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Multiple sclerosis patients show sexual dimorphism in cytokine responses to myelin antigens

Ioana R. Moldovan, MD, PhD^{*,†}, Anne C. Coteleur, MS^{*}, Natacha Zamor, BS^{*}, Robert S. Butler, MS[‡], and Clara M. Pelfrey, PhD^{*,§,1}

^{*} Department of Neurosciences, NC30, Cleveland Clinic Lerner Research Institute, 9500 Euclid Ave., Cleveland, OH 44195

[‡] Department of Quantitative Health Sciences, Cleveland Clinic Lerner Research Institute

[§] Institute of Pathology, Case Western Reserve University School of Medicine, 10900 Euclid Ave., Cleveland, OH 44106

Abstract

Multiple sclerosis affects more women than men. The reasons for this are unknown. Previously, we have shown significant differences in women versus men in inflammatory cytokine responses to the major protein component of myelin, proteolipid protein (PLP), which is thought to be a target in MS patients. Here, using the ELISPOT assay, we examined sex differences in single-cell secretion of Th1 and Th2 cytokines from freshly isolated PBMC between relapsing remitting (RR) MS patients and healthy individuals. Cells were stimulated with MS-associated antigens including proteolipid protein (PLP), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and non-disease related antigens. Our data show a sex bias in the cytokine responses to multiple MS-relevant myelin antigens: Women with MS show IFN γ -skewed responses and men with MS show IL-5-skewed responses. These data extend our previous findings (Pelfrey et al., 2002): (1) by demonstrating gender skewing in cytokine responses to an expanded myelin antigen repertoire, which includes MBP, MOG and PLP; (2) by showing TNF α and IL-10 do not display comparable gender skewing compared to IFN γ and IL5; (3) by defining the patient population as early, untreated RR MS patients to avoid confounding factors, such as different disease stages/disability and immunomodulatory therapy; and (4) by showing HLA type does not appear to underlie the gender differences. These findings may explain increased susceptibility to MS in women and could contribute to the differences in disease severity between men and women.

Keywords

multiple sclerosis; inflammatory T helper-1 (Th1); anti-inflammatory T helper-2 (Th2); cytokines; sexual dimorphism; myelin

1 To whom correspondence should be addressed: Clara Pelfrey, Ph.D., Department of Neurosciences, NC30, Cleveland Clinic Lerner Research Institute, 9500 Euclid Ave., Cleveland, OH 44195. Phone: 216-444-9184. Fax: 216-444-7927. email: pelfrec@ccf.org.

[†] Present Address: Cellular Technology Ltd., 10515 Carnegie Ave., Cleveland, OH 44106.

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Introduction

Many autoimmune diseases are more frequent in women than men, including multiple sclerosis, rheumatoid arthritis, Grave's disease, systemic lupus erythematosus, myasthenia gravis, Sjogren's syndrome and Hashimoto's thyroiditis. The reasons for a sex bias in MS and other autoimmune diseases are poorly understood but may include sex-related differences in immune responsiveness, sex steroid or hormonal effects, and sex-linked genetic factors (Whitacre et al., 1999).

MS is thought to be a T cell-mediated autoimmune disease with a T helper-1 (Th1)-type skewing of the immune response towards proinflammatory cytokines (e.g. IL-2, IFN γ , IL-12 and TNF α). IFN γ has been strongly linked to MS pathogenesis through several findings: increased production of IFN γ prior to clinical attacks (Beck et al., 1988; Lu et al., 1993); treatment of MS patients with rIFN γ induced exacerbations (Panitch et al., 1987); the CNS inflammatory process is characterized by increased IFN γ expression (Woodroffe and Cuzner, 1993). TNF α is another Th1 cytokine that is cytotoxic for oligodendrocytes in vitro and has been implicated in the pathology of multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE) (Selmaj et al., 1991b; Selmaj et al., 1991a; Selmaj and Raine, 1988; Sharief and Hentges, 1991). On the other hand, Th2 cells, which are also associated with autoimmune disease, secrete anti-inflammatory cytokines (e.g. IL-4, IL-5, IL-10), which favor humoral-mediated responses (Lucey et al., 1996). IL-10 has been shown to suppress EAE (Bettelli et al., 1998; Cua et al., 1999; Mathisen et al., 1997) and may underlie the beneficial effects of beta-interferon treatment in RRMS (Rudick et al., 1998). Both IL-4 and IL-5 are key regulators in humoral-mediated and adaptive immunity by acting as co-stimulators for the growth/differentiation of B cells and stimulation of immunoglobulin secretion. Importantly, Th2 cytokines are associated with down-regulation of Th1 cytokines and may provide protection from Th1-mediated autoimmune disease (Bettelli et al., 1998; Cua et al., 1999; Racke et al., 1994).

In these studies, we evaluated the sex differences in cytokine responses that we observed previously (Pelfrey et al., 2002) with an expanded set of myelin antigens that have been implicated in MS, including MBP and MOG. We also evaluated whether the Th1/Th2 differences we observed with IFN γ and IL-5 extend to additional Th1 or Th2 cytokines that have known involvement in MS, including TNF α and IL-10. Since HLA genes are directly linked to susceptibility in MS, we analyzed whether HLA genotype had a direct interactive effect. We observed sex differences in cytokine responses to myelin in *both* men and women with MS. These findings may explain increased susceptibility to MS in women and could contribute to the differences in disease severity between men and women.

Materials and Methods

Study subjects

Sixty patients with mild RRMS (38F, 22M) and 94 healthy controls (53F, 41M) were recruited. All the MS patients were untreated for at least 3 months prior to the study. Careful selection of study subjects avoided the confounding factors of disease stage, immunomodulatory therapy, age and disease duration. For complete study subject characteristics, see Tables I and II. Patients were recruited from the Mellen Center for Multiple Sclerosis Treatment and Research at the Cleveland Clinic. Informed consent was obtained from each study subject. The study was reviewed and approved by the Cleveland Clinic IRB.

