TCR-stimulated changes in cell surface CD46 expression generate type 1 regulatory T cells

One-sentence summary: Defects in the T cell receptor–stimulated processing of a cell surface receptor may contribute to the pathogenesis of autoimmune diseases.

Editor's summary:
Receptor processing dampens inflammation
One of the hallmarks of autoimmune diseases, such as multiple sclerosis (MS), is the lack of regulatory T cells to suppress inflammation. Stimulation of the complement regulatory protein CD46 on T cells triggers the conversion of inflammatory effector cells into interleukin-10 (IL-10)–secreting type 1 regulatory T (Tr1) cells, a process that is defective in MS patients. Ni Choileain *et al*. found that T cell stimulation altered the O-glycosylation status of CD46, changing its mass and enabling its translocation to the immune synapse, the site of T cell activation. The cell surface abundance of CD46 was reduced upon generation of Tr1 cells, which produced IL-10. In contrast, T cells from MS patients showed no change in CD46 abundance and continued to produce the inflammatory cytokine IFN-γ. Together, these data may aid in the design of immunotherapies to treat MS.

TCR-stimulated changes in cell surface CD46 expression generate type 1 regulatory T cells

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Abstract

A lack of regulatory T cell function is a critical factor in the pathogenesis of autoimmune diseases, such as multiple sclerosis (MS). Ligation of the complement regulatory protein CD46 facilitates the differentiation of T helper 1 (T_H1) effector cells into interleukin-10 (IL-10)–secreting type 1

regulatory T cells (Tr1 cells), and this pathway is defective in MS patients. Cleavage of the ectodomain of CD46, which contains three N-glycosylation sites and multiple O-glycosylation sites, enables CD46 to activate T cells. Here, we found that stimulation of the T cell receptor (TCR)-CD3 complex was associated with a reduction in the apparent molecular mass of CD46 in a manner that depended on O-glycosylation. CD3-stimulated changes in CD46 O-glycosylation status reduced CD46 processing and subsequent T cell signaling. During T cell activation, CD46 was recruited to the immune synapse in a manner that required its serine-, threonine-, and proline-rich (STP) region, which is rich in O-glycosylation sites. Recruitment of CD46 to the immune synapse switched T cells from producing the inflammatory cytokine interferon-γ (IFN-γ) to producing IL-10. Furthermore, $CD4^+$ T cells isolated from MS patients did not exhibit a CD3-stimulated reduction in the mass of CD46 and thus showed increased amounts of cell surface CD46. Together, these data suggest a possible mechanism underlying the regulatory function of CD46 on T cells. Our findings may explain why this pathway is defective in patients with MS and provide insights into MS pathogenesis that could help to design future immunotherapies.

Introduction

The presence of autoreactive T cells and a lack of regulation of autoreactive T cells both contribute to the pathogenesis of autoimmune diseases (1). Defects in both conventional $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells and Tr1 regulatory cells, which are characterized by their secretion of interleukin 10 (IL-10) and the low amounts of interferon-γ (IFN-γ) that they produce, have been demonstrated. The critical role of IL-10 in the maintenance of immune homeostasis was demonstrated in a study that showed that myelin-reactive T cells from healthy donors and those from patients with multiple sclerosis (MS) differ in their production of IL-10 (2), implying that

IL-10 suppresses autoreactive T cells in healthy donors. Indeed, induction of IL-10 or regulatory T cell function has some promising clinical applications (3, 4).

The complement regulator CD46 modulates the adaptive immune response by controlling T cell activation, differentiation, and polarity. CD46 stimulation regulates inflammation (5-9) and T cell homeostasis (9-12), and it controls T cell metabolism by sustaining signaling to mammalian target of rapamycin (mTOR) complex 1 (mTORC1) (13) and activating the inflammasome (14). Although CD46 costimulation stimulates the differentiation of naïve CD4+ T cells into T helper 1 (T_H1) cells, as IL-2 accumulates, CD46-costimulated T cells reduced their secretion of IFN-γ and increase their production of IL-10, thus switching from Th1 cells to Tr1 cells (10). This Th1-Tr1 switch is defective in chronic inflammatory diseases, such as MS (15), asthma (16) and rheumatoid arthritis (RA) (10) highlighting the importance of CD46 in ensuring T cell homeostasis. However, the molecular mechanisms responsible for the defective CD46 pathway remain ill-defined.

A key event in the activation of primary T cells is the enzymatic processing of CD46, resulting in the matrix metalloproteinase–mediated cleavage of the CD46 ectodomain, which is followed by the cleavage of its cytoplasmic tails by gamma-secretase. CD46 can express one of two cytoplasmic tails, Cyt1 and Cyt2, which are produced by alternative splicing. Processing of Cyt1 is required for IL-10 production and enables T cell activation, whereas cleavage of Cyt2 results in the inhibition of T cell activation, and therefore is pivotal to ensure T cell homeostasis (11, 17). The cleaved cytoplasmic tails translocate to the nucleus where they likely control target genes (13). Hence, the processing of CD46 is a critical factor in the control of effector T cells and the amount of remaining cell surface CD46 on activated T cells is tightly controlled (18-20).

CD46 is a highly glycosylated type I transmembrane protein. N-glycosylation of CD46 is required for CD46 complement regulatory function and its O-glycosylation, although not critical, contributes to cytoprotection (21). The CD46 ectodomain consists of four short-consensus repeat domains (SCR1 to SCR4), which are followed by a region rich in serine, threonine, and proline residues (the STP region), which is encoded by the A, B, and C exons, and then by one of the two short cytoplasmic tails, Cyt1 or Cyt2. The four major isoforms of CD46, which are produced by alternative splicing, express either a BC- or C-encoded STP region, and either Cyt1 or Cyt2 (22). There is a single N-glycosylation site in SCR1, SCR2, and SCR4, whereas there are multiple O-glycosylation sites in the STP region. Here, we demonstrated that T cell receptor (TCR) activation is a key regulator of the proteolytic cleavage of CD46 through the modulation of CD46 glycosylation. We showed that the STP domain of CD46, which is rich in O-glycans, was critical to its regulatory function and signaling capabilities and we propose that a defect in the regulation of CD46 glycosylation contributes to the impaired CD46 pathway observed in MS patients.

