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# CYFIP2 is highly abundant in CD4+ cells from multiple sclerosis patients and is involved in T cell adhesion

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DNA microarray profiling of CD4+ and CD8+ cells from non-treated relapsing and remitting multiple sclerosis (MS) patients determined that the cytoplasmic binding partner of fragile X protein (CYFIP2, also called PIR121) was increased significantly compared to healthy controls. Western analysis confirmed that CYFIP2 protein was increased approximately fourfold in CD4+ cells from MS compared to inflammatory bowel disorder (IBD) patients or healthy controls. Because CYFIP2 acts as part of a tetrameric complex that regulates WAVE1 activation we hypothesized that high levels of CYFIP2 facilitate T cell adhesion, which is elevated in MS patients. Several findings indicated that increased levels of CYFIP2 facilitated adhesion. First, adenoviral-mediated overexpression of CYFIP2 in Jurkat cells increased fibronectin-mediated adhesion. Secondly, CYFIP2 knock-down experiments using antisense oligodeoxynucleotides reduced fibronectin-mediated binding in Jurkat and CD4+ cells. Thirdly, inhibition of Rac-1, a physical partner with CYFIP2 and regulator of WAVE1 activity, reduced fibronectin-mediated adhesion in Jurkat and CD4+ cells. Finally, inhibition of Rac-1 or reduction of CYFIP2 protein decreased fibronectin-mediated adhesion in CD4+ cells from MS patients to levels similar to controls. These studies suggest that overabundance of CYFIP2 protein facilitates increased adhesion properties of T cells from MS patients.

**Key words:** Human / T lymphocytes / Multiple sclerosis / Adhesion molecules and signal transduction

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## 1 Introduction

Multiple sclerosis (MS) is an autoimmune event associated with a chronic inflammatory response, demyelination and axonal loss [1]. Based on neuropathological examination, CD4+ and CD8+ T lymphocytes are present in high numbers in MS lesions [2], and these cells play a prominent role in the pathophysiology of MS [3]. Activated T cells within MS lesions are associated with elevated production of pro-inflammatory cytokines and chemokines, which are associated with demyelination, blocking of remyelination and the induction of axonal loss [4]. Although the driving force behind these events remains unclear, it is believed that reduction of T cell movement into the central nervous system will protect MS patients.

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**Abbreviations: CYFIP:** Cytoplasmic binding partner of fragile X protein **PIR121:** p53-inducible response protein **MS:** Multiple sclerosis **IBD:** Inflammatory bowel disorder

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Because CD4$^+$ and CD8$^+$ T cells are among the major peripheral blood cell types found in MS lesions [2], we hypothesized that an analysis of their immune gene expression patterns would identify novel markers involved in MS pathogenesis. Although others have used this approach to determine changes in gene expression in MS lesions [5–7], there have been no reports about the changes in gene expression in specific immune cell types. Accordingly, we purified CD4$^+$ and CD8$^+$ cells from both MS patients that had disease activity within the past 6 months and were not receiving any therapeutic intervention and healthy age-matched controls and determined gene expression profiles. Our bioinformatic analysis identified several genes that were upregulated in both CD4$^+$ and CD8$^+$ cells. A novel observation was that the cytoplasmic binding partner of fragile X protein (CYFIP2, also called PIR121) was upregulated in CD4$^+$ cells from MS patients; Western analysis confirmed that CYFIP2 levels were increased significantly, whereas its closely related family member CYFIP1 was not.

The function of CYFIP proteins in the central nervous system is unknown, as they lack any known motifs or functional domains. However, it is speculated that CYFIP are involved in regulating the translational machinery at the synaptic terminal that is disrupted in fragile X syndrome [8]. CYFIP may also regulate axon growth, guidance or branching [9]. It was recently shown that CYFIP2 is part of a heterotetrameric protein complex that negatively regulates WAVE1 [10]. The four proteins in this complex are Nck-associated protein (Nap125), HSPC300, an orthologue of CYFIP2 (PIR121) and WAVE1. Rac-1 activation leads to Nck activation and the subsequent release of WAVE1 and HSPC300 from the complex, which promotes actin nucleation [10]. Rac-1 plays an important role in regulating T cell adhesion, which is increased in the highly active leukocytes found in MS patients [11, 12].

Several reports indicate that the Rac-1 pathway may be disrupted in T cells from MS patients. Specifically, Rac-1 can be activated by Vav-1, which is closely coupled with the activation of CD28 [13] and is an important co-activator of T cell receptor responses in MS pathogenesis [14]. In addition, inhibition of a direct activator of Rac-1, PI3K [15], using wortmannin or LY249002 significantly reduces the transendothelial migration rate of V$\delta$1$^+$ cells from relapsing and remitting MS patients [12]. Finally, in the animal model of MS, experimental allergic encephalomyelitis (EAE), blocking the functional activation state of Ras proteins (highly similar in function to Rac-1) using trans-trans-farnesylthiosalicylic acid (which destabilizes the attachment of Ras and Rho-like proteins at the plasma membrane) significantly reversed the development of EAE and reduced T cell proliferation and the activation state of myelin-reactive T cells [16].