HLA typing

The HLA class II typing was performed by the Allogen Laboratories at the Cleveland Clinic Foundation, Cleveland, OH. Genomic DNA was extracted from peripheral blood and HLA class II DR/DQ typing was performed by sequence-specific oligonucleotide probing after polymerase chain reaction amplification of gene segments of interest (PCR-SSOP). In some cases DRB1* typing was performed by direct sequencing of PCR amplified gene segments using dye-labeled terminators (Applied Biosystems, Foster City, CA).

ELISPOT assay

The ELISPOT method was used as previously described (Pelfrey et al., 2000; Pelfrey and Moldovan, 2005) with the following modifications: the ELISPOT plates (7770–0052, Whatman, Clifton, NJ) and the cell number (300,000 cells/well). Each antigen was tested in duplicate wells. The individual background value (mean of media wells) was subtracted from the value obtained in the presence of each stimulating agent. A response was considered positive if the reactivity exceeded the cut-off value, calculated by subtracting individual subjects' background values.

Antibodies, reagents and antigens

The anti-human capture/detection monoclonal antibodies were obtained from the following sources: IFN γ (Pierce-Endogen); TNF- α (BD Pharmingen), IL-10 and IL-5 (eBiosciences). We purchased the following reagents: streptavidin-HRP (DAKO, Carpinteria, CA); 30% H₂O₂, AEC substrate and BSA (fraction V) (Sigma, St. Louis, MO); DMF (Acros); Tween-20 (polyoxyethylene 20-sorbitan monolaurate) (Fisher, Pittsburgh, PA); PBS, RPMI-1640 (Cleveland Clinic, Central Services Media Lab). Complete culture medium consisted of RPMI-1640 supplemented with 100 IU/ml penicillin, 100 μ g/ml streptomycin, 2mM L-glutamine, 25 mM HEPES, and 10% newborn bovine serum (Life Technologies, Gaithersburg, MD). Antigens consisted of: PHA-P (10 μ g/ml) (Sigma); Streptokinase (1,250 IU/ml; final 1:5 dilution) (Astra USA, Inc., Westborough, MA); Tetanus toxoid (1:100, 1:1,000 dilutions) (Accurate Chemical, Westbury, NY); *Candida albicans* whole cell extract (1:50 dilution) (Hollister Stier, Spokane, WA); Anti-CD3 mAb (final, 5 μ g/ml) (BD Pharmingen, San Diego, CA). Control antigens were chosen as previously described (Pelfrey CM and Moldovan, 2005; Pelfrey et al., 2000).

Myelin-derived proteins and peptides

Myelin peptides for these experiments followed the sequence of human PLP, MBP and MOG. Myelin peptides relevant to MS are as follows: MBP 87–106 (Martin et al., 1990; Zhang et al., 1994); PLP 40–60 (Markovic-Plese et al., 1995; Pelfrey et al., 1993; van der Veen et al., 1993); PLP 89–106 (Pelfrey et al., 1994; Trotter et al., 1991); PLP 103–120 (Correale et al., 1995; Ohashi et al., 1995; Trotter et al., 1991); PLP 139–158 (Chou et al., 1992; Correale et al., 1995; Zhang et al., 1994); PLP 172–191 (Markovic-Plese et al., 1995); PLP 195–206 (Tuohy et al., 1997); MOG 1–22 (Kerlero et al., 1997); MOG 34–56 (Kerlero et al., 1997); MOG 64–96 (Kerlero et al., 1997). We purchased human MBP (100 μ g/ml) from Advanced Immuno Chemical, Inc. (Long Beach, CA). Human PLP (10 or 50 μ g/ml) was prepared as previously described (Folch et al., 1957; Tuohy et al., 1989). The peptides derived from PLP (40–60, 89–106, 103–120, 139–158, 172–191, and 195–206), MOG (1–22, 34–56, and 64–96), Tetanus toxoid (830–844 and 947–967) and MBP 87–106 were synthesized by Fmoc solid phase method with > 96% purity (Protein Core Facility, Cleveland Clinic). Synthetic peptides were reconstituted in DMSO or water at a stock concentration of 1mg/ml and stored in 20 μ L aliquots at –20°C until use. Peptides were thawed and diluted in media to a final concentration of 10 μ g/ml.

Statistical analysis

The measurements in this study are the result of ELISPOT assays of developed spots, where each spot represents a single cytokine-secreting cell. They are the average count of the number of spots present in duplicate culture wells after the background (media) counts have been subtracted. The distribution of count data is Poisson, not normal, therefore analyses of the number of cytokine-secreting cells was performed using the negative binomial regression comparing MS patients to healthy controls (disease effect), males to females (gender effect) and interaction between diagnosis (MS/Control) and gender (Male vs. Female). Age, HLA type, and disease duration were also included as covariates. When a significant interaction was observed, pair-wise comparisons were performed to determine which groups contributed significantly to the interaction. The test statistic for each of the variables was the chi-square. The variable was considered statistically significant if the P value associated with the chi-square statistic was < 0.05 . Analyses were adjusted for multiple comparisons using the Bonferroni correction. All analysis was performed using SAS (9.1). Negative binomial regressions were run using SAS Genmod Procedures. Bootstrap methods were applied to the data used to compute sample values of the IFN γ /IL-5 ratios for control and MS males and females for each of the cytokines. The resultant sample ratios for male and female control and male and female MS were differenced. For each cytokine one thousand such differences were generated. The means and standard deviations of this sample population of differences were computed and tested against the observed difference of the male and female ratios.

