

Tight junction proteins expression and modulation in immune cells and multiple sclerosis

Ilana Mandel^a, Tamar Paperna^a, Lea Glass-Marmor^a, Anat Volkowich^a, Samih Badarny^{a, b},
Ilya Schwartz^b, Pnina Vardi^{c, d}, Ilana Koren^c, Ariel Miller^{a, e, *}

^a Rappaport Faculty of Medicine and Research Institute, Technion-Israel Institute of Technology, Haifa, Israel

^b Movement Disorders Clinic, Carmel Medical Center, Haifa, Israel

^c Pediatric Endocrinology and Diabetes Center, Armon Clalit Health Services Clinic, Haifa, Israel

^d Felsenstein Medical Research Center (FMRC), Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

^e Division of Neuroimmunology & Multiple Sclerosis Center, Carmel Medical Center, Haifa, Israel

Received: March 20, 2011; Accepted: July 1, 2011

Abstract

The tight junction proteins (TJPs) are major determinants of endothelial cells comprising physiological vascular barriers such as the blood–brain barrier, but little is known about their expression and role in immune cells. In this study we assessed TJP expression in human leukocyte subsets, their induction by immune activation and modulation associated with autoimmune disease states and therapies. A consistent expression of TJP complexes was detected in peripheral blood leukocytes (PBLs), predominantly in B and T lymphocytes and monocytes, whereas the *in vitro* application of various immune cell activators led to an increase of claudin 1 levels, yet not of claudin 5. Claudins 1 and 5 levels were elevated in PBLs of multiple sclerosis (MS) patients in relapse, relative to patients in remission, healthy controls and patients with other neurological disorders. Interestingly, claudin 1 protein levels were elevated also in PBLs of patients with type 1 diabetes (T1D). Following glucocorticoid treatment of MS patients in relapse, RNA levels of *JAM3* and *CLDN5* and claudin 5 protein levels in PBLs decreased. Furthermore, a correlation between *CLDN5* pre-treatment levels and clinical response phenotype to interferon- β therapy was detected. Our findings indicate that higher levels of leukocyte claudins are associated with immune activation and specifically, increased levels of claudin 5 are associated with MS disease activity. This study highlights a potential role of leukocyte TJPs in physiological states, and autoimmunity and suggests they should be further evaluated as biomarkers for aberrant immune activity and response to therapy in immune-mediated diseases such as MS.

Keywords: autoimmunity • biomarker • blood–brain barrier • interferon- β • multiple sclerosis • tight junctions

Introduction

Autoimmune diseases are associated with aberrant activity and enhanced migratory capacity of immune cells [1, 2]. In multiple sclerosis (MS), the extravasation of immune cells through the blood–brain barrier (BBB) elicits CNS inflammation and de-myelinating lesions, which culminate in the clinical dysfunctions characteristic of the disease [3, 4]. Accordingly, MS treatments, such as glucocorticoids (GCs), interferon- β (IFN- β) and natalizumab,

suppress leukocyte migration into the CNS, either by reducing BBB permeability or blockade of leukocyte–endothelial cell (EC) interactions [5–8].

The initial stages of leukocyte migration, including interactions with ECs in the stages of rolling and adhesion are well characterized [9]. Less clear is how leukocytes transverse through the EC layers of the vascular wall at various tissues during their physiological patrol or while infiltrating target organs in autoimmune diseases such as MS.

TJs between endothelial and epithelial cells form the structural basis of physiological barriers such as the BBB, which serves as a physical and metabolic barrier between the CNS and the systemic circulation. They are composed of the membranal TJPs occludin, claudins and the junctional adhesion molecules (JAMs), as well as various scaffold and cytoplasmic proteins such as the zonula occludens (ZO) proteins [10]. Their function is crucial for maintenance of

*Correspondence to: Ariel MILLER, M.D., Ph.D.,
Division of Neuroimmunology & Multiple Sclerosis Center,
Carmel Medical Center, 7 Michal St.,
Haifa 34362, Israel.
Tel.: +972-4-8250851
Fax: +972-4-8250909
E-mail: millera@tx.technion.ac.il or milleras@netvision.net.il

Table 1 Demographic and clinical characteristics of the study participants at the time of enrolment

Participant group	Gender	Age	Disease	Relapses in past 2 years	EDSS*
	F/M (% females)	(years)	Duration (years)	Number	Median [range]
		Mean \pm S.D.	Mean \pm S.D.	Mean \pm S.D.	
MS patients in relapse	35/16 (69%)	34 \pm 9.7	4.9 \pm 4.4	2 \pm 1.4 [†]	3 [0–5.5] [‡]
MS patients in remission	39/14 (74%)	35 \pm 10	6 \pm 5.7	1.2 \pm 1.1	2 [0–5]
Healthy control participants	24/16 (60%)	36.5 \pm 9.5			
OND [§] control participants	9/6 (56%)	47.7 \pm 11.1	4.7 \pm 3.8		
T1D control participants	13/3 (81%)	27.4 \pm 9	15.3 \pm 7.3		

*EDSS: expanded disability status scale.

[†]Not including the relapse at the time of enrolment.

[‡]EDSS score at the last remission period before the relapse.

[§]OND: other neurological diseases.

tissue homeostasis, while malfunction can cause severe disease and lethality [11–15]. Altered expression of claudins has also been reported in various tumours, suggesting that claudins may also be involved in other processes apart from their structural role [16]. The differential expression of distinct claudin proteins in subtypes of epithelia and endothelia, including the BBB, is suggested to account for the distinct permeability characteristics of these cellular layers [17–19]. Claudin 1, 3, 5 and 12 were reported to be expressed in brain endothelia, with a distinct expression of claudin 5 in ECs but not epithelial cells [15]. Knockout experiments in mice demonstrated the importance of claudin 5 in regulation of the BBB permeability for small but not large non-charged solutes [20]. Recently it was reported that claudins can be expressed by cells such as oligodendrocytes, astrocytes and neurons [21, 22]. JAMs are also expressed on various types of leukocytes and were suggested to be involved in the extravasation process through EC layers [23]. However, the expression of TJPs such as claudins and occludin by human leukocytes, and their possible role specifically in association with human disease states, to date, has not gained attention.

