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Original Research Paper

Human interferon regulatory factor 5 homologous epitopes of *Epstein-Barr* virus and *Mycobacterium avium* subsp. *paratuberculosis* induce a specific humoral and cellular immune response in multiple sclerosis patients

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Abstract

Background: A large number of reports indicate the association of *Epstein-Barr* virus (EBV), and *Mycobacterium avium* subsp. *paratuberculosis* (MAP) with multiple sclerosis (MS).

Objective: To gain a better understanding of the role of these two pathogens, we investigated the host response induced by selected antigenic peptides.

Methods: We examined both humoral and cell-mediated responses against peptides deriving from EBV tegument protein BOLF1, the MAP_4027 and the human interferon regulatory factor 5 ($IRF5_{424–434}$) homolog in several MS patients and healthy controls (HCs).

Results: Antibodies against these peptides were highly prevalent in MS patients compared to HCs. Concerning MS patients, $BOLF1_{305–320}$, $MAP\_4027_{18–32}$ and $IRF5_{424–434}$ peptides were able to induce mainly Th1-related cytokines secretion, whereas Th2-related cytokines were down-regulated. Flow cytometry analyses performed on a subset of MS patients highlighted that these peptides were capable of inducing the release of pro-inflammatory cytokines: IFN-γ and TNF-α by $CD4^+$ and $CD8^+$ T lymphocytes, and IL-6 and TNF-α by $CD14^+$ monocyte cells.

Conclusion: Our data demonstrated that both EBV and MAP epitopes elicit a consistent humoral response in MS patients compared to HCs, and that the aforementioned peptides are able to induce a T-cell-mediated response that is MS correlated.

Keywords: *Epstein-Barr* virus, *Mycobacterium avium* subsp. *paratuberculosis*, humoral response, antigen-specific T-cell stimulation, cytokines

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Introduction

The etiology of multiple sclerosis (MS) is unknown, and it is generally believed to be the result of interaction among different factors. Several studies are ongoing with the main objective of investigating the role played by infectious agents.[1] Since initial exposure to numerous viruses or bacteria may occur during childhood, and considering a possible involvement of viruses in triggering demyelination and inflammation,[2] it is postulated that microorganisms could ignite MS-associated immune dysregulation. Indeed, several viruses and bacteria have been associated with MS,[3,4] but none of them has been definitively confirmed as the etiological agent behind MS. A recent study supports the hypothesis that *Epstein-Barr* virus (EBV) and *Mycobacterium avium* subsp. *paratuberculosis* (MAP), two infectious agents linked to MS,[5–7] might be able to trigger MS-related autoimmunity most likely acting through a common target.[8] In fact, it was demonstrated that peptides deriving from these

pathogens could be cross-recognized by antibodies (Abs) targeting self-epitopes.[8] Moreover, a strong humoral response was detected against Epstein Barr Nuclear Antigen 1 (EBNA1)$_{400–413}$, the homologous mycobacterial peptide MAP_0106c$_{121–132}$ and the human myelin basic protein (MBP)$_{85–98}$ in MS patients compared to healthy controls (HCs).[8] To investigate further the role of MAP and EBV in human MS, we explored the humoral response against a potentially relevant EBV epitope, which derives from EBV tegument protein (BOLF1$_{305–320}$) and two peptides homologous to BOLF1$_{305–320}$, MAP_4027$_{18–32}$ and human interferon regulatory factor 5 (IRF5)$_{424–434}$.

Noteworthy is the fact that EBV BOLF1 is one of the lytic antigens produced during EBV primary infection, asymptomatic or infectious mononucleosis (IM).[9] It was demonstrated that Abs against BOLF1$_{305–320}$ epitope cross-react with the homologous self-epitope belonging to human transaldolase, one of the candidate autoantigens in MS.[10] It is important to investigate EBV lytic antigens, as having a previous history of IM increases dramatically the risk of developing MS.[11,12] The immune reactivity toward EBV in MS patients suggests that IM could contribute to MS through multiple mechanisms, including molecular mimicry.[13]