We report here two novel observations. First, through the use of a non-biased microarray screening approach, we find that CYFIP2 is increased in CD4$^+$ cells from MS patients but not inflammatory bowel disorder (IBD) patients, another autoimmune-affected population. Secondly, the increased levels of CYFIP2 in CD4$^+$ cells from MS patients facilitate fibronectin-mediated binding.

## 2 Results

### 2.1 DNA microarray screening identifies several genes highly expressed in CD4$^+$ and CD8$^+$ T cells from MS patients

We used a non-biased DNA microarray screening approach [17] to determine gene expression profiles in T cells from MS patients and healthy controls (Table 1 and 2). Briefly, the mean and standard error of the mean was determined by dividing the average expression level (pixel density) of each gene in cells from MS patients by the level in healthy controls. Gene profiles of CD4$^+$ or CD8$^+$ cells from MS patients clustered together but separated from CD4$^+$ or CD8$^+$ cells isolated from healthy controls (Fig. 1a; only CD4$^+$ profiles shown). Venn diagram analysis identified several genes that were upregulated in both CD4$^+$ and CD8$^+$ cells in MS patients as compared to controls (Fig. 1). Many pro-inflammatory genes, including CD40 binding receptor, IL-16, RANTES, CCR-5 and CCR-6, were increased in both CD4$^+$ and CD8$^+$ cells from MS patients (changes in gene expression in CD4$^+$ cells from MS patients are reported in Table 3 and 4). We also observed that several genes were down-regulated in CD4$^+$ and CD8$^+$ cells from MS patients (not shown; raw data for CD4$^+$ and CD8$^+$ analysis reported at www.sbrc.ca/dnnd/Immuno-microarray.htm).

Because it was reported that intracellular calcium homeostasis is disrupted in T cells from MS patients prior to clinical relapse [18], we induced this event by treating CD4$^+$ cells from healthy controls with thapsigargin. Venn diagram analysis identified several genes in CD4$^+$ and CD8$^+$ cells from MS patients that were also elevated in thapsigargin-treated CD4$^+$ cells from healthy controls (Fig. 1b). Thus, genes were also categorized based on their response to elevations in intracellular calcium (Fig. 1b and Table 4).

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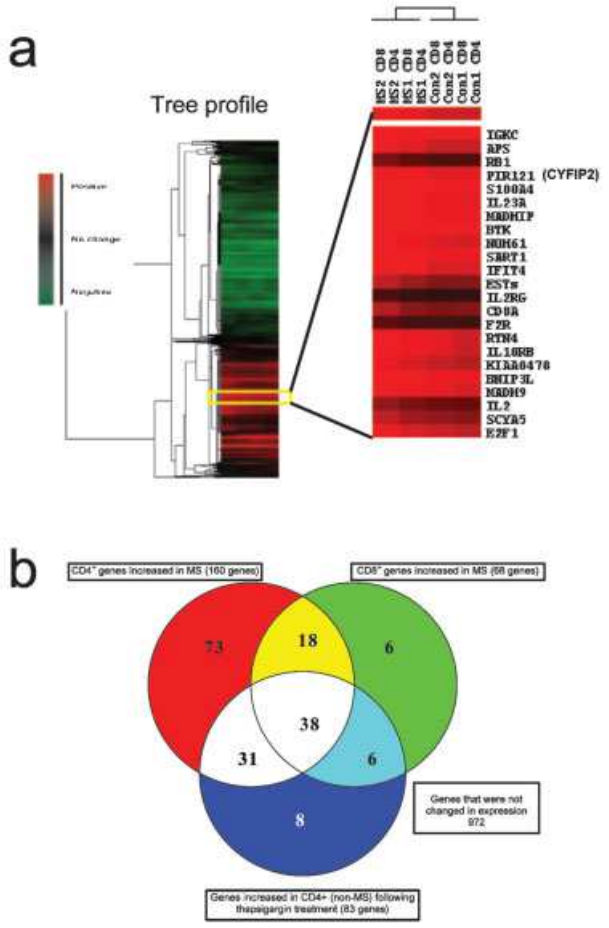


*Fig. 1.* Gene expression profiles of CD4+ and CD8+ cells from MS patients. (a) Averaged linkage hierarchical clustering indicated that gene profiles of control and MS patients cluster separately. The yellow box indicates the expanded area to the right. Clustering diagram represents data from arrays of two control patients and two MS patients. (b) Venn Diagram summarizing groups of genes highly expressed in CD4+ (red) or CD8+ (green) cells from MS patients compared to healthy controls or thapsigargin-treated *vs.* untreated CD4+ cells from healthy controls (blue). Gene lists for each section of the Venn Diagram were generated by identifying the gene most highly expressed (*p* value 2-tailed *t*-test analysis [17]) and identifying all genes with a similar expression profile (minimal correlation coefficient of 0.99; Genespring analysis). Raw data from experiments can be found at http://www.sbrc.ca/dnnd/Immuno-microarray.htm.

