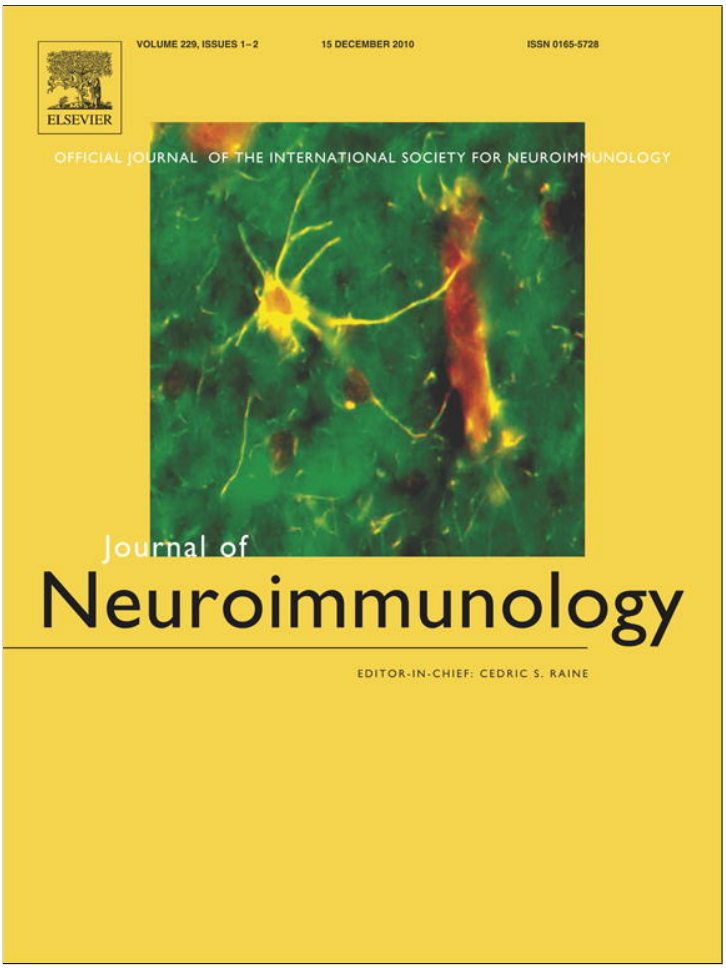

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# A novel galectin-1 and interleukin 2 receptor β haplotype is associated with autoimmune myasthenia gravis

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ABSTRACT

Galectin-1 (LGALS1) and interleukin receptor 2β (IL2Rβ) are regulators of T-cell activation. Here we evaluated the association of regulatory region polymorphisms of the *LGALS1* (rs4820293, rs4820294) and *IL2Rβ* (rs743777, rs228941) genes in 146 Caucasian myasthenia gravis patients compared to 291 ethnically matched controls. A significant difference was found in the distribution of the rs4820293/rs743777 polymorphism haplotypes ($p<0.01$). The rs4820293 polymorphism, previously not described to be associated with any disease, does not affect LGALS1 expression in peripheral mononuclear cells and skeletal muscle. Pathway analysis revealed interaction between LGALS1 and IL2Rβ suggesting a role of these proteins in this rare disease.



## 1. Introduction

Myasthenia gravis (MG) is a tissue-specific, T-cell mediated autoimmune disease characterized by autoantibodies against different components of the neuromuscular junction. In 70–90% of the patients these antibodies target the nicotinic acetylcholine receptor (AChR). In addition to environmental risks, genetic factors also contribute to the development of MG. Both HLA and non-HLA gene polymorphisms have been reliably associated with this disease, although their precise function at the molecular level still remains elusive (Giraud et al., 2008).

Galectins belong to a family of carbohydrate binding proteins with high affinity to β-galactosides. Based on the prophylactic and therapeutic activities of a lectin from the electric eel in experimental MG in rabbits (Levi et al., 1983), members of the respective lectin family, the human adhesion/growth-regulatory galectins (Villalobo et al., 2006; Schwartz-Albiez, 2009), are attractive candidates as players in the pathomechanism of MG. Of note, the recently described role of galectin-1 (LGALS1) from regulatory T-cells in autoimmune suppression via crosslinking of ganglioside GM1 of effector T-cells and also its abundant presence in muscle tissue, even useful for fibre typing, direct interest to the prototype LGALS1 (Bardosi et al., 1989; Wang et al., 2009). It is also highly expressed in the thymus, a tissue often showing pathology in MG, where it can induce apoptosis in thymocyte subsets (Earl et al., 2010). Its physiological relevance is further underscored by serving as transcriptionally upregulated effector of the tumor suppressor $p16^{INK4a}$ and binding partner of the oncogenic H-Ras (Villalobo et al., 2006; André et al., 2007). Though LGALS1 is a key player in immunomodulation, polymorphisms of its gene have not been associated with disease to date.

