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## 1 **$\alpha$ -Gal-based vaccines: Advances, opportunities, and perspectives**

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14

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### 16 **ABSTRACT**

17 Humans and crown catarrhines evolved with the inability to synthesize the oligosaccharide  
18 galactose- $\alpha$ -1,3-galactose ( $\alpha$ -Gal). In turn, they naturally produce high quantities of the  
19 glycan-specific antibodies, which can be protective against infectious agents exhibiting the  
20 same carbohydrate modification on their surface coat. The protective immunity induced by  $\alpha$ -  
21 Gal is ensured through an antibody-mediated adaptive and cell-mediated innate immune  
22 response. Therefore, the  $\alpha$ -Gal antigen represents an attractive and feasible target for  
23 developing glycan-based vaccines against multiple diseases. In this review article, we provide  
24 an insight into our current understanding of the mechanisms involved in the protective  
25 immunity to  $\alpha$ -Gal and discuss the possibilities and challenges in developing a single-antigen  
26 pan-vaccine for prevention and control of parasitic diseases of medical and veterinary  
27 concern.

## 28 **The rationale for developing an $\alpha$ -Gal-based vaccine against parasites**

29 Carbohydrates are abundantly expressed on the surface of nearly all cells in both prokaryotic  
30 and eukaryotic organisms, where they exist in the form of complex glycans, polysaccharides,  
31 and oligosaccharides linked to proteins and lipids [1,2]. Indeed, many pathogens causing  
32 diseases in humans and animals contain unique carbohydrate structures on their surface coat  
33 that serve as receptors by which the pathogenic organisms attach to and invade host cells [3-  
34 11]. The pathogen carbohydrate molecules are often structurally different from those  
35 displayed on mammalian host cells and are thereby recognized by the host immune system  
36 [2]. Natural infections and exposure to the carbohydrate antigens on infectious agents can  
37 elicit an innate immune response and activate B cells to produce protective glycan-binding  
38 antibodies (Abs) in the host. The potential of the surface **glycotopes** (see **Glossary**) to induce  
39 such a potent immune response makes carbohydrates attractive and feasible targets for  
40 vaccine development [1,2,12]. Accordingly, the scientific interest in exploiting the  
41 immunogenic glycan antigens as vaccine constituents has considerably increased in the last  
42 years and it resulted in the development of efficient glycoconjugate vaccines targeting  
43 encapsulated bacteria strains such as *Haemophilus influenzae* type B, *Streptococcus*  
44 *pneumoniae*, and *Neisseria meningitidis* [1]. Significant progress has also been made with  
45 conjugate vaccine formulations for control of other viral, parasitic, and fungal diseases  
46 [2,13,14].

47

48 The oligosaccharide galactose- $\alpha$ -1,3-galactose ( $\alpha$ -Gal) is currently one of the most interesting  
49 carbohydrates that attracts growing attention from the scientific community after it was  
50 recognized as a major allergen responsible for the production of specific immunoglobulin  
51 (Ig)E that mediates delayed and severe anaphylactic reaction to mammalian meat  
52 consumption in humans previously exposed to tick bites [15-20]. This novel type of tick-

53 induced food allergy is better known as red meat allergy or the  $\alpha$ -Gal syndrome [21-23]. The  
54 terminal  $\alpha$ -Gal residues have recently been found in the saliva of several tick species, but the  
55 mechanism by which ticks promote an anti- $\alpha$ -Gal response and high-level sensitization in  
56 humans is not known [24-28]. It has been, however, hypothesized that tick salivary  
57 prostaglandin E2 (PGE2) may induce class switch recombination on B cells leading to IgE  
58 production [29]. Another possible explanation is that  $\alpha$ -Gal from tick salivary  
59 glycoconjugates is presented to **antigen-presenting cells (APCs)** and B lymphocytes in the  
60 context of T helper (Th) 2 cell-mediated immunity, which leads to the differentiation of  $\alpha$ -  
61 Gal-specific B cells into IgE secreting plasma cells [29]. The way how  $\alpha$ -Gal is captured,  
62 processed and presented to CD4+ T cells seems to determine whether the antigen is  
63 recognized by the host immune system as harmless, like possibly in case of helminths and  
64 fungi, or hazardous [9,23]. By contrast to the detrimental effect of the IgE immune responses  
65 to  $\alpha$ -Gal, the production of IgG and IgM isotypes induced by gut microbiota seems to be  
66 beneficial as these Abs can be protective against different pathogens exhibiting carbohydrates  
67 with the similar  $\alpha$ -Gal modification on their surface [4-6,8,10,11,30]. In this review, we  
68 provide a summary of the recent advances in our understanding of the mechanisms involved  
69 in the protective immunity to  $\alpha$ -Gal and discuss the possibilities in developing an  $\alpha$ -Gal-  
70 based vaccine for prevention and control of multiple parasitic diseases of medical and  
71 veterinary importance.