Results

T cell activation modulates the glycosylation of CD46

Expression of CD46 at the surface of T cells is tightly controlled and CD46 processing is important for its effect on T cell functions, most notably for the release of IL-10 (11, 19, 20). We observed that the activation of CD3 by immobilized anti-CD3 antibodies on primary human CD4+ T cells from healthy donors led to a systematic decrease in the molecular mass of CD46 by ~3 kDa (Fig. 1A, arrow). Most of the donors expressed either the BC isoform of CD46 (65%) or both BC and C isoforms (29%), which can be distinguished by Western blotting analysis (23). In our

representative example, the T cells expressed both the BC and C isoforms of CD46 (giving rise to two bands), both of which exhibited a reduction in molecular mass upon CD3 activation (Fig. 1A). As expected, costimulation of CD3 and CD46 by immobilized antibodies resulted in the reduced abundance of CD46, due to the shedding of the CD46 ectodomain. Nevertheless, the residual CD46 proteins were of reduced molecular mass (Fig. 1A, arrow). In contrast, the molecular mass of the glycoprotein CD28 did not change upon CD3 activation (Fig. 1B). The decrease in the mass of CD46 was related to the strength of TCR stimulation (Fig. 1C). Indeed, TCR activation was required for this effect because CD46 stimulation in the absence of CD3 stimulation did not affect the mass of CD46 (Fig. 1D). The decrease in the mass of CD46 in response to CD3 stimulation was enhanced in the presence of IL-2, which is required for the transition of Th1 cells to Tr1 cells (Fig. 1E). A slight shift in the mass of CD46 was observed after 4 to 5 hours of activation (Fig. 1F). Moreover, the shift in CD46 mass was more pronounced in memory than naïve CD4+ T cells (fig. S1).

Because CD46 is highly glycosylated, we hypothesized that one mechanism for the reduction in the molecular mass of CD46 upon TCR stimulation was through modification of CD46 glycosylation. To test this hypothesis, CD46 from either unstimulated or CD3-stimulated T cells was immunoprecipitated and deglycosylated in vitro (Fig. 2A). For the glycosylated samples, a decrease in the mass of CD46 was observed upon CD3 stimulation. Upon removal of both N- and O-glycans with glycanase, the mass of CD46 was reduced to 42 kDa in both the unstimulated and CD3-stimulated samples, corresponding to the reported MW of deglycosylated CD46 (Fig. 2A, bottom arrow) (24), suggesting that the reduction in CD46 mass (Fig. 2A, top arrow) observed upon CD3 stimulation was a consequence of glycosylation changes. To determine whether

activation-induced changes in N- or O-glycosylation of CD46 were involved, we next examined the effects of either N- or -O glycanases upon CD46 mass after CD3 stimulation (Fig. 2B). A CD3-stimulated shift in CD46 mass was still observed after removal of the N-glycans, but not the O-glycans, of CD46, suggesting that TCR stimulation caused a change in the O-glycans of CD46. To confirm a role for O-glycans, we treated CD4+ T cells before activation with swainsonine, which inhibits mannosidase 2 and therefore modifies N-glycan branching (25), or with Benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADG), which modifies O-glycan elongation depending on the extent of their sialylation. The cell surface amounts of CD46 were similar after overnight incubation of primary CD4+ T cells with either inhibitor (Fig. 2C). A reduction in CD46 mass was still observed upon CD3 stimulation in the presence of swainsosine. In contrast, the CD3-stimulated shift in the mass of CD46 was much reduced upon modification of O-glycosylation by BADG (Fig. 2D). Together, these data suggest that CD3 stimulation causes a change in the O-glycosylation of CD46 in activated T cells.

The modification of O-glycosylation by BADG is dependent upon the extent of sialylation (26). When sialylated O-glycans are low in abundance, BADG blocks elongation beyond the initial GalNac residue, whereas when sialylated O-glycans are high in abundance, BADG inhibits O-glycan sialylation, therefore increasing the exposure of nonsialylated O-glycans, which can be detected because of their increased binding to the lectin PNA (peanut agglutinin) (26). Compared to control T cells, BADG-treated T cells exhibited increased binding of PNA (fig. S2), consistent with the increased exposure of nonsialylated O-glycans. We next assessed the binding of a panel of lectins, including conconavalin A (ConA), *Dolichos biflorus* agglutinin (DBA), wheat germ agglutinin (WGA), *Ulex europaeus* agglutinin (UEA), *Sambucus nigra* agglutinin (SBA), and

Ricinis communis agglutinin (RCA), to activated T cells. We found that CD3 stimulation and both CD3 and CD46 stimulation resulted mainly in the increased of binding of WGA, which binds to sialic acids (27). Indeed, BADG reduced the amount of WGA that bound to activated T cells (fig. S2). Together, these data are consistent with the idea that CD3 stimulation modifies the pattern of sialylation of O-glycans on T cells. To determine the effect of CD3 stimulation on CD46 ectodomain shedding, we next compared the cell surface abundance of CD46 on T cells stimulated through CD3, CD46, or both, and found that CD3 activation, in conjunction with CD46 stimulation, enhanced the reduction in CD46 abundance (fig. S3).

Inhibition of O-glycan maturation reduces shedding of CD46 and promotes IFN-γ production

To determine the effects of O-glycans on T cell activation, we pretreated CD4+ T cells overnight with BADG (to prevent O-glycan elongation) before they were activated. Inhibition of O-glycosylation resulted in a reduction in the loss of cell surface of CD46 after 2 hours of CD3 and CD46 costimulation (fig. S4). Inhibition of O-glycosylation enhanced the production of IFN-γ by the cells, but had no substantial effect on cell proliferation or IL-10 secretion. BADG also lead to increased IFN-γ production by CD3-stimulated T cells (fig. S4), suggesting that BADG has effects on other glycoproteins. Nevertheless, these data suggest that O-glycosylation is important in the control T of cell activation and cytokine production.

Deletion of the STP domain in CD46 reduces CD46 shedding and promotes IFN-γ secretion

To specifically assess the role of the O-glycans in CD46 that are localized in the STP domain of the protein, we expressed a glycosylation mutant of the CD46 BC1 isoform in which the STP

region was deleted [ΔSTP, previously characterized in CHO cells (21)] in primary human CD4+ T cells. As a control, we used the wild-type CD46 BC1 isoform. We tagged both proteins with green fluorescent protein (GFP) to assess their relative abundance. We first expressed BC1-GFP and ΔSTP-GFP in NIH 3T3 cells, which do not express endogenous CD46. We stained GFP+ cells with an anti-CD46 antibody and showed that GFP-CD46 BC1 and the GFP-ΔSTP mutant reached the cell surface (fig. S5), consistent with a previous report (21).