In up-to-date literature there is no information relative to MAP_4027$_{18–32}$, except that obtained in silico. MAP_4027$_{18–32}$ peptide belongs to a membrane protein and it is one potential transmembrane segment that spans from residues 21 to 43. MAP_4027$_{18–32}$ being exposed may continuously stimulate the host immune system in MAP-infected individuals, mainly because of the persistent nature of the infection.[7] IRF5 is expressed in microglia following peripheral nerve injury. It was recently demonstrated that the IRF8-IRF5-P2X4R axis in microglia results in induction of IRF5-mediated P2X4R expression;[14] IRF5 is also involved in the polarization of M1 macrophages, which exhibit progressive expressions of inflammatory molecules, such as pro-inflammatory cytokines.[15] Furthermore, several studies demonstrated that polymorphisms in the *IRF5* gene are significantly associated with MS susceptibility.[16,17]

The molecular mimicry phenomenon involving immunodominant epitopes deriving from bacterial and viral persistent antigens may be a decisive factor in directing autoimmunity toward self-antigens in MS patients. It was therefore important to explore whether BOLF1 and the other homologous MAP antigens are able to induce a significant humoral response in MS individuals when compared to HCs.

T-cell-mediated immune response against these epitopes in MS patients was also evaluated to assess if the same T-cell clones are capable of recognizing the peptides investigated in this study.

Methods

Patients

The protocol (PG/2014/2158) was approved by the ethics committee of the University of Cagliari, Italy, and a written consent for inclusion in the study was obtained from all participants. Peripheral blood samples were obtained from 47 MS patients (11 male and 36 female) diagnosed according to the revised McDonald criteria[18] and 53 age-/gender-matched HCs with no history of autoimmune diseases. The clinical characteristics of the MS patients were the following: 42 (89.4%) relapsing–remitting (RR), two (4.2%) secondary progressive (SP) and three (6.4%) primary progressive (PP). The age of the patients was 41 ± 11 years, the duration of disease was 7 ± 6.9 years and the mean age at MS onset was 32 ± 9. The Expanded Disability Status Scale (EDSS) scores of these patients were a mean of 1.8 ±1.8 and ranged from 0 to 8. Patients did not receive any kind of immune/inflammatory-related treatment for at least six months prior to sampling. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation, using Ficoll-Paque PLUS (GE-Healthcare Bio-Sciences, Piscataway, NJ, USA) from sodium-heparinized blood samples.

Peptides

Synthetic peptides BOLF1$_{305–320}$ (AAVPVLAFDAAR LRLLE) from BOLF1 protein (UniProt accession no. I1YP08), MAP_4027$_{18–32}$ (AVVPVLAYAAARLLL) from MAP_4027 protein (UniProt accession no. Q73SP6) and IRF5$_{424–434}$ (VVPVAARLLLE) from IRF5 protein (UniProt accession no. Q13568), were synthesized with purity greater than 90% and purchased from LifeTein (South Plainfield, NJ, USA). All the peptides were reconstituted at 10 mM in dimethyl sulfoxide (DMSO) and kept frozen at –80°C (Table 1).

Enzyme-linked immunosorbent assay (ELISA)

An ELISA for the detection of anti-EBNA-1 Abs was performed using the commercially available anti-EBV EBNA-1 immunoglobulin (Ig)G ELISA (Bio-Rad, USA) kit, according to the manufacturer's protocols. Indirect ELISAs were carried out to detect

Table 1. Peptides alignment obtained using Clustal W 2. The results show the region of identity with a star (*), strong similar amino acid with a dot (.) and a missing region in dashes (-).

Peptide name	Sequence	Source
BOLF1$_{305–320}$	**AAVPVLAFDAARLRLLE**	*Epstein-Barr* virus BOLF1 protein
MAP_4027$_{18–32}$	**AVVPVLAYAAARL-LL-**	*Mycobacterium paratuberculosis* MAP_4027 protein
IRF5$_{424–434}$	**-VVPV—AARL-LLE**	Human interferon regulatory factor 5 protein
	.*** **** **	

MAP: *Mycobacterium avium* subsp. *paratuberculosis*; IRF5: human interferon regulatory factor 5.

