

## IVIg attenuates T cell-mediated killing of human neurons

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### Abstract

Beneficial effects of intravenous immunoglobulin (IVIg) in relapsing-remitting multiple sclerosis (MS) have been described, including a decrease of brain atrophy. We have previously shown that activated T cells kill neurons in culture. In this manuscript, we show that the pretreatment of activated T cells with IVIg attenuates T cell neurotoxicity. This is attributed to the ability of IVIg to decrease the adhesion of T cells onto neurons, possibly through an effect on LFA-1, and by lowering the levels of Fas and FasL on T cells. Our results are relevant to understanding how therapies affect the MS disease process.

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### 1. Introduction

Intravenous immunoglobulins (IVIg) are purified preparations of immunoglobulins from plasma of healthy human donors. IVIg has been used as an immunomodulatory therapy in autoimmune disorders, including inflammatory diseases of the CNS. Randomized controlled trials indicate beneficial effects on disease activity in the relapsing-remitting form of multiple sclerosis (MS), but not for the secondary progressive type (Fazekas et al., 1997; Sorensen et al., 1998; Hommes et al., 2004; Haas et al., 2005; Fazekas et al., 2005; Stangel and Gold, 2005). Similarly, IVIg has been shown to alleviate disease severity in experimental autoimmune encephalomyelitis (EAE), an animal model of MS, and its effectiveness is more pronounced when administered before the development of clinical signs than during a clinical attack (Achiron et al., 2000; Jorgensen et al., 2005; Humle Jorgensen and Sorensen, 2005). The mechanisms of IVIg in MS and EAE are unclear, and hypotheses include the neutralization of circulating autoantibodies through an anti-

idiotype network, interference with antigen presentation, the blocking of Fc receptors and cytokine production, protection against antibody-mediated complement injury of oligodendrocytes, and the enhancement of remyelination (Warrington et al., 2001; Humle Jorgensen and Sorensen, 2005).

MS is now recognized as a disease with substantial degeneration of axons and neurons (Trapp et al., 1998; Cifelli et al., 2002; Grigoriadis et al., 2004; Evangelou et al., 2005) in addition to the destruction of oligodendrocytes and myelin. Indeed, the loss of axons is thought to correlate best with the progression of disability in the disease (Trapp et al., 1999). The mechanisms by which axons and neurons are destroyed are unclear, but there is correspondence between foci of inflammation and axonal injury (Bitsch et al., 2000; Kornek et al., 2000; Kuhlmann et al., 2002; Hemmer et al., 2002; Giuliani et al., 2005), suggesting that inflammatory cells may be the source of molecules that cause injury and toxicity to axons and neurons. Indeed, a variety of inflammatory cell types, including antigen-specific T cells, macrophages/microglia and neutrophils can produce neurodegeneration in cell culture models (reviewed in Neumann et al., 2001; Yong, 2004). These inflammatory cells may produce injury through several factors, including the

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elaboration of free radicals, proteases and excitotoxic glutamate (Bjartmar et al., 2001; Rodriguez, 2003).

Recently, we provided evidence that activated human T cells can kill human neurons in an antigen-independent manner that is not restricted by major histocompatibility complex molecules (Giuliani et al., 2003). The neurotoxicity of T cells is mediated in a cell–cell contact dependent manner, and is decreased by blockade of the molecules CD40L, LFA-1 and FasL. These results suggest that if enough activated T cells, regardless of antigen-specificity, accumulate in any areas of the CNS, they can inflict significant neurodegeneration. There is thus a need to reduce T cell trafficking into the CNS in MS, or to reduce their neurotoxic potential when T cells come into contact with neurons.

Because the toxicity of T cells on neurons involves cell–cell contact mechanisms (Giuliani et al., 2003), we investigated whether the polyclonal nature of the immunoglobulins present in IVIg would serve as a barrier for cellular communication and thus reduce T cell neurotoxicity. Here, we report that the treatment of activated T cells with IVIg decreases the T cell-mediated killing of neurons and we address associated mechanisms. Our results have implications for understanding how therapies affect the MS disease process in general, and how IVIg may affect MS in particular.