Despite its strict structural organization, the TJ complex is highly dynamic and can be regulated by various extracellular and intracellular stimuli including phosphorylation [24], matrix metalloproteinases (MMPs) proteolysis [25], ubiquitination [26, 27], endocytosis [28] and cell signalling factors such as VEGF [29], transforming growth factor- β [30], tumour necrosis factor- α and glucocorticoids [31]. Expression of TJPs in brain endothelia also appears to be regulated by interactions with surrounding cells such as pericytes [32]. These multiple regulation pathways converge to effectively determine and modulate barrier properties of the BBB and other endothelial layers.

In this study, we describe differential expression of membranal TJPs in human leukocytes, in different MS disease states and in comparison to another autoimmune disease (T1D), other neurological diseases (OND) and healthy individuals, as well as in

response to GC immunosuppressive treatment for MS relapse. In addition, our findings indicate that the levels of claudin 5 prior to IFN- β treatment are associated with the drug response phenotype in MS.

Materials and methods

Study population

Participants with confirmed relapsing remitting (RR) MS, according to Poser and McDonald criteria [33, 34], OND and healthy control volunteers were recruited at the MS Center, Carmel Medical Center, Haifa. T1D patients were recruited at the Armon Clalit Health Services clinic in Haifa. Recruitment of patients was conducted during clinic visits that were part of their health care plan. Participants were excluded if symptoms of acute infection were present at time of enrolment. The study was approved by the Helsinki Committee of Carmel Medical Center and signed informed consent was obtained from all study participants.

Blood samples were collected from MS patients in remission, before and after 3–6 months of IFN- β (IFN- β -1a or IFN- β -1b) treatment or already while under IFN- β therapy. Patients were excluded if GC therapy was used less than a month before recruitment. Blood samples were also collected from a separate group of MS patients presenting in an acute relapse before initiation of GC therapy-intravenous methylprednisolone and following 7 days of treatment with GCs, as previously described [35]. The group of MS patients in relapse included two subgroups: a group of patients who were treatment-naïve prior to immunomodulatory treatment at the time of recruitment and a group of patients already treated with IFN- β .

Demographic and clinical data were recorded from all participants (Table 1). The healthy control group was recruited as a matched group for age and gender to both relapse and remission MS patient groups. The OND group included ten patients with focal dystonia, two patients with facial synkinesis, one patient with hemifacial spasm, one patient with Parkinson's disease and one patient with Huntington's disease. The age of the OND and T1D groups was statistically different from the other groups, according to

the availability of patients at the corresponding participating clinics: T1D patients were younger, whereas the OND patients had a later disease onset and the population was more elderly. There were no significant differences between the groups in the other characteristics listed in Table 1. PBLs were prepared from blood samples as described previously [36]. PBLs for immunofluorescence and cell activation analyses were isolated by Ficoll-Hypaque gradient (Novamed, Jerusalem, Israel) from heparinized blood.

Definition of the clinical response for IFN- β -treated patients

IFN- β -treated patients were classified as 'good responders' if no relapses or no increase in expanded disability status scale (EDSS) [37] scores were documented in the 2-year period following treatment initiation as previously defined by us and others [38, 39]. IFN- β -treated patients that did not meet this criteria for good response were pooled into the 'others' group.

PBL activation procedure

For PBL activation assays, cells isolated from healthy donors were grown in the presence of either 2 μ g/ml of PHA-P, 20 units/ml of IL-2 combined with 2.5 μ g/ml of PHA-P (Sigma-Aldrich, St. Louis, MO, USA) or 10 μ g/ml of mouse anti CD3 antibody (R&D Systems, Minneapolis, MN, USA) for 5 days. Cells grown without any activators served as a control. The cells were cultured in RPMI-1640 medium containing 10% FCS, penicillin-streptomycin (100 U/ml) and L-glutamine (2 mM) (all from Biological Industries, Beit HaEmek, Israel).

RNA preparation and cDNA synthesis

TRI Reagent (MRC, Cincinnati, OH, USA) was used for RNA preparation according to the manufacturer's protocol, followed by DNase treatment to prevent genomic DNA amplification (DNA-free™; Ambion, Austin, TX, USA). cDNA synthesis was performed with moloney murine leukaemia virus (M-MLV) reverse transcriptase (Promega Corporation, Madison, WI, USA) with random hexamer primers (Amersham Biosciences, Piscataway, NJ, USA).

Quantitative real-time RT-PCR

For real-time RT-PCR analyses TaqMan technology was employed using primer-probe sets and recommended protocols from Applied Biosystems (Foster City, CA, USA). The *GAPDH* and *UBE2D2* genes were used as reference genes. Relative quantification of mRNA expression was calculated by the comparative C_T method [40]. The relative quantification value of target gene, normalized to the average of the reference genes and relative to a calibrator (*e.g.* a sample from a patient before drug treatment) was expressed as $2^{-\Delta\Delta C_T}$ (fold change). To compare the expression levels between unpaired sample groups, such as comparisons between samples from patients and controls, the quantification value of the target gene was expressed as $2^{-\Delta C_T}$, which is proportional to the amount of RNA in the samples tested. The fold change between the medians of the independent groups was calculated by the equation $(2^{-\Delta C_{T, \text{group 1}}} / 2^{-\Delta C_{T, \text{group 2}}})$, where $2^{-\Delta C_{T, \text{group 1}}}$ is the median value for the group of samples indicated.