Abs specific for all the synthetic peptides included in the study, as previously described.[7]

Competitive inhibition assays

Three MS EBV$^+$ sera and an HC serum were subjected to ELISA on plates coated with BOLF1$_{305–320}$ or MAP_4027$_{18–32}$, respectively. Competitive assays were performed by pre-incubating the sera overnight at 4°C with saturating equimolar concentrations of peptides (BOLF1$_{305–320}$, MAP_4027$_{18–32}$ and IRF5$_{424–434}$), a sero-negative peptide MAP_2694$_{38–46}$[7] and a positive control.

Extracellular cytokine expression assay

PBMCs isolated from four MS (MS# 1–4) fresh blood samples (one male SP and three female RR) and two HCs (one male and one female) were cultured with single peptides (25 μM) and cytokines secreted during the 48-hour culture were measured by a Multi-Analyte ELISArray Kit (QIAGEN). PBMCs without peptide stimulation were used as negative control.

PBMCs were re-suspended in complete medium (CM); Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) and 10% heat-inactivated human serum (Sigma-Aldrich). Cell viability was examined with 0.4% trypan blue solution (Life Technologies) and was always >95%. One ×10^6 cells per well were cultured in a 24-well flat bottom plate. On day 2 of the culture, the single identified peptides (25 μM each peptide) and a negative control were added in the presence of CD28/CD49d co-stimulatory antibodies (BD Biosciences) and 50 U/ml recombinant human interleukin (IL)-2 (Sigma-Aldrich), in order to expand the antigen-specific T-cells. On day 4 of the culture, 1 ml of culture supernatant was harvested for the cytokine secretion assay. Secreted cytokine levels (IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, interferon (IFN)-γ, and tumor necrosis factor alpha (TNF-α)) were measured by a Multi-Analyte ELISArray Kit (QIAGEN) according to the manufacturer's protocol.

Peptide-specific cytokine expression

The stimulation of samples was performed on PBMCs from patients MS #1–4 and HCs #1–2 as previously described.[19] Briefly, 6 ml of peripheral blood was collected in Na$^+$ heparin tubes. Samples were divided into four 0.5 ml aliquots. Three aliquots were incubated with 25 μM of each peptide and the final aliquot served as a non-stimulated control. Anti-CD28/CD49d co-stimulatory antibodies (BD Biosciences, San Diego, CA, USA) were added to each aliquot. The blood was incubated at 37°C for eight hours; brefeldin A (10 μg/ml) was added during the last six hours of culture. After extensive wash with cold phosphate-buffered saline (PBS), the cells were fixed and permeabilized using Cytofix/Cytoperm according to the manufacturer's instructions. The cells were then stained with phycoerythrin (PE) anti-CD3, peridinin chlorophyll (PerCp) anti-CD8, fluorescein isothiocyanate (FITC) anti-IFN-γ and allophycocyanin (APC) anti-TNF-α for 30 minutes. To characterize monocytes, the samples were stained with PE-conjugated anti-CD14, APC anti-IL-6 and PerCp anti-TNF-α. Subsequently, cells were washed and analyzed on fluorescence-activated cell sorting (FACS) Calibur using CellQuest software (BD Biosciences, CA, USA); 10^5 events for each sample were acquired.

Statistical analysis

Data are presented as mean ± standard deviation (SD), median and interquartile range; with p values below 0.05 indicating a statistical significance. Comparisons between MS patients and HC groups were performed using Fisher exact test for non-parametric data, while receiver-operating characteristic (ROC) curves were utilized to evaluate the accuracy of the experiments. Correlation analyses among continuous variables were calculated too. Statistical analysis of data was

evaluated using GraphPad Prism 6.0 software (San Diego, CA, USA).

Results

Peptide analyses

Basic local alignment search tool (BLAST) analysis identified three putative mimicry peptides ($BOLF1_{305–320}$ $MAP\_4027_{18–32}$, $IRF5_{424–434}$) originating from EBV, MAP and human proteins. $BOLF1_{305–320}$ is a 17-mer with 11 amino acids overlapping with $MAP\_4027_{18–32}$, while $BOLF1_{305–320}$ and $IRF5_{424–434}$ boast 11 conserved amino acids even if gaps are present. The alignment of the three peptides revealed that they share nine identical amino acids. Concerning the nature of antigenic determinants recognized by Abs, both linear and conformational epitopes are capable of acting as an antigen-binding molecule, for this reason the three peptides under scrutiny were capable of eliciting the secretion of cross-reactive Abs, probably via molecular mimicry.