## 2.2 CYFIP2 is elevated in CD4+ cells from MS patients, and expression is increased following rises in intracellular calcium

A novel finding of our microarray analysis was that CYFIP2 mRNA is increased by approximately ninefold in CD4+ cells from MS patients as compared to controls (Table 4). RT-PCR analysis confirmed that CYFIP2 mRNA levels, but not CYFIP1 levels, were increased in CD4+ cells from MS patients (not shown). Western analysis determined that CYFIP2 protein was increased significantly in CD4+ cells in MS patients but not in IBD patients or healthy controls (Fig. 2a). Co-immunprecipitation analysis of lysates from Jurkat and CD4+ cells confirmed that Rac-1 and CYFIP2 interact physically [10] and that relative CYFIP2 levels were increased in MS patients (Fig. 2b). CYFIP2 gene expression was increased in Jurkat and CD4+ cells following treatment with thapsigargin, which confirmed our bioinformatic Venn Diagram interpretation that CYFIP2 gene expression was regulated by increases in intracellular calcium (Fig. 2c). In addition, dantrolene treatment, which prevents release of calcium from the endoplasmic reticulum, blocked thapsigargin-mediated increases in CYFIP2 (Fig. 2d).

## 2.3 Rac-1 activity or an increase in CYFIP2 protein levels enhances fibronectin-mediated binding in CD4+ and Jurkat T cells

In *Drosophila*, CYFIP, a homologue of human CYFIP1 and CYFIP2, acts as an effector of Rac-1 [19]. Because we observed that CYFIP2 was increased in CD4+ cells in MS patients and CYFIP2 and Rac-1 interacted physically (Fig. 2) and others have shown that Rac-1 regulates T cell actin polymerization [13], we hypothesized that the Rac-1/CYFIP2 pathway regulates fibronectin-mediated binding. We chose to study fibronectin, as this protein interacts physically with $\alpha$4 and $\beta$1 family integrin proteins [20], and recent clinical trials have shown that Antegren® (an antibody to $\alpha$4 protein) reduced new brain lesions in MS patients as determined by MRI [21, 22]. In Jurkat cells, wortmannin dose-dependently reduced Rac-1 activity (Fig. 3a) and fibronectin-mediated binding (Fig. 3b). Among control Jurkat cells, 14±1.2% of the cell population adhered to fibronectin, whereas 41±2.6% of thapsigargin-treated cells bound. In order to ensure that we were assessing Rac-1 mediated events, we delivered wild-type or mutant versions of Rac-1 to T cells. Adenoviral-mediated delivery of dominant active Rac-1 (V12) increased fibronectin-mediated binding significantly (21.6±3.2% of the cells bound), whereas dominant negative Rac-1 (N17) did not affect binding (Fig 3d). These results indicated that active Rac-1 is involved in fibronectin-mediated binding by T cells. Furthermore, CYFIP2 antisense treatment of Jurkat (1 $\mu$M) or primary CD4+ cells (5 $\mu$M) indicated that reduction of CYFIP2 protein inhibits fibronectin-mediated binding (Fig. 3c). Among control CD4+ cells, 13±1.6% bound to fibronectin, whereas 22±2.6% of cells treated with thapsigargin bound. Western analysis of Jurkat cell lysates confirmed that antisense treatment reduced CYFIP2 levels (inset Fig. 3c). Finally, overexpression of CYFIP2 via

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**Table 1.** Characteristics of MS patients and cell isolation[a)]

| Subject | Age (years) | Sex | Cell isolation | EDSS | Months since diagnosis |
|---|---|---|---|---|---|
| MS1 | 23 | F | PBMC/PBL | ND[b)] | 2 |
| MS2 | 49 | F | PBMC/PBL | 2 | 216 |
| MS3 | 38 | F | PBMC/PBL | ND | 10 |
| **MS4** | **28** | **F** | **CD4/CD8** | **ND** | **12** |
| **MS5** | **35** | **M** | **CD4/CD8** | **ND** | **15** |
| **MS6** | **40** | **F** | **CD4/CD8** | **2.5** | **29** |
| **MS7** | **32** | **F** | **CD4/CD8** | **2** | **109** |
| **MS8** | **50** | **F** | **CD4/CD8** | **3.5** | **72** |
| **MS9** | **32** | **F** | **CD4/CD8** | **0** | **12** |
| **MS10** | **39** | **F** | **CD4** | **2.5** | **6** |
| **MS11** | **34** | **F** | **CD4** | **4** | **19** |
| MS12 | 47 | F | CD4 | ND | 18 |
| MS13 | 25 | F | CD4/CD8 | ND | 5 |
| MS14 | 28 | F | CD4 | 0 | 3 |
| MS15 | 40 | M | CD4/CD8 | 2 | 36 |
| MS16 | 40 | F | CD8 | 3 | 8 |
| MS17 | 47 | F | CD8 | 0 | 17 |
| MS18 | 31 | F | CD4 | ND | 21 |
| MS19 | 25 | M | CD4 | ND | 6 |
| MS20 | 37 | M | CD4/CD8 | 4 | 20 |
| MS21 | 37 | F | CD4/CD8 | ND | 108 |
| Mean ± SEM | 36±1 | 17F/4M | | 2.13±1.5 | 35±11 |

[a)]Blood sample was 50 mls. Patient samples highlighted in bold were examined by microarray analysis. All CD4$^+$ samples were used in confirmation studies in which protein determination was conducted.
[b)]ND: not detected within the past 6 months.