## 2. Methods

### 2.1. Isolation and treatment of T cells

Mononuclear cells were isolated from the blood of healthy volunteers by Ficoll-Hypaque centrifugation as previously described (Chabot et al., 1997; Giuliani et al., 2003) and suspended in serum-free AIM-V medium (Gibco/BRL, Burlington, Canada). To activate T cells, 1 µg/ml of an anti-CD3 antibody (OKT3) was added once for a period of 3 days. The floating cells were then removed from any adherent monocytes, and 100,000 cells in 100 µl of fresh AIM-V were added to 100,000 neurons prepared previously (see below). Flow cytometry analyses (mean±S.E.M., 3 subjects) of the floating cells collected after 3 days of initiation of OKT3 treatment indicated that the cellular populations consisted of 91±1% of CD3+T cells, 5.3±1.1% of CD19+B cells, 2.5±0.3% of CD56+natural killer cells, and 0.03±0.02% of CD14+monocytes. As T cells constituted over 90% of the total cell population, the floating cell preparations that we used for cytotoxicity experiments are henceforth referred to as activated T cells. Some mononuclear cell preparations did not receive OKT3 and the floating cells that were collected 3 days after were referred to as unactivated T cells.

In syngeneic interactions, fetal human T cells were isolated from the spleen of the same donor that provided the brain. The spleen was meshed into minute fragments, and

the released cells were subjected to Ficoll centrifugation and anti-CD3 activation as described above.

When IVIg was used to treat T cells, this was added once, 3 h after the initiation of anti-CD3 exposure so that the initial activation of T cells is unimpaired; cells then remained incubated with IVIg for 72 h before being collected, centrifuged, washed and then co-cultured with neurons. No IVIg was further added except when otherwise stated (Table 1). The treatment of T cells but not neurons with IVIg better simulates the clinical situation in MS where peripheral but not central cells are more likely to be exposed to IVIg because of the latter's uncertain penetration into the CNS. Nonetheless, we did include groups where previously IVIg-treated T cells were further exposed to IVIg at the time of co-culture with neurons, or groups that received IVIg only at the time of co-culture. IVIg was used at 5 mg/ml final concentration except in the concentration–response experiment of Fig. 3. IVIg (Gamunex®) was a clinical commercial preparation kindly provided by Talecris (Research Triangle Park, North Carolina, USA).

### 2.2. Human fetal neuron cultures

Brain tissues from human fetuses of 16–20 weeks fetal age were obtained following therapeutic abortion according to guidelines approved by local institutional ethics committees. Neuronal cultures in excess of 90% purity were prepared as previously described (Vecil et al., 2000; Giuliani et al., 2003) and 100,000 cells were plated onto each well of a 16 well Lab-tek (Nunc) slide coated with poly-ornithine (10 µg/ml). Two to four days after, T cells prepared above were added to the neurons.

### 2.3. Immunocytochemistry and evaluation of cytotoxicity

After a 24 h incubation of neurons with T cells, cultures were fixed with 4% paraformaldehyde and labelled with a mouse monoclonal antibody to microtubule associated protein (MAP)-2 (1:5000, Sigma, St; Louis, MO). A secondary antibody to mouse Ig conjugated to Cy3

Table 1  
Experimental groups

Groups	Activation status for T cells	Further treatment of activated T cells
Unactivated T cells	None	None
Activated T cells	Anti-CD3	None
Activated T cells	Anti-CD3	(I) Pretreatment group (IVIg added to activated T cells before co-culture) (II) Pretreatment+Co-culture group (IVIg added to T cells and also at co-culture with neurons) (III) Co-culture group (IVIg added only at time of co-culture)

(Molecular Probes, Leiden, The Netherlands) was then applied. Cultures were counterstained with Nuclear Yellow (Hoechst dye) to label all nuclei.

To evaluate cytotoxic effects on neurons, and to achieve consistency in quantitation between samples, each well was assessed along its equatorial axis counting 6 random fields using the 40× microscope objective. All the MAP-2 positive cells in each field were enumerated. For every condition in each set of experiments, 4 wells were counted to obtain the mean number of neurons remaining in culture.

