes (TILs)[7], impaired T cell activation and their effector function  
41 (Fig.1) [1,8–11]. Removal of Gal3 or blocking of cell interactions with Gal3 has in some  
42 cases restored T cell effector function and their ability to control tumour burden[1,8–11],  
43 however, Gal3 levels are not typically predictive of the level of immunosuppression.  
44 Nevertheless, Gal3 holds great potential in immunotherapy applications[12,13] but the  
45 underlying mechanisms are often complex and require further investigations.

### 46 *1.1. Biological function of galectin-3.*

47 Gal3 is part of the galectin family, whose members are involved in a broad range of cellular  
48 functions including cell-cell interactions, cell-matrix adhesion, endocytosis, intracellular  
49 trafficking and signalling[12,14,15]. The concentration range at which Gal3 impacts on these  
50 cellular functions appears to be both context and function specific. Thus, the organisations  
51 and processes induced by Gal3 at high concentrations may be different than those at low  
52 concentration and functional impacts at the same concentration could depend on the cell type  
53 and tissue. In serum, Gal3 concentrations are typically in the upper picomolar range, while  
54 cell-based experiments have often been performed at much higher concentrations (see Ref.  
55 [15] for a review).

56 In mammals, the galectin family consists of 15 members that bind to  $\beta$ -galactosides through  
57 their conserved carbohydrate recognition domain (CRD)[15,16]. There are three structural  
58 forms of galectin: a dimeric form with two identical CRDs (found in Gal-1, 2, 5, 7, 10, 11,  
59 13, 14, and 15), a tandem form with two unique CRDs connected via a polypeptide (found in  
60 Gal-4, 6, 8, 9, and 12), and a chimeric form with one CRD attached to a non-lectin

61 polypeptide domain (Gal3) [15–18]. Different galectins have different affinities towards  
62 glycans [17]. They interact with other proteins primarily *via* their lectin domains, while direct  
63 protein-protein interaction also occurs[15]. The bifunctionality of the galectin structures  
64 enable multivalent interactions with their ligands, and subsequently the formation of  
65 oligomers and clusters[19–23]. Gal3 contains a single CRD and a non-lectin N-terminal  
66 domain (NTD) enabling it to engage in both lectin-carbohydrate and protein-protein  
67 interactions (Fig. 2a). Instead of dimers, Gal3 can form diverse oligomeric structures. This  
68 oligomerisation and the ligand-induced clustering is influenced by a number of parameters  
69 (Fig. 2b). Moreover, Gal3 has contrasting functions when expressed cytosolically or  
70 extracellularly[24–26]. Here, our focus will be on the physiological roles of extracellular  
71 Gal3 and how it interacts with the plasma membrane to modify T cell activation.

72 Gal3 is secreted into the extracellular space by a number of different cell types, including T  
73 cells and tumour cells[1–5,27–30]. Thus, Gal3 functions as an autocrine or paracrine  
74 signalling agent. In cancer, Gal3 mostly acts in a paracrine manner, where tumour-secreted  
75 Gal3 inhibits T cell function by binding to their cell surface[1,8,10,11,24,26,31]. In chronic  
76 infections, Gal3 acts in an autocrine manner where T cells themselves secrete Gal3[32]. In  
77 both cases, Gal3 is thought to inhibit T cell function *via* altering the spatial organization of  
78 immune synapses (Fig. 3)[2,24,31–33]. Under physiological conditions, immune suppression  
79 *via* Gal3 is necessary to prevent autoimmunity[24,31,32]. Extracellular Gal3 also binds to  
80 secreted cytokines to inhibit the recruitment of T cells to diseased tissues or tumours and to  
81 induce T cell apoptosis (Fig. 1)[7,21,26,34–36]. In this review, we will be focusing on how  
82 Gal3 enables altering the spatial organization of the plasma membrane and the  
83 immunological synapse to overall promote immune suppression.

#### 84 *1.2. Gal3 structure and ligands.*

85 Human Gal3 is a small 26 kDa size lectin that has a single CRD attached to an unstructured  
86 NTD[15]. The CRD is composed of a  $\beta$ -sandwich made from a 5-stranded  $\beta$ -sheet, or F-face,  
87 and a 6-stranded  $\beta$ -sheet which composes a carbohydrate binding pocket[37].  $\beta$ -galactosides,  
88 such as lactose and N-acetyl-D-lactosamine (LacNAc) bind within the CRD via non-covalent  
89 interactions[37]. This carbohydrate binding pocket forms a cassette or cleft which opens at  
90 both ends enabling Gal3 to bind to long oligosaccharides such as N-glycans and some  
91 glycolipid-type glycans[17,38,39]. The F-face, the reverse side of the CRD, has a conserved  
92 hydrophobic surface, which may aid oligomer formation[15].

93 Gal3 binds carbohydrates with variable affinities depending on their sugar sequence, bond  
94 type, linear or branched structure, and the size of the carbohydrate[17]. Gal3 has ~5 times  
95 higher affinity for LacNAc than lactose due to interaction of the NAc group primarily with  
96 Arg186 in Gal3; mutation of this abolishes preference for LacNAC and binding of  
97 glycoproteins[37,39]. The affinity of Gal3 can also be altered by adding additional  
98 saccharides. For example, the affinity of Gal3 to lactose can be increased by addition of a  
99 blood group determinants[37], another LacNAc unit, or decreased by addition of sialic  
100 acid[38,39].