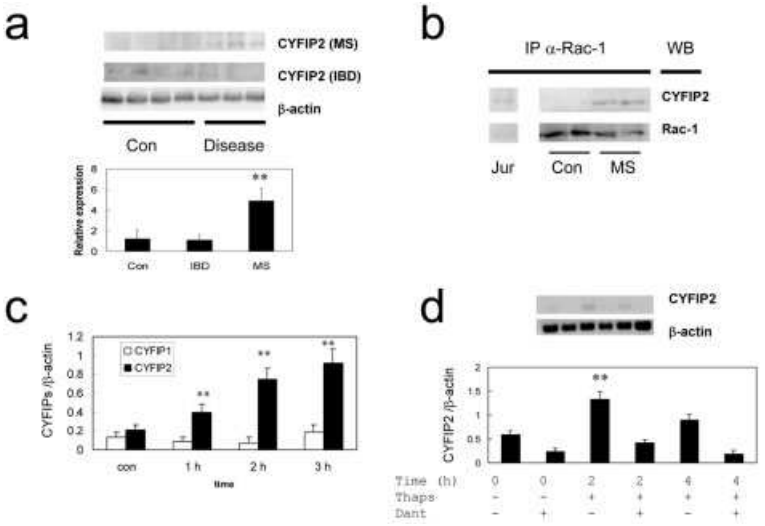


*Fig. 2.* CYFIP2 protein is increased in CD4$^+$ cells from MS patients but not IBD or healthy controls. (a) Representative Western analysis of CD4$^+$ cells isolated from MS patients (analysis conducted on samples 4–11 in Table 1 and 2; $n$=8; samples 4–7 shown), IBD patients (analysis conducted on samples 5–11; $n$=6; samples 5–7 shown) and healthy controls (analysis on samples 7, 9–11, 13, 15–18; $n$=8; samples 9–11 and 13 shown). Graph shows increase relative to β-actin levels. (b) Immunoprecipitation (IP) was conducted using anti-Rac-1 antibody (1:500) overnight (MS samples 18 and 19 and control 17 and 18 shown). Similar results were observed when IP was conducted using anti-CYFIP2 antibody (not shown). Figure is representative of experiments using CD4$^+$ cells from 6 control and 6 MS patients (MS samples 16–21 and control samples 11, 13, 15–19). (c) CYFIP2 but not CYFIP1 mRNA is increased in Jurkat cells treated with thapsigargin (100 nM). (d) Treatment of Jurkat cells with dantrolene (10 μM) blocks CYFIP2 gene expression induced by increased intracellular calcium levels. Figure is representative of three separate experiments. $^{**}p<0.001$, significantly different from healthy controls or control samples.

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**Table 2.** Characteristics of normal control and IBD control patients and cell isolation[a)]

| Subject | Age (years) | Sex | Cell isolation |
|---|---|---|---|
| CON1 | 35 | M | PBMC/PBL/CD8 |
| CON2 | 32 | F | PBMC/PBL/CD8/CD4 |
| **CON3** | **35** | **M** | **CD4/CD8** |
| **CON4** | **48** | **F** | **CD4/CD8** |
| CON5 | 34 | F | PBMC |
| CON6 | 32 | F | PBMC |
| **CON7** | **26** | **M** | **CD4/CD8** |
| CON8 | 27 | M | PBMC |
| **CON9** | **28** | **F** | **CD4/CD8** |
| **CON10** | **29** | **M** | **CD4/CD8** |
| **CON11** | **35** | **F** | **CD4/CD8** |
| CON12 | 42 | F | PBMC |
| **CON13** | **28** | **F** | **CD4/CD8** |
| CON14 | 27 | F | PBL |
| **CON15** | **33** | **M** | **CD4/CD8** |
| CON17 | 40 | F | CD4 |
| CON18 | 25 | F | CD4 |
| CON19 | 23 | M | CD4/CD8 |
| Mean ± SEM | 32±6 | 11F/6M | |
| IBD1 | 41 | F | CD4s |
| IBD2 | 56 | M | CD4s |
| IBD3 | 50 | M | CD4s |
| IBD4 | 29 | F | CD4s |
| IBD5 | 55 | F | CD4s |
| IBD6 | 18 | M | CD4s |
| IBD7 | 58 | F | CD4s |
| IBD8 | 23 | F | CD4s |
| IBD9 | 18 | F | CD4s |
| IBD10 | 53 | F | CD4s |
| IBD11 | 19 | M | CD4s |
| Mean ± SEM | 38±5 | 7F/4M | |

[a)]Blood sample was 50 mls. Patient samples highlighted in bold were examined by microarray analysis. All CD4+ samples were used in confirmation studies in which protein determination was conducted.

**Table 3.** Genes up-regulated in CD4+ cells in MS patients compared to controls[a)]

| Increase | SEM | Unigene | Description | Function |
|---|---|---|---|---|
| 6.64 | 1.55 | Hs.25648 | TNFSF member 5 | Binds CD40L |
| 6.63 | 1.14 | Hs.54443 | Chemokine (C-C) receptor 5 | Chemokine 5 signaling |
| 6.19 | 1.89 | Hs.82127 | IL-16 | Lymphocyte chemoattractant |
| 5.04 | 1.65 | Hs.173936 | IL-10 receptor | Anti-inflammatory actions |
| 4.56 | 1.48 | Hs.125124 | EphB2 (transcription factor) | Ephrin signaling |
| 4.52 | 1.37 | Hs.348669 | CDC28 protein kinase 1 | Regulation of CDK cell cycle |
| 3.54 | 0.95 | Hs.241392 | Small inducible cytokine A5 (RANTES) | Lymphocyte chemoattractant |
| 3.08 | 1.21 | Hs.46468 | Chemokine (C-C) receptor 6 | Lymphocyte recruitment |
| 2.91 | 0.75 | Hs.96055 | E2F transcription factor 1 | Cell cycle transcription |

[a)] Mean (increase) and SEM in gene expression changes were determined by dividing the average expression of each gene from MS patients by that from healthy controls. Genes listed in Table 3 or 4 were increased significantly in both CD4+ and CD8+ cells from MS patients compared to controls (yellow box, Fig. 1b).