#### 2.4. T cell adhesion assay

Unactivated or activated T cells were prelabelled for 10 min with carboxy-fluorescein diacetate, succinimidyl ester (CFSE). T cells (100,000 cells) were then added for 6 h to neurons (100,000 cells per well of 16 well Lab-Tek slide). The medium was removed, the co-culture was washed gently once with saline, the cells were then fixed with 4% paraformaldehyde, and neurons were stained as described above. Using an immunofluorescence microscope, the number of T cells (green) adhering onto neurons (red) was evaluated. Each well was assessed along its equatorial axis counting 6 random fields using the 40× objective. All CFSE-positive cells in each field were enumerated.

#### 2.5. Flow cytometry

Untreated and treated cells were collected for flow cytometry. Staining was performed in a two step process by incubating the cells for 40 min at 4 °C with primary antibody {mouse anti-CD11a (LFA-1, leukocyte function antigen-1), mouse anti-human Fas (CD95) or mouse anti human FasL (CD95 ligand)} followed by 15 min incubation with a goat anti-mouse-IgG-FITC conjugated secondary antibody. Mouse anti-human IgG1 and FITC antibodies were used as isotype controls. All antibodies were from Becton Dickinson (Mississauga, Ontario, Canada).

#### 2.6. Statistical analysis

Since all experiments involved multiple groups, statistical analysis was conducted using 1-way ANOVA with Tukey's post-hoc test. Statistical analysis was performed using GraphPad Instat.

### 3. Results

#### 3.1. IVIg decreases the toxicity of activated T cells on neurons

Activated T lymphocytes from adult human donors caused significant death to neurons by 24 h of co-culture, as determined by the decreased number of MAP-2 positive neurons in culture (Figs. 1 and 2A), and in correspondence

with previously published results (Giuliani et al., 2003). In contrast, unactivated T cells did not kill neurons.

When adult human T cell preparations were treated with 5 mg/ml IVIg after their activation with anti-CD3 and prior to incubation with neurons, the toxicity of T cells was attenuated (Fig. 2A). The decrease of T cell killing by IVIg pretreatment occurred in a concentration-dependent manner, and became evident at concentrations above 1 mg/ml (Fig. 2B). IVIg was still effective when applied only at the time of T cell — neuron co-culture (Fig. 2A).

We had the opportunity to evaluate T cells obtained from the spleen of the same donor that provided brain for neural culture. Fig. 3 shows that the syngeneic T cells kill neurons, and this too was attenuated by the pretreatment of the T cells with IVIg.

#### 3.2. IVIg decreases adherence of activated T-cells on neurons

As the toxicity of activated T cells on neurons requires cell–cell contact (Giuliani et al., 2003), and given that IVIg was still neuroprotective when applied only at the time of co-culture, we examined whether IVIg affected the adherence of activated T-cells on neurons. We found that there was a significant reduction in the number of T cells adhering on neurons when T cells were pretreated with 5 mg/ml IVIg but not control BSA (Fig. 4).

#### 3.3. IVIg downregulates LFA-1, FasL and Fas expression on activated T cells

We (Giuliani et al., 2003) previously demonstrated that blockade of the cell surface molecules LFA-1 and FasL on activated human T cells attenuated their ability to kill human fetal neurons. Thus, we examined by flow cytometry whether IVIg modulated the expression of these molecules on T cells.

Whether activated or not, over 90% of T cells expressed LFA-1. The activation of T cells induced an increase in the level of expression of LFA-1 compared to unactivated T cells, as evident by a rightward shift of flow histogram and an increase in mean fluorescence intensity. IVIg (5 mg/ml) pretreatment of activated T cells for 3 days did not reduce the proportion of T cells that expressed LFA-1, but it decreased the level of LFA-1 on activated T cells (Fig. 5A).