The *Interleukin receptor 2β* gene (*IL2Rβ*) is found in the 22q13 chromosomal region, 500 kilobases upstream of *LGALS1*. Its protein product is a component of the IL2 signalling pathway, which is essential for T-cell activation. The rs743777 single nucleotide polymorphism (SNP) of *IL2Rβ*, localised in the 5′ near gene region, has been shown to be associated with rheumatoid arthritis in the Caucasian population (Barton et al., 2008; Wellcome Trust, 2007) using large scale whole genome association studies, while no information on MG is available.

Here we investigate the association of MG with two novel SNPs in the 5′-regulatory region of *LGALS1* and two regulatory region polymorphisms of *IL2Rβ*.

## 2. Subjects and methods

### 2.1. Study population

146 Hungarian MG patients selected from the NEPSYBANK (Hungarian Neurological and Psychiatric Biobank) (Molnar and Bencsik, 2006) and 291 controls enrolled from Caucasian blood

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donors and individuals participating on routine check-ups without any known autoimmune disease were the subjects of our study. Diagnosis of MG was based on standard criteria including symptoms of fluctuating muscle weakness supported by an electromyographic pattern of neuromuscular transmission dysfunction by repetitive stimulation. Data of patients and controls are summarized in Table 1. Five MG patients also suffered from rheumatoid arthritis defined by the criteria of the American College of Rheumatology (Arnett et al., 1988). As an association of the rs743777 SNP with rheumatoid arthritis was described earlier (Barton et al., 2008), thus, we excluded these patients from the detailed statistical analysis. Muscle samples without morphological changes were obtained from the biobank of The Centre for Molecular Neurology, Semmelweis University. The biopsies, carried out due to muscle pain ($n=6$), presented no muscle pathology by standard histochemical and immunohistochemical protocols (Sewry and Molnar, 2010). All patients and controls gave written informed consent. This study was carried out according to the Helsinki Declaration and was approved by the local Ethical Committees.

### 2.2. Determination of anti-AChR antibody concentration

AChR-specific antibody levels were measured with radioreceptor-immunoassay (DRG Diagnostics, Germany) according to the manufacturer's instructions. In the assay $^{125}$I labelled α-bungarotoxin was incubated with test sera and complexes of labelled receptor and antibodies were immunoprecipitated with anti-human IgG and counted in a γ counter. The amount of radioactivity was directly proportional to the concentration of anti-AChR autoantibodies of the sample.

### 2.3. Prediction of functional SNPs

We used the ELDORADO software of the Genomatix website (www.genomatix.de) to search for alterations in transcription factor binding sites coinciding with SNPs within the 5′-regulatory region of the human *LGALS1* gene and the regulatory regions of the *IL2Rβ* gene.

### 2.4. DNA isolation and polymorphism analysis

Genomic DNA was extracted from peripheral blood and human muscle biopsy samples using the QIAamp DNA blood and tissue mini kit (Qiagen, Valencia, California, USA), according to the manufacturer's recommendations. Genotypes were determined using TaqMan Allelic Discrimination Assays (Assay IDs: C_2495792_10, C_2495793_10, C_11885292_10 and C_2403397_10 for galectin-1 rs4820293, and rs4820294 as well as IL2Rβ rs228941 and rs743777, respectively; Applied Biosystems, Foster City, CA, USA) using a standard protocol (Srivastava et al., 2010). Briefly, polymerase chain reactions (PCR) were carried out in 96 well format with the ABIPRISM 7000 Sequence Detector (Applied Biosystems) with mixes consisting of 10 ng of genomic DNA, 5 μl of TaqMan Universal PCR Master Mix, 0.25 μl of 40× Assay Mix, and double-distilled water up to 10 μl final volume. Thermal cycle conditions were as follows: denaturation at 95 °C 10 min, followed by 40 cycles of denaturation at 92 °C for 15 s, and annealing and extension at 60 °C for 1 min. After PCR the endpoint fluorescence intensity was read in each well. The allelic specific intensity data of each plate were analysed using the ABI PRISM 7000 software (Applied Biosystems) to automatically determine the genotype of each sample.