Major histocompatibility complex (MHC) class I molecules bind short peptides of nine residues, whose N- and C-terminal ends are anchored into the pockets located at the ends of the peptide-binding groove.

Longer peptides such as $MAP\_4027_{18–32}$, $BOLF1_{305–320}$ and $IRF5_{424–434}$ can be accommodated by the bulging of their central portion, resulting in binding peptides of 8 to 15 residues in length.[20] Conversely, the optimal length for peptides binding to class II proteins span from 12 to 16 residues.

T-cell receptors (TCR) are capable of binding linear determinants, and only two or three amino acids mediate peptide MHC binding. Moreover, many class I molecules have a hydrophobic pocket that recognizes one hydrophobic amino acid; in $MAP\_4027_{18–32}$, $BOLF1_{305–320}$ and $IRF5_{424–434}$ peptides the interaction might be due to the two leucine (L) residues at the C-terminal end of the peptides.

ELISA

EBV positivity in all individuals was evaluated by a commercially available anti-EBV EBNA-1 IgG ELISA kit. Of the 47 MS patients, 40 (85%) were EBV^+ and seven (15%) $EBV^–$, while concerning the 53 HCs, 40 (75%) were EBV^+ whereas 13 (25%) were $EBV^–$.

Abs against $BOLF1_{305–320}$ were found in 20 out of 40 (50%) EBV^+MS patients and only in one out 40 (2.5%) EBV^+ HC (area under the curve (AUC) = 0.9, $p > 0.0001$); none of the seven $EBV^–$ MS and 13 HCs reacted against $BOLF1_{305–320}$ (Figure 1 (a)).

Regarding $MAP\_4027_{18–32}$, 32 out of 40 (82.5%) EBV^+ MS patients and two out of 40 (5%) EBV^+ HCs were positive for this peptide (AUC = 0.9, $p > 0.0001$) whereas two out of seven (28.5%) $EBV^–$ MS patients and six out 13 (46%) $EBV^–$ HCs reacted against $MAP\_4027_{18–32}$ (Figure 1 (b)).

Abs against $IRF5_{424–434}$ were found in 11 out of 40 (27.5%) EBV^+ MS patients and in two out of 40 (5%) EBV^+ HCs (AUC = 0.7, $p = 0.001$). Among the $EBV^–$ patients, we observed the following situation: Five out of seven (71%) MS patients and none of the 13 HCs were Abs+ for $IRF5_{424–434}$ ($p = 0.001$) (Figure 1 (c)).

Finally, correlation analysis showed a moderate degree of correlation between $BOLF1_{305–320}$ and $MAP\_4027_{18–32}$ in EBV^+ MS patients ($r^2 = 0.35$, $p < 0.0001$) (Figure 2 (a)) and a weak correlation between $BOLF1_{305–320}$/$MAP\_4027_{18–32}$ and $IRF5_{424-434}$ in EBV+ MS patients ($r^2 = 0.11$, $p = 0.02$) (Figure 2 (b), (c)). As regards EBV^+ HCs, no correlation was found (Figure 2 (d–f)).

Competitive inhibition assays

Three MS patients (MS #1, MS #2 and MS #3) and one HC were screened by two different competitive inhibition assays. Regarding the $BOLF1_{305–320}$-coated competition assay (Figure 3 (a)), in all patients both $MAP\_4027_{18–32}$ and $IRF5_{424–434}$ efficiently inhibited antibody binding to the $BOLF1_{305–320}$-coated peptide. The same result was obtained in the $MAP\_4027_{18–32}$-coated competition assay, where both $BOLF1_{305–320}$ and $IRF5_{424–434}$ efficiently inhibited the binding of antibodies to the MAP_4027_{18-32} coated on the plate in all the MS patients (Figure 3 (b)). A control peptide ($MAP\_2694_{38–46}$) was included in all experiments without causing any decrease in signal.

