

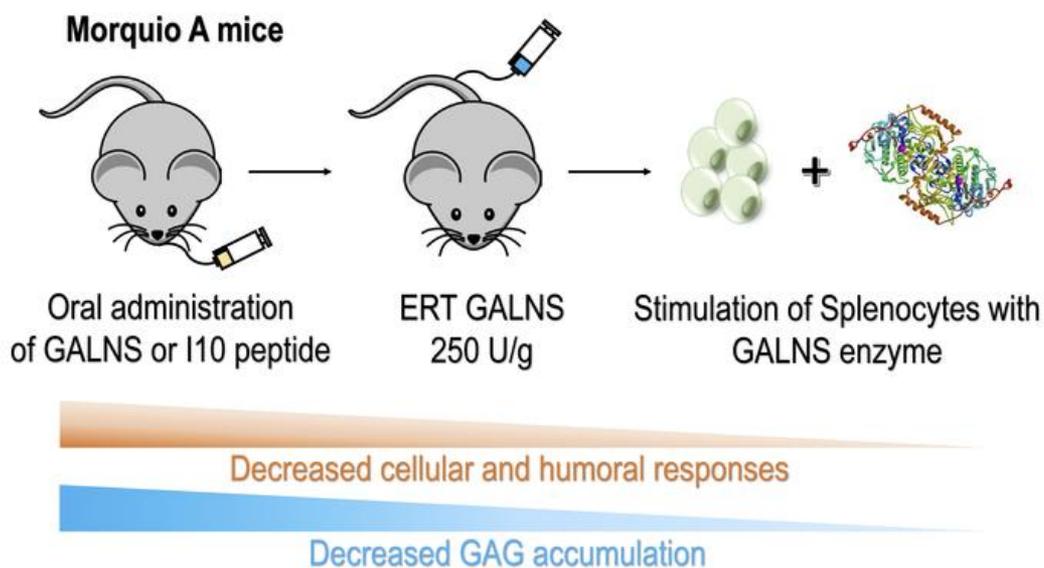
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Oral Immunotherapy Tolerizes Mice to Enzyme Replacement Therapy for Morquio A Syndrome

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Conflict of interest statement

A.C.S., A.P.K., C.J.B., L.A.B., S.T. and A.M.M. declare competing financial interests: they have US patent application 15/876,892. The patent application covers the underlying concept of oral tolerance to treat Mucopolysaccharidosis IVA disease described in the manuscript.

Abstract

Immune response to therapeutic enzymes poses a detriment to patient safety and treatment outcome. Enzyme replacement therapy (ERT) is a standard therapeutic option for some types of Mucopolysaccharidoses including Morquio A syndrome caused by GALNS deficiency. Current protocols tolerize patients using cytotoxic immunosuppressives which can cause adverse effects. Here we show development of tolerance in Morquio A mice via oral delivery of peptide or GALNS during ten days prior to ERT. Our results show that using an immunodominant peptide (I10) or the complete enzyme (GALNS) to orally induce tolerance to GALNS prior to ERT, resulted in several improvements to ERT in mice: *i*) decreased splenocyte proliferation after in-vitro GALNS stimulation; *ii*) modulation of cytokine secretion profile; *iii*) decline in GALNS-specific IgG or IgE plasma; *iv*) decreased GAG storage in liver; and *v*) fewer circulating immune-complexes in plasma. This model could be extrapolated to other lysosomal storage disorders where immune response hinders ERT.

Introduction:

Mucopolysaccharidosis type IVA (MPS IVA) or Morquio A syndrome (MIM ID #253000) is an autosomal recessive disorder caused by the deficiency of N-acetylgalactosamine-6-sulfate sulfatase (GALNS: E.C.3.1.6.4), resulting in accumulation of the glycosaminoglycans (GAGs), keratan sulfate (KS) and chondroitin-6-sulfate in the lysosomes (1, 2). GAG accumulation leads to chronic and progressive deterioration of affected cells, tissues and organs. Clinical manifestations include bone abnormalities, dysostosis multiplex, joint pathology, organomegaly, short stature, pulmonary compromise and valvular heart disease (3, 4). Heterogeneity in patients' phenotype is confirmed by more than 250 mutations reported to date (5-9).

There is no cure for Morquio A, and for many years only palliative treatments have been available (3). Currently, hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT) have been clinically evaluated for Morquio A. HSCT has had limited effectiveness due to graft-versus host disease, risk of infections, and high mortality rate (10, 11). ERT with human recombinant GALNS enzyme or Elosulfase alfa (Vimizim®), was approved by the Food and Drug Administration (FDA) in 2014 (12). Although ERT is known to ease MPS complications, it fails to adequately treat particular tissues, especially the skeletal and cardiovascular systems (13-17). In addition, the major reason behind partial effectiveness of ERT is that the immune system makes antibodies against the infused recombinant protein that neutralize enzyme activity (18-21). In Morquio A clinical trials, study drug-related adverse events were present in 73% to 100% of the patients, and all patients tested positive for anti-elosulfase alfa antibody by week 4 to 6 until the end of the study (17, 22-24). In phase III clinical trials (MOR-004, MOR-005), all patients developed anti-drug antibodies, nearly all developed neutralizing antibodies, and 9.7% of patients developed drug-specific IgE antibodies. Only two patients developed study-drug related serious adverse events (anaphylaxis and hematuria) (17, 25, 26).

These antibodies can cause *i)* treatment resistance, *ii)* type III hypersensitivity reactions, and *iii)* glomerulonephritis due to depositions of immune-complexes in kidney (27). Immune response may depend on factors such as the nature of replaced protein, patient genetic background, structural differences between the infused and the defective protein, and presence or absence of the residual mutant protein or cross reactive immunologic material (CRIM) (28). CRIM-negative patients do not express any residual protein due to large deletions or non-sense mutations, among others. Meanwhile, CRIM-positive patients present mutations that result in circulation of an inactive protein. Therefore, there is a relationship between CRIM status and development of antibodies against the therapeutic protein. It has been shown that CRIM-negative patients could develop a stronger immune response to the protein than CRIM-positive patients (20, 29). To control the potential impact in these patients, immunosuppressive drugs such as cyclosporine A, azathioprine, and methotrexate have been evaluated (30-32). Although non-specific immune suppressive protocols have demonstrated good results inhibiting immune responses to the infused protein, side effects include damage to bone marrow, gastrointestinal tract, liver and kidney, and risk of bacterial and virus infections, among others (33-35). The current challenge is to replace chronic treatments of non-specific immunosuppression and their toxicities with new therapies that safely induce specific immune tolerance (36).

To establish new strategies to modulate immune response to ERT, we evaluated GALNS antigenicity by selecting the most immunogenic epitopes in the protein. In this study, we have developed a protein- or a peptide-based oral immunotherapy to induce specific tolerance to GALNS in Morquio A mice treated by ERT. Oral immunotherapy was chosen as the approach because it is natural, non-toxic, and has demonstrated potential in allergy research. Beyond inducing tolerance to ERT, the treatment improved outcomes, reducing GAG accumulation in the liver and reducing circulatory immune-complexes.

Results

Ten regions in GALNS enzyme were predicted as highly immunogenic in-silico

To comprehensively profile the most immunogenic regions in GALNS enzyme, we used algorithms designed to predict B- and T-cell epitopes (37, 38). Ten 20-mer peptides containing the immunogenic regions in GALNS enzyme were selected: five peptides (A2, B3, G6, H7 and J1) were predicted to contain B-cell epitopes, and three peptides (D9, E8 and I10) were predicted as regions containing T-cell epitopes. In addition, two peptide sequences (C4 and F5) were predicted to contain both B- and T-cell combined epitopes (Table 1). We also predicted the human HLA binding sites within the sequence of the ten peptides (Supplemental figure 1). The location of the predicted peptides are displayed within the structural model of GALNS (Figure 1A), and the presence or absence of structural domains was confirmed (Table 1) (2, 39, 40).

Immunological evaluation of GALNS and synthetic peptides after enzyme replacement therapy showed preferential immunogenicity.

The combination of *in-silico* predictions of immunogenic regions of GALNS with in-vitro and in-vivo experiments were aimed to select the best peptides as representative epitopes within GALNS. Here, we used a knockout Morquio A mouse model (MKC; *Galns*^{-/-}), which corresponds to CRIM-negative patients having the strongest humoral and cellular response against the therapy. MKC mice were treated with 16 intravenous weekly infusions of recombinant human GALNS or PBS. Cellular responses were measured by looking at splenocyte proliferation and secretion of pro-inflammatory cytokines after in-vitro stimulation of isolated splenocytes with GALNS or individual peptides (Figure 1B-F). Splenocyte proliferation in ERT-treated MKC mice was significantly higher than in PBS-treated control mice after in-vitro stimulation of isolated splenocytes with peptides C4, E8, I10, or GALNS (Figure 1B). In immunotolerance studies, it has

been demonstrated that after the antigen is taken up in the gut by dendritic cells, and in the presence of tolerization, Th1 (IFN- γ) and Th2 (IL-4, IL-5, and IL-13) cytokine production is decreased. To differentiate the peptides' capacity to modulate a Th1 or Th2 response, a profile of cytokines was characterized. IFN- γ was measured to define Th1 response, and IL-4, IL-5 and IL-13 for Th2 response (41). Quantification of IFN- γ in GALNS-stimulated cells of ERT-treated MKC mice were significantly higher than PBS controls for peptides C4, E8, I10, and for GALNS (Figure 1C). High IL-4 secretion was observed with the three peptides (C4, E8 and I10) or with GALNS in ERT treated mice. The differences were statistically significant when compared with PBS controls. Higher IL-4 secretion was detected after stimulation with GALNS or peptide I10 (Figure 1D). Only peptides C4, I10 and GALNS protein induced high secretion of IL-5 after the in-vitro stimulation in ERT treated mice compared with the PBS controls (Figure 1E). Significant increase in IL-13 secretion was observed exclusively with peptide I10 and GALNS after the in-vitro stimulation (Figure 1F). As in proliferation assays, splenocytes secreted significantly more cytokines after in-vitro stimulation only with peptides C4, E8, I10 or with GALNS in the ERT-treated MKC mice when compared with PBS control mice (Figure 1C-F). In summary, peptide C4 induced the most IFN- γ secretion among the ten, even when compared with GALNS upon the in-vitro stimulation (Figure 1C). These results suggested that peptides C4, E8 and I10 were the most immunogenic of the ten examined.