**Table 1**
Characteristics of the study population.

| | Patients | Controls |
|---|---|---|
| Number of population | 146 | 291 |
| Gender *n* (%) | | |
| Male | 27 (18) | 84 (29) |
| Female | 119 (82) | 207 (71) |
| Age (mean ± SD, years) | 50 ± 13.2 (range:18–78) | 37.5 ± 13.7 (range:18–83) |
| Age at onset (mean ± SD, years) | 36.2 ± 13.4 (range:7–73) | – |
| AChR antibody positivity *n* (%) | 104 (71) | – |
| Thymus pathology *n* (%) | 101 (69) | – |
| Thymus hyperplasia | 89 (61) | |
| Thymoma | 9 (6) | |
| Thymus carcinoma | 3 (2) | |
| Distribution of weakness *n* (%)[a] | | |
| Ocular | 32 (19) | – |
| Generalized | 114 (81) | – |

SD = standard deviation, AChR = acetylcholine receptor. Out of the 32 ocular myasthenia patients, to date 21 developed generalized symptoms during the course of their disease.
[a] Distribution of weakness at disease onset.

### 2.5. Isolation and stimulation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated with Histopaque (Sigma-Aldrich, St Louis, MO, USA) from healthy controls of AA and GG genotypes for the galectin-1 rs4820293 SNP ($n=4$–4, respectively), and grown in OPTIMEM medium with 2% FCS for 48 h. Cells ($5\times10^6$/5 ml) were grown in the presence or absence of 3 μg/ml concanavalin A (Sigma-Aldrich) and used subsequently for isolation of RNA.

### 2.6. RNA isolation and real-time PCR

RNA was isolated from cells using the RNeasy mini kit (Qiagen) following the manufacturer's protocol. First strand cDNA was produced using random hexamers (Promega, Madison, WI, USA). Real-time PCR was carried out on a ABIPRISM 7000 Sequence Detector (Applied Biosystems) using galectin-1, CTCF and GUSB expression assays from Applied Biosystems, assay IDs: galectin-1:Hs00355202_m1, CTCF: Hs00902007_m1, and GUSB: Hs99999908_m1, respectively. In brief, 1.2 μl of cDNA was added to 5.52 μl $H_2O$. Also, 0.76 μl of Taqman gene expression assay and 7.5 μl of the 2× PCR mastermix (Applied Biosystems) was added to the above mixture to a final total reaction volume of 15 μl/well. The reactions were amplified for 15 s at 95 °C and 1 min at 60 °C for 45 cycles followed by the thermal denaturation protocol. All reactions were run in duplicate and included no template and no reverse transcription controls for each gene. Analyses of real-time quantitative PCR data were performed using the comparative threshold cycle method. The relative amount of mRNA was referred to the one of Glucuronidase B (GUSB).

### 2.7. Immunohistochemistry (IHC)

Immunohistochemical staining was performed on tissue samples snap-frozen in liquid nitrogen and isopentane. The frozen sections were fixed in a 1:1 ice-cold ethanol/acetone mixture for 5 min and then rinsed in PBS for 5 min. Goat anti-human galectin-1 antibody (R&D Systems, Minneapolis, MN, USA) was used in a 1:10 dilution for 1 h. Negative controls were run without primary antibody (not shown). Biotinylated anti-goat antibody (Vector Laboratories Inc., Buringame, CA, USA) was used at 1:200, while streptavidin-FITC (DAKO A/S Denmark) antibody was used at 1:1000 dilution for 45 min and 30 min, respectively. After washing three times, sections were mounted with Prolong-Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA), and stored in the dark at 4 °C. Next day they were examined with a Nikon Eclipse 80i fluorescence microscope using the Spot Advanced software (version 4.6, Diagnostic Instruments Inc., Sterling Heights, MI, USA). Micro-photographs were recorded at 40× magnification.