Immunofluorescence analysis of PBLs

PBLs were fixed in 3.7% formaldehyde for 15 min., washed twice in PBS and adhered to microscope slides with suitable sample chambers and cytopads and centrifugation of 600 r.p.m. for 5 min., using a cytospin centrifuge (all from Wescor, South Logan, UT, USA). Following permeabilization in 0.1% Triton X-100 (Sigma-Aldrich) for 5 min., cells were incubated in blocking buffer containing 4% donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) and 1% BSA (Sigma-Aldrich) for 1 hr, followed by exposure to one or a combination of primary antibodies from different species diluted in blocking buffer [rabbit and mouse anti-claudin 5 1:150 (34-1600, 35-2500), mouse anti-ZO-1 1:50 (33-9100), rabbit anti-ZO-2 1:50 (38-9000); Invitrogen, Grand Island, NY, USA; goat anti-claudin 1 1:50 (SC-17658), rabbit anti-CD14 1:50 (SC-9150) rabbit anti-CD4 (SC-7219) and anti-CD8 (SC-7188) 1:150; Santa Cruz, Biotechnologies, Santa Cruz, CA, USA; rabbit anti-CD3 1:40 (A0452), mouse anti-CD20 1:50 (302301); Biolegend, San Diego, CA, USA] for 1.5 hrs. Slides were then washed in 0.05% Tween-PBS, and exposed to the secondary antibodies, diluted in blocking solution for 1 hr [Cy2- or rhodamine-conjugated donkey anti-rabbit IgG (1:100), rhodamine- or DyLight 488-conjugated donkey anti-mouse IgG (1:100, 1:500) or FITC-conjugated donkey anti-goat IgG (1:50), all from Jackson ImmunoResearch]. For labelling of the nuclei, TO-PRO-3 (Invitrogen) dye was added to the secondary antibody solution (1:100). The confocal system included a Bio-Rad Radiance 2000 confocal set-up hooked to an upright fluorescent microscope (Nikon E600). Quantification of cells expressing either only the CD markers or both the CD markers and the TJPs was performed by counting the number of these cells in randomly selected fields (approximately 30 cells counted for each stain type per experiment) viewed in 60 \times objective magnification from three independent experiments.

Cells stained with only secondary antibodies served as negative controls. For positive controls for TJPs labelling HEK-293 cells transiently transfected with pcDNA constructs with either claudin 1 or claudin 5 were employed; the non-transfected cells present alongside the transfected cells were not stained by the claudin antibodies, verifying each primary antibody's specificity. In addition, single labelling was performed for each antibody separately in parallel to the use of these antibodies in double labelling experiments. The percentage of labelled cells was similar between single- and double-staining experiments.

Protein analysis

Crude membrane fractions were isolated by homogenizing cell pellets in Tris Acetate 10 mM pH 7.3 buffer containing protease inhibitors (Aprotinin, Leupeptin, PMSF; Sigma-Aldrich, or complete protease inhibitor tablets; Roche, Basel, Switzerland). Proteins were separated by denaturing 12% SDS-PAGE and transferred to nitrocellulose membrane for immunodetection. Western blots were performed with the appropriate antibodies [mouse anti-claudin 5 1:500 (35-2500), rabbit anti-claudin 1 1:200 (71-7800); Invitrogen], secondary antibodies (HRP-conjugated goat anti-mouse 1:2500, HRP-conjugated goat anti-rabbit 1:1000; Jackson ImmunoResearch) and ECL detection (Biological Industries, Beit HaEmek, Israel). Quantification of relative expression levels was performed by densitometric analysis using TINA 2.09 software (Raytest Isotopenmegerte GmbH, Straubenhardt, Germany). β -Actin (mouse anti- β -actin 1:5000, A5316; Sigma-Aldrich) served as an internal reference for protein loading. Positive controls were HEK 293 transfected with expression plasmids for either claudin 1 or claudin 5.

Statistical analyses

Statistical analyses of the results were performed with SPSS v.15 software (SPSS Inc. Chicago, IL, USA). Data from IFN- β -treated and treatment-naive MS patients were grouped together for all analyses, because no significant differences were observed between these groups (Mann–Whitney analysis). For comparisons of RNA ($2^{-\Delta\Delta C_T}$) or protein levels in non-paired groups, the Mann–Whitney test was used using a case control study design. Outliers were removed for comparisons of RNA levels between the independent groups at the level of $-\Delta C_T$. When comparing paired groups, the Student's *t*-test was used for samples with a normal distribution (protein levels in activated PBLs compared to control) and the Wilcoxon signed rank test was used to compare between samples that were not normally distributed ($2^{-\Delta\Delta C_T}$ RNA values). Bonferroni correction was applied for multiple testing; corrected *P*-values are presented as *P_B*. Clinical and demographic continuous variables were evaluated by ANOVA and Student's *t*-tests, and categorical variables by chi-square tests. Significant differences for all tests were inferred when *P* < 0.05.

Results

In a preliminary screen for TJ genes expressed in leukocytes using a qualitative RT-PCR assay, we discovered that several claudin genes are consistently expressed in leukocytes from healthy and MS affected individuals (results not shown). The study described herein focused on TJ genes which either showed the most consistent leukocyte expression pattern (*CLDN1* and *CLDN5*) or were previously reported to be expressed in rodent and human leukocytes (*JAM1*, *JAM3*, *OCLN*) [23, 41, 42].

TJPs are expressed by B cells, monocytes, CD4 and CD8 T cells

To determine which of the cell types that comprise the PBL population express claudins 1 and 5, we performed double staining immunofluorescence analyses with leukocyte subtype-specific cell surface markers for T cells (CD3), B cells (CD20) and monocytes (CD14). Lack of commercial anti-claudin antibodies at the time experiments were performed excluded the use of flow cytometry for co-expression analyses, and use of immunofluorescence staining allowed the additional advantage of observing the cellular localization of the TJ protein expression. The expression of both claudins 1 and 5 was detected in CD20⁺ B cells (Fig. 1A and B; 46.8 ± 9.5% of the B cells expressed either claudin 1 or claudin 5), in CD14⁺ monocytes (Fig. 1C and D, 67.5 ± 10.4% of the monocytes expressed either claudin 1 or claudin 5) and in CD3⁺ T lymphocytes (Fig. 1E and F, 45.3 ± 17.7% of the T cells expressed either claudin 1 or claudin 5). Further, characterization of the T cells expressing claudins 1 and 5 demonstrated their expression on both CD4 and CD8 T cell subtypes (Fig. 1G–J; 38.6 ± 12.5% of the CD4⁺ cells and 52.5 ± 10.6% of the CD8⁺ cells expressed either claudin 1 or claudin 5). Expression of claudin 1

or claudin 5 was not observed in cells with polymorphonuclear morphology, typical of granulocytes, in preparations with other positively staining leukocytes (not shown).