### 101 *1.3. Gal3 oligomers.*

102 The structure and morphology of Gal3 oligomers have attracted much attention (Fig. 2a) and  
103 several types of interactions between monomers have been proposed: (1) NTD-NTD, (2)  
104 NTD-CRD, and (3) CRD-CRD. It is generally thought that the NTD is required for the  
105 formation of large oligomers, as many galectin-3 activities, such as hemagglutination and  
106 cell signalling are lost if the NTD is removed. While Gal3 mutants with NTD deletion retain  
107 their ability to bind to carbohydrates, they have a strongly reduced oligomerization  
108 capacity[15,23], as also demonstrated by chemical crosslinking and NMR studies in which  
109 the NTD of Gal3 was shortened, mutated or removed[20,22,40,41]. Indeed, NTD-NTD  
110 interactions occur in a lectin-independent manner. Electron micrographs have revealed long  
111 fibril structures of NTD[23] indicating that oligomeric Gal3 may not adopt a defined  
112 structure. However, it should be noted that different studies often use very different  
113 concentrations of Gal3, and it is difficult to relate Gal3 structures in the high micromolar  
114 range to Gal3 structures in the nanomolar or high picomolar range.

115 The interaction between two NTDs of different Gal3s can lead to oligomerization. NTD-NTD  
116 interactions have been directly measured by chemical crosslinking and NMR spectroscopic  
117 analysis[20,22,41]. NTD-CRD interactions involve parts of the CRD that are not involved in  
118 carbohydrate recognition[20]. For both NTD interactions with itself and NTD-CRD  
119 interactions, fuzzy interactions may enable variable degrees of oligomerization and may  
120 allow Gal3 to undergo liquid-liquid phase separation[20].

121 The third type of Gal3 oligomers is formed through CRD-CRD interactions. However, these  
122 oligomers are small and may be partially blocked when the CRD is bound to a  
123 carbohydrate[23,42–44]. Electron micrographs show that CRDs can form monomers, dimers,  
124 and trimers in the absence of the ligand[23]. Due to the many types of Gal3 oligomers and

125 their multivalent structure, it is unlikely that Gal3 has a single oligomeric form and may  
126 adopt different oligomeric states at different concentrations.

#### 127 *1.4. Ligand-induced Gal3 lattices.*

128 In addition to Gal3 self-association, Gal3 can also form ligand-induced networks or lattices  
129 on the surface of cells or in solution, depending on the spatial organization of its ligands (Fig.  
130 2b)[44]. The size and structure of Gal3 networks are dependent on (1) the concentration of  
131 Gal3, (2) concentration of ligand, (3) valency of the ligand (i.e. number of repeat units of  
132 Gal3 binding moieties), (4) structure of the ligand (i.e. linear or branching), and (5) the  
133 spatial arrangement of ligands (either two-dimensional or 3-dimensional). Due to the large  
134 number of parameters, a systematic study of Gal3 arrangements is yet to be performed.  
135 However, a few guiding principles have been proposed and validated across multiple  
136 laboratories. According to the current consensus, Gal3 without ligand exists as a monomer in  
137 solution up to ~100  $\mu$ M, and only forms large oligomers when the corresponding ligands (e.g.  
138 lactose or LacNAc) are in multivalent states[44–47]. Thus, there are two different processes  
139 of Gal3 interacting with ligands: repetitive lactose or LacNAc units enhance Gal3 binding  
140 affinity to these ligands because of the higher occupation of the tetra-saccharide-binding site,  
141 and lateral clustering of more than one Gal3 molecule to another antenna of the same glycan  
142 or another glycan on the same glycoprotein, leading to higher order organization into lattices  
143 or endocytic pits. The latter may not be necessarily be caused by enhanced affinity but  
144 appears to be a cooperativity[15,44,48].

145 Gal3 has a higher avidity for some branched oligosaccharides as opposed to linear  
146 oligosaccharides[17]. However, directly visualising Gal3 lattices and clusters[47,49], either  
147 in solution or on the cell surface has been challenging given the diversity and complexity of  
148 Gal3 arrangements.

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153 **2. Gal3 influences the organization of the plasma membrane.**

154 The cell membrane contains many glycoproteins (e.g. CD44, CD45, and the T cell receptor  
155 (TCR)) and glycolipids (GSLs and GPI-anchored proteins) that possess carbohydrate ligands  
156 for Gal3. When Gal3 binds to its corresponding multivalent ligand on the cell surface and  
157 oligomerizes, it rearranges the spatial organization of these glycoproteins and glycolipids,  
158 which results in changes to the membrane organisation and endocytosis[49]. Consequently,  
159 this will alter cell migration and cell signalling[14–16,50,51]. Super-resolution microscopy  
160 and single molecule detection techniques have been utilised to visualise nanoscale  
161 organization of Gal3 lattices on the cell surface (by incubating cells with 4 µg/mL of  
162 fluorescently labelled Gal3 for 20 min), revealing forms of Gal3 clusters with a size of  
163 ~0.018 µm<sup>2</sup> and molecular density of ~200 molecules per µm<sup>2</sup>[49]. When cells were depleted  
164 of GSLs, the size of Gal3 clusters was only ~0.016 µm<sup>2</sup> and their molecular density increased  
165 to up to 260 molecules per µm<sup>2</sup>, suggesting GSLs make Gal3 clusters larger but less dense  
166 than Gal3-glycoprotein clusters which are formed when Gal3 binds to glycoproteins, like  
167 CD44[49]. Gal3-glycoprotein clusters are reportedly smaller than Gal3-GSL clusters. These  
168 clusters further decrease in size and in molecular density when the carbohydrate  
169 modifications of CD44 were removed, implying that they are no longer able to incorporate  
170 into the Gal3 lattices[49].