Extracellular cytokines expression levels

After stimulation of MS patients' PBMCs (MS# 1–4) with the identified peptides ($BOLF1_{305–320}$, $MAP\_4027_{18–32}$ and $IRF5_{424–434}$), we observed production of IL-6, IL-10, IL-17A, IFN-γ, and TNF-α secretion (Figure 4 (a–c)), whereas secretion of IL-4, IL-5 and IL-13 in response to stimulation with all peptides was generally weak for all individuals (Figure 4 (d–f)).

Background values of cytokine levels (no peptide) expressed as mean ± SD were the following: IL-6

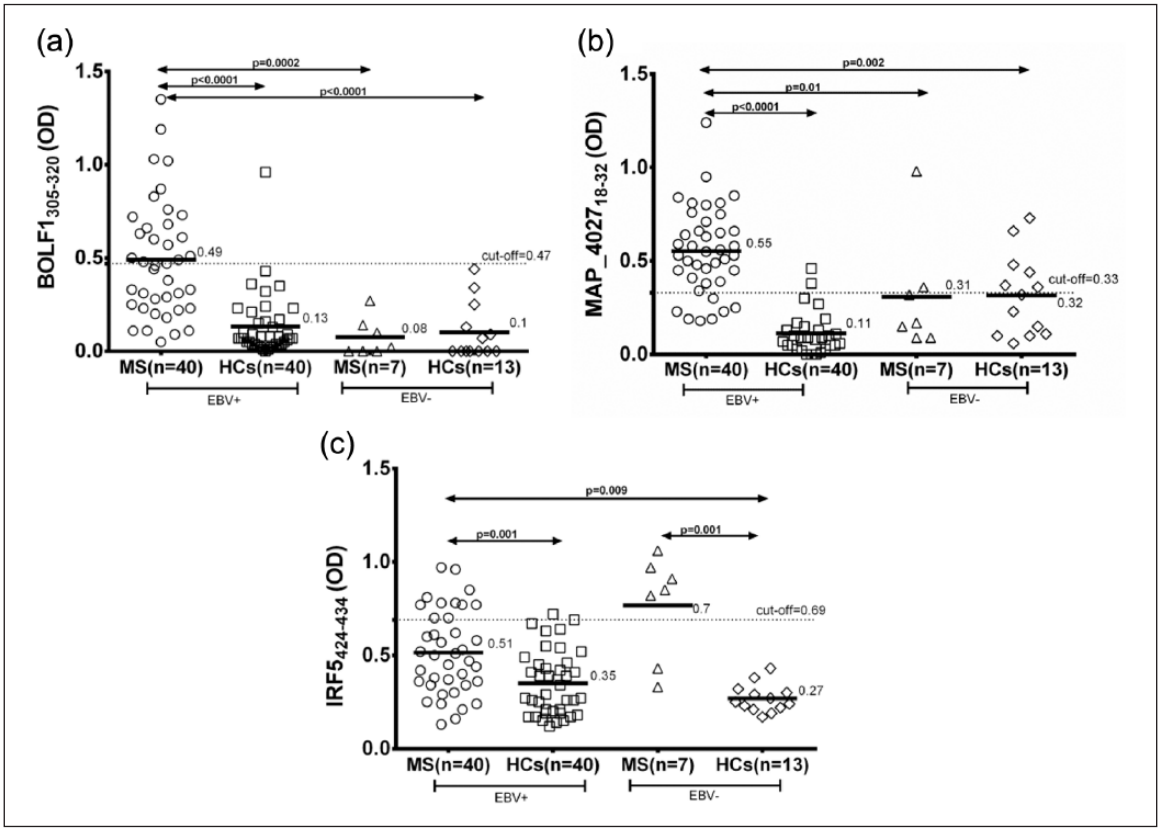

Figure 1. Antibody titers against $BOLF1_{305–320}$, $MAP\_4027_{18–32}$ and $IRF5_{424–434}$ measured by indirect ELISA. Forty-seven MS patients (40 EBV^+; seven $EBV^–$) and 53 healthy controls (40 EBV^+; 13 $EBV^–$) were tested for their reactivity against plate-coated with $BOLF1_{305–320}$ (a), $MAP\_4027_{18–32}$ (b) and $IRF5_{424–434}$ (c) peptides. The horizontal black bars represent the mean value, while *p* values are indicated by two-headed arrows drawn on the top of each distribution. Cutoff values for positivity, calculated by ROC analysis, are indicated by dashed lines. MAP: *Mycobacterium avium* subsp. *paratuberculosis*; IRF5: human interferon regulatory factor 5; ELISA: enzyme-linked immunosorbent assay; MS: multiple sclerosis; EBV: Epstein-Barr virus; ROC: receiver-operating characteristics.