For FasL, unactivated T cells did not express this molecule (percentage of positively stained cells: 0.15). The activation of cells caused an increase of positively stained cells to approximately 50%. IVIg treatment of T cells decreased the percentage of positively stained cells to 18% and also reduced the mean fluorescence intensity from 41 to 28 (Fig. 5B).

For Fas (Fig. 5C), the activation of T cells increased the percentage of positive cells from 30% to 95%; mean fluorescence intensity also rose with activation. IVIg treatment of activated T cells reduced the percentage of

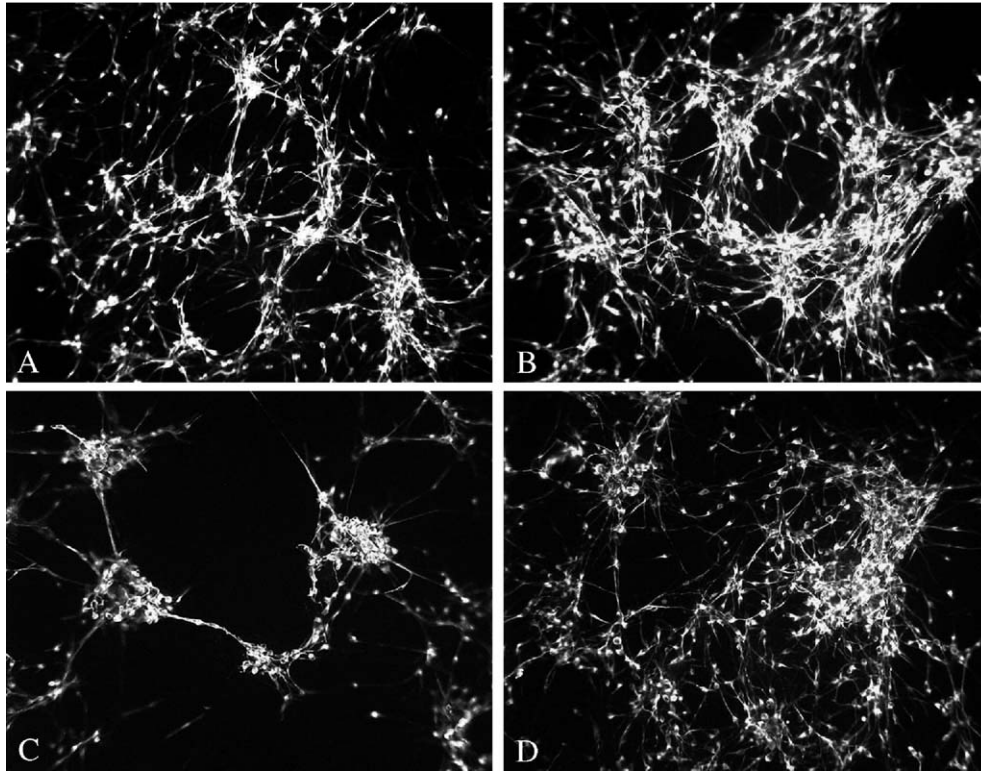


Fig. 1. MAP-2 immunoreactivity to document toxicity of activated T cells and protection by IVIg. Panel A shows a control neuronal culture that is not exposed to T cells. Panel B depicts a neuronal culture exposed to unactivated T cells where no toxicity manifests. Panel C illustrates the loss of neurons following co-culture for 24 h with activated T cells. The clumping of cells is commonly observed prior to their death. In panel D, IVIg treatment (5 mg/ml) of activated T cells prevents the death of neurons.

positively stained cells back to the level of unactivated T cells and also decreased the mean fluorescence intensity.

Thus, IVIg attenuates the levels of cell surface molecules that contribute to T cell contact and toxicity for neurons.