171 Another fluorescence technique used to study the Gal3 network is Förster resonance energy  
172 transfer (FRET)[40]. In this study, Gal3 was labelled with either AF488 or AF555 and the  
173 two conjugates were added to neutrophils. Gal3-AF88 and Gal3-AF55 both bound to the  
174 plasma membrane and formed a tight network close enough to produce a FRET signal (within  
175 the proximity of ~10 nm), suggesting oligomerization of the two sub-populations of Gal3.  
176 However, when the same experiment was done with CRD instead of Gal3, CRD appeared to  
177 be colocalized but were not close enough to produce a FRET signal, implying that CRD alone  
178 was unable to form oligomers. Hence, the authors concluded that NTD is required for Gal3  
179 oligomerization. Further, fluorescence recovery after photobleaching (FRAP) of the Gal3  
180 lattice showed that the Gal3 network is rigid and restricts lateral movement of membrane  
181 proteins[40].

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185 *2.1. Gal3 interaction with membrane proteins.*

186 Gal3 interacts with a wide-range of proteins implicating it in diverse cellular functions. It  
187 binds to these proteins in a lectin-dependent manner. Gal3 has a dissociation constant range  
188 of 0.8–5.6  $\mu\text{M}$  for N-linked glycans, similar to those found for the corresponding intact  
189 glycoproteins[52]. Proteomic analysis reveals that Gal3 interacts with transmembrane  
190 proteins, particularly adhesion proteins such as integrins, CD44 and N-cadherin[49,53,54].  
191 Blocking lectin activity or glycosylated sites of proteins impairs Gal3 binding to these  
192 proteins[49]. Gal3 can change the spatial organization of its ligand through self-  
193 oligomerization. This induces nanometre to micron-scaled clustering of glycosylated  
194 proteins[24,31,49]. However, due to the complexity in both Gal3 oligomerization and Gal3  
195 binding to glycoproteins and glycolipids, there is no generalised effect exerted by Gal3 on the  
196 spatial organization of the plasma membrane. For example, Gal3 induces clustering and  
197 endocytosis of  $\beta 1$  integrins and CD44 which then increases the lateral mobility of N-  
198 cadherin[49,51,53,54]. Mathew and Donaldson described a nuanced role of glycans for Gal3  
199 interactions and clathrin-independent endocytosis[55]. While excessive quantities of Gal3  
200 can erroneously alter cellular function, a baseline level of Gal3 is required for physiological  
201 processes.

202 *2.2. Gal3 interaction with membrane lipids.*

203 Although direct measurements of Gal3 binding to GSLs has not been done, glycans typical of  
204 GSLs have been analysed by methods such as inhibition assays[56], frontal affinity  
205 chromatography[17], fluorescence polarization combined with mutagenesis[39], and x-ray  
206 diffraction[17,38]. Some GSL-derived glycans bind Gal3 with  $K_d$  in the low  $\mu\text{M}$  range,  
207 similar to N-glycans; these include the lacto- and lactoneoseries. However, other GSL-  
208 derived glycans bind the Gal3 CRD poorly or not at all, including ganglioseries and  
209 globoseries[15]. N-glycans tend to have higher numbers of Gal3 binding motifs per  
210 oligosaccharide and the branched geometry may aid Gal3 oligomerization. Additionally, Gal3  
211 may also be able to directly cross the lipid bilayer without the use of lectin activity[57]. On a  
212 cell membrane, Gal3 colocalizes with GSL-rich regions and can affect the mobility of  
213 GSLs[24,49,53]. We previously reported that GSLs were not required for Gal3 binding to  
214 cells but needed for clustering, membrane bending and the formation of endocytic structures  
215 termed clathrin-independent carriers (CLICs)[49]. The mechanism to generate these  
216 endocytic pits has been termed the GlycoLipid-Lectin (GL-Lect) hypothesis[58]. Of note,



217 Gal3 drives CD44 endocytosis at concentrations as low as 0.3 nM, which is in the range of  
218 Gal3 concentrations found in human serum[15,49].