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**Table 4.** Genes upregulated in CD4+ cells from MS patients and thapsigargin-treated CD4+ cells from healthy controls[a)]

| Increase | SEM | Unigene | Description | Function |
|---|---|---|---|---|
| 9.17 | 0.98 | Hs.258503 | FMRP interacting protein CYFIP2 | Binds to fragile X protein |
| 7.75 | 1.34 | Hs.408442 | TGFβ inducible early growth response | Transcription repressor |
| 5.22 | 0.56 | Hs.2554 | Sialyltransferase | Glycotransferase activity |
| 4.91 | 0.75 | Hs.76252 | Endothelin Receptor type A | Endothelin signaling |
| 4.78 | 1.45 | Hs.101850 | Retinol binding protein | Vitamin A action |
| 2.68 | 0.64 | Hs.1613 | Adenosine $A_{2A}$ receptor | Adenosine signaling |
| 2.25 | 0.93 | Hs.7957 | Adenosine deaminase | Adenosine metabolism |
| 2.12 | 0.79 | Hs.159494 | Bruton agammaglobulinemia tyrosine kinase | Associated with immunodeficiency |
| 2.08 | 1.08 | Hs.194236 | Leptin | Endocrine functions |
| 2.04 | 0.89 | Hs.285115 | IL-13 receptor | IL-13 signaling |
| 1.98 | 0.71 | Hs.169294 | Transcription factor 7 (T cell specific) | Regulation of EphB2 mRNA |

a) Genes listed in this table were increased in CD4+ and CD8+ cells from MS patients as compared to healthy controls as well as in control CD4+ cells treated with thapsigargin, indicating that the gene expression is associated with increased intracellular calcium levels (white box, Fig. 1b). The gene with the greatest increase in expression in CD4+ cells from MS patients that also correlated with increases in intracellular calcium was CYFIP2.

adenoviral-mediated delivery of CYFIP2 to T cells significantly increased T cell adhesion (24.5±3.2% of Jurkat cells bound to fibronectin, Fig. 3d).

### 2.4 Inhibition of Rac-1 activity or reduction of CYFIP2 protein levels reduces fibronectin-mediated binding in CD4+ cells from MS patients

CD4+ cells from MS patients had significantly (twofold) higher fibronectin-mediated binding (21.2±2.5%) than those from healthy controls (10.5±1.1%) or IBD patients (9.9±1.3%), and wortmannin treatment reduced binding to the levels observed in cells from healthy controls (Fig. 4a). Thapsigargin treatment, which increases CYFIP2 levels (Table 3), increased fibronectin-mediated binding in CD4+ cells from control and MS patients, and wortmannin effectively reduced fibronectin binding activity to basal levels (Fig. 4b). Finally, reduction of CYFIP2 protein levels using CYFIP2 ODN decreased fibronectin-mediated binding by primary CD4+ cells from MS patients to levels similar to healthy controls (Fig. 4c).

## 3 Discussion

DNA microarray profiling provides a non-biased approach for the identification of novel pathways associated with disease pathogenesis. We report that several genes in CD4+ and CD8+ T cells are upregulated in relapsing and remitting MS patients not currently receiving therapeutic intervention (Table 1 and 2). Many genes have been previously implicated. In particular, we observed that CD40 binding receptor expression is increased on CD4+ and CD8+ cells from MS patients (Table 3). Others have reported that expression of CD40 binding receptor and CD40 ligand are increased in the peripheral blood of MS patients [23, 24]. It was recently shown that marmoset monkeys are protected from EAE when treated with anti-CD40 antibody [25]. Our findings are also in agreement with studies noting that CCR-5 is increased in T cells from MS patients [26, 27], RANTES is up-regulated in T cells (γ/δ subset) [28], and MS cytotoxic T cell lines treated with myelin peptide increase IL-16 [29].

In agreement with others [12, 30, 31], we report here that CD4+ cells from MS patients have higher adhesion properties compared to healthy controls and patients with IBD. Although we have not looked at other "neuroinflammatory" control patients, these studies indicate that CYFIP2 levels and T cell adhesion are regulated differently between MS and IBD patients, suggsting that our observation may be specific to MS patients. A caveat is that we have not determined the extent to which CYFIP2-mediated binding is elevated in memory or naive T cells from MS patients. Memory T cells from MS