Both claudins 1 and 5 were co-localized with the cytoplasmic TJ scaffold proteins ZO-1 and ZO-2, along the leukocyte apparent cell membrane (Fig. 1K–N), suggesting that in leukocytes, claudins can form a TJ complex with similar structure or composition as reported in endothelial or epithelial TJ complexes [43]. Interestingly, although claudins 1 and 5 were mostly co-expressed on the same cells, only approximately 50% of the cells expressing either ZO proteins co-expressed claudin 1 or claudin 5.

Claudin 1 expression is up-regulated in activated leukocytes

Activation of immune cells is an integral part of the inflammatory process underlying autoimmune diseases [44]. We therefore sought to determine the effect of T cell activation on the expression of claudins 1 and 5 in PBLs of healthy donors, as T cells comprise the major cell population within the PBLs. Claudin 1 protein expression was significantly elevated following activation with anti-CD3 antibody (fourfold, *P* = 0.04) or with combined treatment of PHA and IL2 (3.8-fold, *P* = 0.04) as could be deduced from results of both immunofluorescence (Fig. 2A) and Western blot analyses (Fig. 2B and C). Claudin 1 expression was also elevated following PHA treatment (3.4-fold), although the change did not reach statistical significance. These results indicate that activation of T cells up-regulates claudin 1 expression. From a qualitative analysis of the immunofluorescence results, it seemed that the up-regulation of claudin 1 was mainly due to an increase in the expression level per cell and not from an increase in the total number of cells expressing this protein. Claudin 5 expression levels in PBLs did not appear to change following any of the activation protocols (*n* = 3, results not shown).

Claudins 1 and 5 levels are elevated in MS patients in relapse

Following our observations that *in vitro* activation of leukocytes modulates TJP expression, *ex vivo* expression analysis was performed for PBLs from MS patients, OND, T1D and healthy control individuals. Claudin 1 protein levels were significantly higher in MS patients in relapse when compared to healthy controls (3.3-fold change, *P_B* = 1.5 × 10⁻⁵), and to patients in remission (3.1-fold change, *P_B* = 5.9 × 10⁻⁴; Fig. 3A). Claudin 1 protein levels were also higher in the T1D group when compared to healthy controls (2.4-fold change, *P_B* = 7 × 10⁻⁵), but not in the OND group. The higher levels of claudin 1 observed in PBLs from the active autoimmune diseases, MS and T1D, is in line with the effects observed for the *in vitro* activations the expression of this protein in leukocytes. The RNA levels of *CLDN1* could not be reliably analysed, because its transcript

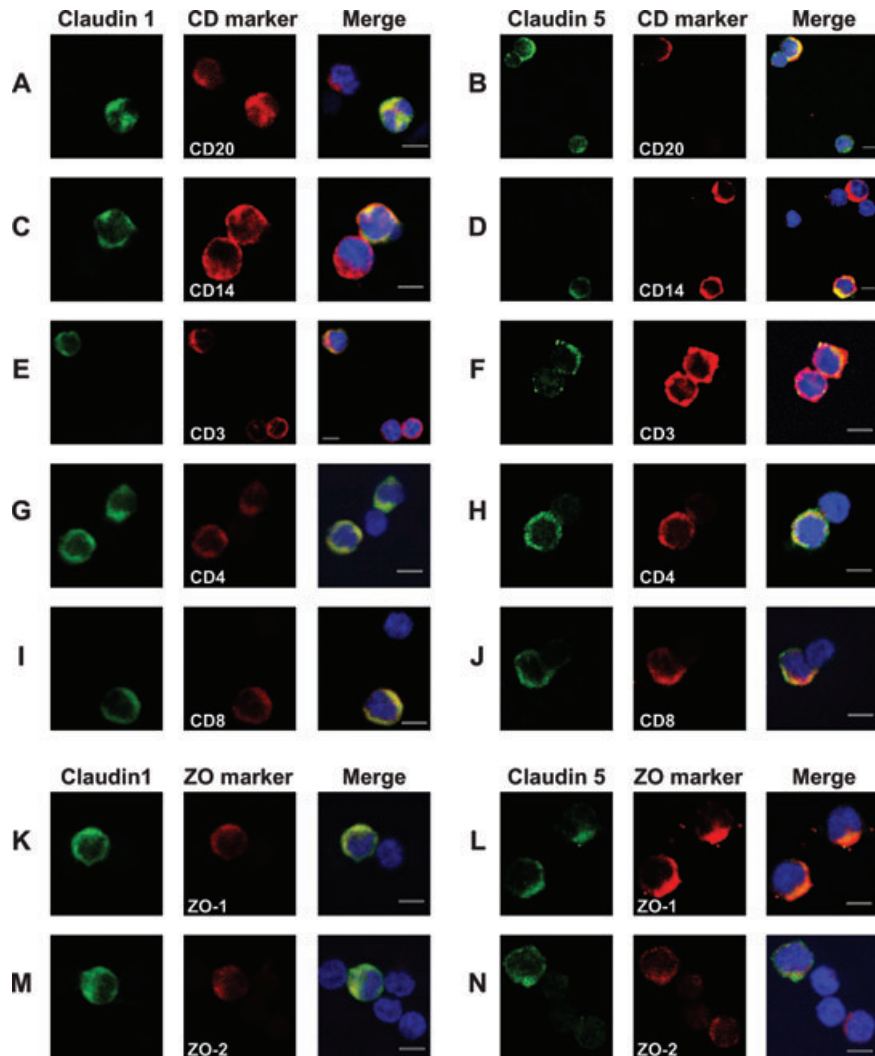


Fig. 1 Expression of TJPs in different leukocyte subsets. Immunofluorescence staining of PBLs from healthy controls with antibodies against claudin 1 or claudin 5 (green) and CD20 (A,B), CD14 (C,D), CD3 (E,F), CD4 (G,H), CD8 (I,J), ZO-1 (K,L) and ZO-2 (M,N) (all red) and a nuclear dye (TO-PRO 3-blue). Scale bar: 5 μ m. Representative results from three independent experiments are shown.

appeared to be very labile using the study's standard RNA extraction procedures and in the quantitative RT-PCR assay (as opposed to the qualitative assay used in the preliminary screen) gave very variable results.