72

### 73 **The $\alpha$ -Gal epitope and anti- $\alpha$ -Gal antibodies**

74 The  $\alpha$ -Gal epitope is a unique oligosaccharide naturally produced on glycoproteins and  
75 glycolipids of non-primate mammals, prosimians, and New World monkeys [31]. The  
76 glycosylation enzyme  $\alpha$ -1,3-galactosyltransferase ( $\alpha$ 1,3GT), encoded by the *ggtal* gene in  
77 mammals, catalyzes the synthesis of the epitope by transferring galactose molecule from

78 uridine diphosphate (UDP)-Gal to N-acetyllactosaminide [32]. Conversely, humans and Old  
79 World primates lack the synthetic machinery to produce the  $\alpha$ -Gal due to the functional  
80 inactivation of the *ggtal* gene, caused by several deletions in the DNA sequence that encodes  
81 premature stop codons [33-35]. Non-mammalian vertebrates (e.g. birds, fish) do not have the  
82 *ggtal* gene and also lack the  $\alpha$ -Gal [10,11,36]. In humans, the  $\alpha$ -Gal epitope is expressed  
83 only in the blood group B glycoconjugates (Gal $\alpha$ 1-3[Fuc $\alpha$ 1-2]Gal $\beta$ 1-4Glc-ceramide) [37].  
84 Therefore, humans and other vertebrates with this *ggtal* gene modification lost immune  
85 tolerance to non-cryptic  $\alpha$ -Gal moieties and are consequently able to produce large quantities  
86 of Abs that specifically bind to the non-self-antigen [38,39]. However, due to the structural  
87 similarity between antigen B and  $\alpha$ -Gal, individuals with blood groups AB and B produce  
88 lower Ab levels against the related antigens  $\alpha$ -Gal (Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc) and gal2 (Gal $\alpha$ -  
89 1,3-Gal) [40]. The disruption of the *ggtal* gene in our primate ancestors, estimated to have  
90 occurred almost 28 million years ago, was likely exerted by a strong selective pressure of an  
91 infectious agent and the subsequent acquisition of immune-resistance to pathogens expressing  
92  $\alpha$ -Gal [31,39]. This evolutionary scenario can be further supported by the following facts: (i)  
93 enveloped viruses express  $\alpha$ -Gal when propagated in cells with active *ggtal* gene; (ii)  
94 helminths, protozoa, bacteria, and fungi also express carbohydrates with terminal  $\alpha$ -Gal  
95 moieties; and (iii) anti- $\alpha$ -Gal Abs can neutralize pathogenic organisms exhibiting  $\alpha$ -Gal  
96 epitopes [37].

97

98 Anti- $\alpha$ -Gal Abs, in particular IgG, IgM, and IgA isotypes are naturally generated in healthy  
99 human individuals as an immunological response to continuous antigenic stimulation by  
100 Gram-negative bacteria of gut flora, which express very diverse terminal and non-reducing  $\alpha$ -  
101 Gal-linked glycans, predominately in Gal $\alpha$ 1,2-, Gal $\alpha$ 1,4-, and Gal $\alpha$ 1,6-R forms [41,42]. A  
102 high proportion of individuals (>70%) in healthy populations exhibit  $\alpha$ -1,3-

103 galactosyltransferase sequences in the bacteria of their gut microbiome [43]. Notably,  $\alpha$ -1,3-  
104 galactosyltransferase genes in bacteria and mammals were not evolutionarily related.  
105 However, other immune-mediated mechanisms may also be activated in response to  $\alpha$ -Gal  
106 [44]. For instance, high levels of anti- $\alpha$ -Gal Abs have also been reported in human patients  
107 infected with *Trypanosoma* spp. (Kinetoplastida), *Leishmania* spp. (Kinetoplastida), and  
108 *Plasmodium* spp. (Apicomplexa), indicating the polyreactive nature of the Abs [4,45-48].  
109 However, pathogen-specific anti- $\alpha$ -Gal seems to have different specificities than Abs  
110 produced towards the  $\alpha$ -Gal from enterobacteria [3-5,45,49]. Nevertheless, IgG2 and IgM  
111 Abs induced by  $\alpha$ -Gal expressed on pig cells are a major immunological barrier preventing  
112 the transplantation of pig organs into humans [50].