#### 4. Discussion

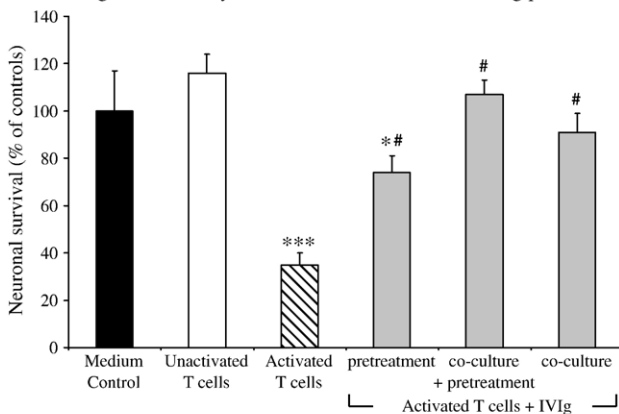
The role of neuroinflammation in causing the degenerative changes in MS is highlighted by the correspondence in foci of inflammation and areas of axonal damage in MS and EAE, and by the reports that antigen-specific T cells (Medana et al., 2001) and other inflammatory cell types can produce neurotoxicity (reviewed in Neumann et al., 2001; Yong, 2004). Antigen non-specific T cells are also lethal to neurons, through mechanisms that require T cells to become activated and then contacting neurons (Giuliani et al., 2003). Thus, important means to decrease T cell neurotoxicity would be to decrease their influx into the CNS, and/or to modulate the properties of T cells such that their subsequent encounter with neurons would not allow toxicity to manifest. These means are being achieved by the commonly used immunomodulators in MS: interferon- $\beta$  reduces the influx of potentially neurotoxic T cells into the CNS while glatiramer acetate generates T cells that are of the T helper 2 subtype that are non-neurotoxic (reviewed in Yong, 2002; Dhib-Jalbut et al., 2002; Farina et al., 2005).

In this work, we show that another treatment used in MS, IVIg, also affects the neurotoxic properties of T cells. We demonstrate that the treatment of allogeneic or syngeneic T cells with IVIg protects human fetal neurons from T cell-mediated killing. As T cell toxicity requires their contact with neurons (Giuliani et al., 2003), this suggests that a mechanism of IVIg resides in its affecting T cell contact with neurons. In support, we find that T cells are impaired by IVIg in adhering onto neurons. Furthermore, T cells treated for 3 days with IVIg decrease their expression of LFA-1, which is involved in the adhesion of T cells onto neurons. The reduction in levels of FasL on T cells by IVIg-pretreatment likely contributes to the mechanisms of IVIg given the important roles of Fas–FasL in controlling cell death in general (Linkermann et al., 2005).

We found that concentrations of IVIg of 1 and 5 mg/ml attenuated T cell neurotoxicity. These concentrations are similar to those used in the experimental literature, and are relevant to those given therapeutically in patients (0.4 g/kg, for a 70 kg subject, represents 28 g in a blood volume of 5–6 l). IVIg concentrations up to 20 mg/kg also were neuroprotective (data not shown).

In the current study, IVIg reduced the levels of Fas on T cells. It is not immediately apparent if this is a desirable or detrimental effect. There is increasing literature on the role of Fas in T cell elimination and immune surveillance (Fortner and Budd, 2005; Dalton et al., 2004). For example,