### 219 *2.3. Gal3 interactions with lipid rafts and lipid raft-associated proteins.*

220 As described above, Gal3 interacts with GSLs for the generation of narrow membrane  
221 curvature and the biogenesis of CLICs[49]. GSLs have molecular characteristics such as  
222 lateral hydrogen bonding capacity and long acyl chains that favour their lateral interactions  
223 for the formation of membrane domains, so-called lipid rafts[59]. Initially the lipid raft  
224 hypothesis proposed that lipids domains form spontaneously, and specific membrane proteins  
225 partition into lipid rafts[60]. Subsequent studies on the dynamic nature of biological  
226 membranes suggest that pure lipid-based rafts that grow to significant sizes under biological  
227 conditions are rather unlikely[61]. Current evidence suggests that raft assemblies are induced,  
228 for example by receptor-ligand interactions, thereby generating conditions under which the  
229 connectivity between raft constituents is enabled[59,62,63]. Oligomeric GSL-binding  
230 proteins such as the bacterial Shiga and cholera toxins, capsid proteins from polyoma and  
231 noroviruses, or Gal3 have molecular characteristics to induce the assembly of lipid rafts or  
232 raft-like domains. In the context of endocytic uptake, the induced lipid domains have a  
233 distinct function by creating an entry portal into cells[64].

234 At the plasma membrane, both exogenous and endogenous Gal3 is found in lipid rafts via  
235 binding to both glycoproteins and glycolipids[41–43,47–50]. Gal3 association with lipid rafts  
236 appears to be cholesterol dependent as shown by studies using methyl- $\beta$ -cyclodextrin  
237 (M $\beta$ CD) to deplete membranes of cholesterol[51,65,67]. Many of the older studies on raft  
238 association have been performed using detergent extraction, which is problematic (discussed  
239 in Ref.[59]). Based on this tool, it was concluded that some Gal3 binding partners such as  $\beta$ 1  
240 integrins are raft associated[43,44], and others such as p75 neurotrophin receptor (p75<sup>NTR</sup>)  
241 and lactase phlorizin hydrolase (LPH) are not[68]. It has also been argued that Gal3  
242 dynamically induces translocation of proteins such as CD45 into such detergent resistant  
243 membrane fractions, while excluding others like the T cell receptor (TCR) and co-receptor  
244 CD4[24]. More recent methodologies for raft studies will need to be used to confirm these  
245 earlier findings, and to determine the biological consequences of raft assembly and  
246 association.

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### 251 **3. Gal3 and the immunological synapse.**

252 Gal3 can alter cell signalling through modifying the spatial organization of the plasma  
253 membrane. This is particularly important in T cell activation[69–71]. Gal3 has been shown to  
254 suppress T cell activation in autoimmune diseases and in cancer by blocking proximal T cell  
255 signalling or inducing apoptosis[1–3,11,26,31–35]. Below, we will discuss some of the  
256 proposed mechanisms by which Gal3 alters plasma membrane organization to negatively  
257 regulate T cell activation.

#### 258 *3.1. T cell signalling at the immunological synapse.*

259 The activation of a T-cell is initiated by the specific binding of the T-cell antigen receptor  
260 (TCR) to a cognate peptide bound to the Major Histocompatibility Complex (pMHC) on the  
261 surface of an antigen-presenting cell (APC). This leads to the spatial reorganization of the  
262 TCR and many accessory receptors to form a specialized area of intimate contact between T  
263 cell and APC, the immunological synapse (Fig. 3), where signals are coordinated and  
264 integrated, to promote or inhibit further T cell activation. The formation of an immunological  
265 synapse results in polarised intracellular trafficking in the T cell[72].

266 The immunological synapse is a highly dynamic organisation, canonically resembling a  
267 bull's-eye structure that is separated into the central, peripheral, and distal concentric zones.  
268 These are collectively referred to as supramolecular activation cluster (SMAC)[73]. The  
269 central zone, cSMAC, contains the TCR-CD3 complex, co-stimulatory and co-inhibitory  
270 molecules, and co-receptors. The co-receptors, either CD4 or CD8, are glycoproteins that aid  
271 T cell activation through stabilizing the interactions of the TCR with pMHC molecules. The  
272 peripheral zone (pSMAC) enhances and further stabilises the physical interaction between T  
273 cell and APC through adhesion receptors such as LFA-1 (receptor for ICAM-1) and CD2  
274 (receptor for LFA-3)[74,75]. The distal zone (dSMAC) consists of CD45, which is a  
275 transmembrane tyrosine phosphatase, and F-actin. There is considerable turnover of TCR  
276 signalling proteins at the immunological synapse with ongoing endocytosis mainly from the  
277 cSMAC and exocytosis mainly to the pSMAC. The precise spatiotemporal arrangement of  
278 signalling receptors within the immunological synapse is vital for the activation and  
279 subsequent responses of T cells[76]. Upon TCR-pMHC ligation, TCRs rearrange themselves

280 into microclusters to initiate signalling. These microclusters have been proposed to originate  
281 at the dSMAC and are transported towards the cSMAC in an F-actin-dependent manner[77–  
282 82]. This process concludes with the internalisation of the TCR in the cSMAC that may also  
283 terminate TCR signals[82,83].

284 After TCR-pMHC ligation, a cascade of intracellular signalling events occurs involving many  
285 kinases and adaptor proteins. In the first step, Lck phosphorylates the TCR-CD3 complex on  
286 immunoreceptor tyrosine-based activation motifs (ITAMs). Phosphorylated ITAMS recruit  
287 and activate the tyrosine-kinase, ZAP-70 which then subsequently activates adapter proteins  
288 like linker for activation of T cells (LAT) and SH2 domain containing leukocyte protein of 76  
289 kDa (SLP-76). This then follows a series of downstream signalling events and results in  
290 upregulation of several genes which ultimately leads to T cell activation and clonal  
291 expansion. Overall, the formation of immunological synapse is crucial for sustained TCR  
292 signalling and T cell activation.