Primary CD4+ T cells were then transfected with two different amounts of the ΔSTP and BC1-GFP constructs and flow cytometric analysis showed similar amounts of both proteins in each case (Fig. 3A). However, the reduction in CD46 upon CD3 and CD46 costimulation was impaired in cells expressing the ΔSTP mutant (Fig. 3, B and C). Expression of a ΔSTP mutant lacking the GFP tag also resulted in impaired loss of cell surface CD46 (fig. S6A). Moreover, in BC1- and ΔSTP-expressing CHO cells (21), which had similar amounts of CD46 at the cell surface, CD3 and CD46 costimulation reduced the cell surface abundance of BC1, but had less of an effect on the ΔSTP mutant (fig. S6B). These data suggest that the STP domain plays a role in determining the extent of loss of CD46 from the cell surface after it is stimulated. We next assessed the shift in the molecular masses of the GFP-tagged CD46 constructs. CD4+ T cells expressing either the BC1-GFP or ΔSTP-GFP constructs were stimulated with anti-CD3, lysed, and analyzed by Western blotting with horseradish peroxidase (HRP)–conjugated anti-GFP antibodies. As expected, BC1-GFP exhibited a reduction in mass upon CD3 activation, whereas the ΔSTP-GFP construct did not (Fig. 3D). Lastly, we measured the number of GFP-expressing cells after 1 day of activation. Whereas we observed a decreased in the number of BC1-expressing T cells that were positive for GFP, the extent of the decrease in GFP+ cells among those expressing the ΔSTP mutant was

reduced (with no effect in cells expressing GFP alone), suggesting aberrant proteolytic processing of ΔSTP (Fig. 3E).

Next, to determine the effect of the STP domain on CD46 function, transfected T cells were stimulated with antibodies against CD3 alone or with anti- CD46 antibodies. We only analyzed data from experiments in which the cells showed similar amounts of BC1-GFP and ΔSTP-GFP to ensure that any effects were construct-specific and not a result of differences in abundance. The increased cell surface expression of CD69, an early activation marker, was reduced in T cells expressing the ΔSTP-GFP mutant compared to that in T cells expressing BC1-GFP (Fig. 4, A to C). Proliferation was not reduced in ΔSTP-GFP expressing T cells (Fig. 4D and fig. S7). The expression of ΔSTP-GFP was associated with the increased secretion of IFN-γ, but not IL-10 (Fig. 4E). A similar increase in IFN-γ–expressing T cells was detected by flow cytometric analysis of cytokine production (Fig. 4F). These data suggest that the STP region of CD46 is required for the switch from Th1 cells to Tr1 cells.

Deletion of the STP domain of CD46 impairs CD46 recruitment to the immune synapse

We next considered whether the altered CD46-dependent functional responses of T cell that we observed may be due to aberrant recruitment of CD46 to the immune synapse. CD4+ T cells were activated with immobilized anti-CD3 and anti-CD46 antibodies or anti-CD3 and anti-CD28–coated beads (CD3/CD28 beads) to mimic contact with antigen-presenting cells (APCs). T cells were stained to detect actin and CD46 to assess their colocalization and then were visualized by confocal microscopy. Whereas uniform surface staining for CD46 was detected in unstimulated T cells, the cellular distribution of CD46 was markedly changed after T cell activation (Fig. 5A).

Redistribution of CD46 was observed after stimulation with anti-CD3 and anti-CD46 antibodies and also after stimulation with CD3/CD28 beads (Fig. 5A), suggesting that this was a consequence of T cell activation rather than antibody-induced redistribution. We next investigated whether the CD46 STP domain was necessary for the recruitment of CD46 to the immune synapse in transfected cells. BC1-GFP or ΔSTP–GFP expressing T cells were activated by CD3/CD28 beads and analyzed using the Flowsight Amnis imaging flow cytometer. The extent of CD46 recruitment to the immune synapse was calculated by analyzing the colocalization of green (CD46-GFP) and red (F-actin, stained with phalloidin-458) after masking and excluding any bead-associated fluorescence. T cells expressing ΔSTP-GFP exhibited less colocalization of CD46 with actin than did BC1-GFP–expressing T cells (fig. S8). To confirm these data, we analyzed colocalization of actin and CD46 in activated T cells by confocal microscopy. The cell surface expression of BC1-GFP and ΔSTP-GFP were similar in unstimulated T cells (Fig. 5, B/C and fig. S9A). Upon stimulation with CD3/CD28 beads, actin was redistributed to the point of contact with the bead in control untransfected T cells (fig. S9B), as well as in cells expressing either BC1-GFP or ΔSTP-GFP. However, colocalization of CD46 and actin was observed only in cells expressing BC1-GFP and not with the ΔSTP-GFP mutant (Fig. 5, B and C). Dynamic actin rearrangements were also observed in cells that underwent CD46 costimulation, consistent with previous observations (8), and colocalization of CD46 with actin was also observed in cells expressing BC1, but less so in cells expressing the ΔSTP mutant (figs. S9 and S10).

Deletion of the STP domain of CD46 increases NF-κB activation

Data from an RNAi screen investigating genes that modulate CD46 cell surface expression in CD3/CD28–activated T cells (19) indicated that knockdown of *CHUK* (which encodes IKKα)

leads to decreased amounts of CD46 at the cell surface. IKKα phosphorylates the inhibitor of NF-κB (IIκB), enabling its degradation by the proteasome and therefore activating NF-κB. These data suggest that activation of the transcription factor nuclear factor κB (NF-κB) is critical for the control of CD46 expression (Fig. 6A). Hence, we first confirmed these data by chemically blocking the NF-κB pathway with the IKKα inhibitor BAY 11-7082. The reduction in cell surface CD46 in response to CD3 and CD46 costimulation was enhanced in T cells treated with the IKKα inhibitor compared to that in control cells (Fig. 6B). A similar effect was observed in cells treated with MG132, a proteasome inhibitor that prevents NF-κB activation by blocking degradation of the NF-κB inhibitor protein IκBα (fig. S11). We next analyzed the activation of NF-κB in T cells expressing either BC1 or ΔSTP. T cells expressing the ΔSTP mutant had increased activation of NF-κB as determined by measurement of p65 phosphorylation, whereas phosphorylation on tyrosine as detected by anti-P-Tyr antibodies was decreased (Fig. 6C and fig. S12). This suggested that TCR signaling was overall reduced in ΔSTP-expressing T cells. Moreover, we found that phosphorylation of ERK was reduced in ΔSTP-expressing T cells compared to that in BC1-expressing cells (Fig. 6D), suggesting that the STP domain of CD46 is required for optimal ERK activation in T cells, which is consistent with the phosphorylation of ERK upon CD3 and CD46 costimulation (8) and the requirement for ERK signaling for IL-10 production by Th1 cells (28).