113

#### 114 **Mechanisms of the protective $\alpha$ -Gal immunity against parasites**

115 In general, the  $\alpha$ -Gal epitope has significant clinical potential in the possibility of developing  
116 an  $\alpha$ -Gal glycovaccine or other interventions such as pro- and postbiotics to induce a  
117 protective  $\alpha$ -Gal immune response against multiple diseases (**Figure 1, Key Figure**)  
118 [4,5,8,10,11,13,14,30]. The immunity induced by  $\alpha$ -Gal influences pathogen infection and its  
119 multiplication by various mechanisms including B cell maturation, antibody-mediated  
120 **opsonization** of  $\alpha$ -Gal-containing pathogens, activation of the **complement system**, Fc-  
121 receptor (FcR)-mediated phagocytosis, activation of macrophage response, antibody-  
122 mediated interference with the  $\alpha$ -Gal antagonistic effect to promote **toll-like receptor 2**  
123 **(TLR2)/nuclear factor kappa-light-chain-enhancer (NF- $\kappa$ B)**-mediated immune response  
124 and upregulation of proinflammatory **cytokines (Table 1)**.

125

126 *Plasmodium* spp.

127 Several epidemiological studies uncovered a strong correlation between  $\alpha$ -Gal-specific IgM  
128 Abs and protection from *Plasmodium* infections in humans [47,48,51,52,53], but the first  
129 experimentally proved evidence for the protective role of  $\alpha$ -Gal-induced immunity was  
130 provided by Yilmaz et al. [4]. In their seminal study, C57BL/6 *ggtal*-knockout (*ggtal*-KO)  
131 mice, which like humans do not express  $\alpha$ -Gal on their cells, were used as an animal model to  
132 assess the protective effect of anti- $\alpha$ -Gal Abs against *Plasmodium berghei* and *Plasmodium*  
133 *yoelii* infections. *Plasmodium* parasites express the  $\alpha$ -Gal carbohydrate possibly bound to  
134 glycosylphosphatidylinositol (GPI)-anchored surface proteins [4,54]. The results of the  
135 experimental study showed that gut colonization by *Escherichia coli* O86:B7 that expresses  
136 high levels of  $\alpha$ -Gal inhibits the parasite transmission by *Anopheles* mosquitoes. In particular,  
137 anti- $\alpha$ -Gal IgM Abs produced in response to *E. coli* O86:B7 blocked infection with  
138 *Plasmodium* spp. in 60% of the mice, but this was not the case when the mice were or were  
139 not exposed to *E. coli* K12, a serotype which does not produce  $\alpha$ -Gal. Immunization of  
140 *ggtal*-KO mice with rabbit red blood cell membranes (rRBCM) or synthetic  $\alpha$ -Gal linked to  
141 bovine serum albumin ( $\alpha$ -Gal-BSA) elicited the production of serum IgM and IgG (IgG1,  
142 IgG2b, and IgG3 subclasses) Abs, but the levels of circulating IgA and IgE Abs were not  
143 detectable. A significant increase in anti- $\alpha$ -Gal Ab production was also observed when mice  
144 were immunized with rRBCM supplemented with TLR9 agonist adjuvant and this was  
145 associated with an 88% reduction in the relative risk of *Plasmodium* infection compared to  
146 61% risk reduction without adjuvant in combination. Further experiments revealed that the  
147 protective effect of  $\alpha$ -Gal immunization is mediated via B cell-dependent mechanisms, as  
148 well as that both IgM and IgG Ab classes confer protection against malaria transmission,  
149 thereby providing **sterile immunity**. The cytotoxic effect of Abs produced towards  $\alpha$ -Gal  
150 appears to be mediated by the classical complement activation. Furthermore, the protective  
151 activity of anti- $\alpha$ -Gal Abs was not observed when *Plasmodium* sporozoites were introduced

152 intravenously, suggesting that the protection induced by  $\alpha$ -Gal immunization is only exerted  
153 in the dermis, where the pathogen is inoculated by mosquitoes. Once the parasite reaches the  
154 blood, the protective effect of the  $\alpha$ -Gal immunity is no longer productive, and the  
155 parasitaemia, disease severity, as well as mortality rate were similar among infected *ggtal*-  
156 KO mice regardless of the source of the  $\alpha$ -Gal residues, i.e. oral administration of *E. coli*  
157 O86:B7 or  $\alpha$ -Gal immunization. Based on these findings, it has been proposed that  $\alpha$ -Gal-  
158 induced immunity protects against *Plasmodium* transmission, but not against the blood stages  
159 of this parasite. These results suggest that anti- $\alpha$ -Gal immunity can influence malaria  
160 incidence, but not disease severity or protection once the disease is established. In agreement  
161 with the hypothesis, individuals from Mali and Senegal exposed to mosquito bites were not  
162 infected by *Plasmodium falciparum* when having high anti- $\alpha$ -Gal Ab levels [4,48]. In  
163 addition, the frequency of blood type B in African countries was positively correlated with  
164 the incidence of malaria, possibly due to weak anti- $\alpha$ -Gal immunity in populations with a  
165 high frequency of blood type B [48]. By contrast, a negative correlation was observed  
166 between the frequency of blood type A and the incidence of malaria [48]. A 4-year  
167 prospective cohort study in childhood malaria in Mali showed that children having blood  
168 types B and AB had higher incidence rate (blood type B: 1.63 and blood type AB: 1.65)  
169 compared to those children with blood types A and O (blood type A: 1.57 and blood type O:  
170 1.45). This supports the role of blood type B in reducing anti- $\alpha$ -Gal Abs, which in turn  
171 increases the incidence of malaria [55].