CLDN5 RNA levels were elevated in MS patients in relapse, compared to healthy controls (1.5-fold change, $P_B = 0.042$) and to patients in remission (1.4-fold change, $P_B = 0.009$; Fig. 3B).

In concordance with RNA levels, claudin 5 protein levels were also elevated in MS patients in relapse compared to healthy controls (4.4-fold change, $P_B = 7 \times 10^{-4}$) and to patients in remission (4.1-fold change, $P_B = 2 \times 10^{-4}$; Fig. 3C). Interestingly, the increase in claudin 5 levels appeared to be specific for the MS relapse state, because no such findings were detected among the autoimmune, OND, and the healthy control groups.

The RNA levels of other TJ gene *OCLN*, *JAM1* and *JAM3* were not significantly different between PBLs from healthy

controls and PBLs from MS patients in relapse or in remission (results not shown).

TJ expression following GC treatment in PBLs from MS patients

The higher levels of claudins observed in leukocytes from MS patients in relapse led us to examine *ex vivo* the effect of GC treatment, which is used at relapse to suppress the heightened immune activity, on TJ expression. *CLDN5* RNA expression was significantly reduced following GC treatment in MS patients in relapse (2.4-fold change, $P = 1.5 \times 10^{-5}$; Fig. 4A). *JAM3* RNA expression levels were reduced as well (1.4-fold change, $P = 0.023$), but *JAM1* (Fig. 4A) and *OCLN* (not shown) RNA levels remained unchanged. In corroboration with the results obtained at

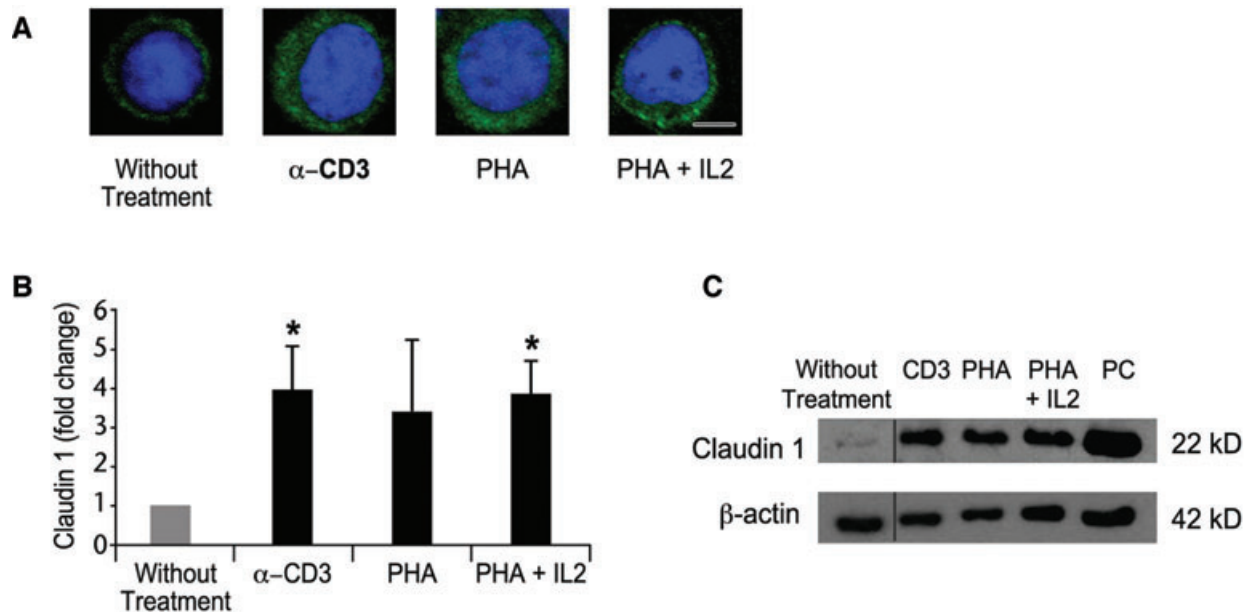


Fig. 2 Claudin 1 is up-regulated in activated PBLs. PBLs from healthy volunteers were activated *in vitro* with either anti-CD3 antibody (α -CD3), PHA, PHA and IL2 or left without treatment (non-activated) and analysed by (A) immunofluorescence analysis with anti-claudin 1 antibodies (green) and a nuclear dye (TO-PRO 3 – blue). Scale bar: 5 μ m. (B) Western blot analysis of claudin 1 protein levels. Bar graph depicts the fold change of claudin 1 protein levels relative to cells without treatment. The protein levels of claudin 1 were normalized to β -actin levels for each activation protocol using densitometry analysis. The data are presented as the mean \pm S.E. of three independent experiments. * $P = 0.04$ (Student's *t*-test). (C) Representative Western blot of claudin 1 expression in PBLs. PC: a positive control of extracts from HEK 293 cells transfected with a claudin 1 expression vector.

RNA level, we detected a reduction in claudin 5 protein expression ranging from 1.6- to 21-fold in MS patients in relapse following GC treatment (Fig. 4B). Claudin 1 protein levels did not change significantly in PBLs of MS patients following GC treatment ($n = 6$, results not shown).

Pre-treatment *CLDN5* mRNA levels are lower in 'good responders' to IFN- β treatment

IFN- β treatment of 3–6 months did not appear to affect the RNA levels of *JAM1*, *JAM3*, *CLDN5* and *OCLN* in PBLs of MS patients in remission ($n = 17$, results not shown). Nevertheless, the elevated levels of *CLDN5* RNA in PBLs collected during the acute relapse, and the reduction following GC treatment, prompted us to test *CLDN5* expression levels as a function of the clinical response of MS patients to IFN- β .