Altered molecular mass and cell surface expression of CD46 in T cells from MS patients.

The CD46 pathway is dysregulated in a number of chronic inflammatory diseases, including MS (15). We next examined the cell surface expression of CD46 in activated T cells purified from patients with relapsing-remitting MS (RRMS). In contrast to the reduction in the molecular mass of CD46 observed upon CD3 stimulation of T cells from healthy controls, no such shift in CD46

mass was apparent in CD3-stimulated RRMS T cells (Fig. 7A). The impaired shift in CD46 MW observed upon CD3 stimulation did not substantially alter the activation of the RRMS T cells as assessed by analysis of proliferation (fig. S13). These data suggest that TCR-mediated changes in CD46 are aberrant in MS T cells. Our previous data suggest that the change in CD46 mass reflects a change in O-glycosylation that increases CD46 shedding. We therefore compared the cell surface expression of CD46 on CD3 and CD46 costimulated T cells from healthy donors and patients with RRMS. Although cell surface CD46 was decreased on costimulated MS T cells compared to unstimulated MS cells, the residual cell surface abundance of CD46 was greater on costimulated MS T cells than on costimulated T cells from healthy controls (Fig. 7B). Because shedding of the CD46 ectodomain is necessary for the cleavage of either of the two potential cytoplasmic tails of CD46, Cyt1 and Cyt2 (11, 17), we next investigated changes in cytoplasmic tail abundance between unstimulated and stimulated T cells, and we compared the effects of CD3 stimulation alone and CD3/CD46 costimulation. Whereas we detected decreases in the abundances of Cyt1 (at day 2) and Cyt2 (at day4) in costimulated T cells from healthy donors, these decreases were impaired in costimulated T cells from MS patients (Fig. 7C). A similar trend of impaired CD46 expression was observed for both untreated and IFN-β–treated patients (fig. S14).

Discussion

Despite a lack of an animal model for analysis of the physiological role of CD46 (due to the restricted expression profile of CD46 in mice to the testis), it is now established that the CD46 pathway is defective in several chronic inflammatory conditions in humans. The switching of Th1 effector cells to regulatory Tr1 cells is impaired upon CD46 stimulation in patients with MS, RA, and asthma, further highlighting the crucial role of the CD46 pathway in exerting control over

exacerbated immune responses. It was reported that the production of C3b, an endogenous ligand for CD46, by T cells through intracellular cathepsin-mediated cleavage of C3 is aberrant in T cells from RA patients (29). Here, we showed a direct effect of TCR stimulation in controlling the extent of CD46 glycosylation and, as a consequence, the cell surface abundance of CD46, which further feeds back to TCR-mediated signaling. We demonstrated an interplay between CD46 abundance and T cell fate, and how it determined the Th1 to Tr1 switch. We propose that this receptor crosstalk mechanism may explain the aberrant pathway in different diseases, because only T cells activated by their specific antigen will be affected by the impaired CD46 pathway.

Glycosylation is one of the most prominent posttranslational modifications of cell surface proteins and defective protein glycosylation is observed in many human diseases. However, glycosylation is a complex process that is highly regulated by cellular activation (30), and specific glycans regulate distinct T cell functions (31). For example, specific changes in the glycosylation of CD43 and CD45 regulate T cell survival and function (32, 33), and the N- and O-glycans of CD45 exhibit antagonistic roles on CD45-induced apoptosis (34). Our data suggest that glycosylation of CD46 is important for the control of human T cell differentiation. We found that inhibition of O-glycosylation maturation or expression of the ΔSTP mutant CD46 reduced the extent of the reduction in CD46 cell surface abundance in response to TCR stimulation, raising the possibility that the STP region influences the accessibility of CD46 to proteases. This effect was observed for both primary human T cells and transfected CHO cells expressing CD46 glycosylation mutants. Comparison of CHO cells expressing the ΔSTP mutant CD46 or the WT BC1 form was complicated by differences in the cell surface abundance of these proteins. However, in a series of experiments, we observed that CHO cells expressing the N-glycan mutants of CD46 (whose

abundances were equivalent to that of ΔSTP CD46), shed CD46 in similar fashion as did cells expressing the WT BC1 CD46 protein, supporting the notion that the reduced cleavage of ΔSTP CD46 was independent of its abundance. Indeed, the STP region is believed to affect the conformation of CD46, and splicing of some of the exons that encode the STP region affects the binding of complement to CD46 (35, 36). The effect observed on the intensity of shedding of CD46 in GFP+ cells also suggests that the expression of ectopic GFP-CD46 affected the behavior of endogenous CD46.

Expression of neuraminidases is increased by T cell activation (37), and Neu1 is found at the surface of activated T cells (38). It is therefore possible that CD3 stimulation affects the extent of sialylation of CD46 as a means to control its regulatory pathway, although additional studies are required to fully establish the role of sialic acid in Tr1 cell differentiation. Differential patterns of protein glycosylation have been reported for mouse Th1, Th17, and Th2 cell subsets (39), and our data suggest that there is differential glycosylation of CD46 on Th1 versus Tr1 cells. We propose that glycosylation changes in CD46 occur after T cell activation that subsequently promotes T cell activation by affecting CD46 recruitment to the immune synapse, signaling, and CD46 processing. We also observed that the shift in the molecular mass of CD46 was more readily detected in memory T cells than in naïve T cells, and that memory cells had more cell surface CD46 than did naïve cells, which suggests that CD46 may play a specific role in memory cells. It is possible that the readily observed shift in the mass of CD46 in memory T cells favors CD46 recruitment to the immune synapse, hence resulting in stronger TCR activation compared to that of naïve T cells.