Oral tolerance reduces cellular and humoral responses to GALNS enzyme replacement therapy

We selected the peptide I10 and GALNS enzyme to induce oral tolerance, due to the similar response after in-vitro stimulation with GALNS. MKC mice received either GALNS or peptide I10 via oral gavage at three different doses (50, 100 or 500 μ g) prior to the regular intravenous ERT

regimen (tolerized group). Two control groups were given PBS (non-tolerized groups). The positive control group was treated by ERT (non-tolerized, treated), and the negative control group received intravenous PBS (non-tolerized, untreated group) (Table 2). The effect of inducing GALNS tolerance was evaluated by measuring cellular and humoral response in the mice treated by ERT. Cellular responses were evaluated by assessing splenocyte proliferation and secreted cytokines (IFN- γ , IL-4, IL-5, IL-10 and IL-13) after GALNS in-vitro stimulation in the tolerized, non-tolerized and untreated groups. Mice fed with peptide I10 showed significantly less splenocyte proliferation after GALNS in-vitro stimulation in two groups (50 μ g or 500 μ g), than non-tolerized mice. The three groups of mice that received GALNS orally presented a statistically significant decline in splenocyte proliferation (50 μ g, 100 μ g, or 500 μ g) (Figure 2A). These results demonstrated that oral administration of peptides affected the cytokine profile in treated mice. Less IFN- γ was secreted in mice treated orally with peptides I10. IFN- γ decrease was statistically significant (50 μ g or 500 μ g of the peptide I10) when compared with the non-tolerized group, suggesting the modulation of Th1 response. Interestingly, mice that received GALNS orally did not show any modulation in the IFN- γ secretion after splenocytes' in-vitro stimulation with GALNS (Figure 2B).

IL-4 (Th2-biased cytokine) production by splenocytes after in-vitro stimulation with GALNS was evaluated. The results showed a statistically significant decrease in the secreted IL-4 by splenocytes of the tolerized groups with peptide I10 50 μ g or 500 μ g, and with GALNS 100 μ g or 500 μ g if compared with the non-tolerized treated mice. The difference in the IL-4 levels of the non-tolerized untreated mice was statistically significant when compared with all groups except mice treated with peptide I10 100 μ g (Figure 2C). Inducing tolerance did not affect Th2 cytokine levels (IL-5 and IL-13). All tolerized groups showed elevated IL-5 and IL-13, but not significantly different from non-tolerized mice (Figure 2D,F). IL-10 is an immunoregulatory cytokine required to induce tolerance. IL-10 did not increase in some tolerized groups that showed inhibition in the

GALNS-specific splenocyte proliferation or pro-inflammatory cytokine secretion. Contrarily, significant IL-10 downregulation was observed in groups treated with peptide I10 50 µg and GALNS 500 µg, compared with non-tolerized mice. This divergent result may relate to the experimental system used (time of detection), or the observed induction of tolerance was IL-10 independent (42). Higher IL-10 in non-tolerized mice could be a mechanism to counteract the inflammation from Th1 and Th2 responses induced post in-vitro stimulation with GALNS (Figure 2B,E).

The effect of oral tolerance on humoral response was examined by measuring the levels of specific IgG and IgE to GALNS. Plasma levels of GALNS-specific IgG were significantly lower in mice fed with peptide I10 50 µg, or GALNS 500 µg than in non-tolerized mice (Figure 3A). GALNS-specific IgE in plasma was also reduced for mice treated with peptide I10 50 µg or with GALNS 500 µg when compared with the non-tolerized group (Figure 3B). In summary, we conclude that tolerization with either GALNS or peptide I10 could protect against harmful formation of both the enzyme-specific neutralizing IgG and anaphylaxis-inducing IgE antibodies during ERT, improving treatment efficiency.

Altogether, these studies indicate that peptide I10 at a concentration of 50 µg and GALNS protein at a concentration of 500 µg have the strongest effect ameliorating the immune responses of ERT.

Mesenteric lymph nodes play a role in oral tolerance to GALNS

To elucidate the tolerogenic role of gut-associated lymphoid tissue (GALT), we measured Forkhead box P3 (Foxp3) and transforming growth factor β (Tgf- β) gene expression in mesenteric lymph nodes (MLN) and Peyer's patches (PP) of tolerized and non-tolerized mice. There was

upregulation in Foxp3 and Tgf- β (molecules associated with immune suppression) in MLN samples, confirming that oral tolerance is GALT originated and requires MLN (Fig 4 A, C). In contrast, Foxp3 and Tgf- β did not show any change in expression in PP of tolerized and non-tolerized mice (Fig 4 B, D). This suggests that PP are not required for oral tolerance (43).

Decreased circulating immune complexes after oral tolerance

Antibodies against proteins used for ERT often cause glomerulonephritis due to immune-complex deposits in the kidney. We measured plasma levels of circulating immune complexes (CICs) to evaluate whether oral tolerance had reduced neutralizing antibodies. As expected, CICs plasma levels were higher in the group that underwent ERT without tolerization (non-tolerized treated mice or PBS-ERT). In contrast, the lowest levels were found in mice treated with vehicle (PBS) and in naïve animals. Plasma CICs were reduced significantly in groups treated orally with peptide I10 50 μ g, and I10 100 μ g, when compared with non-tolerized ERT-treated mice (Figure 5). Our results show that oral tolerance with GALNS or peptide I10 can be used to prevent the development of Type III hypersensitivity reactions in MKC mice treated with ERT.

Vacuole reduction in liver samples of tolerized Morquio A mice

To measure the effect of the oral tolerization protocol in the treated mice, we performed a pathological evaluation of GAGs in liver tissues. Sections were stained with toluidine blue and evaluated by light microscopy. The number of cells with cytoplasmic vacuoles and the total number of vacuoles were counted in eight random microscopic high-power fields as described in the methods section (Figure 6 A-H). Vacuoles were observed in endothelial and Kupffer cells. The highest GAG score and vacuole number were in non-tolerized untreated control mice (PBS-PBS). Although mice that were not tolerized and received ERT (PBS-ERT) showed decrease in GAG

score and vacuole number, their GAG levels were significantly higher than the tolerized mice. All tolerized groups showed a significantly lower GAGs accumulation than the non-tolerized or the untreated group. The improvement was observed in both GAG score (Figure 6I), and GAG vacuole number (Figure 6J). These results were confirmed by electron microscopy studies. At ultrastructural level, sinusoidal endothelial cells and Kupffer cells demonstrated cytoplasmic vacuolar accumulation with finely granular material consistent with GAG storage. Cells with the highest number of vacuoles were present in the untreated and non-tolerized groups. No significant changes were seen in cellular organelles or lipid content (Figure 7).

Reduction of keratan sulfate in plasma samples of tolerized Morquio A mice

The primary biomarker of Morquio A treatment outcomes is KS. We measured plasma levels of KS to evaluate whether oral tolerance affected GAG levels in circulation. We found significantly higher KS plasma levels in the group that underwent ERT without tolerization (non-tolerized treated mice or PBS-ERT) compared to each of the tolerized groups (Figure 8). The highest levels of KS were present in untreated mice (PBS-PBS).

Altogether, these results suggest tolerance achieved by this protocol could reach improved therapeutic levels when compared to regular ERT.

Discussion

Oral tolerance is induced mainly in the GALT and in other mucosal surfaces such as the respiratory tract. It has been defined as the specific suppression of B- and T-cell responses to an antigen administered orally (44, 45). Antigenic immune response represents a significant

obstacle to ERT efficacy (17-20, 46, 47). Oral tolerance constitutes an ideal approach to prevent immune response, and to eliminate side effects of immunosuppressive drugs.

In the present study, we report a novel approach to improve ERT efficacy while reducing the immune response by using a protein- or a peptide-based oral immunotherapy to induce specific tolerance to GALNS in Morquio A mice treated by ERT. The results provide first evidence that oral tolerance can decrease immune response, and improve ERT outcomes. In particular, we show that an immunodominant peptide (I10) or the complete enzyme (GALNS) were used to orally induce tolerance to GALNS prior to ERT. The experiment resulted in several improvements to ERT in mice, including: *i*) decreased splenocyte proliferation after in-vitro GALNS stimulation; *ii*) modulation of cytokine secretion profile; *iii*) decline in GALNS-specific IgG or IgE plasma; *iv*) decreased GAG storage in liver; and *v*) fewer circulating immune-complexes in plasma.

Immunodominant epitopes are specific subunits of the antigenic regions easily recognized by the immune system. The identification of immunodominant epitopes in a specific protein is based on the synthesis of overlapping peptides that result in hundreds of molecules to evaluate (48). In this study, the combination of *in-silico* prediction of immunogenic regions of GALNS with in-vitro experiments aimed to speed up the screening of immunodominant epitopes in GALNS. Ten peptides were selected by the predicted presence of T and B cells epitopes in GALNS sequence.

Th1-driven responses are regarded mainly to cell-mediated immune response; whereas, Th2-type cytokines are predominantly related to antibodies and/or immune-complexes mediated response (49). Although, there is not a clear characterization of the type of response developed in patients undergoing ERT, immune response to infused enzymes is recognized as one of the major complications for the treatment success (18, 19, 21, 32, 50). The degree of immune

response is related to: *i*) presence or absence of residual mutant protein in the individual, and *ii*) structural differences between the infused and defective proteins.

CRIM-negative patients cannot produce any precursor form of the native enzyme, or at least it cannot be detected in circulation. CRIM positive patients produce some precursor forms of abnormal enzyme in circulation (20, 47, 51). CRIM status could predict immune response. A strong immune response against the therapy could be expected in CRIM-negative patients, if the immune system recognizes the infused protein as foreign molecule. This activates strong inflammation, predicting poor clinical outcome. CRIM-positive patients could present a more heterogeneous response against the therapy (52). This would depend mainly on structural differences between the abnormal and native proteins. Other factors could include surrounding environment, patient genetics, route of administration, dosage, frequency, and/or duration of the treatment (19, 32).

Since the mechanisms of oral tolerance are determined by the dose of antigen fed (44), we used low and high doses (peptide I10 or GALNS enzyme). In this study, we used 50, 100 or 500 μg of peptide I10 or GALNS enzyme for tolerization. At any given concentration, the molarity of the peptide I10 is 25.5-fold higher than that of GALNS because the molecular weights are different. Oral administration of peptide I10 or GALNS enzyme prior to ERT promoted a significant reduction in splenocyte proliferation after in-vitro GALNS stimulation. (Except for mice fed peptide I10 100 μg ; Figure 2A.) These results could be explained by induction of T cell anergy, a condition in which T cells exposed to a specific antigen fail to proliferate upon re-stimulation with the same specific antigen (53).

Modulation of cytokine secretion varied by type of oral antigen. Secretion of IFN- γ after in-vitro splenocytes stimulation with GALNS was dramatically lower for mice fed peptide I10 (50 and 500 μg) than the non-tolerized group (Figure 2B). Additionally, a statistically significant decline in

IL-4 secretion was observed in the same groups of mice (Figure 2C). In these groups, there was clear suppression of the Th1-biased (IFN- γ) and Th2-biased (IL-4) responses. Mice fed peptide I10 100 μ g did not show modulation of these cytokines.

Although mice fed GALNS presented a reduction in splenocyte proliferation, they did not show a significant decrease of IFN- γ secretion (Figure 2B). In contrast, decreased IL-4 was detected in mice fed with GALNS (100 and 500 μ g) (Figure 2C). These observations indicated a predominant Th1-biased (IFN- γ) response that counteracts the Th2-biased (IL-4) response. IFN- γ is a versatile cytokine in the immune system, and could play a role in inflammation and tolerance as well. The immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) is mainly induced by IFN- γ to establish immune-tolerance or to induce T cell unresponsiveness (54). In tolerance to allografts, there is evidence that IDO induction is mediated by CD8⁺ T regs secretion of IFN- γ (55). In models of induction of tolerance to alloantigens, it was reported that CD4⁺ T regs require secretion of IFN- γ for immune regulation (56). Induction of IFN- γ has been reported in other models of oral tolerance (57). Therefore, it could be suggested that immune modulation in mice treated with GALNS orally may be mediated by IFN- γ and activation of IDO.