### 293 *3.2. Does Gal3 impact on T cell signalling via lipid rafts?*

294 The immunological synapse not only has a distinct spatial arrangement, it also has  
295 characteristic membrane and lipid properties. A number of studies have described the  
296 condensation of plasma membrane at the immunological synapse as well as an enrichment of  
297 raft lipids, cholesterol and sphingolipids[84–90]. Further, CD4+ and CD8+ T cells have  
298 different ganglioside compositions that are necessary for effective T cell signalling[91]. In  
299 resting T cells, TCR exists in nanoclusters that concatenate upon antigen engagement[92,93].  
300 A number of studies have shown that these TCR clusters are dependent on the membrane  
301 cholesterol levels, as the removal of cholesterol results in a disassembly of TCR clusters in  
302 both resting and activated T cells[94–97]. Moreover, an increase in plasma membrane  
303 cholesterol level was shown to further increase TCR clustering[98–100]. TCRs colocalizes  
304 with GM1[97,101,102] when labelled with cholera toxin B-subunit which most likely induces  
305 GM1 clustering[103–105]. How the lipid composition of T cells impacts on different  
306 inducers and raft assemblies is currently unknown.

307 It is likely that Gal3 induces lipid rafts through binding to glycosylated proteins and  
308 GSLs[58]. This will undoubtedly alter the membrane organisation of the many TCR  
309 signalling proteins associated with raft-like domains as well as the dynamic cycling within  
310 and to or from the immunological synapse. This may be best illustrated at the example of the  
311 co-receptors that reside in GM1-rich areas in T cells[24]. Co-receptors primarily enhance the

312 stability of TCR-pMHC complex. In the absence of co-receptors, the proximal signalling  
313 efficiency is reduced mainly due to the decreased level of Lck recruitment to phosphorylate  
314 ITAMs [32,106]. It has been shown that Gal3 reduces colocalization of TCR with either CD4  
315 or CD8 in T cells[28,32,107]. FRET studies were able to show that the presence of Gal3  
316 reduced co-receptor-TCR interaction and signalling efficiency in T cells while removal of  
317 membrane bound Gal3 restored the colocalization of TCR with co-receptors[28,32,107–109].

### 318 *3.3. Gal3 alters TCR signalling at the immunological synapses*

319 The first study that investigated the effect of Gal3 on the immunological synapse and  
320 subsequent suppression of T cell activation was done by Demetriou *et al* [31]. In this study,  
321 Gal3 was able to inhibit TCR recruitment to the immunological synapses by binding to the  $\alpha$ -  
322 subunit of the TCR (Fig. 3). Mice in which the N-acetylglucosaminyltransferase V (MGAT5)  
323 gene is knocked out and lack a third antenna on N-glycans[110] exhibited reduced cancer  
324 growth and automimmunity[31]. It was proposed that Gal3 was an important binding partner  
325 for the tri-antennary glycan, and that lack of Gal3 binding was explaining the observed  
326 phenotypes, which was not shown directly, however. Since those early experiments,  
327 MGAT5<sup>-/-</sup> cells have been frequently used in other studies [24,31,53,111,112] but the  
328 molecular details of Gal3 interactions with these cells are still unknown. The immune-  
329 suppressing effects of Gal3 seen with wild-type T cells such as the impaired T cell signalling  
330 activities were not observed with MGAT5<sup>-/-</sup> T cells[31], encouraging future studies to  
331 investigate how Gal3 regulates T cell activation. Given the frequent endocytosis and  
332 recycling of T cell signalling proteins[113], Gal3 may impact on retrograde trafficking and  
333 polarized secretion to the immunological synapse of T cell signalling proteins such as  
334 LAT[114].

335 Gal3 can bind to several different glycoproteins that play a role in T cell proximal signalling.  
336 For example, Gal3 binds to CD45, a heavily glycosylated phosphatase, with a dual role in T  
337 cell signalling (Fig. 3)[24,26]. CD45 acts as an inhibitor of T cell activation through  
338 dephosphorylation of CD3-ITAMs and Lck catalytic domain pY394, promoting Lck into a  
339 “closed” or inactive configuration. CD45 also promotes T cell activation through  
340 dephosphorylation of Lck-pY505, inducing its “open” or active configuration[106,115–117].  
341 In MGAT5<sup>+/+</sup> T cells, Gal3 erroneously retains CD45 at the immunological synapse where it  
342 dephosphorylates Lck-pY394 with no change in the Lck-pY505 phosphorylation state[24].  
343 The downstream effects of this was the loss of activation of pZAP70 and pLAT[24].

344 Downstream signalling was restored by either using MGAT5<sup>-/-</sup> cells or lactose treated  
345 MGAT5<sup>+/+</sup> T cells. Alternatively, Gal3 binding to CD45 may also suppress T cell activation  
346 through induction of apoptosis (Fig. 1)[26].