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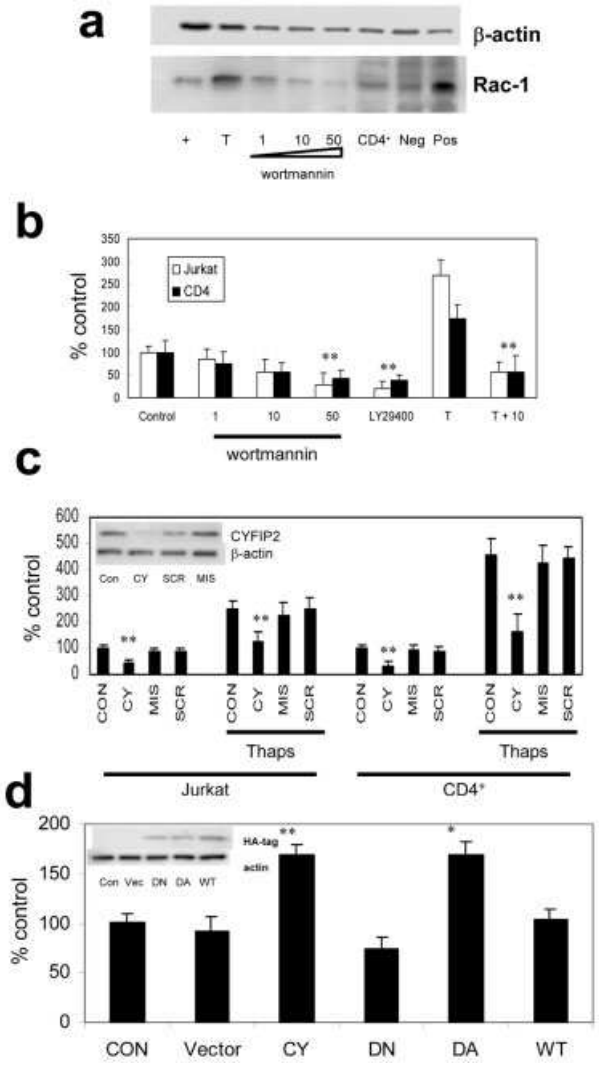


◀ *Fig. 3.* Rac-1/CYFIP2 pathway regulates fibronectin-mediated binding of Jurkat and CD4+ cells. (a) Jurkat cells were treated with thapsigargin (100 nM; T) for 1 h and subsequently with wortmannin (1, 10 or 50 nM) for 15 min, and Rac-1 activity was determined. (b) Identical experiments determined that a blockade of Rac-1 activity via wortmannin (as above) or LY294000 (25 μM)) in Jurkat and CD4+ cells reduced fibronectin-mediated binding. (c) Reduction of CYFIP2 protein inhibited Jurkat and CD4+ fibronectin-mediated binding. Western analysis of CD4+ cells [inset, lane 1 (Con): untreated control; lane 2 (CY): +CYFIP2 ODN (5 μM); lane 3 (SCR): +scrambled CYFIP2 ODN (5 μM); lane 4 (MIS): +mismatched CYFIP2 ODN (5 μM)] confirmed that CYFIP2 protein was reduced. At 24 h after ODN delivery, Jurkat or primary CD4+ cells were treated with thapsigargin (100 nM) for 1 h and adhesion determined. (d) Adenoviral-mediated delivery of CYFIP2 or dominant active Rac-1 significantly increased adhesion (Vector: adenoviral vector only; CY: CYFIP2; DN: N17 Rac-1; DA: V12 Rac-1; WT: wild-type adenoviral vector that contains HA tag). The inset shows HA tag of Rac-1-infected Jurkat cells. Rac-1 activity was increased by 2.5±1.1-fold as determined by PAK binding assay (not shown). CYFIP2 protein levels were increased by 2.1±0.8-fold (not shown). (Results represetative of three experiments; $^{**}p<0.001$, $^{*}p<0.01$ compared to untreated control.)

patients exhibit increased infiltration into the central nervous system compared to naive T cells or memory cells from other inflammatory controls [32]. Of interest, we observed that CCR7 was increased in CD4+ cells from MS patients as compared to controls (2.08±0.15, $p$=0.03, not shown: please see raw data at www.sbrc.ca/dnnd/Immuno-microarray.htm), and recent studies indicate that CCR7 can be used as an indicator of trafficking memory T cells [33]. Future studies that focus on purified memory and naive cells will help to clarify this question.

Our studies clearly indicate that CYFIP2 is involved in Rac-1-mediated T cell adhesion. This conclusion is based on our observations that Rac-1 and CYFIP1 interact physically (Fig. 2b), blockade of Rac-1 activity decreases fibronectin-mediated binding (Fig. 3a, b), dominant-active expression of Rac-1 enhances adhesion (Fig. 3d), and reduction of CYFIP2 protein levels inhibits CD4+ cell binding to fibronectin (Fig. 3c). CYFIP2 is a member of a larger tetrameric complex that regulates WAVE1 protein [10], but its role in this complex remains unclear. CYFIP2 does not have any known enzymatic activity or functional domains [8]. Our antisense experiments indicate that CYFIP2 plays an important role in facilitating T cell adhesion; overexpression of CYFIP2 using adenovirus increased T cell adhesion. We propose two mechanisms by which CYFIP2 might function. Because CYFIP2 binds to Rac-1, it may be an effector molecule of Rac-1. Active Rac-1 would act upon CYFIP2 (and NCK) in order to facilitate WAVE action and thus increase T cell adhesion. Facilitation of cell mobilization was clearly implicated by Eden and colleagues [10]. Alternatively, CYFIP2 could act as a stabilization protein within the tetrameric complex that regulates its activity. Indeed, increasing CYFIP2 levels in T cells enhanced fibronectin-mediated adhesion (Fig. 3d). There is recent evidence to suggest that specific proteins within tetrameric complexes provide stability and enhance enzymatic function [34].