### A. Killing of neurons by activated T cells: kinetics of IVIg protection



### B. Dose-response of IVIg neuroprotection

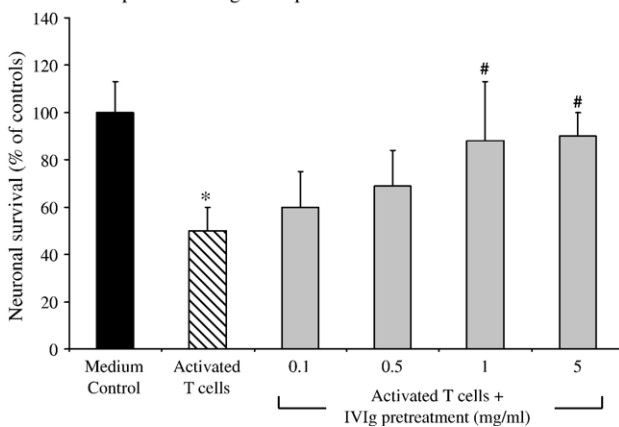


Fig. 2. In allogeneic co-culture, IVIg prevents the toxicity of adult human T cells on fetal human neurons. Medium control refers to neuronal cultures fed culture medium and not exposed to activated or unactivated T cells. Following the counting of the number of MAP-2 positive neurons remaining in culture, the result is expressed as a % of that seen in medium control cultures. Values are means  $\pm$  S.D. of 3 or 4 wells of neurons per condition, and this result has been reproduced 3 times. In panel A, the different means of treatment with 5 mg/ml IVIg were determined, while in panel B, different concentrations of IVIg were incubated with activated T cells for 3 days prior to their co-culture with neurons. Panel A: \* $p < 0.05$  or \*\*\* $p < 0.001$  compared to controls; # $p < 0.001$  compared to activated T cells alone. Panel B: \* $p < 0.05$  compared to controls; # $p < 0.05$  compared to T cells alone.

an increased frequency of autoimmune diseases like diabetes type 1 and MS has been observed in humans with a genetic defect of Fas (reviewed by Dianzani et al., 2003). FasL expression by neurons is a mechanism to kill infiltrating Fas-expressing T cells (Flugel et al., 2000). Moreover, the percentage of Fas-positive T cells in the peripheral blood and cerebrospinal fluid from patients with active MS was decreased compared to patients with inactive disease, suggesting that the lowering of Fas on T cells resulted in their increased survival and pathogenicity (Okuda et al., 2005, Saresella et al., 2005). Nonetheless, the IVIg reduction of Fas expression on T cells could be a beneficial outcome, since the Fas-associated death domain is also known to promote mitosis of T cells (Fortner and Budd, 2005). It is interesting to note that in EAE the

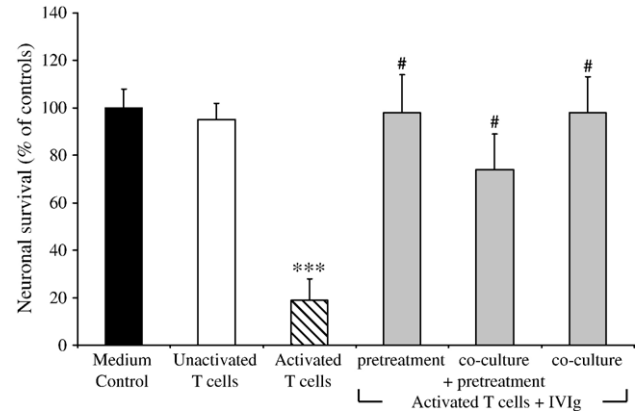


Fig. 3. In syngeneic co-culture, activated T cells kill neurons from the same donor source, and this is attenuated by IVIg. Different means of treatment with 5 mg/ml IVIg were determined. \*\*\* $p < 0.001$  compared to controls; # $p < 0.001$  compared to activated T cells alone.

blockade of the Fas-associated death domain in T cells prevents the development of disease, suggesting that downregulation of Fas on T cells must not necessarily be detrimental (Sun et al., 2005).

The current series of experiments used predominantly T cells exposed to IVIg prior to their encounter with neurons, as this simulates better the condition in MS patients where T cells are more likely to be exposed to IVIg prior to their infiltration into the CNS; this is because the amount of IVIg that enters the CNS in MS is uncertain, although MRI studies with ultrasmall superparamagnetic iron oxide particle (USPIO)-labeled antibodies suggest that antibodies can penetrate demyelinating lesions of the CNS effectively (Pirko et al., 2004). In this regard, it is remarkable that IVIg is effective in reducing neurotoxicity even when applied at the time of co-culture of neurons and T cells, a situation that suggests that if IVIg enters the CNS, it can also confer its effect within.