347 Alterations in the spatial distribution of TCR or phosphatases can clearly affect T cell  
348 signalling. However, less intuitively Gal3 can also induce changes in cytoskeletal and  
349 adhesion protein organization that affect T cell function[33]. For example, in CD8<sup>+</sup> cytotoxic  
350 T cells (CTLs), Gal3 creates two physical barriers that prevent CTLs from delivering  
351 cytotoxic load to target cells[33]. First, Gal3 inhibits the docking of the microtubule-  
352 organizing centre (MTOC) at the immunological synapse, which prevents the delivery of  
353 lytic granules to target cells. Second, Gal3 destabilises the formation of the synapse by  
354 preventing the recruitment of adhesion protein LFA-1, because incorporation into the Gal3  
355 lattice reduces the lateral mobility of LFA-1[33]. The inability to recruit LFA-1 to the  
356 immunological synapse also prevents actin clearing, a necessary process to provide physical  
357 space to release lytic granules from the T cell to the target cell[33].

358

#### 359 **4. Gal3 influence on co-inhibitory receptors in T cells.**

##### 360 *4.1. Biological function of co-inhibitory receptors.*

361 T cell mediated immune responses are tightly regulated to maintain tolerance for self-  
362 antigens and prevent autoimmunity. The balance between co-stimulatory and co-inhibitory  
363 receptors primarily sets this immunological tolerance at the molecular level[118]. Their  
364 expression levels and function can dictate cell proliferation to cytokine expression capacity in  
365 T cells[118–120]. Here, our focus will be on co-inhibitory receptors and their function in T  
366 cell suppression.

367 Most co-inhibitory receptors are transmembrane glycoproteins which belong to the  
368 immunoglobulin superfamily and have elevated expression levels in both effector and  
369 memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells[121]. Co-inhibitory receptor-based suppression of TCR  
370 signalling can occur through the extrinsic APC ligand binding and intrinsic TCR signalling  
371 inhibition mechanisms[119,121]. In the intrinsic mechanisms of T cell suppression, co-  
372 inhibitory receptors can outcompete co-stimulatory receptors in binding to common ligands  
373 by displaying a higher affinity, resulting in TCR suppression. Cytotoxic T-lymphocyte-  
374 associated protein 4 (CTLA-4) is a classic example for this mechanism. Here, CTLA-4

375 competes with CD28 co-stimulatory receptor to bind to their common ligands CD80 and  
376 CD86 expressed on APCs[122–124]. CTLA-4 also employs an extrinsic mechanism of T cell  
377 suppression known as trans-endocytosis, a process which captures and removes common  
378 ligands for both co-inhibitory and co-stimulatory receptors from the surface of the antigen-  
379 presenting or cancer cells and make them unavailable for binding[125]. Other co-inhibitory  
380 receptors like TIGIT can inhibit dimerization of co-stimulatory receptor CD226 and  
381 subsequently impairs its function[126]. Further, it can downregulate TCR components at  
382 transcriptional level[127].

383 In general, most co-inhibitory receptors possess immunoreceptor tyrosine-based inhibitory  
384 motifs (ITIM) or immunoreceptor tyrosine-based switch motifs (ITSM) which aid the  
385 negative regulation of TCR signalling, intrinsically[119,121]. Concurrently, the molecular  
386 mechanism behind suppression of TCR and downstream signalling follows the  
387 phosphorylation of ITIM domains, via the recruitment of Src homology region 2 domain-  
388 containing phosphatases (SHP-1 and SHP-2) to ITSM regions. In some cases, TCR signalling  
389 inhibition can occur in an ITIM-independent manner. LAIR-1, for example can utilise C-  
390 terminal Src kinase (Csk) to inhibit TCR signalling[128]. Inhibitory receptor CTLA-4 is  
391 constitutively expressed by regulatory T cells (Treg) upon activation [129,130]. In mouse  
392 models it has been shown that the absence or blockade of CTLA-4 can cause spontaneous  
393 autoimmunity. The likelihood of this spontaneous autoimmunity progressing into a systemic  
394 disease has been prevented by other compensatory mechanisms within T cells[131,132]. On  
395 the contrary, during chronic infections and cancer, inhibitory receptor expression levels are  
396 being elevated in T cells as an immune evasion mechanism used by the pathogen or  
397 malignant cells to spread throughout the host environment[133,134]. Overexpression of  
398 inhibitory receptors can induce CD8+ T cells to become non-responsive, exhausted T cells  
399 and can promote expansion of Treg cells[135–138]. Hence, these inhibitory receptors have  
400 become attractive drug targets for a number of tumour and cancer immunotherapy treatments,  
401 collectively known as checkpoint blockade which can effectively restore some effector  
402 functions of CD8+ T cells[134,139].