Results

Cytokine responses reveal highly significant sex differences

To examine sex differences in the cytokine responses to myelin proteins, peptides and control antigens, we used the ELISPOT assay to compare all male responses versus all female responses for each cytokine. Table 3 demonstrates several highly significant sex differences in IFN γ and IL-5 responses. Women showed strong IFN γ skewing to PLP 40–60 ($p = 0.0356$) and to PLP 195–206 ($p = 0.0124$). In 12/20 or 60% of all antigens tested, the women with MS show the highest mean IFN γ responses, although this reached significance with only 5 antigens (Table 3, right side, labeled MS F, in bold). Where men showed significantly higher IFN γ responses than women (MOG 34–56, PLP 89–106), the differences were attributable to the control men, and not the men with MS. The mitogens, anti-CD3 and PHA, did not induce IFN γ skewing by gender. For control antigens, only diphtheria toxoid and candida extract showed IFN γ skewing in all females (Table 3, All M vs. F).

Interestingly, 11/13 myelin antigens showed statistically significant IL-5 skewing in all males, whereas no control antigens or mitogens showed IL-5 gender skewing (Table 3, All M vs. F). To examine the male IL-5 skewing further, we separated the responses by disease status and sex. Table 4 shows 9 of the myelin antigens with the most significant male IL-5 skewing, and of these, 7/9 demonstrated that the men with MS gave the highest mean IL-5 response (Table 4, bold numbers marked with asterisks).

We also examined sex-by-disease interactions, where neither sex nor diagnosis (DX) *alone* were sufficient to explain the cytokine outcome. For PLP 40–60, IFN γ responses were highly skewed only to *women with MS* (Sex vs. DX, $p = 0.001$). Comparisons between groups show the MS female IFN γ response to PLP 40–60 is significantly greater compared to men with MS, control men and control women (Fig. 1). Although we predicted that TNF α and IL-10 responses would show similar gender skewing, TNF α responses did not show any female skewing compared to IFN γ , with the exception of the TNF α response to tetanus toxoid and its peptide TT 830–844 (TT, $p < 0.0048$; TT 830–844, $p < 0.018$). With one exception, MOG 1–22 ($p < 0.05$), IL-10 responses did not show significant male skewing similar to IL-5 (data not shown).

Within MS patients, women with MS show IFN γ skewing and men with MS show IL-5 skewing in response to peptides of PLP, MOG and MBP

To understand the contribution of sex more clearly, we divided the subjects into 4 categories: Control males, Control females, MS males and MS females. Although we observed significant *female* skewing in the IFN γ response to multiple myelin peptides and *male* skewing in IL-5 responses to the same myelin epitopes (Table 3), this data is not able to show the interplay between IFN γ and IL-5 responses that we observed in the women with MS, nor is it able to show the important cross-regulation that occurs between Th1 and Th2 cytokines. Th1 and Th2 cytokines rarely occur in high amounts in the same cell or in the same person. This is because they often have opposing immune functions as well as cross-regulatory function on the other type of cytokine. The ratio between IFN γ /IL5 demonstrates this Th1 skewing in women with MS, revealing IFN γ skewing in MS females' responses to 11/12 myelin epitopes (only whole PLP protein did not show this skewing)(Table 5). Five of these showed highly significant increased ratios in women with MS compared to the men with MS: PLP 40–60, PLP 195–206, MOG 64–96, $p < 0.0001$; PLP 172–191, MOG 34–56, $p = 0.01$ (Table 5). Of these myelin antigens, only PLP 195–206 also showed an increased ratio in control women compared to control men.

Control antigens/mitogens do not show significant gender skewing

To determine if the IFN γ -skewed responses in MS females were unique to myelin antigens, we also tested cytokine responses simultaneously to mitogens and a series of control antigens consisting of non-MS-associated antigens including tetanus toxoid, diphtheria toxoid, *Candida albicans* extract, and streptokinase. In contrast to the myelin epitopes, mitogen-stimulation with anti-CD3 mAb and PHA failed to show significant differences between Male vs. Female IFN γ (Table 3, $p = 0.18$ and 0.65 , respectively). Like wise, IL-5 responses to mitogens and control antigens did not reveal sex differences (Table 3). When we examined the IFN γ /IL-5 ratio for sex/disease combinations, MS females did show a significantly elevated IFN γ /IL-5 ratio compared to MS males for anti-CD3 and PHA (Table 5). Tetanus toxoid stimulation resulted in virtually identical ratios between women and men (Table 5). Although some control antigen responses showed IFN γ -skewed responses in women (e.g. diphtheria, $p = 0.0001$ vs. males) none showed parallel IL-5 skewing in men (Table 3). For 4/6 control antigens, IFN γ /IL-5 ratios still showed significantly higher ratios in women with MS compared to men with MS. This was not true for control females vs. control males, with the exception of TT 830–844 (Table 5).

The HLA DR composition of our study subjects does not explain the gender differences

To determine whether the observed sex differences might be explained by particular HLA types, we genotyped HLA DR/DQ in the majority of our study subjects (Tables 1 and 2). As expected, HLA DRB1*1501 (DR15) was more highly represented among the MS patients, with 22/43 (51%) of DR15+ MS patients and 23/72 (32%) of DR15+ healthy controls. We performed a negative binomial regression analysis examining whether the presence of DR15, DR4 or DR3, or any combination of those alleles, correlates with cytokine responses, while controlling for gender, disease and age. This allowed us to see whether gender-, disease-, or age- effects were confounded by disproportionate representation of particular HLA types among the responders. Almost no cytokine responses could be attributed *exclusively* to HLA type (data not shown). As might be expected, the IFN γ response to MBP was highly associated with the presence of all 3 HLA types (DR3 $p < .0001$; DR4 $p < .0001$; DR15 $p < .0003$), with MBP 87–106 strongly associated with DR15 ($p < 0.003$). Neither MBP nor MBP 87–106 showed IFN γ gender or -disease associations (Table 3, All M vs. F, and not shown). IFN γ responses to PLP 40–60 also appeared to be associated with DR15 ($p < 0.0007$). The IL-5 response to MBP showed an association with DR3 ($p < 0.0001$). Other DR3 associations

included MOG 1–22, PLP 139–158, PLP 195–206 (all $p \leq 0.004$). These very few associations do not appear to be sufficient to explain our gender differences. Thus, these data suggest that the HLA DR composition of our study subjects does not explain the gender differences observed for the majority of the myelin antigens.