Although CD46 has not been identified in large GWAS studies for MS, variants of CD46 have been reported in a Spanish cohort (40). Polymorphisms in genes involved in the regulation of CD46 abundance or signaling, including the gene encoding STAT3, which acts on the *CD46* promoter (41), the gene encoding IL-2RA, whose binding to IL-2 is required for the switch from TH1 cells to Tr1 cells (10), and the gene encoding EP4, which is one of the receptors for PGE_2 and whose cell surface abundance is specifically increased by CD46 (19), have also been identified (42). Defects in the N-glycosylation pathway lead to autoimmunity (43). Dysregulated N-glycosylation has been observed in MS patients, and variants in Mgat5, an enzyme catalyzing the synthesis of beta-1,6 GlcNAc-branched N-linked glycans, have been identified in MS patients and linked to disease severity (44, 45). The role of N-glycosylation of CD46 in T cell activation and differentiation remains to be elucidated. Moreover, defects in IgG glycosylation have also been detected in MS patients (46, 47), which suggests a more global defect in glycosylation.

Our data suggest that TCR activation promotes the differential glycosylation of CD46, which facilitates the shedding of CD46 upon ligation, thereby critically promoting the production of IL-10 (11). Pre-clinical studies with anti-CD3 monoclonal antibody have shown their efficacy in inducing tolerance, including increasing the production of IL-10 and ameliorating type 1 diabetes (48). The modulation of CD46 glycosylation and cell surface abundance, and of its subsequent regulatory functions by anti-CD3 antibodies may, in part, explain the beneficial effect of these therapeutic antibodies in vivo.

The defective reduction in CD46 cell surface abundance observed in T cells expressing the ΔSTP mutant was associated with an altered cytokine production profile, which is similar to the cytokine

profile of CD46-costimulated T cells from MS patients, which produce more IFN-γ than IL-10 (15). This is consistent with our previous studies that identified the importance of CD46 cleavage in promoting IL-10 secretion (11). T cells from MS patients did not show any shift in CD46 molecular mass upon activation, suggesting the extent of O-glycosylation of CD46 in these cells was impaired, hence affecting downstream signaling and processing. Indeed, T cells from MS patients had increased amounts of residual cell surface amounts of CD46 after costimulation than did cells from healthy donors and they exhibited aberrant amounts of the two cytoplasmic tails of CD46. Because cleavage of Cyt1 is necessary for IL-10 production (11), the translocation of Cyt1 to the nucleus is likely required to promote *IL10* expression, and Cyt1 translocation may be dysregulated in T cells from MS patients.

Our data demonstrate the key role of the STP domain in controlling CD46 cleavage and its regulatory function. Stimulation of CD46 induces ERK phosphorylation (8), and we showed that the STP domain promoted ERK activation. The highest concentrations of anti-CD3 antibody were the most effective in inducing a shift in the mass of CD46. Our data suggest a potential mechanism to account for previously published observations that IL-10 production by Th1 cells required strong TCR stimulation by high antigen concentrations and ERK phosphorylation (28), and with the requirement for strong TCR stimulation to generate IL-10–producing regulatory T cells (49). The STP domain was required for the recruitment of CD46 to the immune synapse and for signaling downstream of the receptor. CD46 is present in lipid rafts (12) and CD46-Cyt1 binds to Discs Large MAGUK Scaffold Protein 4 (DLG-4) (50). DLGs are part of a family of scaffolding proteins called MAGUKs (membrane-associated guanylate kinase homologues), and DLG1 is recruited to the immune synapse in response to TCR stimulation, including in Tregs (51, 52).

Localization of DLG1 to neuronal synapses is mediated by the serine/threonine kinase Ca2+/calmodulin-dependent protein kinase II (CAMKII) (53). CAMKII mediates NF-κB activation in response to TCR stimulation by being recruited to the immune synapse and phosphorylating the adaptor protein B-cell CLL/lymphoma 10 (BCL10), which stimulates activation of the CARMA1-BCL10-Malt1 (CBM) complex. The CBM complex in turn activates the IKKs, resulting in NF-κB activation (54). We hypothesize that the recruitment of CD46 to the immune synapse may involve a TCR-dependent DLG-CD46 complex, perhaps through CAMKII activation, which promotes CD46 signaling and its further cleavage leading to IL-10 secretion. Indeed, CAMKII stimulates IL-10 production by T cells through the activation of the transcription factor myocyte enhancer factor-2 (MEF2) (55). Variants of NF-κB and MEF2 have been identified in MS patients, and T cells from MS patients have enhanced NF-κB activation and exhibit increased responses to inflammatory signals (56, 57), a similar profile to that of T cells expressing the ΔSTP CD46 mutant. Because T cells from MS patients secrete more pro-inflammatory cytokines and less IL-10 than do T cells from healthy donors (2), this supports our idea that the aberrant IL-10 response in MS is due to altered modification of glycosylation in CD46 leading to increased NF-κB signaling. Overall, these studies demonstrate the key role of the STP domain of CD46 in the functions of this receptor, and suggest that CD46 glycosylation is regulated in response to TCR stimulation (Fig. 8). Dysregulation of this pathway likely contributes to the impaired regulatory function of Tregs occurring in MS. Further elucidation of the molecular mechanisms involved could provide new therapeutic targets that might correct these defects.

Materials and Methods

Study design

This study was designed to determine why the regulatory pathway triggered by CD46 ligation on primary human T cells is dysfunctional in MS. We first characterized mechanistic insights into regulation of the abundance of CD46 in healthy T cells (ethical approval AMREC 15-HV-013) and then compared sex- and age-matched healthy donors to patients with relapsing-remitting MS (RRMS, ethical approval SR258, see patient description in Table 1). T cells were isolated from peripheral blood after informed consent. Ethical approval was obtained from the Lothian Health Board Ethics Committee. Sample sizes were defined on the basis of our previous investigations. Studies were not blinded. Numbers of donors are indicated in the legends.

Cell purification and activation

PBMCs were isolated from the blood of age and sex-matched healthy donors or RRMS patients. Total CD4+ T cells or CD4+ memory T cells were purified from PBMCs with the EasySep Human CD4+ T Cell Isolation Kit (StemCell, Grenoble, France) and then activated with immobilized anti-CD3 (OKT3, 5 µg/ml), anti-CD46 (MC120.6, 10 µg/ml), or both anti-CD3 and anti-CD46 together, and 10 U/ml of rhIL-2 was added to all activating conditions (Cambridge Bioscience, Cambridge, UK) (6). In some experiments, cells were treated with swainsonine, which inhibits mannosidase 2 (25), or with Benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADG), which prevents O-glycan elongation (3 mM, Sigma, Gillingham, UK), or its respective vehicle control, methanol, before activation, or the NF-κB inhibitor (Bay 11-7082, 0.5 µM, Sigma) was added to the culture. BC1 and ΔSTP-expressing CHO cells and CHO cells expressing the 3 N-glycosylation mutants of CD46, all previously described (21), were kindly provided by Dr. John Atkinson (Washington University, St-Louis, MO).