Secretion of IL-5 or IL-13 was not down-regulated in any groups treated by oral tolerance. Levels did not differ from the non-tolerized group. In mice treated orally with GALNS, increased levels of IFN- γ may suppress and counteract IL-4 but not IL-5 and IL-13 (Figure 2D,F).

IL-10 is an important cytokine for tolerance induction. Surprisingly, IL-10 secretion after splenocytes stimulation was markedly reduced, especially for the groups feeding with peptide I10 50 μ g or GALNS 500 μ g, compared with the non-tolerized group (Figure 2E). This divergent result may be related to the experimental system used (time of detection), or it could be speculated that induction of tolerance was IL-10 independent (58). Higher IL-10 levels in non-tolerized mice could be a mechanism to counteract inflammation due to the higher levels of Th1 and Th2-type

cytokines induced after in-vitro stimulation with GALNS. Interestingly, though splenocytes of GALNS-fed mice produced higher levels of IFN- γ , no IL-10 increase was detected.

The effect of oral tolerance induction on humoral response was determined. As expected, and in accord with the cellular response profile, plasma levels of GALNS-specific IgG in mice fed peptide I10 50 μ g or GALNS 500 μ g were significantly lower than in non-tolerized mice (Figure 3A). Cytokines like IL-4 and IFN- γ are important in B cell biology and in mediating regulation of antibody isotypes by B cells. Previous reports demonstrate that IL-4 secretion is critical for the optimal maturation and selection of high affinity IgG1 antibodies (59). GALNS-specific IgE plasma levels significantly decreased in mice treated with peptide I10 50 μ g or with GALNS 500 μ g, when compared with the non-tolerized group (Figure 3B). Instant hypersensitivity reactions (anaphylaxis, urticarial, allergic asthma) are mediated by IgE antibodies, and infiltration of eosinophils directs chronic inflammation (60). Also, IL-4 signaling regulates immunoglobulin class switching to IgE through STAT6 activation (61, 62), whereas IFN- γ is mainly associated with switching to IgG2a (63). Therefore, down-regulation of IL-4 and IFN- γ in mice treated orally reduces GALNS-specific IgG and IgE in plasma. Although mice fed GALNS secreted higher levels of IFN- γ after GALNS in-vitro stimulation, levels of IgG and IgE were significantly lower than the non-tolerized mice. This result agrees with our hypothesis that in mice treated orally with GALNS, IFN- γ could be tolerogenic.

We could speculate that induction of tolerance in groups of mice fed with peptide I10 is regulated by a different mechanism than in mice fed with the complete enzyme. Induction of anergy and down-regulation of Th1/Th2 response in mice treated with peptide I10 could be mediated by induction of CTLA-4. On the other hand, it seems that induction of tolerance in mice treated by GALNS orally could be mediated by IFN- γ . Interestingly, both mechanisms may induce the immunomodulatory enzyme IDO (54, 64-66). IDO is a cytosolic enzyme that catalyzes the first

step of oxidative tryptophan catabolism. Tryptophan metabolism suppresses effector T cells (66, 67). However, confirming this hypothesis requires additional evaluations of regulatory markers.

We measured Foxp3 expression to confirm the hypothesis that MLN support the generation of Foxp3⁺ regulatory T cells in tolerized animals (68-70). We observed high Foxp3 expression in MLN and not in PP. These findings confirm that MLN are indispensable to induce oral tolerance. On the other hand, PP may be important to transport the antigen from the gut lumen to the lymphoid areas of the GALT (69, 70) but are not required to induce oral tolerance to GALNS. We found changes in Foxp3 expression in MLN, which could be related to the dose of antigen used (71) (Figure 4).

We also evaluated how oral tolerance affected pathological improvement in mice treated by ERT. Circulating immune-complexes (CICs) in plasma and GAG accumulation in liver were evaluated in tolerized and non-tolerized mice. Glomerulonephritis has been reported in ERT patients, due to deposits of immune-complexes in kidney as a secondary effect of the immune response (21, 27). Inducing tolerance to GALNS used in ERT reduced immune-complex deposits in the glomerula (data not shown) and in plasma (Figure 5). There was significant reduction of CICs in mice fed with 50 µg and 100 µg of I10, reaching levels of non-tolerized, untreated mice (PBS-PBS). This result presents a clinical impact – the potential to avoid nephrotic syndrome in ERT patients.

There was a significant reduction of liver GAGs in all tolerized groups with I10 (Figure 6C-E) and GALNS (Figure 6 F-H) when compared to the non-tolerized, ERT treated group (Figure 6B) and untreated mice (Figure 6A). Measuring GAGs by a score system or by counting vacuoles confirmed the results (Figure 6 I,J). In addition, there was a significant reduction of plasma KS in all tolerized groups compared to the non-tolerized group (Figure 8). These results suggest that

build-up of tolerance towards GALNS can decrease even further GAGs levels in liver and in circulation when compared to regular ERT.

Conclusions

Our present study established a protocol to evaluate immunogenicity of a lysosomal enzyme, namely GALNS enzyme. This evaluation was based on the combination of *in-silico* predictions with in-vitro and in-vivo studies. The main goal was to induce oral tolerance to GALNS prior to ERT. We demonstrated that repeated administration of GALNS immunogenic peptides or the complete enzyme decreased response to GALNS in mice treated by ERT. Beyond inducing tolerance to ERT, the treatment improved outcomes, reducing GAG accumulation in the liver and circulation, and reducing circulatory immune-complexes. This is the first immunogenicity evaluation of GALNS enzyme and characterization of immune response to ERT in Morquio A mouse models. It is also the first report that demonstrates induction of oral tolerance to a lysosomal enzyme used for ERT with a significant improvement.

Methods

Production and purification of recombinant human GALNS enzyme.

Supernatants of Chinese Hamster Ovary (CHO) cells over-expressing human GALNS were cultured in protein-free media and purified by a two-column procedure as described previously (72). Enzyme activity of GALNS was determined by using 4-methylumbelliferyl- β -D-galactopyranoside-6-sulfate as substrate (73). One unit of GALNS enzyme activity is defined as

the amount of enzyme that catalyzes the conversion of 1 nmol of 4-methylumbelliferyl- β -D-galactopyranoside-6-sulfate per hour.

Prediction of GALNS epitopes.

Human GALNS sequence was available in the NCBI protein database (www.ncbi.nlm.nih.gov). Signal peptide prediction was determined using the proteomics and sequence analysis tools of the ExPASy Proteomics Server (<http://au.expasy.org>) to ensure that the epitopes were not located in the signal peptide region. To predict the immunogenic peptides in GALNS we used a combination of two computational algorithms to predict potential T and B cell epitopes: Immune Epitope Database analysis resource (IEDB www.immuneepitope.org) and RANKPEP (<http://bio.dfci.harvard.edu/RANKPEP/>) (37, 38). Prediction of B-cell epitopes were evaluated by the IEDB analysis resource. This algorithm is based on the predictions of surface accessibility and flexibility of the molecule, and the presence of β -turns and linear epitopes. MHC-II epitopes (H2-IAb) were selected by the best scores of IC₅₀nM (concentration of peptide that inhibits binding of a standard peptide by 50%) and binding potential. HLA binding sites were predicted by using the IEDB analysis resource. We selected ten candidate peptides (Table 1), which were synthesized by Biomatik Corporation.

Computational model of GALNS and peptide location.

Structural model of GALNS was previously constructed based on the homology and X-ray crystal structures of N-acetylgalactosamine-4-sulfatase (4S) and arylsulfatase A (ASA) (2). I-TASSER server was used to predict a computational model of GALNS structure (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>). Rasmol program was used for molecular visualization and peptide location.

In-vitro evaluation of immunodominant peptides.

The selected peptides were evaluated in a Morquio A mouse model (knock-out mice, *Galns*^{-/-}, MKC). This model was produced by targeted disruption of the murine *Galns* gene. MKC mice do not express *Galns* mRNA, and no enzyme activity of GALNS is detected (74). Morquio A mice were treated by enzyme replacement therapy (ERT), receiving 16 weekly infusions of human GALNS at a concentration of 250 U/g of body weight through the tail vein. A control group received PBS (75). Ten days after the last infusion, mice were euthanized and their spleens were aseptically removed. The tissues were homogenized with a syringe plunger in complete RPMI 1640 medium (10% fetal bovine serum, 2 μM glutamine, 50 U penicillin/ml, 50 μg streptomycin/ml, 100 μM non-essential amino acids, 50 μM 2-mercaptoethanol). The suspensions were centrifuged at 1,000 rpm for 10 minutes. The red blood cells were lysed using a Lysis buffer (Sigma). The specificity of cellular response against the peptides or the complete enzyme in the in-vitro stimulation was determined by splenocyte proliferation and cytokine secretion.

Splenocyte proliferation.

In a 96-well plate, 5×10^5 splenocytes / well were stimulated with the individual peptides (100 μg/ml), GALNS (150 μg/ml), concanavalin A (Con A) (3 μg/ml) or media in triplicates for 72 hours at 37°C, saturated humidity and 5% CO₂. Cells were pulsed with 1 μCi of radioactive thymidine for the last 18 hours of incubation. Thymidine incorporation was measured by β-Scintillation counter (Trilux Microbeta Counter) (76).

Secreted cytokines.

Cytokines were determined in the cell culture supernatants. In a 96-well plate, 1×10^6 splenocytes / well were stimulated with the individual peptides (100 μg/ml), the complete GALNS (150 μg/ml), Con A (3 μg/ml) or media in triplicates for 72 hours at 37°C, saturated humidity and 5% CO₂. Cells were centrifuged at 1,000 rpm during 10 minutes. Secreted cytokines (IL-4, IL-5, IL-13, and IFN-

γ) in the collected supernatants were detected by LUMINEX technology, using a Millipore Milliplex™ kit according to manufacturer's instructions.

Detection of IgG and IgE antibodies α -GALNS by ELISA.