172

### 173 ***Leishmania spp.***

174 *Leishmania spp.* is another group of important and widespread protozoan parasites containing  
175 terminal  $\alpha$ -Gal epitopes and thus they represent potential targets for vaccine development  
176 against human visceral and cutaneous **leishmaniasis** [5,6,46]. Variable levels of the  $\alpha$ -Gal



177 moieties have been so far observed in *Leishmania major*, *Leishmania infantum*, and  
178 *Leishmania amazonensis* [5,6,56]. *Leishmania major*, and perhaps other *Leishmania* species,  
179 synthesize Type-II glycoinositolphospholipids (GIPL)-2 and GIPL-3, which are capped with  
180 terminal, non-reducing and highly immunogenic  $\alpha$ -galactopyranosyl residues with different  
181 structural configurations [5,57]. These glycolipids are expressed in abundance in the  
182 amastigote stages, residing in macrophages of mammalian hosts [57]. In a recent study, three  
183 different neoglycoproteins (NGPs) containing synthetic  $\alpha$ -Gal in different configurations,  
184 namely Gal $\alpha$ (1,6)Gal $\beta$ -BSA (NGP5B), Gal $\alpha$ (1,4)Gal $\beta$ -BSA (NGP12B), and Gal $\alpha$ (1,3)Gal $\alpha$ -  
185 BSA (NGP17B), were evaluated as potential prophylactic vaccine candidates against *L.*  
186 *major* [5]. The *ggtal*-KO mice immunized with the three NGPs tested produced high levels  
187 of specific anti- $\alpha$ -Gal IgG Abs. However, only NGP5B administered alone or in combination  
188 with CpG adjuvant displayed a significant complement-independent lytic activity against  
189 infective metacyclic *L. major* promastigotes. Incubation of NGP5B and NGP5B + CpG  
190 immunized mice sera with *L. major* promastigotes caused lysis of 44% and 60%  
191 promastigotes, respectively. In contrast to *Plasmodium* parasites [4], the lytic effect was  
192 documented only when the complement was heat inactivated. Furthermore, mice immunized  
193 with NGP5B had significantly reduced parasite load and the size of footpad lesions by 96%  
194 compared to control groups. Similarly, a significant reduction in *L. infantum* and *L.*  
195 *amazonensis* parasite load in liver and spleen along with increased levels of anti- $\alpha$ -Gal IgG  
196 were observed in another study in which *ggtal*-KO mice were immunized with Q $\beta$  virus-like  
197 particle bearing  $\alpha$ -Gal trisaccharide [6]. In NGP5B immunized mice, a strong protective Th1  
198 cellular immune response with increased levels of proinflammatory cytokines such as  
199 interleukin (IL)-12p40, IL-2, and interferon (IFN)- $\gamma$  were recorded. NGP5B and NGP5B +  
200 CpG in mice induced a robust CD4<sup>+</sup> and a CD8<sup>+</sup> T cell response, which is essential for the  
201 protection against *L. major*. Taken together, immunization with this NGP5B alone or with

202 CpG in combination induce partial, but significant protective  $\alpha$ -Gal immunity against the *L.*  
203 *major* infection in mice.