Because we and others observed that wortmannin blocks Rac-1 activity (Fig. 3a, [15]), Rac-1 activity levels are likely increased in CD4+ cells from MS patients. Unfortunately, our samples of CD4+ cells drawn from MS patients were too small to accurately detect changes in Rac-1 activity. Nevertheless, the finding of Rac-1 regu-

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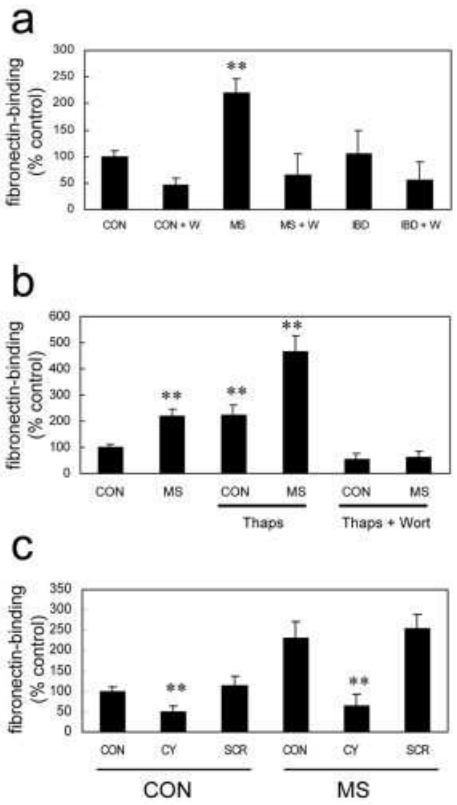


*Fig. 4.* CYFIP2 is involved in enhanced adhesion of CD4$^+$ cells from MS patients. (a) CD4$^+$ cells from MS patients displayed a twofold increase in fibronectin-mediated adhesion compared to healthy controls or IBD patients ($n$=12 MS patients, 11 IBD patients, 12 healthy controls). Wortmannin treatment (50 nM) significantly inhibited binding activity. (b) Thapsigargin treatment (100 nM) elevated fibronectin-mediated binding by CD4$^+$ cells MS and healthy controls, which was inhibited significantly by wortmannin treatment (50 nM) ($n$=6 MS patients, 6 controls). (c) CYFIP2 ODN treatment reduced fibronectin-mediated binding by CD4$^+$ cells from MS patients to levels similar to those of healthy controls ($n$=4 MS patients, 4 controls; CON: untreated CD4$^+$ cells from control or MS patients; CY: treated with 5 μM CYFIP2 ODN; SCR: treated with 5 μM scrambled ODN). (**$p<0.001$, significantly different from non-stimulated or untreated control.)

lates fibronectin-mediated binding of T cells from MS patients may help to bring together several previous findings. Specifically, Vav, a guanine exchange factor that negatively regulates the active (GTP-bound) state of Rac-1 [35], and Cbl-b, which negatively regulates Vav in the development of autoimmunity [36], have been associated with MS. Although our efforts here focused on downstream functional implications of Rac-1/CYFIP2 activation (cell adhesion), studies have indicated that activation of any of several signaling events involved in the T cell receptor or CD28-mediated activation (and thus, Rac-1 activation) plays a prominent role in driving the T cell activation associated with MS.

Clinically, our observations are compatible with involvement of the α4β7 integrin in increased fibronectin-binding, as α4β7 activity is blocked by wortmannin [37]. Preliminary experiments in our laboratory showed that anti-α4 antibody (1 μg/ml, 90 min pretreatment) blocks Jurkat T cell binding to fibronectin by 56±9% ($n$=2, data not shown) and that Jurkat cells have high expression of α4 protein (Western analysis, not shown). Clinical trials have determined that Antegren®, a monoclonal antibody specific for the α4 integrin, significantly reduces the number of new brain lesions [21, 22]. It is speculated that Antegren® interferes in α4β1 or α4β7 interactions with the immunoglobulin superfamily member VCAM-1. It will be important to determine the actions of Rac-1/CYFIP2 on regulation of α4 integrin activity.

In summary, our microarray studies indicate that CYFIP2 is highly abundant in CD4$^+$ cells in MS patients. Although we did not determine the exact mechanism of action of CYFIP2, it appears that high levels of CYFIP2 facilitate T cell adhesion and thus that interventions that reduce CYFIP2 gene expression or function may benefit MS patients.

## 4 Materials and methods

### 4.1 Patient population

Heparinized blood was analyzed from healthy volunteers ($n$=19), patients diagnosed with relapsing remitting MS ($n$=21) [38] followed at the MS Clinic at the University of Manitoba, or patients diagnosed with IBD ($n$=11) followed at the University of Manitoba Inflammatory Bowel Disease Clinical and Research Centre. MS patients were not receiving anti-immune therapy and had experienced a relapse within the previous 6 months. Patients were examined using the expanded disability status scale (EDSS) and MRI scans. IBD patients were not receiving any anti-immune therapy or, minimally, 5-aminosalicylates. All protocols were approved by the University of Manitoba Research Ethics Board. Groups were age-matched (Table 1 and 2).