Discussion

Previously, we have shown significant differences in women versus men in inflammatory cytokine responses to the major protein component of myelin, proteolipid protein (PLP), which is thought to be a target in MS patients (Pelfrey et al., 2002). In the present study, we examined sex differences in single-cell secretion of Th1 and Th2 cytokines from freshly isolated PBMC between MS patients and healthy individuals. Our data show a sex bias in the cytokine responses to multiple potentially MS-relevant myelin antigens: Women with MS show IFN γ -skewed responses and men with MS patients IL-5-skewed responses. These data extend our previous findings (Pelfrey et al., 2002): (1) by demonstrating gender skewing in cytokine responses to an expanded myelin antigen repertoire, which includes MBP, MOG and PLP; (2) by showing TNF α and IL-10 do not display comparable gender skewing compared to IFN γ and IL5; (3) by defining the patient population as early, untreated RR MS patients to avoid confounding factors, such as different disease duration, age and immunomodulatory therapy; and (4) by showing HLA type does not appear to underlie the gender differences.

Previous studies have examined sex differences, but have not observed similar skewing as we report here. One study showed no significant differences for any cytokines between men and women with MS (Eikelenboom et al., 2005). A possible explanation for these results is that the authors used PMA to stimulate cells before measuring cytokines, and the very strong, non-specific, mitogenic stimulus is not able to distinguish between the sexes. Our data agree, since neither anti-CD3 nor PHA was able to distinguish sex differences with any of the cytokines we tested, although ratios of IFN γ /IL-5 did reveal some skewing with mitogenic stimulation, but only in women with MS. In another study, women were more likely than men to have increased T-cell reactivity to immunodominant PLP peptides (PLP 184–199 and 190–209), but did not show increased responses to MBP or MBP 82–100 (Greer et al., 2004). We also were unable to show significant female IFN γ reactivity to MBP or MBP 87–106. Instead, our results suggest that the primary factor in observing a significant MBP IFN γ response is the HLA type of the responders. Jacobs and coworkers used PMA to stimulate PBMC and analyzed cytokines by flow cytometry (Nguyen et al., 2003). The percentage of TNF α -producing CD3 positive cells was significantly higher in male compared to female RR-MS patients. In contrast, we observed increased TNF α in men only for whole PLP, but were not able to detect mitogen-induced differences in any cytokine responses. These authors also observed that the percentage of CD3 positive cells producing IFN γ was significantly correlated with EDSS in females but not in males (Nguyen et al., 2003). This agrees with our previous observation showing a positive correlation between IFN γ responses in MS patients and disability measured by the MS Functional Composite (Moldovan et al., 2003). Notably, 75% of the MS patients in our study were women. One other group has observed a sex bias in the cytokine responses among MS patients (Greer et al., 2004). The common feature between these studies that relates to sex differences in cytokine expression is the use of myelin peptides rather than mitogen stimulation. Thus, the key to observing sex differences among cytokines that distinguish between Th1 responses and Th2 responses in MS is the use of myelin-specific antigens. It is important to note that several studies have reported an association between HLA DR2 (DRB1*1502 and *0501) and female gender in MS (Al-Shammri et al., 2004; Duquette et al., 1992; Fukazawa et al., 2000; Van et al., 1986), highlighting the importance of studying male and female patients separately. We were not trying to repeat these studies, but rather to assess whether our gender-skewed cytokine responses can be explained by a disproportionate representation of HLA

alleles in our MS patient cohort. Our results do not support HLA as a critical factor in the gender-specific cytokine skewing.

Men with MS tend to have a more aggressive disease course. Disability is more severe in men and mortality is higher, even among men with an initially relapsing remitting course (Weinshenker et al., 1989; Wynn et al., 1990). It is now clear that changes on MRI reflect the underlying pathological process typical of MS, with contrast-enhancing lesions representing areas of active inflammation with blood-brain barrier disruption, whereas T1- hypointense lesions (known as “black holes”) indicate axonal loss (Truyen et al., 1996). In our hands, men with MS showed highly IL-5 skewed responses to a long list of myelin peptides, suggesting that men respond in a Th2/IL-5-dominated manner, which most likely serves to suppress Th1-IFN γ -dominated responses. In men, this may alter the pathological course of MS in a more destructive manner than for women. This theory is supported by a recent MRI study, in which men demonstrated fewer gadolinium contrast-enhancing lesions, but a higher proportion of black holes compared with women, indicating that men with MS are more prone to develop destructive and less inflammatory lesions than women (Pozzilli et al., 2003). Recently, the same group presented another study of gender-related modulation of pathological changes in RRMS where they related serum sex hormone levels to MRI characteristics of brain lesions in MS. In men, they reported a positive correlation between estradiol concentrations and brain damage as measured by both T1 and T2- lesion load (Tomassini et al., 2005). Greater brain damage, as documented by black holes, was associated with higher testosterone in women and higher estradiol levels in men, indicating that sex hormones (both estradiol and testosterone) are involved in the process leading to irreversible tissue damage, but that their role might differ between the sexes. There are other supporting studies that suggest men may have a more destructive and potentially Th2-type, Ab-mediated disease. A recent study of therapeutic plasma exchange as a therapy for MS (Keegan et al., 2002; Keegan et al., 2005; Weinshenker et al., 1999) showed that MS patients with a pattern II pathology, characterized by antibody/complement-associated demyelination, showed substantial neurological improvement following plasma exchange. One of the factors associated with a favorable response in that study was male sex (Keegan et al., 2002). Thus, it appears that men are more likely to develop a Th2/IL-5 skewed immune response to myelin proteins. Evidently, there are more factors involved that remain to be determined, since glatiramer acetate treatment can induce IL-5 and does not induce more severe disease (Duda et al., 2000). We speculate that under certain conditions in the brain, the increased IL-5 response may predispose men with MS to Ab-mediated demyelination.