#### 403 *4.2. Gal3 and T cell co-inhibitory receptors.*

404 The influence of Gal3 on co-inhibitory signalling has not been fully explored. However,  
405 recent work suggests that Gal3 could inhibit T cell activation through binding to lymphocyte-  
406 activation gene 3 (LAG-3). In pancreatic ductal adenocarcinoma, Gal3 was shown to bind to

407 CD8+ T cells in the tumour microenvironment that express programmed cell death protein 1  
408 (PD-1) and LAG-3[2]. Co-immunoprecipitation showed binding of Gal3 to LAG-3, but not to  
409 PD-1, even though both LAG-3 and PD-1 can contain Gal3 ligands[32,140]. Depletion of  
410 Gal3 recovered the ability of T cells to control the tumour burden[2]. However, this  
411 mechanism of Gal3-mediated T cell suppression may be disease specific. In a murine viral  
412 chronic infection, the mechanism of Gal3-induced T cell suppression did not involve  
413 inhibitory receptors PD-1 and LAG-3[32]. A recent study has shown that utilization of  
414 immune check-point blockades also reduces the effect of Gal3 on T cell suppression[9]. In  
415 melanoma, the blockade treatment for anti-CTLA-4 and anti-Vascular endothelial growth  
416 factor A (VEGF-A) elicited a humoral response to Gal3[9]. In addition, antibodies produced  
417 against Gal3 correlated to an improved patient outcome[9]. Overall, Gal3 plays a pleiotropic  
418 function in T cell suppression by influencing inhibitory receptors which negatively regulate T  
419 cell activation and responses[141].

420

## 421 **5. Conclusion.**

422 Understanding the mechanisms how tumours create immunosuppressive environments has  
423 become imperative in developing novel strategies to combat cancer. Certain proteins  
424 expressed on cancer cells that can potentially disrupt T cell activation and promote immune  
425 evasion have become attractive targets for immunotherapy-based treatments. Gal3 has been  
426 shown to be one such target with great potential. Primarily, Gal3 suppresses T cell activation  
427 by altering the spatial organization of the immune synapses. At physiological levels, this  
428 suppression is necessary to prevent autoreactivity of T cells. However, in a disease state, such  
429 as cancer or chronic infection, Gal3-mediated T cell suppression restricts the ability of the  
430 immune system to adequately respond and mitigate the disease state. Recent studies have  
431 proposed several mechanisms for this suppression, some of which are conflicting. The  
432 diversity of these mechanisms can be due to the number of variables that influence Gal3  
433 binding, oligomerization, lattice formation, endocytosis, and disease specific physiology.  
434 Moreover, a study of Gal3 in isolation may be misleading, as other members of the galectin  
435 family may have different, but overlapping carbohydrate-binding specificities and  
436 affinities[15,17]. Additionally, it is also important to consider the multifaceted nature of Gal3  
437 which has been implicated in a number of cellular and biological functions[142].  
438 Nevertheless, our current understanding of how Gal3 alter T cell signalling and functions will

439 be useful in developing novel immunotherapeutic strategies in combating cancer and chronic  
440 infections in the future.

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#### 450 **Author contributions**

451 R.C.G. and S.D.G. contributed equally in reviewing the relevant literature and writing the  
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#### 453 **Competing interests**

454 The authors declare no competing or financial interests.

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914 **Figure 1. Gal3 influence and regulate T cell responses.** Galectin 3 is known to regulate and  
915 influence a range of biological processes in T cells, including signalling, activation,  
916 proliferation, apoptosis and cytokine secretion.

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919 **Figure 2. Gal3 structure and variables that influence oligomerization and cluster**  
920 **formation. A.** The Gal-3 monomer consists of a CRD and an unstructured NTD. The  
921 structure of gal3's three different oligomeric configurations. **B.** Variables that influence gal3  
922 binding to carbohydrates and subsequent cluster formation. Gal3 binding is higher when there  
923 are: more ligands present, higher glycoprotein density, higher lipid density and when  
924 glycosylated proteins are contained within lipid rafts.