In patients classified as 'good responders' to IFN- β , the levels of *CLDN5* mRNA before treatment initiation were significantly lower compared to pre-treatment levels in patients classified as 'others', whose response phenotype did not meet the 'good responders' criteria (1.9-fold change, $P = 0.02$; Fig. 5). These two patient groups did not differ significantly in any of their demographic and clinical characteristics. Stratification by response did not reveal any effect of IFN- β treatment on *CLDN5* RNA expression levels.

Discussion

TJPs are structural proteins expressed predominantly by endothelial and epithelial cells [43]. Research from recent years provides accumulating evidence for the possible role of TJPs expressed on BBB ECs in experimental autoimmune encephalitis (EAE) and MS [45–48]. We report herein for the first time the expression of claudins 5 and 1 by human T cells, B cells and monocytes. These cell types have an important role in the pathogenesis of autoimmune diseases such as MS [3] and T1D [49, 50]. Interestingly, only a portion of each immune cell population expressed the claudins examined, suggesting that only specific subtypes of the leukocytes express these proteins. Generation of anti-claudin antibodies that are compatible with FACS technology will aid the classification of these immune cell sub-types.

Activation of leukocytes underlies the inflammatory process and associated autoimmune processes [44]. Here we demonstrate that claudin 1 protein levels are elevated in *in vitro* activated PBLs and in leukocytes of patients with autoimmune disease—MS patients in relapse and T1D patients. This up-regulation of claudin 1 in the autoimmune disease groups, which was not evident in the OND control group, seems to be the result of general immune cell activation and may be related, among others, to the PKC signalling pathway [51], as previously reported in non-immune cells [52]. Notably, increased levels of claudin 1 in melanoma cells through PKC

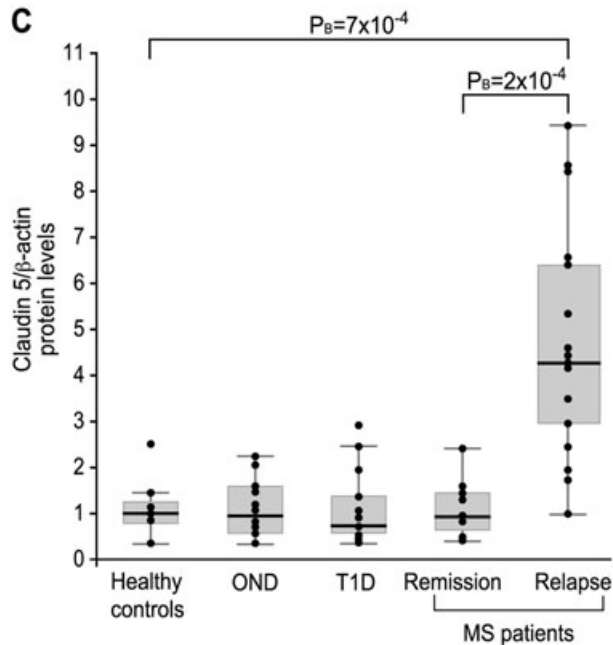
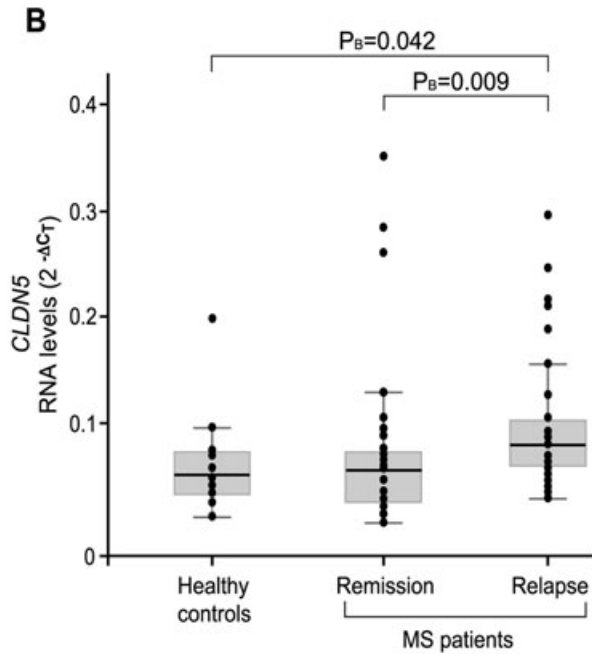
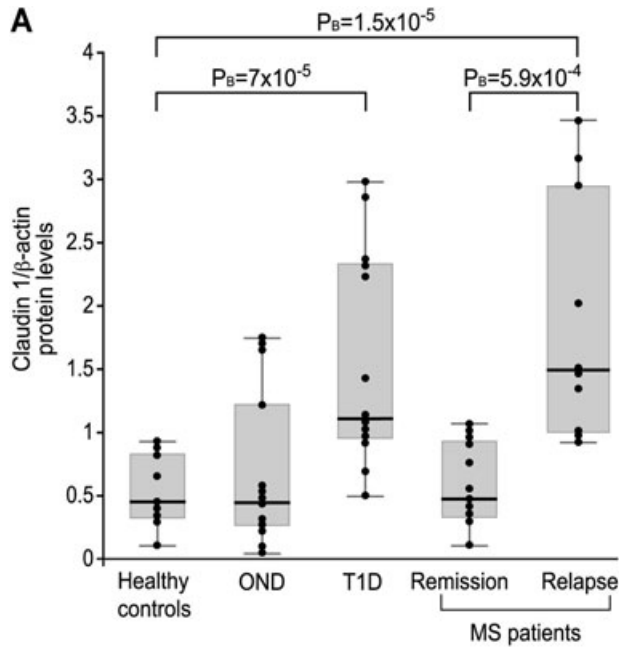


Fig. 3 Expression levels of claudins 1 and 5 in leukocytes from MS and T1D. **(A)** Claudin 1 protein levels in PBLs of healthy controls ($n = 13$), OND controls ($n = 14$), T1D controls ($n = 14$), MS patients in remission ($n = 13$) and MS patients in relapse ($n = 10$). **(B)** The expression levels of *CLDN5* RNA ($2^{-\Delta C_T}$) relative to reference genes were determined by real-time RT-PCR of PBL RNA derived from MS patients in relapse ($n = 33$), MS patients in remission ($n = 39$), and healthy controls ($n = 18$). **(C)** Claudin 5 protein levels in the PBLs of healthy controls ($n = 13$), OND controls ($n = 14$), T1D controls ($n = 14$), MS patients in remission ($n = 11$), and MS patients in relapse ($n = 17$). Box plot and data points display the relative protein levels determined by Western blot analysis. The relative intensity of claudin 1 or claudin 5 proteins compared to β -actin signals was determined by densitometry. Medians are depicted by horizontal bars.

activation are associated with the invasive properties of these cells [53], suggesting a link between TJ expression and cell motility.