An indirect ELISA technique was used to detect plasma levels of IgG and IgE antibodies against GALNS in treated and untreated mice. Ninety-six well polystyrene microplates were coated with 2 μ g/ml of GALNS enzyme in coating buffer (15mM Na₂CO₃, 35mM NaHCO₃, 0.021 NaN₃, pH 9.6) and incubated overnight at 4 °C in a wet chamber. The plates were blocked with 3% Casein in PBS during 1 hour at room temperature in a wet chamber and washed twice, first with TTBS (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5), and then with TBS (10 mM Tris, 150 mM NaCl, 0.05%, pH 7.5), 100 μ l of mice plasma samples were diluted (1:500 for IgE or 1:1,000 for IgG) in TTBS, added to the plate and incubated 2 hours at 37 °C in a wet chamber. Four washes with TTBS were performed. 100 μ l of anti-mouse IgE-HRP (Thermo Scientific) 1:1,000 in TTBS or anti-mouse IgG-Peroxidase (Sigma) 1:5,000 dilution in TTBS were applied. After three washes with TTBS, followed by one wash with TBS, the experiment was developed with the substrate TMB (3,3',5,5'-Tetramethylbenzidine, Sigma). The enzymatic reaction was stopped with 1N HCl solution and the absorbance was measured at 450nm in a Multiskan® EL800 (Bio-Tek Instruments). Plasma concentrations of IgG antibodies α -GALNS were derived by extrapolation of the absorbance values from a calibration curve using the monoclonal antibody α -GALNS (19B2).

Induction of oral tolerance.

Six-week-old MKC mice (*Galns*^{-/-}) were divided into 8 groups (n=3). Oral tolerance was induced by feeding mice with 50, 100 or 500 μ g of peptide I10, or GALNS enzyme. Two control groups received PBS alone (Table 2). The mice were fed by oral gavage with either peptide I10 or GALNS enzyme every other day over a period of 9 days (in total 5 times). One week after the last oral

administration, mice were treated by weekly intravenous infusions of GALNS enzyme with a dose of 250U/kg of body weight or PBS for the control group. The specificity of cellular response against the GALNS was determined by splenocyte proliferation and cytokine secretion as described previously.

Quantitative real-time PCR of Foxp3 and Tgf- β .

Total RNA was isolated from MLN or PP of tolerized and non-tolerized mice using the Qiagen RNeasy Micro Kit (DNA Genotek, Qiagen) according to the manufacturer's instructions. First strand complementary DNA synthesis was performed by using iScript™ Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad). cDNA levels of Foxp3 and Tgf- β were quantitated by qPCR using Prime PCR™ SYBR® Green Assay (Bio-Rad) in a Bio-Rad CFX Connect Real Time PCR system. Both negative and positive controls were included in each plate. Each study sample was analyzed with three technical replicates. Amplification conditions used for qPCR were: 95 °C for 2 minutes, followed by 40 cycles of denaturation and annealing/extension cycles at 95 °C for 5 seconds and 60 °C for 30 seconds. Actb and Gapdh were used as normalization controls with three values averaged for each sample to compare fold regulation of the two experimental genes. Fold change was determined by the $\Delta\Delta C_t$ method. Samples were handled per the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.

Determination of Immuno-complexes in plasma.

A proceptor™ – CIC ELISA kit (ProGen Biologics) was used to detect mouse circulating immune complex in mice plasma samples. The assay was performed according to the manufacturer's instructions. Briefly, 100 μ L of twenty-fold diluted mice plasma sample were placed in an antibody-coated 96-well microplate and incubated for 90 minutes at 25°C. After three washes, 100 μ L of HRP-Conjugated antibody were added to the plate and incubated for 1 hour at 25°C. Three additional washes were performed and 100 μ L of substrate solution were used for color

development. After five minutes of incubation at room temperature, 50 μ L of stop solution was used. Absorbance was measured at 450nm in a microplate reader.

Determination and quantification of Glycosaminoglycans (GAGs) accumulation.

Liver tissues from 24 mice used in the oral tolerance protocol were evaluated for GAGs storage. Tissues were fixed in 4% paraformaldehyde / 2% glutaraldehyde and embedded in Spurr's resin. Assessment of GAGS accumulation was performed on toluidine-stained one-micron sections using light microscopy. Both the number of cells with cytoplasmic vacuoles as well as total number of vacuoles were counted in eight random microscopic high power fields ($\times 100$ oil immersion). A score from 0 to 5 (0 = none, 1 = 1; 2 = 2-3; 3 = 4-5, 4 = 6-7 and 5 \geq 7) was assigned according to the number of cells containing vacuoles per high power field. The total number of vacuoles per high power field was also counted in each of the eight random microscopic power fields. Data was obtained and tabulated blindly. The media of both, number of cells with vacuoles and total number of vacuoles, was calculated and compared among the different groups. Tolerized and non-tolerized MKC mice treated by ERT were compared.

Quantification of Keratan Sulfate (KS) levels in plasma.

A high sensitive Keratan Sulfate ELISA kit (Seikagaku Biobusiness) was used to detect KS levels in mice plasma samples. The assay was performed per manufacturer's instructions. Briefly, 20 μ L of five-fold diluted mice plasma sample were placed in an antibody-coated 96-well microplate and incubated for 1 hour at room temperature. After five washes, 100 μ L of HRP-Conjugated antibody were added to the plate and incubated for 1 hour at room temperature. Five additional washes were performed and 100 μ L of substrate solution were used for color development. After 30 minutes of incubation at room temperature, 100 μ L of stop solution was used. Then the absorbance was measured at 450nm in a microplate reader.

Statistical analysis.

The results were expressed as mean \pm standard deviation for each group of mice. Statistical analyses were performed with IBM SPSS Statistics 23.0. Two tailed paired t-test was used to compare the differences between groups. P values were adjusted for multiple comparisons by controlling the false discovery rate using the Benjamin and Hochberg method (77).

Study approval.

All mice were housed in a pathogen-free environment with normal diet. All procedures were in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines under approved protocols at Saint Louis University and followed the NIH's Guide for the Care and Use of Laboratory Animals (8th ed., National Academies Press, 2011).

Author contributions

A.C.S. and A.M.M. conceived and designed the study; A.C.S., A.M.M., B.K., and Q.G. performed experiments; A.P.K., C.J.B. and A.K.C. provided additional technical guidance and expertise; M.A.G. conducted the pathological evaluation; E.A. performed statistical analyses, L.A.B. and S.T. assisted in manuscript preparation and discussions; A.M.M. supervised the study; and A.C.S. and A.M.M. wrote the manuscript, with input from all authors.

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FIGURES AND FIGURE LEGENDS

Figure 1. Selection of immunodominant peptides within GALNS enzyme. (A) Location of the synthetic peptides in the 3D structure of GALNS enzyme. Peptide A2 (Blue), B3 (Green), C4 (Pink), D9 (Yellow), E8 (Violet), F5 (Orange), G6 (Cyan), H7 (Light blue), I10 (Purple) and J1 (Light green). The active site of the protein (C79) is shown in red. **(B-F). Selection of immunodominant peptides after splenocytes in-vitro stimulation.** MKC mice were treated by 16 intravenous weekly infusions of human GALNS (E: black dots) or PBS (P: red dots). Ten days after the last infusion, mice were euthanized and splenocytes were in-vitro stimulated with GALNS or a single peptide. The background levels from unstimulated cells were subtracted. **(B)** Levels of splenocyte proliferation (n = 6 measurements from two different mice) and **(C-F)** secretion levels of cytokines **(C)** IFN- γ , **(D)** IL-4, **(E)** IL-5 and **(F)** IL-13. (n = 9 measurements from three different mice). Data are shown as scatter plots with mean \pm 95%. *p<0.05; **p<0.01; ***p<0.001 represent statistically significant difference between treated and untreated mice.

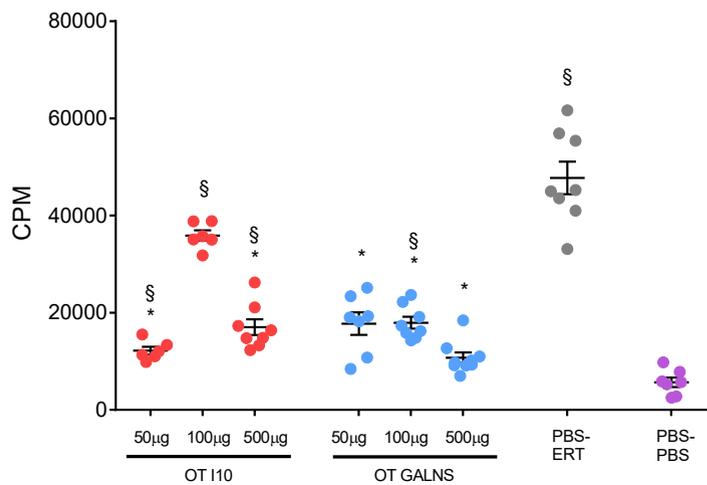
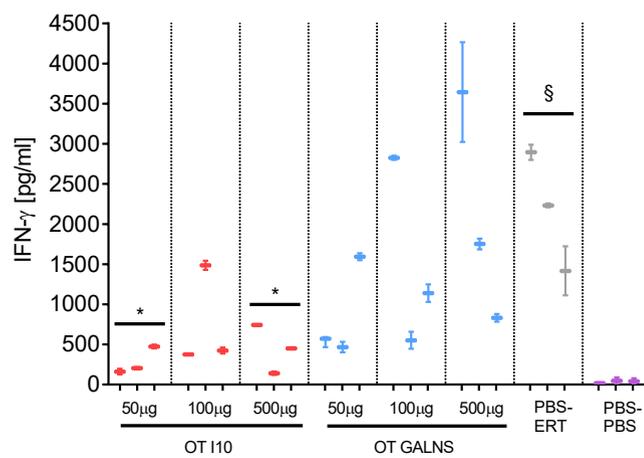
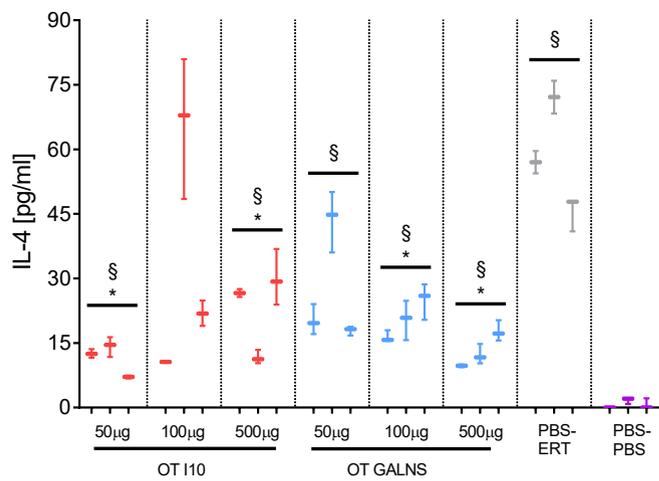
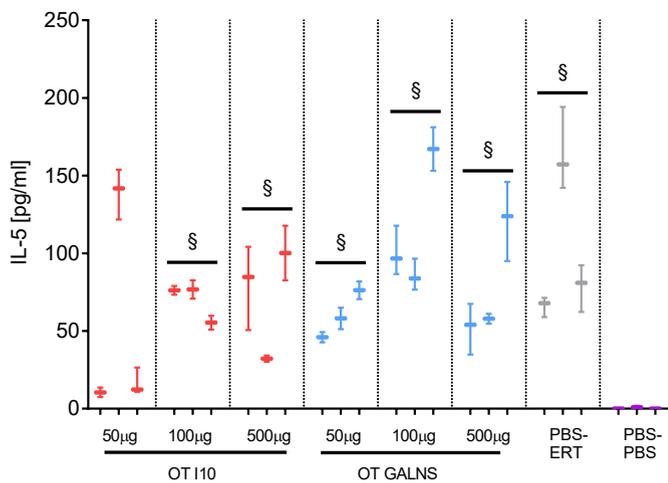
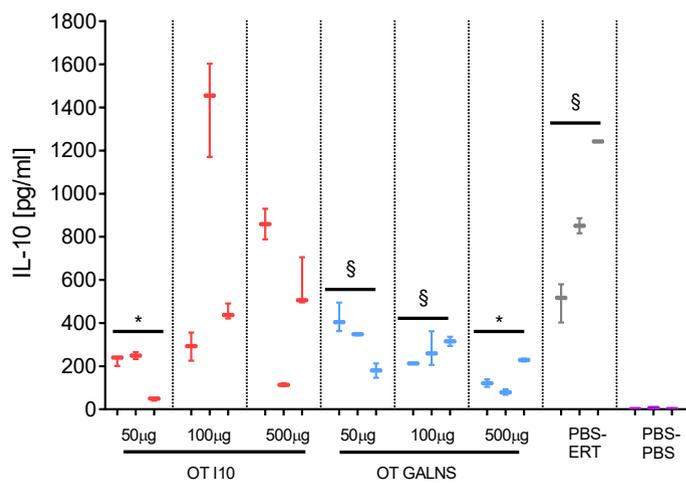
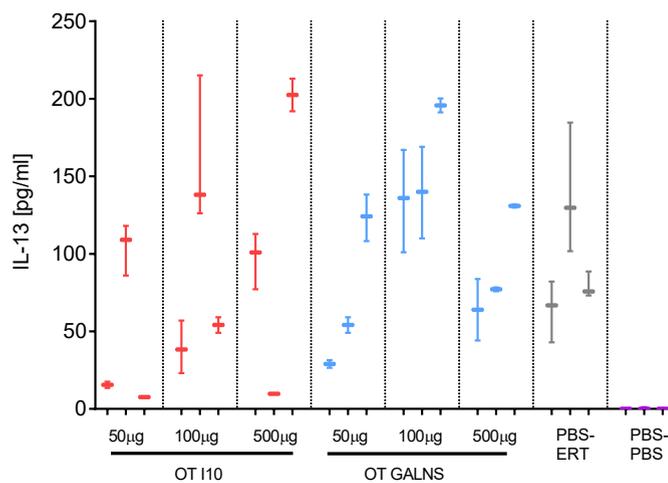
A**B****C****D****E****F**