Results from other laboratories would support the concept that IVIg alters T cell physiology. IVIg treatment of activated, antigen-specific T cell lines was shown to have

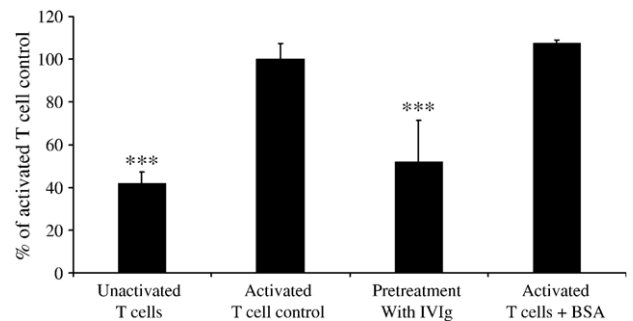


Fig. 4. The adherence of activated T cells onto neurons is reduced by IVIg pretreatment. As T cells acquire the capacity to adhere onto neurons when activated, we normalized all values to the number of activated T cells that adhered onto neurons over 6 h. The pretreatment group refers to activated T cells incubated for 3 days with 5 mg/ml IVIg. \*\*\* $p < 0.001$  compared to activated T cell control. Bovine serum albumin (BSA) control at 5 mg/ml did not interfere with the ability of activated T cells to adhere onto neurons.

a dose- and time-dependent anti-proliferative effect (Aktas et al., 2001). In primary human mononuclear cell preparations, IVIg (1–10 mg/ml) for 3–7 days reduced anti-CD3 induced proliferation (Amran et al., 1994). In addition, IVIg was shown to downregulate the synthesis of the IL-2 receptor and to inhibit proliferation induced by polyclonal stimulation of peripheral blood mononuclear cells (Andersson et al., 1996). Furthermore, in vitro IVIg treatment of lymphocytes reduced the production of pro-inflammatory

cytokines such as interferon- $\gamma$  and tumor necrosis factor- $\alpha$  and increased the production of IL-10 (Reske et al., 2003). IVIg can also modulate the apoptosis of human lymphocytes and monocytes (Prasad et al., 1998). It is important to note that in our experiments, cells after 3 days of IVIg were enumerated so that equal numbers of IVIg- or non-IVIg-treated T cells were added to the neuronal cultures. Also, we added IVIg 3 h after the anti-CD3 exposure of T cells, so the initial activation phase was not altered. These manipulations allowed us to examine the impact of IVIg-treated T cells on neurons, rather than the indirect consequence of IVIg decreasing T cell numbers and thus the exposure of neurons to reduced number of T cells.

This study is limited by not including cells from diseased states, particularly from patients with MS. However, as T cells require prior activation in culture to manifest their neurotoxicity, it is possible that such in vitro activation may abrogate any potential differences in vivo of T cells from MS subjects versus controls. Means to examine T cells and their neurotoxic potential with minimal alteration of their status in vivo would be desirable for future studies.

The efficacy of IVIg has been established in immune-mediated disorders such as Guillain–Barre syndrome, chronic inflammatory demyelinating polyneuropathy, myasthenia gravis and the prevention of graft-versus-host disease in recipients of allogeneic bone marrow transplants (Misra et al., 2005). Its efficacy in MS is still somewhat controversial, and its modest effect on relapses has caused it to be considered a second-line drug in relapsing-remitting MS after interferon- $\alpha$  and glatiramer acetate. In the secondary progressive form of MS, a recent study (Hommes et al., 2004) indicates that IVIg did not alter the clinical course or the T2 lesion load on magnetic resonance imaging (MRI). Nonetheless, from that cohort, MRI-determined brain volume in terms of a partial cerebral fraction decreased significantly less with IVIg over 24 months than placebo treatment (Fazekas et al., 2005). From the same cohort over 24 months, MRI magnetization transfer ratio histogram peak height findings were also suggestive of a reduction of brain tissue loss by IVIg treatment (Filippi et