The fact that activation of leukocytes increases their the expression of claudin 1, indicates the possibility that other conditions involving immune activation, such as viral or parasite exposure, may also lead to changes in the expression of TJPs in leukocytes, as has been demonstrated for EC TJPs [54, 55]. Notably,

viral entry into the CNS occurs in some cases by a 'Trojan Horse' mechanism in which the infected leukocytes modulate the EC TJPs and change the barrier permeability, as recently reported for West Nile virus [54]. Whether the viruses also modulate the leukocyte TJ expression is yet to be demonstrated.

A recent study demonstrated a transcriptional increase in JAM3 levels in T cells following activation [56], which combined

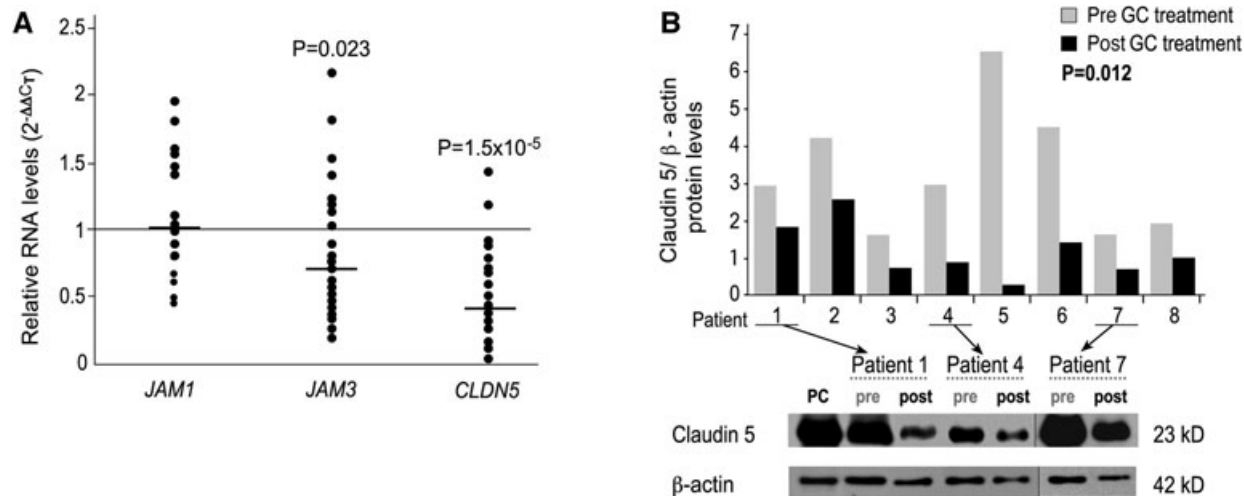


Fig. 4 GC treatment leads to a reduction in TJ expression in MS patients. TJ gene expression in PBLs from MS patients in relapse following therapeutic intravenous methylprednisolone GC treatment as determined by **(A)** real-time RT-PCR analysis of paired samples from before and 7 days after GC treatment initiation for each donor. RNA relative levels described by $2^{-\Delta\Delta C_T}$ values for the genes *JAM1*, *JAM3* and *CLDN5* ($n = 28$ for each gene). **(B)** Bar graph depicting the claudin 5 protein levels before (pre) and following (post) GC therapy ($n = 8$). The relative intensity of claudin 5 compared to β -actin signals was quantified by densitometry using Western blot analysis; representative blots shown in bottom panel for three patients. PC: positive control of extract from HEK 293 cells transfected with a claudin 5 expression construct.

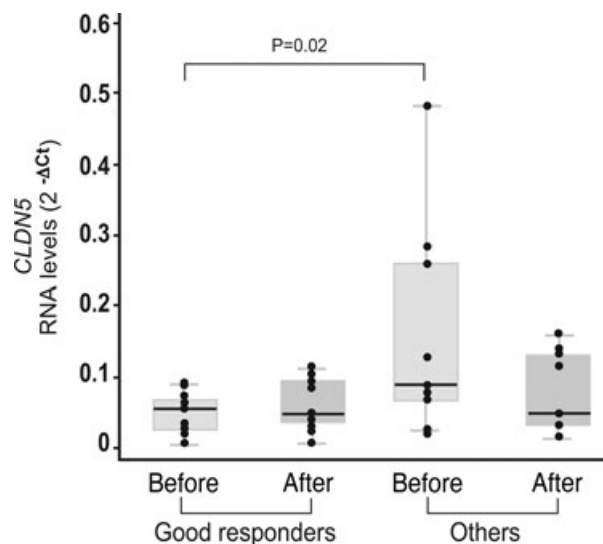


Fig. 5 Pre-treatment *CLDN5* mRNA levels are significantly lower in patients defined as 'good responders' to IFN- β treatment. The relative expression of *CLDN5* RNA ($2^{-\Delta C_T}$) was determined by real-time RT-PCR in PBLs collected from patients defined as 'good responders' ($n = 13$) and 'others'—patients whose clinical response to IFN- β did not meet the criteria for 'good responders' ($n = 9$), before and after 3–6 months of IFN- β treatment.

with the effect of GC we observed of down-regulation of *JAM3* transcript levels, suggests that *JAM3* too is a TJP whose expression is regulated by inflammatory processes, but distinctly from those activated in MS.

The choice to recruit independent MS patient groups for relapse and remission states may be considered a limitation of this study, as opposed to collection of consecutive samples from the same individual in relapse and remission states. However, the significance of the findings attained in spite of the lower power of the matched independent groups *versus* paired samples study design suggest that claudin expression levels in leukocytes indeed vary in disease states and their function may play a role in leukocyte activity.