Figure 2. Effect of tolerance induction on cellular response after the in-vitro GALNS stimulation of splenocytes. Oral tolerance was induced by feeding MKC mice with 50, 100 or 500 μ g of peptide I10 (red) or GALNS enzyme (blue). Control groups were fed with PBS (grey and purple). One week after the last oral dose, mice received 16 weekly intravenous infusions of human GALNS (red, blue and grey) or PBS (purple). Ten days after the last infusion, mice were euthanized and splenocytes were in-vitro stimulated with GALNS. The background levels from unstimulated cells were subtracted. The induction of tolerance was evaluated by **(A)** levels of splenocyte proliferation (Data are shown as scatter plots with mean \pm 95%. Each scatter plot represents the average of three measurements for each mouse, n=3 mice per group), and **(B-F)** secretion levels of **(B)** IFN- γ , **(C)** IL-4, **(D)** IL-5, **(E)** IL-10, and **(F)** IL-13. Quantitative data are represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, median line, and whiskers ranging from 5th and 95th percentile values of the average of two measurements for each mouse, n=3 mice per group. * Benjamini and Hochberg adjusted p values represent statistically significant difference between tolerized and non-tolerized (PBS-ERT) mice. **(A)** p<0.04, **(B)** p<0.025, **(C)** p<0.04, **(D)** p=not significant (ns), **(E)** p<0.03, and **(F)** p=ns. § Benjamini and Hochberg adjusted p values represent statistically significant difference between ERT treated mice and untreated (PBS-PBS) mice. **(A)** p<0.03, **(B)** p<0.008, **(C)** p<0.04, **(D)** p=0.04, **(E)** p<0.02, and **(F)** p=0.02

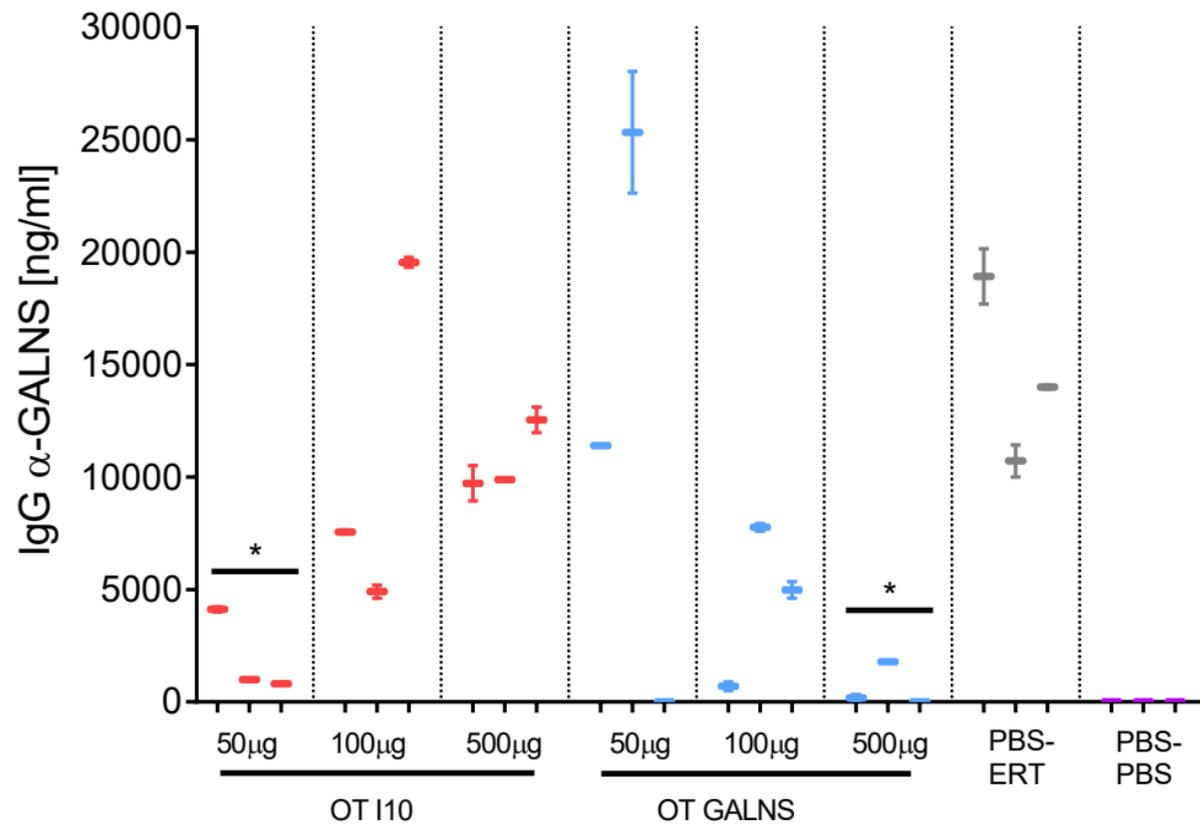
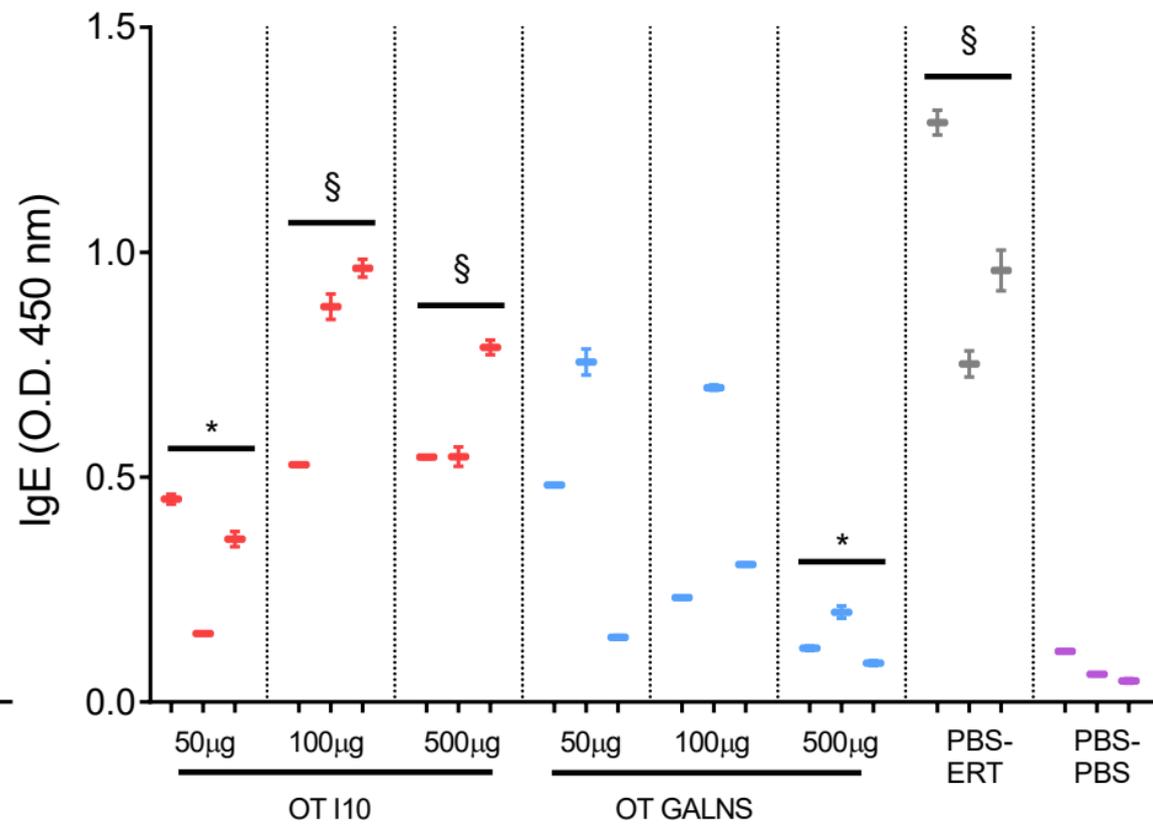
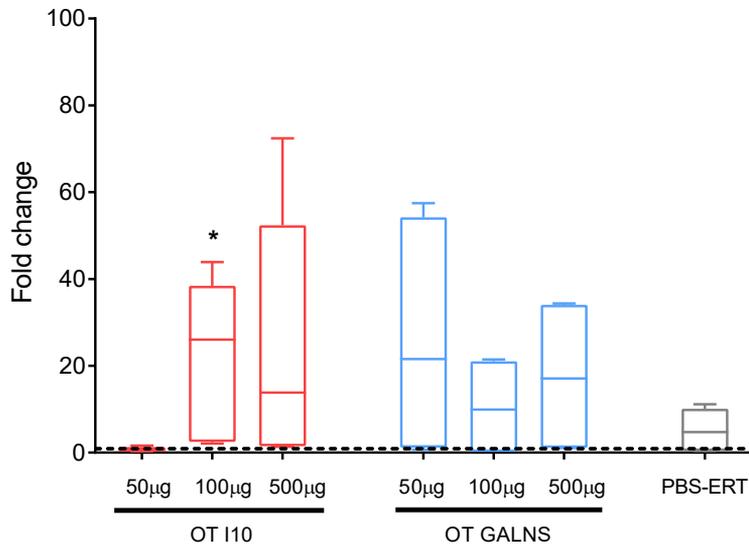
A**B**