The concept of ‘neuroprotective immunity’ might explain why the MS disease course is often milder and less disabling in women than in men. The significantly decreased IFN γ /IL-5 ratio in men may suppress the regulatory or neuroprotective effects provided by IFN γ . A beneficial role for IFN γ in MS has been suggested by several animal models. IFN γ receptor knockout mice developed more severe EAE (Ferber et al., 1996) and neutralizing antibodies against IFN γ increase Theiler's virus-induced demyelination and viral persistence (Rodriguez et al., 2003). Alternatively, genetic variants that affect expression or function of IFN γ might influence the susceptibility to MS and severity of the disease. Weinshenker and colleagues showed that being a carrier of the A allele of a 3'(325)*G→A single nucleotide polymorphism just 3' to the *IFNG* gene is associated with increased susceptibility to MS in men (Kantarci et al., 2005). More recently, we have shown that this polymorphism is associated with decreased IFN γ protein and mRNA expression in men with MS (Kantarci et al., 2007). Thus, genetic variation in IFN γ may partially account for gender differences in susceptibility to MS.

In addition to some protective effects, IFN γ is known for its detrimental activities associated with MS. IFN γ has been strongly linked to MS pathogenesis through increased production of IFN γ prior to clinical attacks (Beck et al., 1988; Lu et al., 1993), treatment of MS patients with

rIFN γ induced exacerbations (Panitch et al., 1987), and the CNS inflammatory process is characterized by increased IFN γ expression (Woodroffe and Cuzner, 1993). Thus, IFN γ displays a complex set of activities that may differ according to location in the body, time of expression in the disease, concomitant expression of sex hormones and activity on different subsets of cells.

Sex hormones can alter the immune response in many ways. What is poorly understood is how endogenous hormone levels affect the quantity and quality of a particular immune response. Cytokine gene expression may be affected directly or indirectly by sex hormones. The IFN γ gene appears to be directly affected by 17-beta-estradiol, which markedly increases activity of the IFN γ promoter in lymphoid cells that express the appropriate hormone receptor, and can augment the effect of T-cell-activating agents (Fox et al., 1991). We are examining endogenous levels of sex hormones to determine whether hormones play a role in the immune response to myelin proteins in MS. There are still many avenues of research to discover with respect to the role of sex differences and the immune response in MS.

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Abbreviations

MS, multiple sclerosis
 MBP, myelin basic protein
 MOG, myelin oligodendrocyte glycoprotein
 RRMS, relapsing remitting multiple sclerosis
 PLP, proteolipid protein
 IL-5, interleukin-5
 IL-10, interleukin-10
 TNF α , tumor necrosis factor alpha
 IFN γ , interferon gamma
 Th1, T-helper-1
 Th2, T-helper-2
 EAE, experimental autoimmune encephalomyelitis

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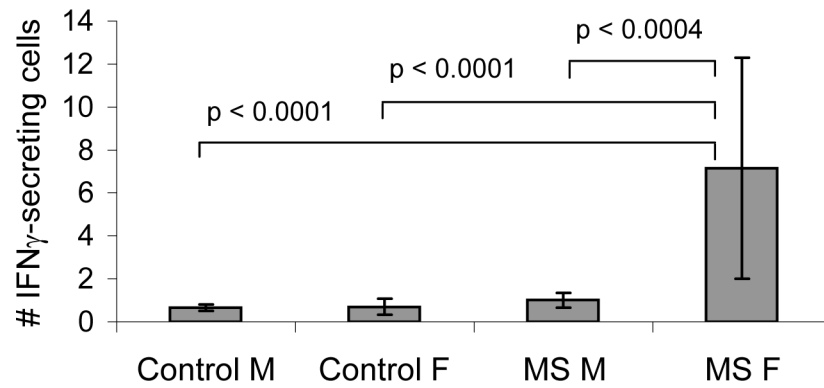


Figure 1. IFN γ response to PLP 40–60 is significantly greater in women with MS
 ELISPOT responses to PLP 40–60 (mean \pm SEM) are shown for healthy controls and MS patients divided by sex. Chi Square P values show pair-wise comparisons between groups and are adjusted for multiple comparisons. MS F, n = 38; MS M, n = 22; Control F, n = 40; Control M, n = 32.