204

### 205 *Trypanosoma cruzi*

206 *Trypanosoma cruzi* has a complex carbohydrate-rich surface coat that contains GPI-anchored  
207 mucins (tGPI-mucins) with the linear and immunodominant  $\alpha$ -Gal glycotope and several as  
208 yet uncharacterized branched terminal  $\alpha$ -Gal *O*-glycans [3]. Previous studies showed that  
209 patients with acute and chronic **Chagas disease** produce high levels of anti- $\alpha$ -Gal Abs that  
210 specifically recognize the tGPI-mucins on trypomastigotes and are considerably different in  
211 terms of specificity and biological activity than natural anti- $\alpha$ -Gal Abs produced in response  
212 to gut microbiota [3,45,49]. The latter also exhibits substantially lower lytic activity against  
213 *Trypanosoma* trypomastigotes [3,45]. Therefore, Portillo et al. [8] recently investigated the  
214 efficacy of the *T. cruzi* immunodominant  $\alpha$ -Gal epitope as a prophylactic glycovaccine  
215 candidate in the acute model of Chagas disease. They immunized *ggtal1*-KO mice by  
216 intraperitoneal injection of synthetic  $\alpha$ -Gal linked to human serum albumin (Gal $\alpha$ 3LN-HSA  
217 or Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc-HSA), which is almost structurally identical to that found on  
218 tGPI-mucins. This comprehensive study clearly demonstrated that mice immunized with  
219 Gal $\alpha$ 3LN-HSA alone or in combination with liposomal-monophosphoryl lipid A (LMPLA)  
220 adjuvant had significantly reduced parasite load (91.7-99.9%) in all tissues tested, reduced  
221 cardiac inflammation, myocyte necrosis, and T cell infiltration in comparison to control  
222 groups. The trypanolytic effect of anti- $\alpha$ -Gal Abs occurred in a complement-independent  
223 manner, similar to that in *Leishmania* parasites [5]. These Abs specifically bind to the  
224 parasites and destabilize the plasma membrane leading to the surface coat disruption,  
225 agglutination, and death. The authors further demonstrated that purified murine IgG Abs to  
226 Gal $\alpha$ 3LN-HSA effectively block the host cell infection and intracellular proliferation of the

227 parasite. Immunization with Gal $\alpha$ 3LN-HSA + LMLP increased the production of IgG1 and  
228 IgG2b subclasses and significantly increased levels of serum cytokines, chemokines, and  
229 growth factors, in particular IL-2, IL-4, IL-9, IL-15, C-C chemokine motif ligand 3 (CCL3),  
230 and vascular endothelial growth factor (VEGF). Furthermore, a significant increase of  
231 antigen-induced CD4<sup>+</sup> and CD8<sup>+</sup> T cells was observed along with a considerable expansion  
232 of memory CD4<sup>+</sup>CD44<sup>+</sup> T cells, but not memory CD8<sup>+</sup>CD44<sup>+</sup> T cells, which are considered  
233 to be critical for protective and long-lasting  $\alpha$ -Gal immunity against *T. cruzi* infection. Based  
234 on the overall results, the authors propose that the production of protective anti- $\alpha$ -Gal Abs to  
235 Gal $\alpha$ 3LN-HSA is mediated through a T cell-dependent B cell memory mechanism.

236

## 237 **Challenges in developing an $\alpha$ -Gal-based vaccine**

### 238 *Carrier protein*

239 The evolutionary adaptation in crown catarrhines and their ability to induce a protective  $\alpha$ -  
240 Gal immune response against infection and multiplication of pathogens suggests the  
241 possibility of developing  $\alpha$ -Gal-based interventions for control of multiple diseases affecting  
242 humans, birds, and fish [4-6,8,10,11,13,14,30]. The prospective vaccine antigen should be  
243 capable to induce antigen-specific plasma cells secreting protective Abs and development of  
244 memory T and B cells in order to provide efficient and long-lasting protection [2,4,5,8].  
245 Nevertheless, the target carbohydrate antigen must not induce autoimmune- or allergy-related  
246 anti- $\alpha$ -Gal IgE Abs following immunization [2,4,5,8]. In contrast to protein antigens,  
247 carbohydrates are classified as thymus-independent antigens with poor immunogenic  
248 reactivity [58]. Due to the lack of T cell activation, B cells predominantly produce IgM and  
249 IgG Abs of low affinity and the immune response induced by carbohydrates is often short-  
250 lived [59-62]. Therefore, covalent conjugation of carbohydrate antigens to carrier protein has  
251 been proposed to overcome the immunoreactive issue [2]. Synthetic  $\alpha$ -Gal-containing NGPs

252 coupled to BSA or HSA have been often used for classical immunization by injection with  
253 great success in mice [4,5,8,11,30]. It is important, however, to note that the protein linker  
254 can sometimes induce the production of anti-linker Abs and influence the immune response  
255 against desired glycan antigen [5]. To minimize the cross-reactions and to achieve a strong  
256 and more specific immune response towards specific glycotope, smaller and more flexible  
257 protein linkers should be used for glycan conjugation [63,64]. It has also been shown that  
258 pre-existing natural anti- $\alpha$ -Gal Abs can enhance the immunogenicity of antigens exhibiting  $\alpha$ -  
259 gal epitopes by increasing a T cell-dependent immune response following immunization [65-  
260 68]. In this sense, combining the  $\alpha$ -Gal carbohydrates with other protein vaccine candidates  
261 could improve the immunogenicity of the  $\alpha$ -Gal vaccine formulations [4,69].