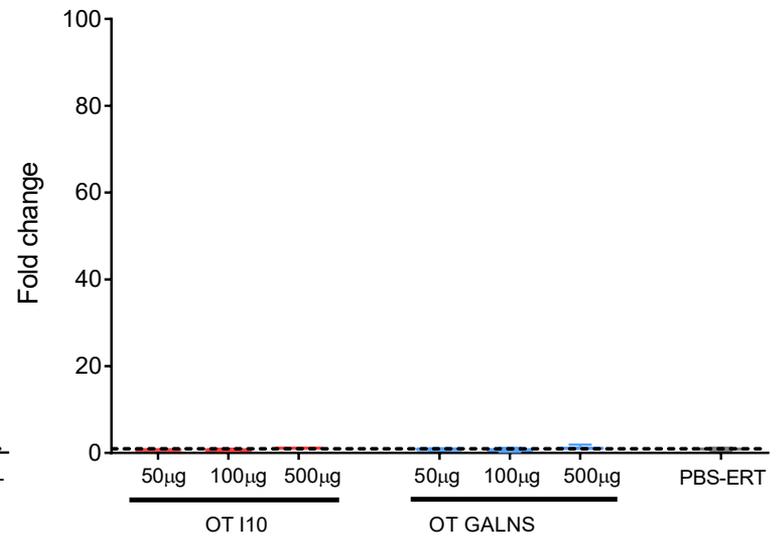
Figure 3. Effect of tolerance induction on humoral response to GALNS. Oral tolerance was induced by feeding MKC mice with 50, 100 or 500 μ g of peptide I10 (red) or GALNS enzyme (blue). Control groups were fed with PBS (grey and purple). One week after the last oral dose, mice received 16 weekly intravenous infusions of human GALNS (red, blue and grey) or PBS (purple). The induction of tolerance was evaluated in plasma samples after 16 intravenous infusions by **(A)** Levels of IgG specific to GALNS and **(B)** Levels of IgE specific to GALNS. Quantitative data are represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, median line, and whiskers ranging from 5th and 95th percentile values of the average of two measurements for each mouse, n=3 mice per group of treatment. * Benjamini and Hochberg adjusted p values represent statistically significant difference between tolerized and non-tolerized (PBS-ERT) mice. **(A, B)** p<0.01. § Benjamini and Hochberg adjusted p values represent statistically significant difference between ERT treated mice and untreated (PBS-PBS) mice. **(B)** p<0.02.

A

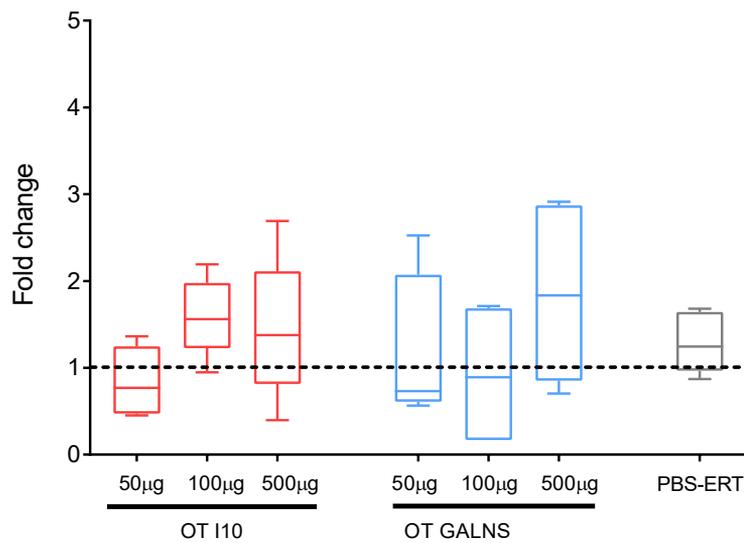
Foxp3 in MLN

**B**

Foxp3 in Peyer's Patches

**C**

Tgf-β1 in MLN

**D**

Tgf-β1 in Peyer's Patches

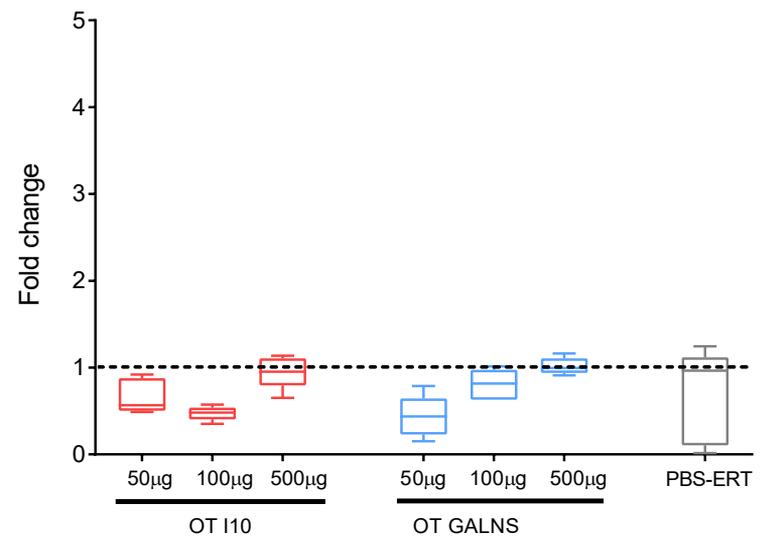


Figure 4. Mesenteric lymph nodes play a role in oral tolerance to GALNS. RNA from MLN and PP of tolerized and non-tolerized mice were used to perform quantitative RT-PCR analysis. Expression levels of Foxp3 were measured in **(A)** MLN and **(B)** PP. Expression levels of Tgf- β were measured in **(C)** MLN and **(D)** PP. Fold change indicates the change in RNA transcripts of tolerized mice and non-tolerized mice normalized by untreated (PBS-PBS) mice. Quantitative data are represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, median line, and whiskers ranging from 5th and 95th percentile values of nine measurements from three mice. * Benjamini and Hochberg adjusted p values represent statistically significant difference between tolerized and non-tolerized (PBS-ERT) mice. **(A)** $p < 0.025$. The dotted horizontal line indicates 1-fold change.

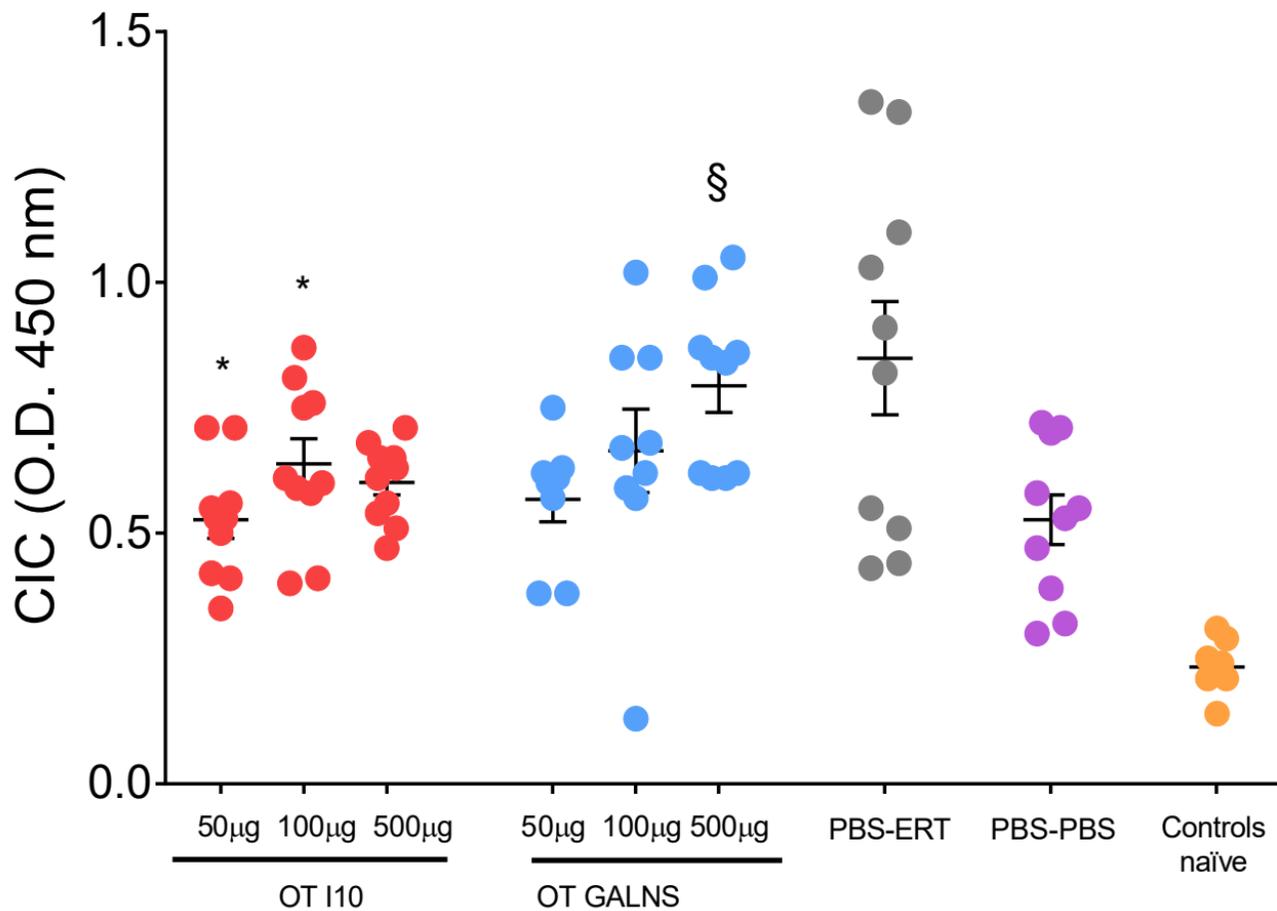


Figure 5. Circulating immune complexes in plasma. Quantities of M complement proteins C1q, C3, C4, C5 bound to circulating immune complexes (CIC) were measured using a preceptor – CIC enzyme-linked immunosorbent assay (ELISA) kit. Oral tolerance was induced by feeding MKC mice with 50, 100 or 500 μg of peptide I10 (red) or GALNS enzyme (blue). Control groups were fed with PBS (grey and purple). One week after the last oral dose, mice received 16 weekly intravenous infusions of human GALNS (red, blue and grey) or PBS (purple). Blood samples were obtained ten days after the last infusion. Controls naïve MKC mice did not undergo any treatment (orange). Data are shown as scatter plots with mean \pm 95%. Each scatter plot represents the average of ten measurements from 5 mice. * Benjamini and Hochberg adjusted p values represent statistically significant difference between tolerized and non-tolerized (PBS-ERT) mice. $p < 0.05$. § Benjamini and Hochberg adjusted p values represent statistically significant difference between ERT treated mice and untreated (PBS-PBS) mice. **(B)** $p < 0.007$.