**Table 2**
Genotyped SNPs, genotype and allele frequencies and deviation from the Hardy–Weinberg equilibrium.

| SNP | Alleles (1/2)[a] | Position[b] | Function | Genotype 11[a] | | Genotype 12[a] | | Genotype 22[a] | | N[c] | Allele2 (%)[d] | HWE[e] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| rs4820293 | G/A | 36400867 | 5′ near gene | 96[f] | 32% | 147 | 51% | 48 | 16% | 291 | 0.42 | 0.51 |
| | | | | 40[g] | 27% | 61 | 42% | 45 | 31% | 146 | 0.52 | 0.05 |
| rs4820294 | G/A | 36400989 | 5′ near gene | 111[f] | 41% | 132 | 47% | 37 | 13% | 280 | 0.37 | 0.82 |
| | | | | 57[g] | 42% | 64 | 47% | 16 | 12% | 137 | 0.35 | 0.76 |
| rs743777 | A/G | 35881553 | 5′ near gene | 138[f] | 50% | 112 | 41% | 24 | 9% | 274 | 0.29 | 0.85 |
| | | | | 55[g] | 39% | 62 | 44% | 23 | 16% | 140 | 0.39 | 0.43 |
| rs228941 | C/G | 35853667 | 3′ UTR | 152[f] | 55% | 102 | 37% | 23 | 8% | 277 | 0.32 | 0.32 |
| | | | | 79[g] | 53% | 57 | 39% | 12 | 8% | 148 | 0.27 | 0.70 |

[a] Alleles on the forward strand; 1, frequent allele; 2, rare allele.
[b] Position according to the HapMap database.
[c] Number of controls or patients successfully genotyped for the SNP.
[d] Frequency for allele2.
[e] HWE: deviation from the Hardy–Weinberg equilibrium ($\chi^2$ test).
[f] Control data—upper row.
[g] Patient data—lower row.

### 2.8. Western blot analysis

Whole tissue lysates from muscle samples were prepared using ProteoJet cell lysis buffer (Fermentas, Burlington, ON, Canada) supplemented with 1 μM leupeptin and aprotinin, 200 μM phenylmethyane sulfonyl fluoride, and 100 mM sodium orthovanadate protease inhibitors (all from Sigma-Aldrich). SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on pre-cast 12% Bis–Tris gels (Lonza, Basel, Switzerland) according to the manufacturer's recommendations on a Mini-Cell electrophoresis system (BioRad Laboratories, Hercules, CA, USA) using 15 μg of protein/lane. For immunoblotting, proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membranes (BioRad Laboratories, Hercules, CA, USA). Anti-human galectin-1 antibody (R&D Systems, Minneapolis, MN, USA) or monoclonal mouse anti-human β-actin antibody (Sigma-Aldrich) were used at 1:1000 and 1:5000, respectively. Rabbit anti-goat-HRP (R&D Systems, Minneapolis, MN, USA) and HRP-conjugated goat anti-mouse IgG (DAKO A/S Denmark) were used as secondary antibodies respectively, both at 1:5000 for 1 h. The proteins were visualized using the ECL chemiluminescence detection system (GE Healthcare, Buckinghamshire, UK).

Semi-quantification of galectin-1 protein expression was carried out using the Fluorochem 5500 gel documentation system with the AlphaEaseFc software version 3.2.2.

#### 2.8.1. Statistical analysis

Both allelic and genotypic association and Cochran–Armitage tests were performed using an online Hardy–Weinberg equilibrium (HWE) calculator (footnote: http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl) based on Wigginton et al. (2005). Age and gender differed significantly in the control and patient groups; correction was made with logistic regression using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Haplotype frequencies were calculated using the PHASE (Donnelly and Stephens, 2003) and Haploview (Barrett et al., 2005) software. The strength of association was represented by odds ratio (OR) or exponentiation of the B coefficient (Exp(B)) in logistic regression models.

#### 2.8.2. Statistical analysis of the expression assays

We used a two-way ANOVA for the comparison of galectin-1 expression normalized to GusB, and a Student *t*-test to compare the expression of galectin-1 in muscle tissues between the AA and GG genotypes. $P<0.05$ was considered statistically significant.