Proliferation assays

Cellular proliferation was determined by pre-labeling purified T cells with eFluor-670 (eBioscience, Hatfield, UK) before activation according to the manufacturer's instructions and then assessing the remaining fluorescence after 4 or 5 days.

Cytokine detection

The amounts of IL-10 and IFN-γ secreted into the cell culture medium were determined with ELISA kits specific for human IL-10 (BD Pharmingen, Oxford, UK) and IFN-γ (Thermo-fisher) as previously described (18-20) or with cytokine secretion assays (Miltenyi, Bisley, UK), according to the manufacturer's instructions.

CD46 detection by Western blotting

Cells were lysed with RIPA buffer. In some instances, CD46 was immunoprecipitated from the samples with an anti-CD46 monoclonal antibody (clone MC120.6, 1 µg/sample). CD46 was analyzed by Western blotting with polyclonal anti-CD46 antibodies (Santa Cruz, Wembley, UK). The membranes were then stripped and incubated with anti-actin antibodies (Abcam).

Deglycosylation

CD46 was immunoprecipitated from cell lysates and deglycosylated with the ProZyme (Hayward, CA) Enzymatic deglycosylation kit (GK80110) and ProZyme prO-Link Extender (GK80115) according to the manufacturer's instructions.

Plasmids and transfection

CD4+ T cells were nucleofected (Lonza, Tewkesbury, UK) with plasmids encoding the WT-BC1 or the ΔSTP glycosylated mutant CD46 constructs as previously described (21). Constructs were kindly provided by Dr. John Atkinson (Washington University, St Louis, MO). These constructs were tagged with GFP by cloning PCR fragments of the BC1 and ΔSTP coding sequences in frame with that coding GFP in the Xho I and Eco RI sites of the pEGFP-N1 plasmid (Clontech), resulting in the fusion of GFP to the C terminus of BC1 and ΔSTP. The resulting constructs were verified by sequencing. CD4+ T cells underwent nucleofection with either the U14 or V24 programs depending on the desired readouts; V24 was used for greater transfection efficiency (50 to 60% of cells) and short time course experiments (such as signaling), whereas the U14 program was less efficient (~20 to 30% of cells transfected), but resulted in greater viability and was used to assess cytokine production and proliferation. After nucleofection, the cells were cultured overnight at 37^{o}C before undergoing stimulation.

Flow cytometry and Phosphoflow

T cells were stained with FITC-conjugated anti-CD46 (Biolegend, London, UK) and PE-conjugated anti-CD69 (Biolegend). After cells were permeabilized with 0.5% saponin, Cyt1 and Cyt2 were detected with specific monoclonal antibodies generously provided by Drs Maggie So and Nathan Weyand (University of Arizona, AZ), as previously reported (11). To account for different basal amounts of Cyt1 and Cyt2 among donors, their relative changes in abundance upon cell stimulation were calculated as follows: [MFI(act) – MFI(US))/MFI(US))*100]. To analyze pERK, pp65, and pTyr, transfected cells expressing either the BC1 or ΔSTP CD46 proteins were incubated on ice for 15 min with anti-CD3 antibodies alone or in the presence of anti-CD28 antibodies, and the antibodies were crosslinked with F(ab')2 rabbit anti-mouse IgG (Jackson

laboratories) followed by incubation of the cells at 37°C for 15 min. The cells were then immediately fixed with BD fix buffer III, permeabilized, and incubated with APC-conjugated anti-pERK (BD Bioscience), FITC-conjugated anti-pTyr (PY20-FITC, Santa Cruz), or PE-conjugated anti-pp65 (BD phosflow, S529-PE) antibodies. Cells were analyzed with a FACScalibur flow cytometer. A lectin screening kit was also used to assess the binding of a panel of lectins after T cell activation (Lectin kit I, Vector laboratories).

Confocal microscopy

Localization of the ectodomain of CD46 and the intracellular tails Cyt1 and Cyt2 was examined by confocal microscopy. Chamber slides (16-well, Nunc, VWR International Ltd, UK) were pre-coated with poly-D-lysine (PDL) (2 μg/ml) before being coated with either mouse IgG1 (15 μg/ml) or anti-CD3 (5 μ/ml) and anti-CD46 (10 μg/ml) antibodies, and CD4+ T cells were then added to the wells. The cells were then fixed with 4% paraformaldehyde (PFA) and quenched with NH_4Cl (50 mM in PBS), before being permeabilized with 0.5% v/v Triton X-100 and blocked with 20% v/v goat serum in PBS (Cambridge Bioscience, UK). T cells were incubated with mouse anti-CD46 (clone MCI20.6) or anti-Cyt1 or anti-Cyt2 monoclonal antibodies overnight, which was followed by labeling with Alexa Fluor 488–conjugated F(ab')2 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA), counterstaining with DAPI nuclear stain, and mounting with ProLong Gold (Invitrogen, Carlsbad, CA, USA). Images were acquired with a Leica SP5 and imported to ImageJ 1.42q for analysis. In some experiments, T cells were activated with CD3/CD46 and CD3/CD28 dynabeads (ThermoFisher, Paisley, UK) for 30 min at 37°C, fixed with 4% PFA, permeabilized, and stained with phalloidin-AF568 (Molecular Probes, Paisley, UK), anti-CD46-FITC (Biolegend), and DAPI and then analyzed by confocal microscopy.

Amnis imaging

Transfected primary T cells expressing either the BC1-GFP or the ΔSTP-GFP mutant CD46 constructs were stimulated with CD3/CD28 dynabeads for 10 and 30 min at 37°C. After fixation and permeabilization, the cells were stained with phalloidin-AF568 and Hoechst and analyzed with the Amnis Flowsight imager flow cytometer. Single cells were identified through the size vs. aspect ratio of the brightfield image and those with attached beads were identified by increased side scatter. The area vs. aspect ratio of the side scatter image enabled refinement to those cells with only one bead. This population was then plotted on Aspect ratio intensity vs symmetry of the brightfield image using a tight object mask. This final population consisted of single cells with one bead attached in the correct orientation (x, y on the image as opposed to z). This population was then assessed for colocalization of CD46-GFP and actin by comparing the bright detail of the two images. The population, R8_synapse, was plotted on Bright detail similarity R3_MC_CD46 GFP_Actin.