Claudin 5 levels were elevated in PBLs of MS patients in the relapse state, yet were unaffected by *in vitro* activation of PBLs. These results suggest that the up-regulation of claudin 5 in leukocytes in MS reflects the interaction with additional cell types beyond those included in the PBL, and is not related *per se* to the effect of immune activation as observed for claudin 1 or *JAM3*. Because claudin 5 levels in T1D were similar to control levels, the increase in claudin 5 levels in the MS relapse may be related to disease activity in MS, as supported by the fact that claudin 5 levels are suppressed by GC administered to treat the acute MS relapse symptoms.

The correlation observed for the pre-treatment levels of claudin 5 RNA with clinical response to IFN- β is of interest, although the overlap between the study groups suggests that as a biomarker for drug response this transcript by itself would not be sufficient. Due to the small sample size available for this study, further studies with larger sample sizes are required to assess the validity of these observations as well as complementary analyses of claudin 5 protein expression in pre-treatment PBL samples, which were not available in this study. Nevertheless, the lack of a direct effect of IFN- β on claudin 5 levels suggests that the difference in pre-treatment levels of claudin

5 we observed may highlight a disease subset more prone to response to IFN- β , but that the claudin 5 is not directly related to the IFN- β mechanisms of action.

What may be the possible role of TJP expressed in leukocytes? The biological role of such complexes in cells that do not form cellular sheets is of interest, as well as the possible functional differences between TJ-expressing and non-expressing leukocytes. The fact that we observed the co-localization of claudin1, claudin 5 and the ZO proteins at the leukocyte cellular membrane suggests that TJ complexes are assembled similarly to the EC TJ [43].

Our proposed explanation is that leukocyte TJs may be involved in leukocyte extravasation. According to this suggestion, leukocyte TJPs may compete with EC TJPs through their exposed extracellular loops, allowing the opening of the endothelial paracellular space in a zipper-like manner and facilitating the penetration of the leukocytes through EC barriers, such as the BBB in the case of MS. A similar model has been proposed to explain the penetration of murine dendritic cells through the gut epithelium [57] and the migration of murine bone marrow dendritic cells through the lung epithelia [58]. Thus, elevated levels of TJPs in the leukocytes of MS patients in relapse, may contribute to increased extravasation of these cells into the CNS, which may manifest as a clinical exacerbation. Consequently, the decreased levels of TJPs in immune cells following GC treatment may reduce the strength of their interaction with the ECs' TJs, reducing their infiltrative potential. A demonstration of a functional effect of interference with the extracellular interactions of the claudins is obviously required to support this hypothesis. However, we have not yet been successful in generating antibodies to epitopes at the extracellular regions of claudin 5, in preliminary experiments conducted (unpublished data), because of the low antigenicity of these claudin regions. Of note, a commercial antibody reportedly binding claudin 5 extracellular domains failed to bind cells expressing claudin 5 constructs in our hands and therefore could also not be used to demonstrate functional effects on leukocyte migration.

In some cells the TJPs appeared to be distributed in a polarized manner (Fig. 1J–L for instance), yet in others TJPs were expressed evenly along the cell membrane (Fig. 1F). Future experiments using electron microscopy can further elucidate these observations. If indeed leukocyte TJPs are involved in their extravasation, this polarized expression may point to a directionality in certain leukocyte subtypes.

Other possible roles of TJPs in leukocytes may be suggested based on their proposed functions in tumours and metastasis [16]. For example, several lines of evidence link between MMP activity and the TJPs. Some claudins are capable of promoting the activation of MMPs and can up-regulate cancer cell invasion activity through this activation [59, 60]. Thus, increased expression of specific claudins in leukocytes may contribute to increased levels of activated MMPs, which is observed in MS as well as in T1D [1, 36, 61, 62]. Furthermore, claudin expression has been linked

also to resistance to apoptosis [63], which is part of the aberrant autoreactivity of immune cells in MS [64]. Accordingly, GC treatment, which reduced TJP expression in MS patients, is known to decrease the levels of MMPs and increase the apoptosis of immune cells [5, 65–67].

As is well-known, ECs can display cognate MHC-peptide complexes and serve also as antigen presenting cells (APCs) [68]. As such, they interact with T cells through the 'immunological synapse' (IS)-a term coined for the recognition site between APCs and T cells, which includes the MHC/TCR complex, co-stimulatory and adhesion molecules, as well as cytokine and chemokine receptors [69]. A similar site is also formed on B cells upon encountering an antigen [70]. We and others have shown that immune cells of MS patients express altered levels of IS components [71, 72]. The recruitment of lipid rafts, in which TJs are known to be dispersed [73, 74], is a key event in the formation of an IS, which determines its composition and function [75]. In this regard, TJPs co-expressed by T cells, B cells and ECs, may be considered as part of the molecular complexes located at or adjacent to the IS and their aberrant expression in MS leukocytes should be evaluated within the context of the IS activity in the process of immune cell activation and migration.

In summary, our results demonstrate that TJPs are expressed by distinct subsets of human leukocytes, and that they can be differentially affected by immune activation, with implications for autoimmune diseases such as MS and T1D, as well as steroid therapy. Further studies are required to assess the functional role and implications of the presence of TJPs on the surface of leukocytes, including assessment of their contribution to the extravasation process through EC layers. Such a role for TJPs in leukocytes' functions has implications with respect to the decoding of the molecular cascade underlying MS pathogenesis and in development of biomarkers for disease activity and prediction of response to treatment, as well as to the future development of novel targeted therapeutics for autoimmune diseases in general and for MS specifically.

Acknowledgements

We thank Dr. Ofra Barnett-Griness for assistance in statistical analysis, Maayan Merhav and Noga Gershoni for expert technical assistance and Sara Dishon for providing continuous patient care. This work was supported by an educational grant of Merck-Serono, by the Israeli Ministry of Health and the Technion's Pen Research Fund.

Conflict of interest

The authors confirm that there are no conflicts of interest.

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