262

### 263 ***Structure of the $\alpha$ -Gal epitopes and immunization route***

264 The structural configuration of the epitope and the immunization route are other important  
265 considerations for the successful  $\alpha$ -Gal vaccine development. Immunization with linear  
266 Gal $\alpha$ 3LN (Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc trisaccharide), which is structurally nearly identical to  
267 native *T. cruzi* surface glycotope, provided the full protection against lethal *T. cruzi* challenge  
268 in *ggtal1*-KO mice [3,8]. The naturally occurring human anti- $\alpha$ -Gal Abs induced by gut  
269 microbiota have a considerably weaker binding activity to the linear Gal $\alpha$ 3LN on tGPI-  
270 mucins of *T. cruzi* and lower trypanolytic effect on metacyclic trypomastigotes than parasite-  
271 specific anti- $\alpha$ -Gal Abs [3,45,49]. Indeed, the immunodominant Gal $\alpha$ 3LN glycan on tGPI-  
272 mucins is a major target for the protective anti- $\alpha$ -Gal Abs and it has not been yet reported in  
273 any enterobacteria [41], suggesting that gut enrichment with  $\alpha$ -Gal-containing bacteria may  
274 not be as efficient as immunization with synthetic NGPs for *T. cruzi* infection [8]. On the  
275 other hand, the experimental study by Mateos-Hernández et al. [10] showed that oral  
276 administration of *E. coli* O86:B7 protects turkeys (*Meleagris gallopavo*) from developing

277 respiratory clinical aspergillosis, while subcutaneous immunization with the synthetic Gal $\alpha$ 1-  
278 3Gal-BSA failed to protect against an infectious challenge with *A. fumigatus*. *Escherichia*  
279 *coli* O86:B7-treated turkeys had substantially lower granulomatous lesion score,  
280 inflammatory response, and lower hyphae score than immunized birds or those from control  
281 groups. Interestingly, the protective effect of *E. coli* O86:B7 was not due to the increased  
282 production of serum anti- $\alpha$ -Gal IgY, but it is likely associated with a considerable reduction  
283 of anti- $\alpha$ -Gal IgA Abs in the lungs of infected birds. The mechanism by which *E. coli*  
284 O86:B7 abrogates anti- $\alpha$ -Gal IgA response in the lungs of immunized turkeys remains to be  
285 elucidated, but it may be associated with the production of  $\alpha$ -Gal-specific **regulatory T cells**  
286 (**Tregs**) in the guts, which can then migrate to the lungs and induce tolerance to the infection.  
287 Based on these findings, it can be suggested that the efficacy of immunization with  $\alpha$ -Gal and  
288 the activation of the specific protective immunity may be dependent on the source, i.e.  
289 configuration of the  $\alpha$ -Gal epitopes and the route of its administration (immunization through  
290 injection vs oral administration).

291

### 292 ***Infection route***

293 Nevertheless, the protective effect of the  $\alpha$ -Gal-induced immunity may also be depending on  
294 the route of pathogen infection. In a recently published study, zebrafish (*Danio rerio*) was  
295 introduced as a new animal model to investigate the possibility of using  $\alpha$ -Gal-based vaccine  
296 formulation for the control of tuberculosis [11]. In two separate experiments, zebrafish were  
297 first immunized by intraperitoneal injection of synthetic Gal $\alpha$ 1-3Gal-BSA either in  
298 combination with ISA 71 VG adjuvant or without it and then challenged with *Mycobacterium*  
299 *marinum*, a bacterium causing chronic tuberculosis-like diseases in fish [70-72]. The  
300 protection against the mycobacterial infection, characterized by increased production of  
301 protective anti- $\alpha$ -Gal IgM Abs, opsonization of *M. marinum*, promotion of FcR-mediated

302 phagocytosis and macrophage activation, was only observed in zebrafish vaccinated with  
303 Gal $\alpha$ 1-3Gal-BSA and infected by mucosal exposure to *M. marinum*, but not in those infected  
304 intraperitoneally.