Statistical analysis

The groups were analyzed with Graphpad Prism software. Flow cytometry data were analyzed using the Wilcoxon t-test, when assessing paired samples, or ANOVA with Dunn's multiple samples correction. ELISA data are the average of duplicate wells, and the averages obtained for the different donors were analyzed with the Bonferoni-corrected Wilcoxon test. A non-parametric Mann-Whitney t-test was used to compare healthy T cells and MS T cells. All *P* values are two-tailed and with a 95% confidence interval.

Supplementary Materials

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Fig. 1. T cell activation affects CD46 MW. (**A** and **B**) $CD4^+$ T cells from healthy donors were left unstimulated (US) or were stimulated with anti-CD3 antibody alone (CD3) or in the presence of anti-CD46 antibody (CD3.46) for 48 hours. The cells were then analyzed by Western blotting with antibodies against the indicated proteins. Western blots are representative of at least 10 experiments. The arrow indicates the lowest mass of the protein. As a control, unstimulated and anti-CD3–stimulated cells were analyzed by Western blotting to detect CD28 (B). Western blots are representative of two experiments. To aid in visualizing whether any shift in the apparent molecular mass of a protein of interest has occurred, the image has been overlaid with a dashed

white line that is centered on bands corresponding to the protein of interest in unstimulated cells. Other Western blot images throughout the manuscript and Supplementary Materials have been similarly treated. (**C**) Primary $CD4^+$ T cells were left unstimulated or were stimulated with the indicated concentrations of anti-CD3 antibody before being analyzed by Western blotting as described in (A). Data are representative of two experiments. The arrow points to the lowest mass of CD46. (**D**) Primary $CD4^+$ T cells were left unstimulated or were stimulated with the indicated antibodies before being analyzed by Western blotting as described in (A). Western blots are representative of four experiments. (**E**) Primary $CD4^+$ T cells were left unstimulated or were stimulated with anti-CD3 antibody (5 μg/ml) in the presence of increasing doses of IL-2 (left) for 2 days or else were incubated with or without IL-2 (10 U/ml) for 2 days (right) before being analyzed by Western blotting as described in (A). Western blots are representative of four experiments. (**F**) Primary $CD4^+$ cells were left unstimulated or were stimulated with anti-CD3 antibody for 1 or 4 hours (donor 1, left) or for 5 hours (donor 2, right) before being analyzed by Western blotting as described in (A). Western blots are representative of three experiments.

Fig. 2. **T cell activation modulates CD46 O-glycosylation.** (**A** and **B**) CD46 was immunoprecipitated from human $CD4^+$ T cells that were left unstimulated (US) or were stimulated with anti-CD3 for 2 days. The cells were lysed, subjected to immunoprecipitation (IP) with anti-CD46 antibody, and analyzed by Western blotting with antibodies against CD46 (A) before (-) or after treatment (+) with a mixture of N- and O-glycanases and exoglycosidases (Glyc), or (B) N-glycanases (N-glyc) or O-glycanases and exoglycosidases (O-glyc) separately. Western blots are representative of two experiments. (**C**) $CD4^+$ T cells were incubated overnight with vehicle (Ctrl), swainsonine, which inhibits mannosidase 2 (N-glyc inh), or Benzyl 2-acetamido-2-deoxy-α-D-

galactopyranoside, which modifies O-glycan elongation (O-glyc inh), and then the cell surface abundance of CD46 was determined by flow cytometry. (**D**) The cells shown in (C) were then left unstimulated or were stimulated with anti-CD3 antibody before being analyzed by Western blotting with the indicated antibodies. Western blots are representative of four experiments.

Fig. 3. **Expression of the ΔSTP CD46 mutant reduces CD46 shedding.** (**A** and **B**) $CD4^+$ T cells were nucleofected with the indicated amounts of plasmids encoding GFP alone, WT BC1-GFP, or the ΔSTP-GFP mutant. (A) The cells were analyzed by flow cytometry to detect the percentage of GFP^+ cells. (B) The cells were then left unstimulated or were stimulated with anti-CD3 antibody alone or in the presence of anti-CD46 antibody. Four hours later, the cells were analyzed by flow cytometry to determine the cell surface abundance of CD46. (**C**) Analysis of the average mean fluorescence intensity of CD46 in GFP^+ cells after 4 hours of activation. Data are means ± SEM of seven experiments analyzed by Wilcoxon test. (**D**) $CD4^+$ T cells expressing the indicated GFP-tagged constructs and left unstimulated or stimulated with anti-CD3 were analyzed by Western blotting with antibodies against the indicated proteins. Western blots are representative of two experiments. Arrowhead indicates the highest mass of CD46. (**E**) $CD4^+$ T cells were transfected with plasmids encoding GFP alone (Ctrl) or the BC1-GFP or STP-GFP mutant constructs, and activated by anti-CD3 antibody alone or with anti-CD46 antibody. One day later, the percentage of GFP+ cells were determined by flow cytometry. Data are means ± SEM of nine experiments. The data were analyzed by one-way ANOVA (Friedman test **p=0.0030) with Dunn's multiple comparison test.

Fig. 4. **Expression of a ΔSTP CD46 mutant decreases CD69 expression and increases IFN-γ production.** (**A**) $CD4^+$ T cells were nucleofected with plasmids encoding the WT BC1-GFP or ΔSTP-GFP mutant constructs and were stimulated with anti-CD3 and anti-CD46 antibodies. Four hours later, the cells were analyzed by flow cytometry to determine the cell surface expression of CD46 and CD69 in GFP+ cells. (**B**) Analysis of the MFI of CD69 in GFP^+ cells. Data are means ± SEM of seven experiments and were analyzed by Wilcoxon test. (**C**) $CD4^+$ T cells were nucleofected with plasmids encoding the WT BC1-GFP or ΔSTP-GFP mutant constructs and were left unstimulated or were stimulated with the indicated combination of anti-CD3 and anti-CD46 antibodies. The percentage of GFP+CD69+ cells were analyzed by flow cytometry. Data are means ± SEM of 11 donors and were analyzed Friedman test (***p<=0.0001) followed by a Bonferroni-corrected Wilcoxon t-test. (**D**) $CD4^+$ T cells expressing the BC1 or ΔSTP constructs were left unstimulated or were stimulated with the indicated combination of anti-CD3 and anti-CD46 antibodies before the percentage of proliferating cells was assessed by flow cytometry. Data are means ± SEM of five donors. (**E** and **F**) $CD4^+$ T cells expressing the BC1 or ΔSTP constructs were costimulated with anti-CD3 and anti-CD46 antibodies before the amounts of IL-10 and IFN-γ secreted into the cell culture medium was determined by ELISA (E) or by secretion assays (F). Data are means ± SEM of six (E) or four (F) experiments and were analyzed by Wilcoxon t-test.