### 4.2 Cell lines and primary blood lymphocytes

Human Jurkat cells were maintained as outlined previously [37]. Highly purified CD4$^+$ or CD8$^+$ cells (greater than 95% by FACS, not shown) were isolated from peripheral blood mononuclear cells (PBMC) by negative selection (StemCell Technologies, Vancouver, BC).

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### 4.3 Total RNA isolation, microarray analysis, and RT-PCR

$CD4^+$ and/or $CD8^+$ cells ($5\times10^6$) were isolated from MS patients or healthy controls as denoted in Table 1 and 2. Total RNA was purified 2 h after thapsigargin treatment (100 nM). Changes in gene expression were determined using the NIA nylon Immune-array according to routine procedures [17]. Significant changes in gene expression were identified if *p* values between groups equaled 0.001 or lower. Cluster analysis was conducted with Gene Cluster [39] and Venn diagrams constructed using Genespring Software (Silicon Genetics, San Francisco, CA). Primers used for CYFIP1 were forward 5'GAGACTTGATGCCAAAATGA and reverse 5'GAAATCCAGCACAGCAAAAC, yielding a product of 414 base pairs. Conditions used were 94/65/72°C for 30 cycles. CYFIP2 primers used were forward 5'GATTTGATATCA-AGGTGCCCCGGC and reverse 5' AACAGGACACTGC-CAGCCATGG, yielding a product of 543 base pairs. Conditions used were 94/71/72°C for 30 cycles. β-actin PCR primers used were forward 5'TGGTGGGCATGGGTCAGAAG and reverse 5'GTCCCGGCCAGCCAGGTCCAG, yielding a product of 385 base pairs. Conditions used were 30 cycles at 94/53/72°C.

### 4.4 Co-immunoprecipitation and antibodies

CYFIP2 and Rac-1 protein interactions in Jurkat or $CD4^+$ cells were determined following overnight co-immunoprecipitation using anti-Rac-1 antibody (1:500, Santa Cruz Biotechnology). Rac-1 or CYFIP2 were detected by Western analysis using anti-Rac-1 antibody (1:500) or anti-CYFIP2 monoclonal antibody (1:100; gift from Dr. J.L. Mandel, University of Strasbourg, France) followed by chemiluminescence detection.

### 4.5 Rac-1 activation

Rac-1 activation levels were determined by binding of active Rac-1 to an effector peptide p21 activated kinase (PAK), and levels of active Rac-1 were determined by Western analysis using anti-Rac-1 rabbit polyclonal antibody (Cytoskeleton, Denver, CO). Unless otherwise indicated, $10\times10^6$ Jurkat or $CD4^+$ cells were used to determine active Rac-1 levels.

### 4.6 CYFIP2 antisense and *in vitro* delivery

In the 5' ATG start region within CYFIP1 and CYFIP2, there are 10 base pair mismatches (within the first 31 nucleotides). ODN designed to target CYFIP2 were CYFIP2 ODN 5'GGGCATCTTCCAGGGTGACGTGCGTGGTCAT3' and mismatched CYFIP2 5'GGGCATCTTCCAGTAC**TGTGTG**-CGTGGTCAT3' (mismatched in bold). ODN were delivered using Lipofectamine (Invitrogen) as described [40]. CYFIP1 and CYFIP2 mRNA levels and CYFIP2 protein levels were determined 24 h following ODN delivery. Fibronectin-mediated binding assays were conducted 24 h following delivery of ODN to $CD4^+$ or Jurkat cells.

### 4.7 Fibronectin-mediated binding

Fluorescent-based fibronectin binding adhesion was determined using calcein-AM-(Molecular Probes) loaded Jurkat cells or primary $CD4^+$ cells. Cells were bound to fibronectin-coated (10 μg/ml) 96-well black plates (assessment of β1-integrin family, αV family, and α4β7 binding [20]) for 30 min, and specific fibronectin-mediated binding was compared to nonspecific binding (uncoated plates). Rac-1 activity was inhibited using wortmannin (1, 10 or 50 nM; Sigma) or LY294000 (25 μM; Sigma) for 15 min prior to calcein loading. Reduction in Rac-1 activity was confirmed using the Rac-1 binding activation assay.

### 4.8 CYFIP2 and Rac-1 adenoviral delivery

Adenoviral infections [CYFIP2, dominant active Rac-1 (V12), dominant negative (N17), wild type and vector only] were conducted using 80,000 MOI for 48 h prior to adhesion analysis (modified protocol [41]). Cells were resuspended in PBS at $1\times10^6$/ml with 2 μg/ml calcein and incubated in the dark at 37° C for 30 min prior to analysis of fibronectin-mediated adhesion. In parallel, cells were analyzed for increases in Rac-1 levels (via HA tag) or activity (via PAK binding) or CYFIP2 levels (via Western). Dantrolene (10 μM; Sigma-Aldrich, St. Louis, MO) was delivered to Jurkat cells 30 min prior to experimentation.

### 4.9 Statistics

Pixel density was analyzed by Scion software. All tests were examined by ANOVA or Student's *t* test and statistical significance followed post hoc using Tukey-Kramer. Significance was considered to be at the $p<0.01$ level (Instat, Graphpad Software, San Diego, CA).

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