305

### 306 ***Bacteria used for oral immunization***

307 In most of the studies reported here, *E. coli* O86:B7 strain was proved to successfully elicit  
308 protective  $\alpha$ -Gal immunity in *ggta1*-KO mouse and bird models [4,10]. No apparent  
309 morbidity or mortality occurred in any of the test or control *ggta1*-KO mice due to oral  
310 gavage with live *E. coli* O86:B7. Mice remained healthy and active, with no detectable  
311 weight loss, diarrhoea, or observable abnormalities [4,73]. Humans orally inoculated with the  
312 parental strain of *E. coli* O86:B7, *E. coli* O86, developed increased blood group B Abs [74].  
313 However, *E. coli* O86:B7 was associated with a gastroenteritis outbreak [75]. Therefore, gut  
314 colonization with probiotic *E. coli* Nissle 1917 (ECN1917) strain offers a much safer and  
315 better alternative [47] also because this strain has higher  $\alpha$ -Gal content compared to *E. coli*  
316 O86:B7 and it is possibly able to induce a stronger  $\alpha$ -Gal immune response. Using Gram-  
317 positive bacteria as a probiotic-based vaccine would also be an option, but in contrast to  
318 Gram-negative enterobacteria, they do not have immunogenic lipopolysaccharide (LPS)  
319 components [76], which may have an impact on the immune response against  $\alpha$ -Gal in the  
320 intestinal mucosa [47]. To overcome the possible low antigenicity, probiotic-based vaccines  
321 using Gram-positive bacteria could be combined with TLR4 agonist for LPS [13,47]. This  
322 can be implemented by transforming candidate Gram-positive bacteria with a plasmid  
323 containing LPS specific peptide mimotopes [77], as previously observed in *Lactobacillus*  
324 *casei* [78]. Alternatively, a plasmid containing bacterial *ggta1* reported in *E. coli* and other  
325 bacteria could be transferred to any bacteria and theoretically used as a probiotic-based  
326 vaccine [47].

327

328 **Concluding remarks**

329 Immunization by  $\alpha$ -Gal either through injection of synthetic NGPs or oral administration of  
330  $\alpha$ -Gal-expressing bacteria represents a promising and innovative strategy for the prevention  
331 and control of parasitic diseases in humans and animals. A combination of protective protein  
332 antigens with  $\alpha$ -Gal-containing compounds may improve vaccine efficacy by activating  
333 complementary immune protective mechanisms. Furthermore, these interventions have the  
334 potential to induce an efficient and long-lasting protective immune response targeting other  
335 pathogenic agents with the surface  $\alpha$ -Gal modifications including *Borrelia burgdorferi* sensu  
336 lato, *Anaplasma phagocytophilum*, Newcastle disease virus, human immunodeficiency virus  
337 (HIV), and measles virus [7,14,23,79]. However, all currently available data are gathered in  
338 experimental studies employing the humanized mouse or turkey/chicken and fish models [4-  
339 6,8,10,11,30], which opens up the question of whether these promising findings in animals  
340 can be reliably translated to humans (see **Outstanding Questions**). Therefore, more clinical  
341 studies are still needed to optimize the vaccine antigen and standardize the immunization  
342 protocols and to better understand the mechanisms underlying the protective immunity  
343 induced by  $\alpha$ -Gal. There is still much to be learned (see **Outstanding Questions**), but in the  
344 era of the -omics technologies, the development of efficient immunization interventions  
345 based on the  $\alpha$ -Gal antigens against multiple pathogens appears to be closer to reach than  
346 ever.

347

348 **Disclaimer Statement**

349 The authors declare that they have no conflicts of interest.

350

351 **References**

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543

## 544 **Glossary**

545 **Antigen-presenting cells (APCs):** are a large group of various cells (i.e., macrophages,  
546 dendritic cells, B lymphocytes) that induce the cellular immune response by processing and  
547 exposing an antigen to T cells

548 **Chagas disease:** a severe tropical disease caused by the parasite *Trypanosoma cruzi*, which is  
549 transmitted to humans and animals mostly by triatomine bugs. It is also referred to as  
550 American trypanosomiasis.

551 **Complement system:** an integral part of the innate immune system that supports antibodies  
552 and phagocytes to clear foreign particles, microbes, and damaged body cells from an  
553 organism. The system also promotes inflammation and opsonization.

554 **Cytokines:** a large group of small signalling proteins, peptides, or glycoproteins that are  
555 secreted by cells of the immune system. They are involved in the interactions and  
556 communications between cells, and the stimulation of the cell movement toward infection  
557 sites. Cytokines include interferons (IF), interleukins (IL), chemokines, and tumour necrosis  
558 factors (TNF).

559 **Glycotope:** a specific part of a carbohydrate antigen that is recognized by the immune  
560 system, in particular antibodies, B cells, or T cells.

561 **Leishmaniasis:** a vector-borne disease of medical and veterinary concern caused by the  
562 protozoan parasites from the genus *Leishmania*. The parasites are transmitted through the  
563 bites of infected phlebotomine sandflies. Over 20 *Leishmania* species infect humans  
564 worldwide and the diseases can be displayed as cutaneous, visceral, and mucocutaneous  
565 forms.