Fig. 5. The STP domain of CD46 is required for its recruitment to the immune synapse. (**A**) Purified human $CD4^+$ T cells were left unstimulated or were stimulated with anti-CD3 and anti-CD46 antibodies or with CD3/CD28 dynabeads for 30 min. The cells were immediately fixed, permeabilized, and stained with anti-CD46-FITC (green), phalloidin (red), and DAPI (blue). Cells were then visualized by confocal microscopy. Images are representative of at least five cells from

each of two experiments. (**B** and **C**) $CD4^+$ T cells were nucleofected with the plasmids encoding WT BC1-GFP (B) or the ΔSTP-GFP mutant (C) and the cells were left unstimulated (US) or were stimulated with CD3/CD28 beads or immobilized anti-CD3 and anti-CD46 antibodies. The cells were fixed, permeabilized, and stained with phalloidin-568 (red) and DAPI (blue). Cells were then visualized by confocal microscopy. Images are representative of at least 10 cells analyzed each in at least four different experiments (see more examples in the supplementary figures). White arrows point to areas of actin polymerization.

Fig. 6. Deletion of the STP domain of CD46 increases NF-κB activation. (**A**) An RNAi screen was performed on primary $CD4^+$ T cells infected by lentiviruses encoding 5000 different shRNAs (19). The Z-score of CD46 abundance at the cell surface obtained after knockdown of *CHUK* compared to that of controls is shown. Infection with lentiviruses encoding two different shRNA constructs targeting CHUK led to strong negative Z scores. (**B**) $CD4^+$ T cells were left unstimulated (US) or were stimulated with anti-CD3 antibody alone or together with anti-CD46 antibody in the presence or absence of the IKK inhibitor Bay 11-7082. Two hours later, the cells were analyzed by flow cytometry to determine the cell surface expression of CD46. Data are representative of three experiments. (**C**) $CD4^+$ T cells expressing the WT BC1-GFP or ΔSTP-GFP mutant constructs were left unstimulated or were stimulated with CD3/CD28 dynabeads and then analyzed by flow cytometry to detect pp65 NF-kB and pTyr-containing proteins. Data are representative of three experiments. (**D**) The relative change in the abundances of phosphorylated NF-κB, ERK, or of tyrosine-phosphorylated proteins was determined by flow cytometric analysis of cells expressing the mutant was normalized to the phosphorylation obtained in the paired samples expressing BC1 (n= 2 or 3 donors).

Fig. 7. CD4$^+$ T cells from MS patients fail to exhibit a CD3-induced shift in CD46 mass and have aberrant CD46 expression. (**A**) CD4$^+$ T cells were purified from the blood of healthy controls (HC) or patients with RRMS (MS) [(MS donors 1 and 2 were untreated; donors 3 and 4 underwent IFN-β treatment]. The cells were left unstimulated (US) or were stimulated with anti-CD3 antibodies for 4 days before being analyzed by Western blotting with antibodies against the indicated proteins. (**B**) CD4$^+$ T cells from healthy controls (HC) or patients with RRMS (MS) were stimulated with anti-CD3 and anti-CD46 antibodies and the MFI of cell surface CD46 was assessed by flow cytometry on the indicated days (top) or after 4 days of activation (bottom). Data are means ± SEM of six donors for HC and 4 donors for MS. See Table 1 for donor details. (**C**) CD4$^+$ T cells from healthy controls (HC) or RRMS patients (MS) were stimulated for 2 (left) or 4 (right) days with anti-CD3 antibody alone or in the presence of anti-CD46 antibody before the percentages of cells expressing either of the two cytoplasmic tails of CD46 (Cyt1 and Cyt2) were determined by intracellular flow cytometry analysis as previously described (11). Data are means ± SEM of 37 (HC, day 2), 25 (MS, day 2; 12 untreated; 13 IFN-β–treated), 28 (HC, day 4), or 20 (MS, day 4, 9 untreated; 11 IFN-β–treated) donors. Data were analyzed with the Kruskal-Wallis test ($P < 0.05$) followed by a Wilcoxon t-test when paired and Mann Whitney t-test to compared HC and MS samples.

Fig. 8. The role of the TCR and CD46 glycosylation in the switch from T_H1 cells to Tr1 cells. TCR activation leads to a change in the O-glycosylation of CD46 (i) that promotes the recruitment of CD46 to the immune synapse (ii). This is turn enhances the T cell response upon CD3 and CD46 costimulation, which leads to the processing of CD46 and the switch from IFN-γ secretion to IL-

10 secretion (iii). This TCR-stimulated change in the glycosylation state of CD46 is defective in $CD4^+$ T cells from MS cells, which leads to aberrant CD46 processing, a dysfunctional signaling pathway, and defective Tr1 cell generation. sCD46, soluble CD46.

Table 1. Description of the donors used in this study. UT, untreated. EDSS, Expanded Disability Status Scale.

	Healthy Controls	**MS Patients**
Surface CD46		
Sex (females:males)	24:9	21:4
Age (years, mean ± SD)	33.5 ± 7.5	42 ± 8.1
Age range	22 to 52	26 to 57
EDSS (mean ± SD)	-	3 ± 1.59
Treatment	-	12 UT; 13 IFN-β
CD46 Cyt1 Cyt2		
Sex (females:males)	24:19	18:8
Age (years, mean ± SD)	33.2 ± 7.6	41.5 ± 9.1
Age range	22 to 52	26 to 57
EDSS (mean ± SD)	-	2.8 ± 1.79
Treatment	-	13 UT; 13 IFN-β