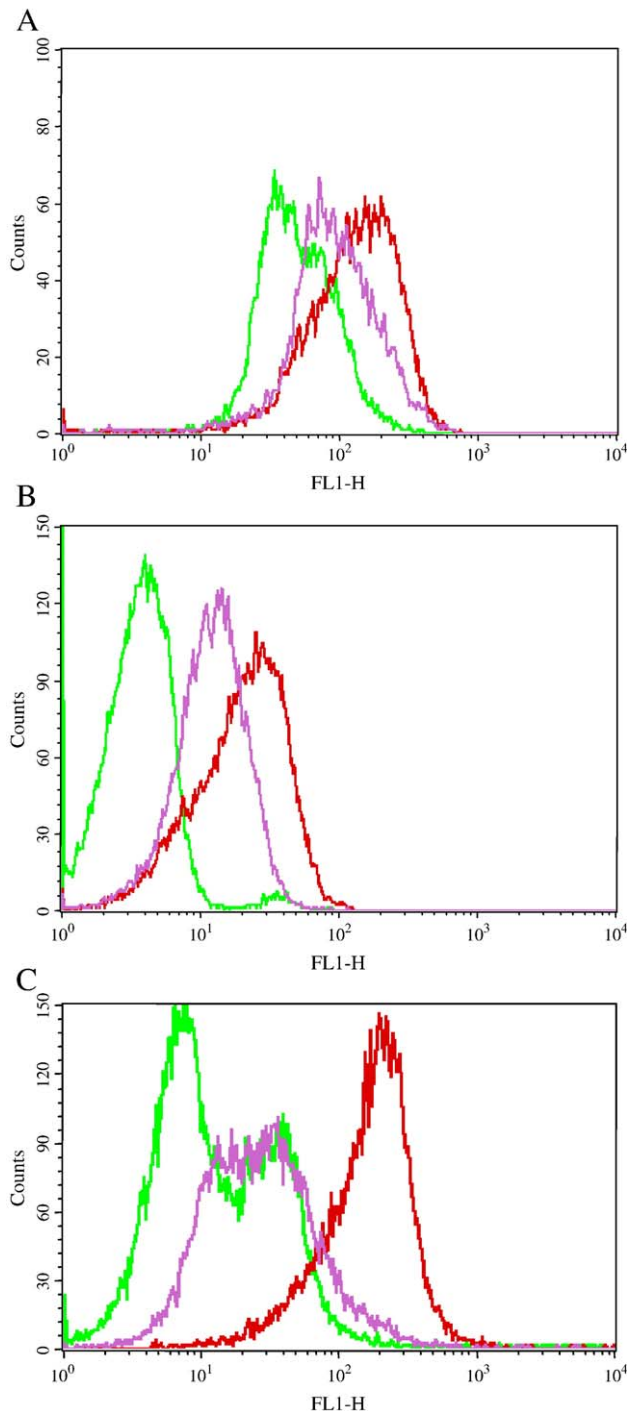


Fig. 5. IVIg pretreatment reduces the expression of LFA-1 (panel A), FasL (B) and Fas (C) on activated T cells. Representative flow cytometry profiles are displayed, for unactivated T cells (green line), activated T cells (red) and activated T cells pretreated with 5 mg/ml IVIg (purple). For LFA-1, while IVIg treatment did not reduce the proportion of T cells that expressed LFA-1, it decreased the level of LFA-1 on activated T cells; in the example shown, mean intensity fluorescence decreased from 141 to 112 after treatment. For FasL, the activation of cells caused an increase of positively stained cells which was reduced by IVIg. Finally, for Fas, the number of positive cells and mean fluorescence intensity rose with activation of T cells; IVIg treatment reduced the percentage of positively stained cells back to the level of unactivated T cells and also decreased the mean fluorescence intensity (203 to 77 in the example shown). These results were reproduced in 2 other series for panels B and C, and 4 times for panel A. There were more subjects in panel A compared to panels B and C since LFA-1 staining represented the initial experiment to assess whether IVIg affected adhesion molecules that could be evaluated by flow cytometry.

al., 2004). Our current results that IVIg reduced T cell neurotoxicity may offer a possible explanation to the observed decrease in brain atrophy documented by Fazekas et al. (2005), and the favorable magnetization transfer results of Filippi et al. (2004).

In summary, we have uncovered a new property of IVIg that is previously unrecognized. Our results indicate that IVIg interferes with properties of activated T cells so that they are less able to kill neurons. These findings have relevance to neurodegenerative diseases of the nervous system.

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