Table 1

MS patient characteristics

Study ID	MS Males				MS Females				
	DD ^a	Age ^b	HLA Class II ^c		Study ID	DD	Age	HLA Class II	
			DRB1	DQB1				DRB1	DQB1
18	1.5	46	-	-	4	0.4	53	-	-
29	4.4	48	-	-	5	4.3	40	-	-
50	4.0	50	-	-	13	0.4	39	1,15*	-
74	0.2	30	12,15	3,6	15	1.5	50	-	5,6
87	0	47	3,7	3,2	17	9	43	-	-
103	2.9	48	7	3	19	0.3	36	-	-
106	0.4	46	4,15	3,6	20	1.3	37	-	-
117	3.1	34	1,15	5,6	31	0.3	37	-	-
122	3.5	29	9,11	3	37	7	46	-	-
133	0.08	40	7,15	2,6	45	1.8	28	8,12	3,4
139	0.1	33	8,15	4,6	46	.3	42	1,13	5,6
140	0.07	35	4,15	3,6*	48	.3	31	-	-
141	0.1	42	3,8	2,3	52	1.1	29	15,14	5,6
143	0.1	29	1,15	5,6	60	3.3	40	10,13	5,3
145	0.3	45	1*,15	3,6	76	2.5	46	-	-
147	0.08	47	7,15	3,6	78	1.0	33	4,16	3,5
160	0	33	-	-	81	0.04	23	3,15	2,6
174	-	46	-	-	83	1.2	46	1,15	5,6
178	0.1	19	1,4	5,3	99	0.1	34	13,14	5,6*
179	2.6	60	11	3	105	0.9	50	1,15	5,6
184	0	57	-	-	107	0.1	41	4,14	3,5
185	0	40	-	-	110	12.9	39	7,15	2,6
					112	0.01	44	13,15	3,6
					113	0.09	33	3,15	2,6*
					119	0.2	43	10,15	5,6
					123	9.5	52	15*	6
					126	1.5	24	1,13	3,6
					127	0.2	33	4,15	3,6
					130	2.7	45	4,15	3,6
					131	3.3	35	7,15	3,6
					134	0.1	30	3,8	4,6
					135	0.2	34	7,11	2,3
					138	0.6	42	-	-
					146	0.1	23	1*,11	3
					148	12.2	41	11,15	3,6
					149	0.5	33	8,15	3,6
					151	0.1	31	13	3,6*
					153	5.4	30	7,11	2,3

^a DD is disease duration in years.^b Average ages: MS males, 41.1 ± 9.9 yrs, MS females, 37.8 ± 7.9 yrs.

^cHLA Class II lists both alleles separated by a comma (homozygous individuals have only one number). Four-digit molecular HLA typing has been simplified to 2 digits (e.g. *1501 is shortened to 15; *1301 is shortened to 13; *0401 is 4; *0101 is 1; *0301 is 3). Numbers followed by “*” are allelic variants: 1* is *0103 (not the same as *0101); 15* is 1503, an allelic variation that is rare in Caucasians; 6* is *0602 but ambiguities exist with 0619 and 0620. Representation of HLA types: DR15: MS M 53%; MS F 43%; DR4: MS M 20%; MS F 14%; DR3: MS M 13%; MS F 11%.

^dDashes are data that were not tested or unavailable.

Table 2

Healthy Control characteristics

Study ID	Control males				Control females			
	Age ^a	HLA Class II ^b		Study ID	Age	HLA Class II		DQBI
		DRBI	DQBI			DRBI	DQBI	
12	45	- ^c	-	8	26	-	-	-
22	35	-	-	10	27	3,7	2	2
28	30	-	-	21	48	13,15	6,6	6,6
32	25	-	-	23	30	-	-	-
35	45	15,1	5,6	24	25	-	-	-
38	40	-	-	25	24	-	-	-
47	34	15,7	6,2	27	45	-	-	-
49	44	-	-	30	38	4,16	3,5	3,5
56	34	13,4	3,6	36	39	4,11	3	3
62	42	-	-	39	35	15	6	6
65	22	-	-	40	48	1,3	5,6	5,6
66	21	-	-	41	45	3,11	2,3	2,3
68	24	1,3	5,2	42	34	15	6	6
71	45	7,4	2,3	43	33	3,13	2,6	2,6
72	32	3,4	3,2	51	50	4	3	3
73	22	-	-	53	47	3	2	2
79	41	3,11	2,3	55	50	13,14	5,6	5,6
80	48	4,7	3,2	58	36	-	-	-
84	24	10,11	3,5	59	20	1,13	5,3	5,3
94	40	4,13	3,6*	70	44	-	-	-
96	27	4,15*	3,6*	75	27	-	-	-
100	40	11,13	6*,6	77	55	3,15	2,6	2,6
101	41	-	-	82	48	1,3	5,2	5,2
102	52	-	-	86	36	11,15	3,6	3,6
108	39	3,15*	4,6	88	30	3	2	2
111	25	14,15	5,6*	89	38	15	6	6
114	38	4,7	3,2	90	51	3,4	3,2	3,2
120	39	4,15	3,6	91	35	4,13	3	3
125	41	11,14	3,5	92	28	3,4	2,3	2,3
129	29	11,15	3,6	93	38	11,16	5,6	5,6
132	27	3,4	2,4	95	21	15*	6	6
150	39	15	6	98	43	11,15*	6	6
152	36	1	5	104	48	-	-	-
159	24	11,13	3,6*	109	43	11,13	5,3	5,3
161	41	11,13	3,6	115	31	3,15	2,6	2,6
162	24	11,15	3,6	116	41	4,11	3,3	3,3
163	39	13,15	3,6	118	49	11,15	3,6	3,6
164	42	4,12	3,3	124	51	-	-	-
165	29	4,15	3,6	128	46	1,9	5,2	5,2
172	54	7,13	2,6*	136	33	11	6	6
173	28	13	6*	137	55	7,15	2,6	2,6
				144	42	11,3	2,3	2,3
				154	34	7,12	3,3	3,3
				155	36	7	2	2
				156	27	1,15	5,6	5,6
				157	36	3,15	2,6	2,6
				166	34	1,10	5	5
				167	30	7,8	2,4	2,4

Control males				Control females			
Study ID	Age ^d	HLA Class II ^b		Study ID	Age	HLA Class II	
		DRB1	DQB1			DRB1	DQB1
				168	45	1,15	5,6
				169	26	4,15	3,6
				170	29	1,3	2,5
				171	49	15,15*	6,6
				181	45	1	5

^a Average ages: Control males (CM), 35.4 ± 8.8 yrs ; Control females (CF), 38.2 ± 9.3 yrs.