566 **Nuclear factor kappa-light-chain-enhancer (NF- $\kappa$ B):** a protein complex found in almost  
567 all animal cell types, which controls transcription of DNA, cytokine production, and cell  
568 survival. NF- $\kappa$ B is involved in rapid cellular responses to various stimuli and plays a key role  
569 in triggering the protective immune response to infections.



570 **Opsonization:** a process by which a microbial agent or a cell is marked for ingestion and  
571 destruction by phagocytes.

572 **Regulatory T cells (Tregs):** subpopulation of T cells, which have a role in modulating other  
573 cells of the immune system. Tregs control the immune response to self-antigens and prevent  
574 autoimmune diseases. In general, they suppress or downregulate the induction and  
575 proliferation of effector T cells.

576 **Sterile immunity:** a unique immune status, which prevents effective pathogen infection into  
577 the host and is different from the immunity that allows infection but with subsequent  
578 successful eradication of the pathogen.

579 **Toll-like receptors (TLRs):** a class of proteins that play a crucial role in the innate immune  
580 system. They are usually expressed on macrophages and dendritic cells that pathogen-  
581 associated molecular patterns derived from various infectious agents.

582

### 583 **Figure caption**

584 **Figure 1, Key Figure.  $\alpha$ -Gal vaccination induces protection against several pathogens.**

585 Parental and/or oral immunization with preparations containing the carbohydrate  $\alpha$ -Gal has  
586 been shown to protect against kinetoplastid (*Trypanosoma cruzi* and *Leishmania* spp.),  
587 apicomplexan (*Plasmodium* spp.), mycobacterial (*Mycobacterium marinum*), and fungal  
588 (*Aspergillus fumigatus*) pathogens in different animal models.

**Table 1. An overview of  $\alpha$ -Gal antigens used as vaccine candidates against parasites and other infectious agents and mechanisms of protective  $\alpha$ -Gal immunity.**

Targeted pathogen	Source of $\alpha$ -Gal epitope	Animal model	Immunization route	Infection route	Mechanism of protective immunity	Reference
<i>Plasmodium berghei</i> , <i>P. yoelii</i>	<i>Escherichia coli</i> O86:B7, rRBCM, $\alpha$ -Gal-BSA	C57BL/6 $\alpha$ 1,3GT- knockout mouse	PO, IP	ID, IV	Lytic activity of anti- $\alpha$ -Gal IgM and IgG antibodies (complement activation)	[4]
<i>Leishmania major</i> , <i>L. infantum</i> , <i>L. amazonensis</i>	Gal $\alpha$ (1,6)Gal $\beta$ -BSA, Q $\beta$ - $\alpha$ -Gal	C57BL/6 $\alpha$ 1,3GT- knockout mouse	SC	FPI, IP	Lytic activity of anti- $\alpha$ -Gal IgG antibodies (complement independent), upregulation of Th1 cytokines, upregulation of memory CD4 <sup>+</sup> CD44 <sup>+</sup> , CD8 <sup>+</sup> CD44 <sup>+</sup> and CD4 <sup>+</sup> CD69 <sup>+</sup> T cells	[5,6]
<i>Trypanosoma cruzi</i>	Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc- HSA	C57BL/6 $\alpha$ 1,3GT- knockout mouse	IP	IP	Trypanolytic activity of anti- $\alpha$ -Gal IgG antibodies (complement independent), upregulation of cytokines, chemokines and growth factors, expansion of antigen-specific memory CD4 <sup>+</sup> CD44 <sup>+</sup> T cells	[8]
<i>Mycobacterium marinum</i>	Gal $\alpha$ 1-3Gal-BSA	Zebrafish ( <i>Danio rerio</i> )	IP, ME	IP	B cell maturation, antibody-mediated opsonisation, FcR-mediated phagocytosis, macrophage response, interference with the $\alpha$ -Gal antagonistic effect of the TLR2/NF- $\kappa$ B-mediated immune response, upregulation of proinflammatory cytokines	[11]

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<i>Aspergillus fumigatus</i>	<i>Escherichia coli</i> O86:B7, Gal $\alpha$ 1-3Gal-BSA	Turkey ( <i>Meleagris gallopavo</i> )	PO, SC	IT	Decreased anti- $\alpha$ -Gal IgA levels in the lungs, production of $\alpha$ -Gal-specific Tregs (putative mechanism)	[10]
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**Abbreviations:** FPI – footpad injection; ID – intradermally; IP – intraperitoneally; IT – intratracheally; IV – intravenously; ME – mucosal exposure (i.e., immersion in water containing *M. marinum*); PO – perorally; SC – subcutaneously.