**Table 3**
The estimated haplotype frequencies and odds ratios (GAGG is the reference haplotype).

| Haplotype | Haplotype freq. | Freq. in cases | Freq. in controls | Chi-square | *p*-value | Odd | OR |
|---|---|---|---|---|---|---|---|
| GAGG | 0.094 | 0.066 | 0.108 | 3.942 | 0.0471 | 0.611111 | 1 |
| GGAG | 0.12 | 0.149 | 0.106 | 3.38 | 0.066 | 1.40566 | 2.300172 |
| CGAA | 0.007 | 0.017 | 0.003 | 5.33 | 0.021 | 5.666667 | 9.272727 |

## 3. Results

### 3.1. Identification of putative functional SNPs

We identified the rs4820293 polymorphism of *LGALS1* gene which establishes a putative CCCTC-binding factor (CTCF) site. Furthermore, the rs4820294 polymorphism *LGALS1* gene was identified which generates a putative E2F binding site on the negative strand, while obliterating an NF1 binding site on the positive one. These SNPs are located on the negative DNA strand 722 and 600 bp 5′ from the transcription start site respectively, in the 5′ near gene regulatory region of the *LGALS1* gene (Salvatore et al., 1995). We also

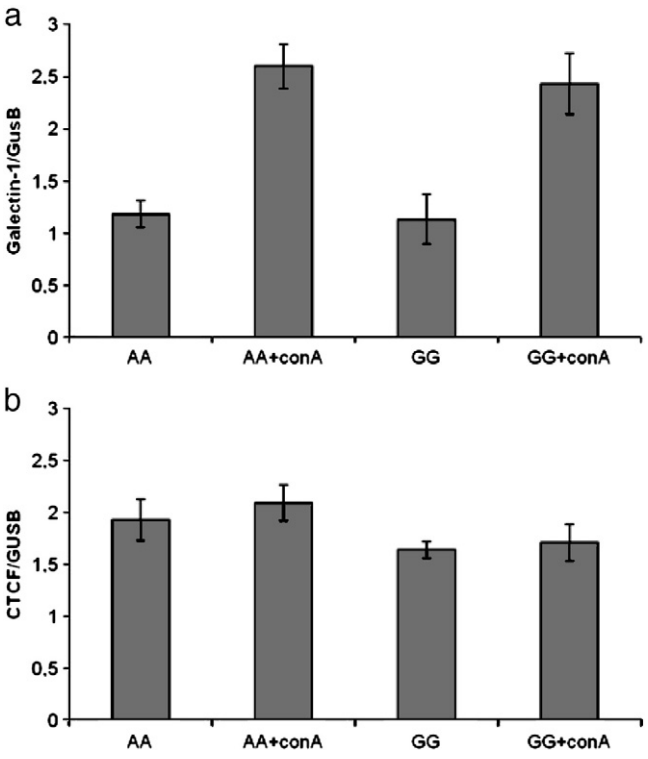
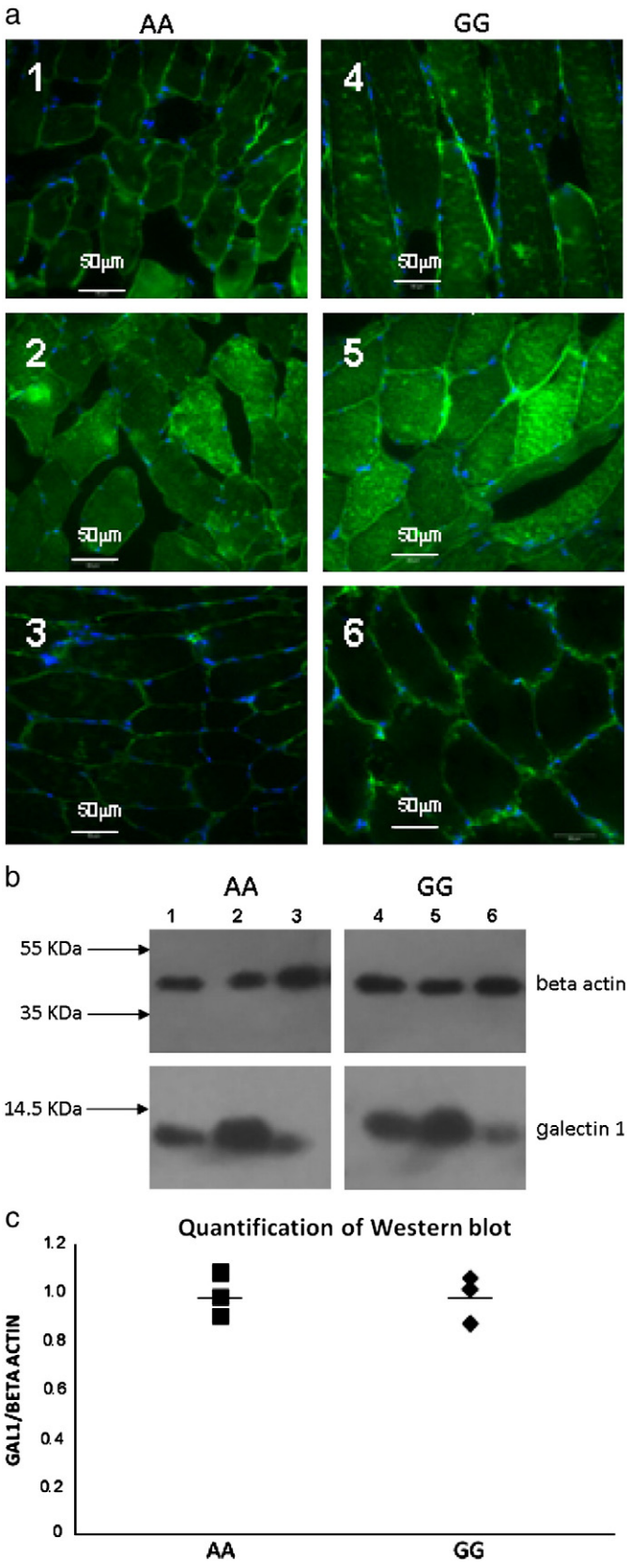