^b HLA Class II lists both alleles separated by a comma (homozygous individuals have only one number). Four-digit molecular HLA typing has been simplified to 2 digits (e.g. *1501 is shortened to 15; *1301 is 13; *0401 is 4; *0101 is 1; *0301 is 3). Numbers followed by "*" are allelic variants: 1* is *0103 (not the same as *0101); 15* is 1503, an allelic variation that is rare in Caucasians; 6* is *0602 but ambiguities exist with 0619 and 0620. Representations of HLA types: DR15: CM 31%; CF 33%; DR4: CM 38%; CF 19%; DR3: CM 17%; CF 30%.

^c Dashes are data that were not tested or unavailable.

Table 3
Summary of IFN γ and IL-5 cytokine responses: Sex and disease differences

Stimulation	Control Males		Control Females		MS Males		MS Females		P values ^b	
	Mean ^d	SEM	Mean	SEM	Mean	SEM	Mean	SEM	All M vs. F	MS F vs. MS M
IFNγ										
Candida	4.8	1.4	5.8	1.7	9.4	4.3	42.9	23.2	0.0452	.0265 MS F
Diphtheria	10.2	3.6	9.9	3.5	4.1	1.7	55.3	33.4	0.0004 F	<.0001 MS F
Streptokinase	23.7	6.5	13.8	3.5	17.0	5.4	25.3	6.1	0.6747	0.7994
Tetanus toxoid	26.3	21.4	21.9	7.6	10.8	3.9	41.9	19.8	0.19	0.0164 MS F
TT 830-844	0.8	0.2	1.2	0.7	1.5	0.6	3.2	1.3	0.0778	0.0778
TT 947-967	1.6	0.5	1.0	0.4	1.0	0.4	3.9	1.0	0.132	0.0108 MS F
anti-CD3	555.4	57.1	612.8	48.9	557.3	70.9	605.1	52.6	0.1799	.1764
PHA	709.0	57.0	724.1	41.6	691.8	57.6	757.0	38.5	0.4343	.6463
MBP	12.4	6.9	30.0	22.3	49.7	40.7	7.1	1.9	0.1727	0.0159 MS M
MBP 87-106	1.7	1.2	0.4	0.1	0.9	0.3	0.5	0.3	0.3989	0.4609
MOG 1-22	2.9	1.5	1.5	0.8	1.6	0.8	1.4	0.4	0.4359	0.6189
MOG 34-56	3.7	2.5	0.5	0.2	2.3	1.0	2.2	1.1	0.0067 M	0.3531
MOG 64-96	2.9	1.8	0.8	0.3	3.1	1.8	3.1	1.1	0.6929	0.1167
PLP-10	0.7	0.2	0.8	0.3	4.5	3.4	5.1	1.7	0.1797	0.1461
PLP-50	5.5	3.1	1.1	0.4	2.8	1.2	3.4	1.3	0.3411	0.4807
PLP 40-60	0.7	0.2	0.7	0.4	1.0	0.3	7.1	5.2	0.0356 F	0.0002 MS F
PLP 89-106	10.6	7.1	0.3	0.1	2.6	1.6	1.3	0.6	0.3962	0.3962
PLP 103-120	0.7	0.2	2.7	1.8	6.5	3.6	4.4	1.9	0.0941	0.9992
PLP 139-158	0.6	0.2	0.7	0.3	3.4	1.4	1.0	0.5	0.4059	0.1009
PLP 172-191	0.6	0.2	0.7	0.2	1.3	0.5	2.8	1.1	0.2688	0.1973
PLP 195-206	0.8	0.3	3.8	3.0	2.0	0.6	4.4	2.1	0.0124 F	0.4152
									All M vs. F	MS M vs. MS F
IL-5										
Candida	0.8	0.3	2.0	0.8	8.8	4.7	3.0	1.6	0.6518	0.039 MS M
Diphtheria	1.6	0.5	2.1	1.6	3.3	2.1	9.3	6.3	0.3022	0.349
Streptokinase	2.8	0.8	2.6	0.7	7.3	3.0	3.8	1.9	0.1133	0.0642
Tetanus toxoid	21.5	8.0	14.4	4.7	3.8	1.7	17.1	7.1	0.2086	0.0784
TT 830-844	0.8	0.4	0.2	0.1	0.6	0.3	0.3	0.1	0.1036	0.4001
TT 947-967	0.6	0.2	0.4	0.1	0.7	0.5	0.4	0.2	0.2954	0.0973
anti-CD3	77.7	15.5	92.6	16.7	107.7	30.7	85.2	13.1	0.903	0.92
PHA	97.5	16.1	119.5	14.4	128.6	29.3	85.0	11.3	0.4301	0.1992
MBP	0.9	0.3	3.5	3.0	4.2	2.0	0.5	0.2	0.0106 M	0.038 MS M
MBP 87-106	1.3	0.8	0.6	0.2	1.7	1.2	0.4	0.1	0.0094 M	0.1856
MOG 1-22	1.2	0.5	0.3	0.1	2.3	1.3	0.9	0.5	<.0001 M	0.0106 MS M
MOG 34-56	0.4	0.1	0.2	0.1	1.6	0.9	0.3	0.1	0.0069 M	0.0023 MS M
MOG 64-96	0.4	0.1	0.3	0.1	1.5	0.8	0.4	0.2	0.3172	0.3579
PLP-10 ^c	5.2	3.9	0.4	0.2	0.5	0.2	6.2	5.8	0.0203 M	0.6358
PLP-50 ^c	6.1	4.8	0.4	0.2	2.4	1.5	6.7	5.2	0.0217 M	0.9666
PLP 40-60	0.8	0.5	0.3	0.1	2.1	1.0	0.3	0.1	0.0008 M	0.0256 MS M
PLP 89-106	1.2	0.8	0.3	0.1	1.5	0.8	0.6	0.4	0.037 M	0.4899
PLP 103-120	1.2	0.6	0.4	0.1	1.1	0.4	0.4	0.1	0.0064 M	0.1602
PLP 139-158	1.2	0.7	0.3	0.1	1.3	0.7	0.4	0.1	0.0023 M	0.0351 MS M
PLP 172-191	1.3	0.7	0.3	0.1	0.5	0.2	0.5	0.2	0.0999	0.8925
PLP 195-206	1.8	0.8	0.5	0.1	1.5	0.7	0.2	0.1	0.0001 M	0.0016 MS M

^aThe measurements in this study represent the mean number and standard error (SEM) of IFN γ - or IL-5-secreting cells present in duplicate culture wells after the background spot counts (media alone) have been subtracted.