925

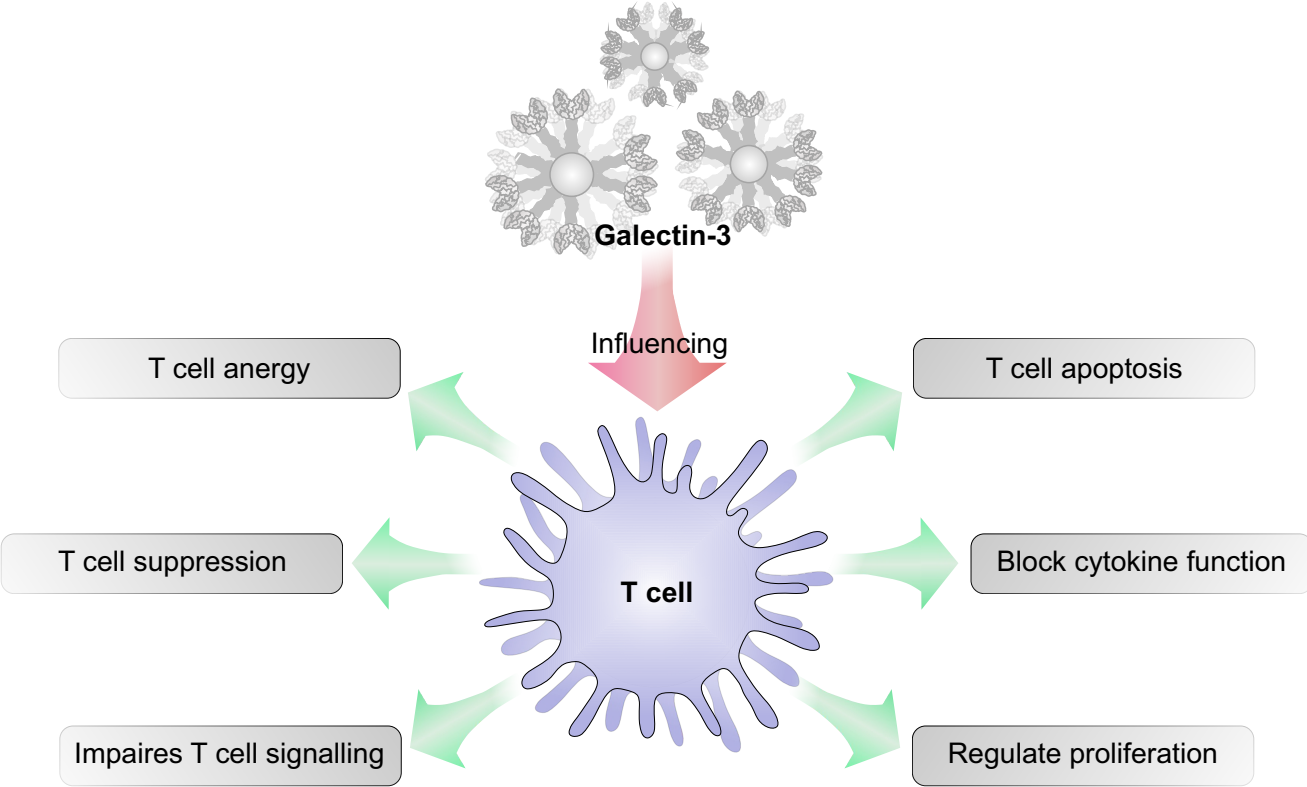
926

927 **Figure 3. Gal3 influence in the formation of immunological synapses.** The immunological  
928 synapse is a highly dynamic organisation which resembles a bull's-eye structure that is  
929 separated into the central (cSMAC), peripheral (pSMAC), and distal concentric (dSMAC)  
930 zones. Each zone contains different signalling proteins which helps to propagate T cell  
931 signalling and in turn promote or inhibit T cell activation. Gal3 is shown to interact with  
932 several key receptors involved in T cell signalling and restricts their recruitment during the  
933 formation of immunological synapses. These signalling proteins include TCR ( $\alpha$ -subunit),  
934 phosphatase CD45 and co-inhibitory receptors like LAG-3 and CTLA-4.

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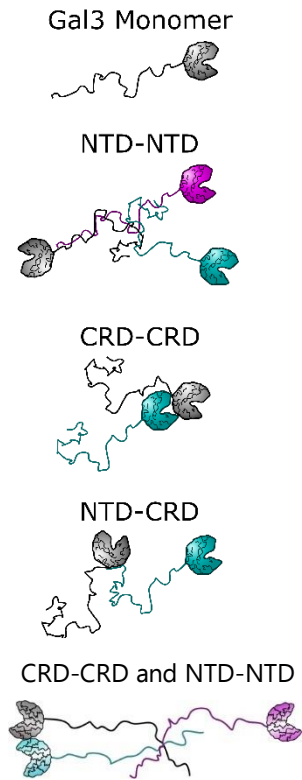


**Figure 1**



**Figure 2**

**A.**



**B.**

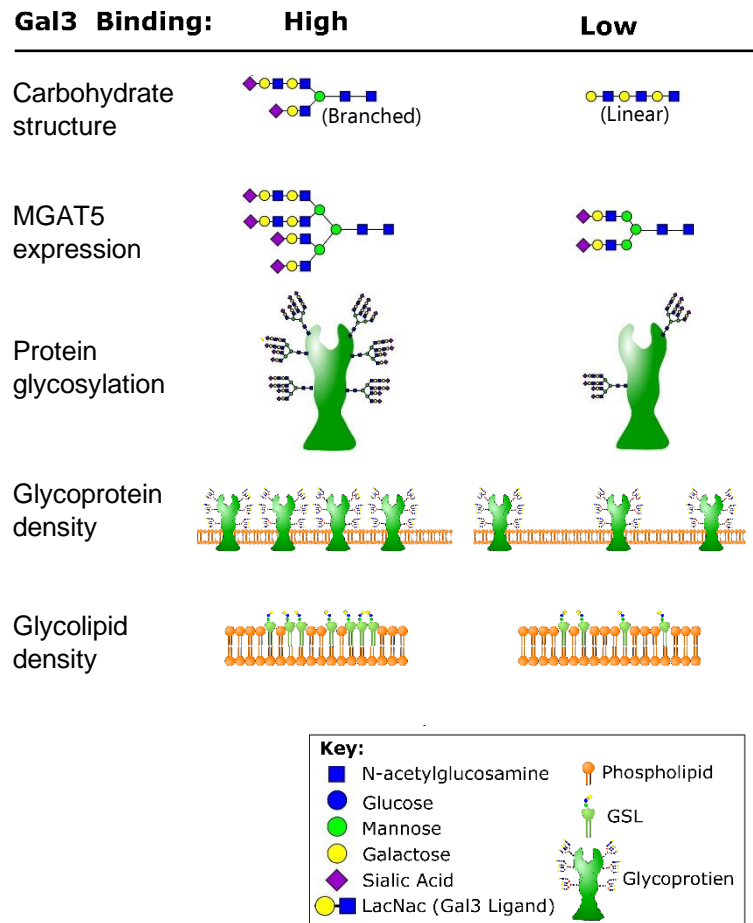


Figure 3