**Fig. 1.** Relative gene expression of PBMCs of individuals with different *LGALS1* genotypes. a. Galectin-1 and b. CTCF expression in human PBMCs of the different galectin-1 rs4820293 genotypes. Relative levels of gene expression were normalized with respect to the extent of expression of GUSB. We used a two-way ANOVA for the comparison of relative galectin-1 expressions in PBMCs. Error bars indicate standard error of mean values.

investigated the regulatory regions of the *IL2Rβ* gene. Our analysis revealed the disruption of a putative homeobox protein F (HOXF) binding site due to the rs743777 polymorphism, while a pleomorphic adenoma gene protein (PLAG) and Z-box binding factor (ZBPF) site is established due to the rs228941 SNP.




### 3.2. Strong association of the rs4820293 and rs743777 LGALS1/IL2Rβ haplotype with MG

Genotypes were determined for all samples, and each SNP was assessed for deviation from HWE (Table 2). Results in Supplementary Table 1 show strong association of MG with rs4820293 and rs743777. However, as age and gender differed in control and MG groups significantly, they were controlled using logistic regression and stratification. Both logistic regression and stratified analysis showed significant association of rs4820293 and rs743777 with the clinical status of MG, AChR positivity, and thymus pathology at allelic levels. In logistic regression models containing Age and Gender, and treating the SNPs as continuous variables, rs4820293 is significant for MG ($p = 0.022$, Exp(B) coefficient (coeff) = 0.701), for thymus pathology ($p = 0.037$, Exp(B) coeff. = 0.691), and for AChR positivity ($p = 0.019$, Exp(B) coeff. = 0.300). In the same logistic regression setup rs743777 is significant in the interaction with Age for MG ($p = 0.007$, Exp(B) coeff. = 0.990), for thymus pathology ($p = 0.021$, Exp(B) coeff. = 0.988), and for AChR positivity (p− = 0.022, Exp(B) coeff. = 0.989). The effect strength and significance are comparable in stratified analyses.

The rs4820293 and rs743777 show a joint effect as well, their interaction rs4820293 rs743777 is significant with $p = 0.014$, Exp(B) coeff. = 0.805 in logistic regression containing Age and Gender.

To investigate the joint effects of these SNPs, we also looked for the association at the haplotype level. Using a single block of the four SNPs in *LGALS1*and *IL2Rβ*, the haplotypes were found to be significantly associated with MG with a corrected *p*-value <0.01 using the PHASE software (Donnelly and Stephens, 2003). The haplotype frequencies in patients with MG and in controls were significantly different (Table 3). The frequencies were also computed by Haploview (data not shown) (Barrett et al., 2005). These frequencies and the derived OR values further support the linked relevance of rs4820293–rs743777 and *LGALS1–IL2Rβ*: the corresponding OR for the GGAG haplotype is 2.3 and for the "complete mutant" haplotype CGAA the OR is 9.27 (Table 3).