^bNumbers represent P values for the negative binomial regression. Numbers in bold are significant P values at $p < 0.05$. Each bold value has a column to the right describing the direction of the response (e.g. males greater than females = M; MS Males greater than MS Females = MS M; MS Females greater than MS Males = MS F). P values have been adjusted for multiple comparisons (Bonferroni Correction). The negative binomial regression model also adjusted for disease duration and age.

^cWhole PLP was tested at either 10 $\mu\text{g/ml}$ or 50 $\mu\text{g/ml}$.

Table 4

IL-5 significant gender responses are male skewed

Antigen	Male vs. Female			By DX and Sex			
	Sex	Avg ^d	SE ^d	P value ^b	Dx,Sex ^c	Avg ^d	SE ^d
MOG 1-22	F	0.59	0.22	<0.0001	C M	1.15** ^d	0.50
	M	1.56	0.57		C F	0.32	0.09
MOG 34-56	F	0.28	0.07	0.0069	MS M	2.26***	1.32
	M	0.83	0.33		MS F	0.91	0.46
MBP 87-106	F	0.51	0.14	0.0094	C M	0.39	0.14
	M	1.48	0.65		C F	0.22	0.07
PLP 40-60	F	0.29	0.07	0.0008	MS M	1.58***	0.85
	M	1.29	0.48		MS F	0.34	0.13
PLP 89-106	F	0.46	0.19	0.037	C M	1.33	0.64
	M	1.33	0.59		C F	0.63	0.12
PLP 103-120	F	0.41	0.09	0.0064	MS M	1.74*	0.38
	M	1.17	0.43		MS F	0.37	0.14
PLP 139-158	F	0.34	0.09	0.0023	C M	0.85*	0.49
	M	1.21	0.50		C F	0.32	0.10
PLP 195-206	F	0.38	0.09	0.0001	MS M	2.05***	1.01
	M	1.65	0.53		MS F	0.26	0.09
MBP	F	2.1	1.64	0.0106	C M	1.21	0.81
	M	2.1	0.78		C F	0.32	0.11
					MS M	1.53	0.79
					MS F	0.63	0.40
					C M	1.24*	0.64
					C F	0.39	0.12
					MS M	1.05	0.38
					MS F	0.43	0.14
					C M	1.15	0.70
					C F	0.32	0.11
					MS M	1.32*	0.68
					MS F	0.37	0.15
					C M	1.76	0.76
					C F	0.54	0.14
					MS M	1.47***	0.65
					MS F	0.20	0.08
					C M	0.94	0.29
					C F	3.46	3.04
					MS M	4.16***	2.04
					MS F	0.51	0.18

^aMean and standard error (SE) of IL-5 secreting cells. Bold numbers highlight where MS Males (MS M, right side) showed the highest IL-5 responses of any disease (DX) or sex combination.

^bNegative binomial regression P values showing significant difference between all male vs. all female responses.

^cDX, Sex: C M, control males (n=33); C F, control females (n=41); MS M, MS males (n= 19); MS F, MS females (n=35).

^dAsterisks represent P values in pairwise comparisons where MS M were significantly greater than MS F (***) p≤0.001, **p≤0.01, *p≤0.05) or C M greater than C F.

Table 5

IFN γ /IL-5 Ratios ^a

Stimulus	Controls			MS Patients			P-value ^b
	CM	CF	P-value	MS M	MS F	P-value ^b	
Candida	5.8	3.0	NS	1.1	14.4	<.0001 ^c	
Diphtheria	6.3	4.7	NS	1.2	6.0	NS	
Streptokinase	8.5	5.4	NS	2.3	6.7	<.0001	
Tetanus tox.	1.2	1.5	NS	2.8	2.4	NS	
TT 830-844	0.9	5.0	<.0001	2.5	10.3	<.0001	
TT 947-967	2.5	2.7	NS	1.5	9.9	<.0001	
anti-CD3	7.2	6.6	NS	5.2	7.1	<.0001	
PHA	7.3	6.1	NS	5.4	8.9	<.0001	
MBP	13.3	8.7	NS	12.0	13.9	NS	
MBP 87-106	1.2	0.6	NS	0.5	1.3	NS	
MOG 1-22	2.5	4.8	NS	0.7	1.5	NS	
MOG 34-56	9.4	2.2	NS	1.5	6.5	0.0148	
MOG 64-96	8.1	3.0	NS	1.2	8.3	<.0001	
PLP-10	0.1	1.8	NS	9.9	0.8	NS	
PLP-50	0.9	3.2	NS	1.2	0.5	NS	
PLP 40-60	0.8	2.2	NS	0.5	27.8	<.0001	
PLP 89-106	8.8	0.9	NS	1.7	2.1	NS	
PLP 103-120	0.6	6.8	0.0010	6.2	10.2	NS	
PLP 139-158	0.5	2.3	NS	2.6	2.8	NS	
PLP 172-191	0.5	2.4	NS	2.5	5.5	0.0100	
PLP 195-206	0.5	7.1	0.0005	1.4	21.8	<.0001	

^aRatios are the mean IFN γ response divided by the mean IL-5 response for all subjects in that group.^bP values were calculated using a bootstrap analysis.^cP values in bold represent IFN γ /IL-5 ratios where the female ratio is significantly increased compared to the male ratio (P \leq .01) by a one-way students' T test. NS = not significant.