(343±263.5 pg/ml), IL-10 (38.6±19.6 pg/ml), IL-17A (79.3±102.4 pg/ml), IFN-γ (149.3±249.1 pg/ml) and TNF-α (5.6±8.9 pg/ml). The levels of cytokine expression observed after stimulation with each peptide ($BOLF1_{305–320}$, $MAP\_4027_{18–32}$ and $IRF5_{424–434}$), followed a very similar trend. For instance, values related to $BOLF1_{305–320}$ are the following: IL-6 (4446.66±1220.33 pg/ml), IL-10 (763.3±475.3 pg/ml), IL-17A (340.6±206.7 pg/ml), IFN-γ (989.3±516.5 pg/ml) and TNF-α (94±55.6 pg/ml) (Figure 4 (a)). Cytokine secretion in response to stimulation with peptides was defined as positive, when the level of each cytokine exceeded 10 times those obtained as a background level, and/or the *p* value calculated by *t* test was <0.05. Compared to background level, a 13-fold increase ($p = 0.0006$) was observed for IL-6, a 20-fold increase ($p = 0.0226$) for IL-10, an 18-fold increase ($p = 0.0196$) for TNF-α, and a six-fold increase ($p = 0.0262$) for IFN-γ. Conversely, only a four-fold increase was observed in IL-17A production, a result that is not statistically significant ($p = 0.0636$). Thus, $BOLF1_{305–320}$, $MAP\_4027_{18–32}$ and $IRF5_{424–434}$ peptides are capable of inducing the secretion of multiple MS-related cytokines.

Concerning the stimulation of HCs' PBMCs (HCs #1–2) with $BOLF1_{305–320}$, $MAP\_4027_{18–32}$ and $IRF5_{424–434}$, the levels of IL-6, IL-10, IL-17A, IFN-γ, TNF-α (Figure 5), IL-4, IL-5 and IL-13 secretion (Figure 4 (a–c)) were generally weak for all participants. The tendency to induce Th1 cytokines expression was the same for both HCs.

Flow cytometric quantitation of peptide-specific circulating T lymphocytes

Flow cytometric evaluation of both peptide-specific CD8+ and CD8– (i.e. CD4) T lymphocytes after eight-hour stimulation of peripheral blood from four MS patients and two HCs was performed. We