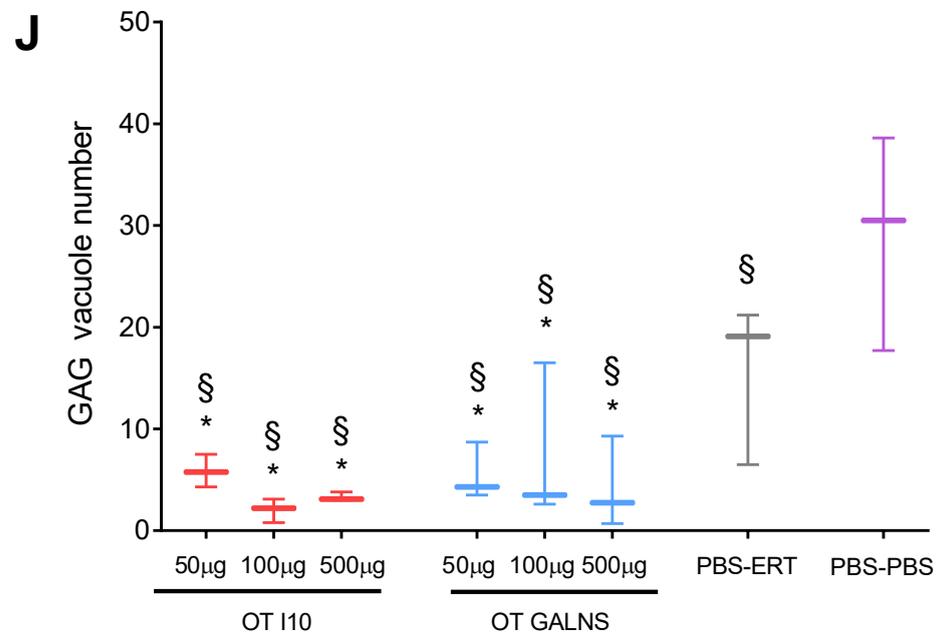
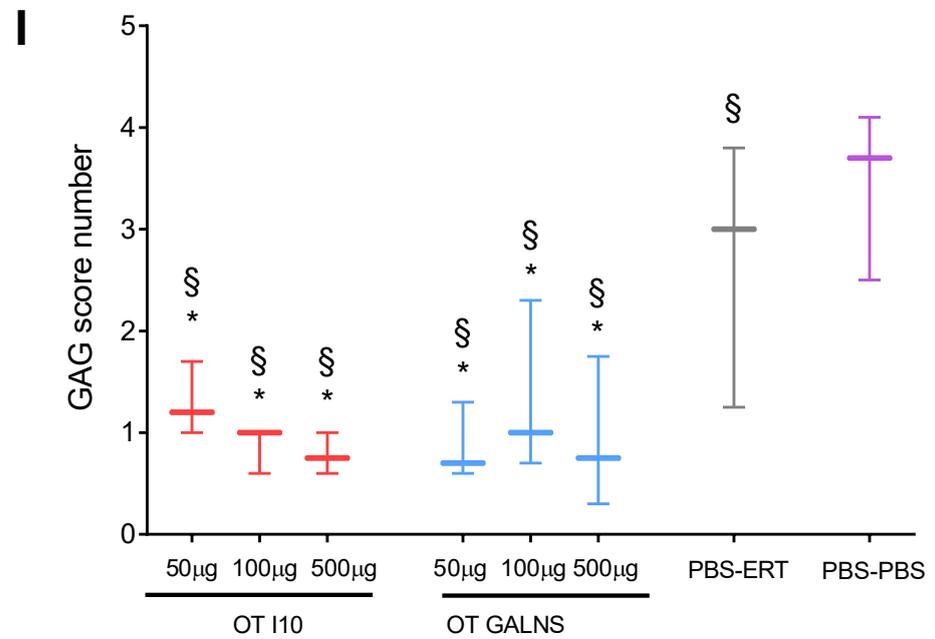
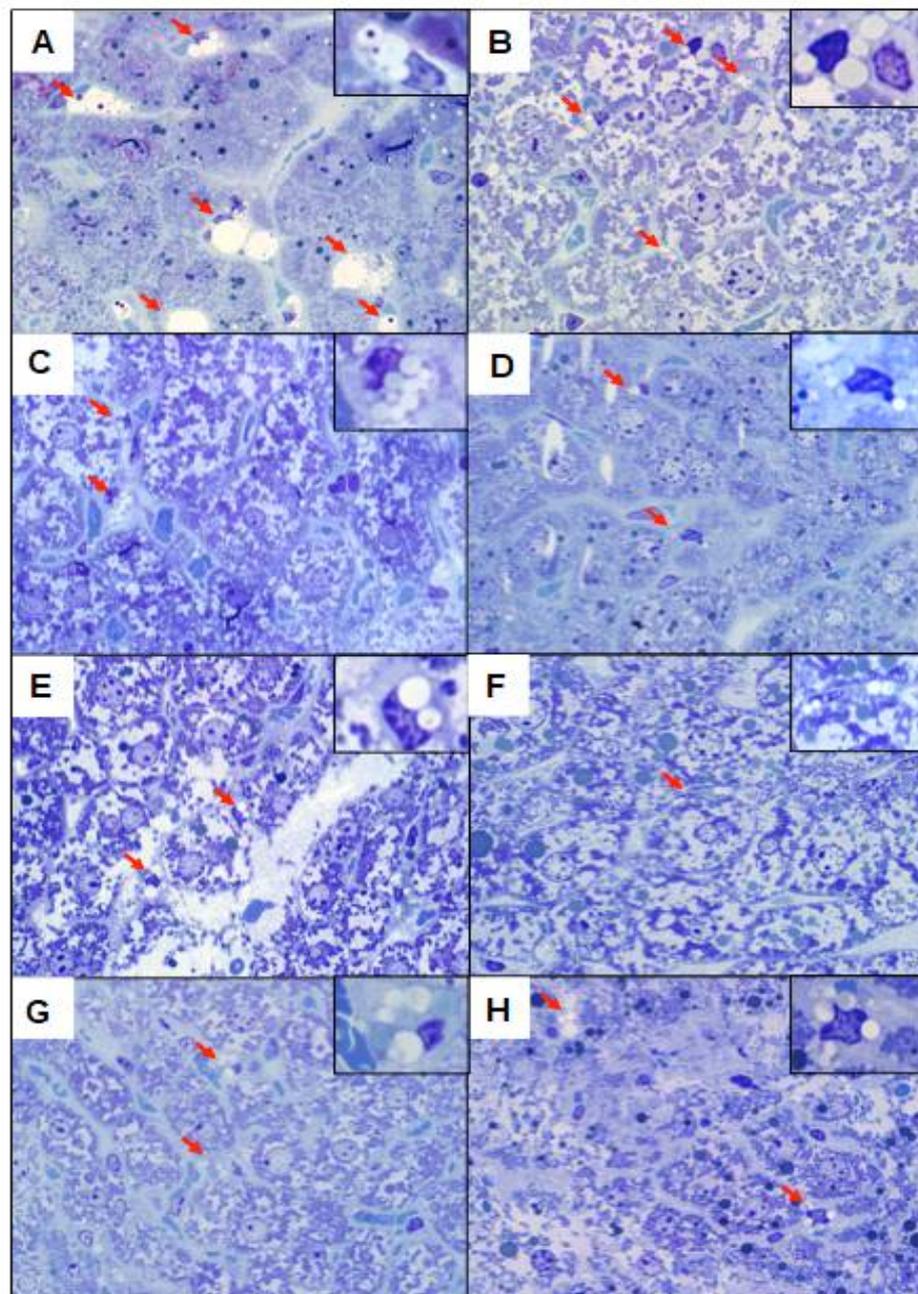


Figure 6. Glycosaminoglycans (GAGs) accumulation in liver. Light microscopic findings of toluidine-blue stained, one-micron thick sections of mice liver from **(A)** PBS control group, **(B)** ERT non-tolerized group, and tolerized groups either with Peptide I10 (**C**, 50 µg; **D**, 100 µg and **E**, 500 µg) or GALNS (**F**, 50 µg; **G**, 100 µg and **H**, 500 µg) prior to ERT. The red arrows and insets show GAGs aggregates. Accumulation of GAGs was significantly higher in control groups **(A,B)** and much less in treated groups **(D,H)**. Wild type mouse cells do not have any GAG accumulation because they have a functional GALNS enzyme. Original magnification, 100X. The number of cells with cytoplasmic vacuoles **(I)** as well as total number of vacuoles **(J)** were counted in eight random microscopic high-power fields (100X). A score from 0 to 5 (0 = none, 1 = 1; 2 = 2-3; 3 = 4-5, 4 = 6-7 and 5 ≥ 7) was assigned according to the number of cells containing vacuoles per high power field. Quantitative data are represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, median line, and whiskers ranging from 5th and 95th percentile values. * Benjamini and Hochberg adjusted p values represent statistically significant difference between tolerized and non-tolerized (PBS-ERT) mice. **(I, J)** p<0.05. § Benjamini and Hochberg adjusted p values represent statistically significant difference between ERT treated mice and untreated (PBS-PBS) mice. **(I, J)** p<0.05.