### 3.3. The rs4820293 polymorphism does not influence galectin-1 expression in human PBMC or muscle

The rs4820293 SNP of *LGALS1* was predicted to establish a novel putative CTCF binding site. CTCF is a zinc-finger protein, shown to alter gene expression by blocking interactions between enhancers and promoters or by altering DNA methylation (Dunn and Davie, 2003; Kim et al., 2007). Thus, we speculated that the rs4820293 polymorphism might alter transcription of the *LGALS1* gene. We tested this hypothesis in PBMCs and skeletal muscle, given that both muscle tissue and the neuromuscular junction express LGALS1 in high quantities (Bardosi et al., 1989; Svensson and Tagerud, 2009). We isolated PBMCs from 4–4 healthy individuals of AA and GG genotypes, and stimulated them for 48 h with the mitogen concanavalin A (ConA). Relative LGALS1 expression was detected by real-time PCR, normalized to GUSB. Although the expression of LGALS1 increased significantly upon stimulation, no significant difference was observed between the different genotypes in either the stimulated or control PBMCs. CTCF expression did not change upon stimulation in either of the genotypes (Fig. 1a and b).

**Fig. 2.** LGALS1 content of muscle tissue in individuals with different *LGALS1* genotypes. a. Representative samples of IHC for galectin-1 detection in normal muscle specimen with different galectin-1 rs4820293 genotypes. b. Galectin-1 Western blot of human muscle samples from individuals with different genotypes (3–3 individuals with AA and GG genotypes of the rs4820293 SNP). Numbers represent the different samples consistently in the IHC and Western blot analysis. Inter-individual variability in muscle galectin-1 expression is pronounced in both examined genotypes. c. Semi-quantification of the WB results. No significant difference in protein content can be seen between the genotypes.

Muscle samples of individuals with muscle pain, but without muscle pathology were also genotyped for the rs4820293 polymorphism. Individuals ($n = 3$–3) of the AA and GG genotypes were analysed for *LGALS1* expression using Western blot (WB) analysis (Fig. 2b) and IHC (Fig. 2a). No difference was found in LGALS1 protein level between the various genotypes normalized to β-actin (Fig. 2c) in accordance with the IHC results. However, in the GG genotype, galectin-1 distribution differs from the AA genotype muscle samples, suggesting that more secreted form can be seen.

## 4. Discussion

In this report we demonstrate for the first time a *LGALS1–IL2Rβ* haplotype association with a human disease. The *LGALS1* rs4820293 polymorphism has not been described previously, thus we tested whether it could alter galectin-1 expression in PBMCs or in muscle tissue. We found no difference in expression in either tissue. It is worth noting however, that in the muscle samples LGALS1 distribution differed between the GG and AA genotypes, as membrane staining was more intense in the former variant suggesting altered routing of LGALS1. Difference of expression of proteins in the muscles may occur upon exercise, which hypothesis was not tested here. Also CTCF may not always alter gene expression by itself (Gombert and Krumm, 2009), it may aid and potentiate the binding of other transcription factors which may exert their effect in myasthenic muscle only. Alternatively, CTCF, a major insulator protein, may act at the epigenetic level and alter methylation profiles. In view of the therapeutical potential of the fish galectin-related galectin-1 however, no evidence for activity at the muscular level had been reported (Levi et al., 1983).

It has been shown previously that LGALS1 expression can be upregulated via IL2R mediated signalling routes in PBMCs (Fuertes et al., 2004). Functional interactions between LGALS1 and IL2Rβ have been described through $Ca^{2+}$ signalling and the H-Ras route (Wang et al., 2009; Rotblat et al., 2004). Identification of such associations with disease development should provide new insight into the pathogenesis of autoimmune MG. The reported haplotype association also gives further research a clear direction, e.g. to test LGALS1 expression of regulatory T-cells, where it is a key regulator of TRPC5 $Ca^{2+}$ channels (Wang et al., 2009).

The following are the supplementary materials related to this article. Supplementary Table 1.

Supplementary materials related to this article can be found online at doi:10.1016/j.jneuroim.2010.07.015.

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