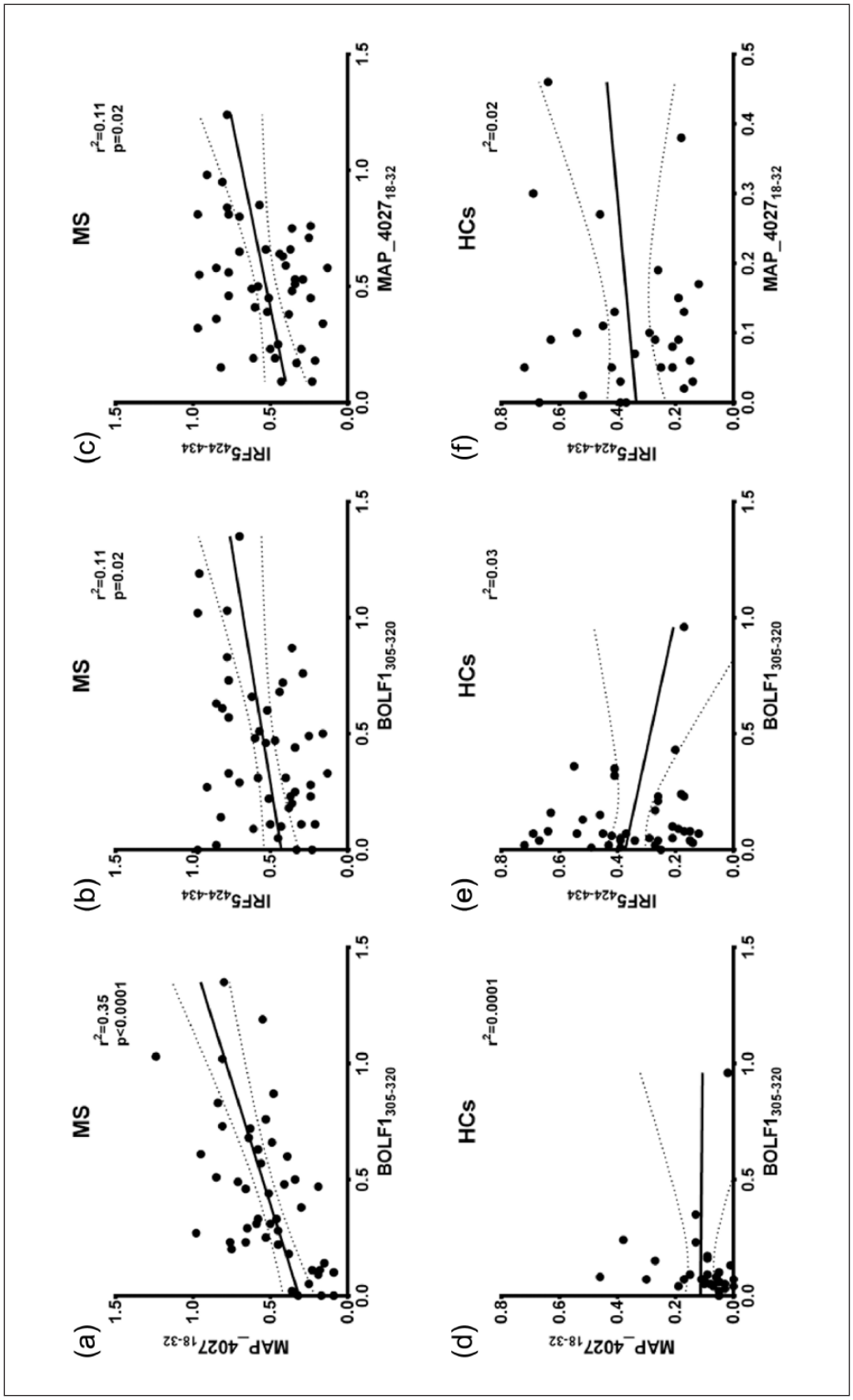

Figure 2. Scatter plot showing the correlations between $BOLF1_{305-320}$, MAP_4027_{18-32} and $IRF5_{424-434}$ antibody titers. The correlation analysis was performed on 40 EBV+ MS patients ((a), (b), (c)) and 40 EBV+ healthy controls ((d), (e), (f)). MAP: *Mycobacterium avium* subsp. *paratuberculosis*; IRF5: human interferon regulatory factor 5; EBV: Epstein-Barr virus; MS: multiple sclerosis.