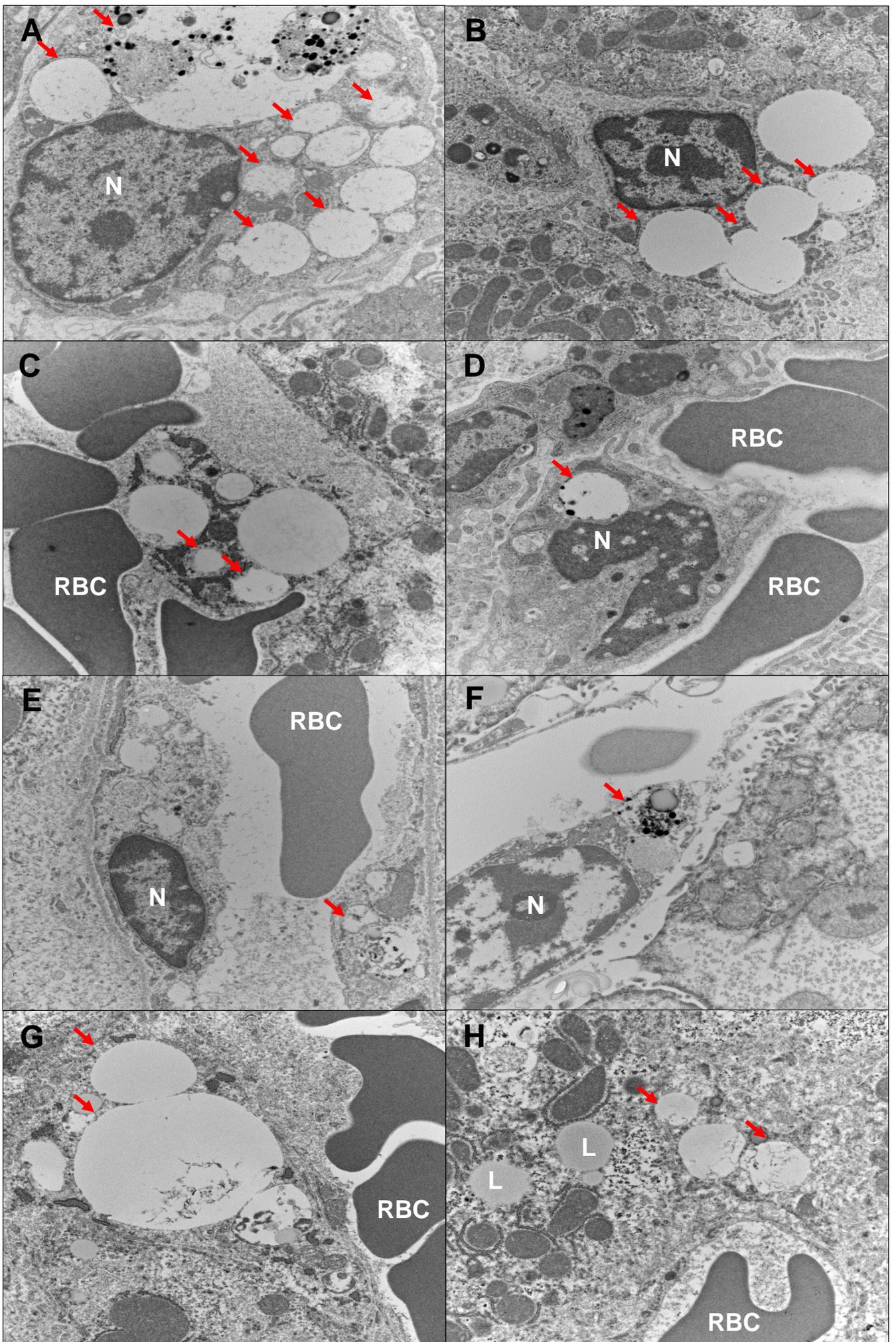


Figure 7. Ultrastructural demonstration of glycosaminoglycans (GAGs) accumulation in liver. Electron microscopic findings of mice liver from **(A)** PBS control group, **(B)** ERT non-tolerized group, and tolerized groups with Peptide 10 (**C**, 50 µg; **D**, 100 µg and **E**, 500 µg) or GALNS (**F**, 50 µg; **G**, 100 µg and **H**, 500 µg). The red arrows and insets show GAGs aggregates and distended lysosomal vacuoles. Accumulation of GAGs was significantly higher in control groups **(A,B)** and much less in treated groups **(D,H)**. Original magnification: 15,000X. RBC: red blood cells; N: nucleus; L: lipid vacuole.

Figure 8

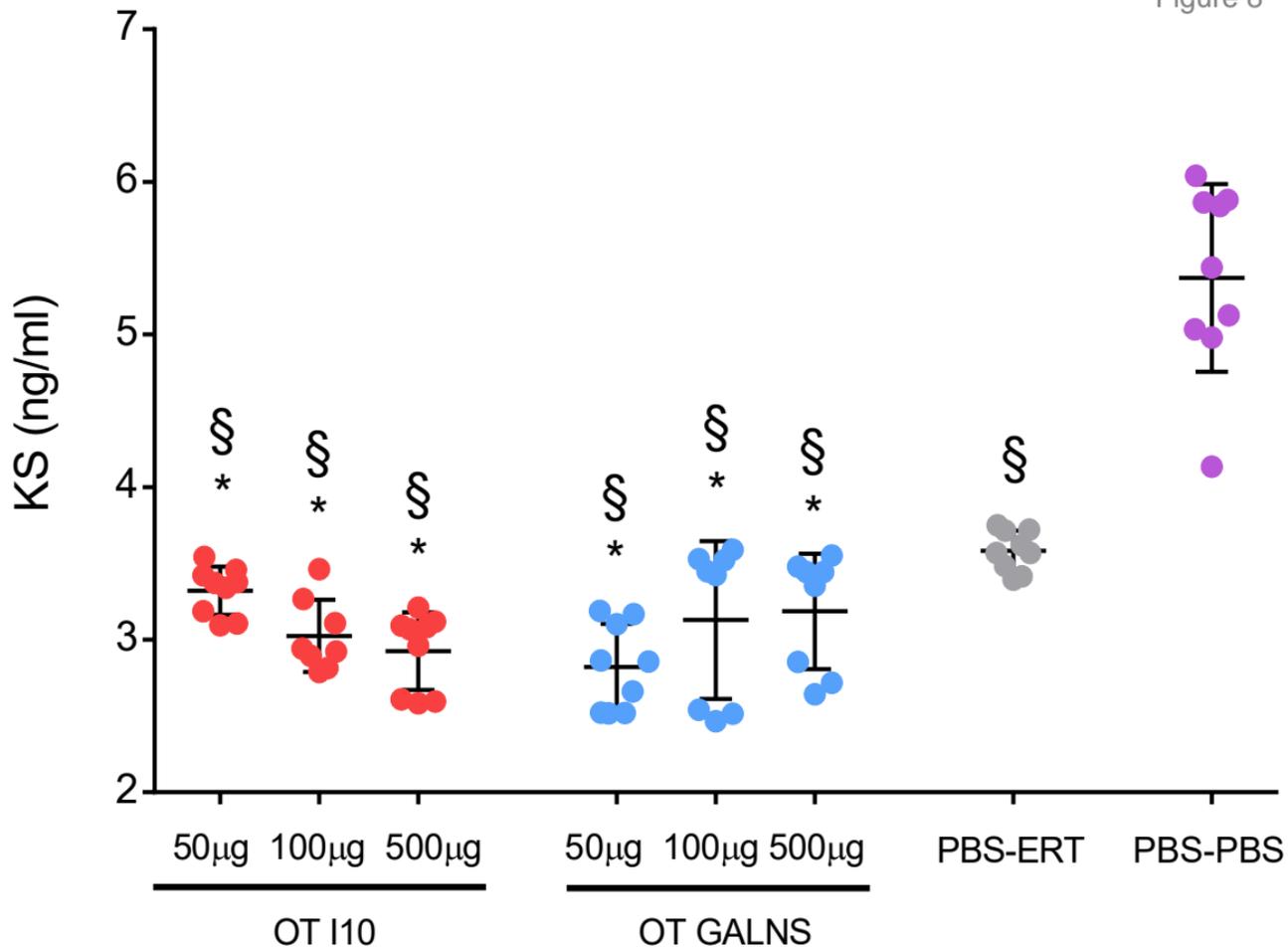


Figure 8. Keratan Sulfate (KS) levels in plasma. Quantities KS in plasma were measured using a high sensitive KS ELISA kit. Oral tolerance was induced by feeding MKC mice with 50, 100 or 500 μ g of peptide I10 (pink dots) or GALNS enzyme (blue dots). Control groups were fed with PBS (green and purple dots). One week after the last oral dose, mice received 16 weekly intravenous infusions of human GALNS (pink, blue and green dots) or PBS (purple dots). Blood samples were obtained ten days after the last infusion. Data are shown as scatter plots with mean \pm 95%. (n=2-3 measurements for each mouse, n=3 mice per group). * Benjamini and Hochberg adjusted p values represent statistically significant difference between tolerized and non-tolerized (PBS-ERT) mice. p<0.05. § Benjamini and Hochberg adjusted p values represent statistically significant difference between ERT treated mice and untreated (PBS-PBS) mice. p<0.05.

Table 1. Amino acid sequences of predicted GALNS synthetic peptides

GALNS epitope-containing synthetic peptides							
Peptide No	GALNS region	Structural domain	Sequence	Type of epitope	MHC Pred - IC50 [#]	RANKPEP	IEDB
A2*	44-63	α -helix/loop	GDLGVYGEPSRETPNDRMA	B	GDLGVYGEPSRETPNDRMA	GDLGVYGEPSRETPNDRMA	GDLGVYGEPSRETPNDRMA
B3 [§]	101-120	loop	NAHARNAYTPQEIVGGIPDS	B	NAHARNAYTPQEIVGGIPDS	NAHARNAYTPQEIVGGIPDS	NAHARNAYTPQEIVGGIPDS
C4 [§]	163-182	loop	PNCHFPGPYDNKARNIPVYR	B/T	PNCHFPGPYDNKARNIPVYR	PNCHFPGPYDNKARNIPVYR	PNCHFPGPYDNKARNIPVYR
D9 [§]	206-225	α -helix	TQIYLQEALDFIKRQARHHP	T	TQIYLQEALDFIKRQARHHP	TQIYLQEALDFIKRQARHHP	TQIYLQEALDFIKRQARHHP
E8 [§]	226-245	β -sheet/loop	FFLYWAVDATHAPVYASKPF	T	FFLYWAVDATHAPVYASKPF	FFLYWAVDATHAPVYASKPF	FFLYWAVDATHAPVYASKPF
F5*	241-260	α -helix/loop	ASKPFLGTSQRGRYGDVAVRE	B/T	ASKPFLGTSQRGRYGDVAVRE	ASKPFLGTSQRGRYGDVAVRE	ASKPFLGTSQRGRYGDVAVRE
G6 [§]	291-310	loop	AALISAPEQGGGNGPFLCGK	B	AALISAPEQGGGNGPFLCGK	AALISAPEQGGGNGPFLCGK	AALISAPEQGGGNGPFLCGK
H7*	347-366	α -helix/loop	TTSLALAGLTPPSDRAIDGL	B	TTSLALAGLTPPSDRAIDGL	TTSLALAGLTPPSDRAIDGL	TTSLALAGLTPPSDRAIDGL
I10 [§]	473-492	α -helix/loop	QQHQEALVPAQPQLNVCNWA	T	QQHQEALVPAQPQLNVCNWA	QQHQEALVPAQPQLNVCNWA	QQHQEALVPAQPQLNVCNWA
J1 [§]	503-522	loop	KLKGKCLTPPESIPKKLWSH	B	KLKGKCLTPPESIPKKLWSH	KLKGKCLTPPESIPKKLWSH	KLKGKCLTPPESIPKKLWSH

* Predicted by IEDB

§ Predicted by RANKPEP

IC50: < 50 nM, <100 nM, < 200 nM

Table 2. Group of mice for the oral tolerance induction

GROUP	ORAL ADMINISTRATION		ERT
	Peptide I10	GALNS	Weekly i.v. infusions
1	50 µg		GALNS
2	100 µg		GALNS
3	500 µg		GALNS
4		50 µg	GALNS
5		100 µg	GALNS
6		500 µg	GALNS
7		PBS	GALNS
8		PBS	PBS