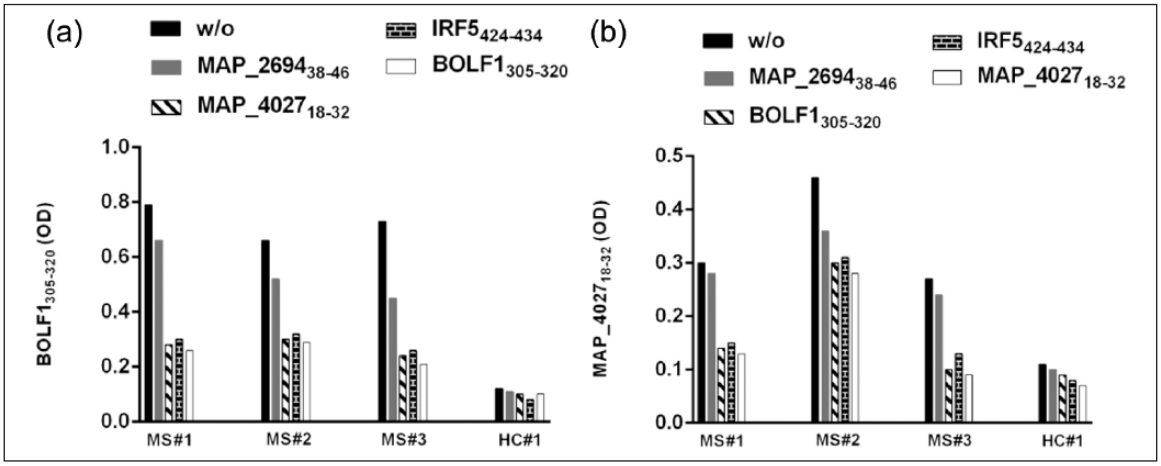

Figure 3. Competitive inhibition assays. Sera from three MS (MS #1–3) patients and one healthy control (HC #1) were subject to ELISA on plates coated with $BOLF1_{305–320}$ (a) and $MAP_{18–32}$ (b), respectively. The same sera were pre-incubated overnight with saturating equimolar concentrations of peptides ($BOLF1_{305–320}$, $MAP\_4027_{18–32}$ and $IRF5_{424–434}$) and a sero-negative peptide ($MAP\_2694_{38–46}$). The first bar (w/o) represents a regularly performed ELISA (at 1/100 serum dilution). MAP: *Mycobacterium avium* subsp. *paratuberculosis*; IRF5: human interferon regulatory factor 5; MS: multiple sclerosis; ELISA: enzyme-linked immunosorbent assay.

analyzed the expression of TNF-α and IFN-γ upon stimulation with the following peptides: $BOLF1_{305–320}$, $MAP\_4027_{18–32}$ and $IRF5_{424–434}$. PBMCs from MS patients showed CD8– (i.e. CD4) and CD8+ T–cells expressing both cytokines at a frequency higher of 0.5% and 1.0%, respectively (Figure 5 (a)). A statistical analysis demonstrated that the frequency of both TNF-α and IFN-γ-expressing CD4 and CD8 T-cells was higher in MS PBMCs stimulated with the homologous peptides compared to PBMCs not stimulated (Figure 5 (b)).

Regarding the HCs, none of the peptides was capable of inducing cytokines production by $CD4^+$ and $CD8^+$ T-cells (Figure 6).

Intracytoplasmic cytokine expression by peptide-stimulated monocytes

The cytokine expression pattern of $CD14^+$ monocytes in four MS patients and in two HCs was further analyzed by FACS in the cytokine-driven assay. We analyzed the expression of TNF-α and IL-6 by CD14+ monocytes following a stimulation of PBMCs with the peptides $BOLF1_{305–320}$, $MAP\_4027_{18–32}$ and $IRF5_{424–434}$.

Increased expression of both IL-6 and TNF-α was observed for the peptide-stimulated $CD14^+$ monocytes from MS patients and increased both IL-6 and TNF-α expression (Figure 5 (c)), whereas IL-6 and TNF-α expression were absent in HCs.

Discussion

The aim of this study was to investigate the presence of specific humoral and T-cell-mediated responses, mounted against peptides deriving from EBV and MAP antigens, which in turn are homologs to host proteins related to MS disease. MS has traditionally been considered a disease mediated by T-cells, in particular by CD4 helper T-cells, mainly due to the reported genetic association between MS and the MHC class II region.[21] Nevertheless, the role of CD8 T-cells has been re-evaluated,[22] and recent studies reassigned an important role to the humoral response driven by B cells,[23] hence it is important to clarify how B- and T-cells interact in the complex pathogenesis of MS.

This study reports: First, an evaluation of the ability of $BOLF1_{305–320}$ and the homologs $MAP\_4027_{18–32}$ and $IRF5_{424–434}$ peptides to induce a strong humoral response in MS patients, and second, the antibody-mediated cross-recognition of these homologuous peptides. These data confirmed that EBV and MAP are capable of inducing the production of autoantibodies targeting different MS-correlated epitopes.

IRF5 polymorphisms have been associated with MS in different studies.[16,24] *IRF5* gene variation has a role in the pharmacological and clinical outcome of IFNβ therapy.[17] Furthermore, *IRF5* is an important regulator of IFN activity; It modulates immune signaling cytokine expression, apoptosis, cell cycle and it has been linked to MS development.[14,15,18] Moreover, *IRF5* regulates the toll-like receptor (TLR)-dependent

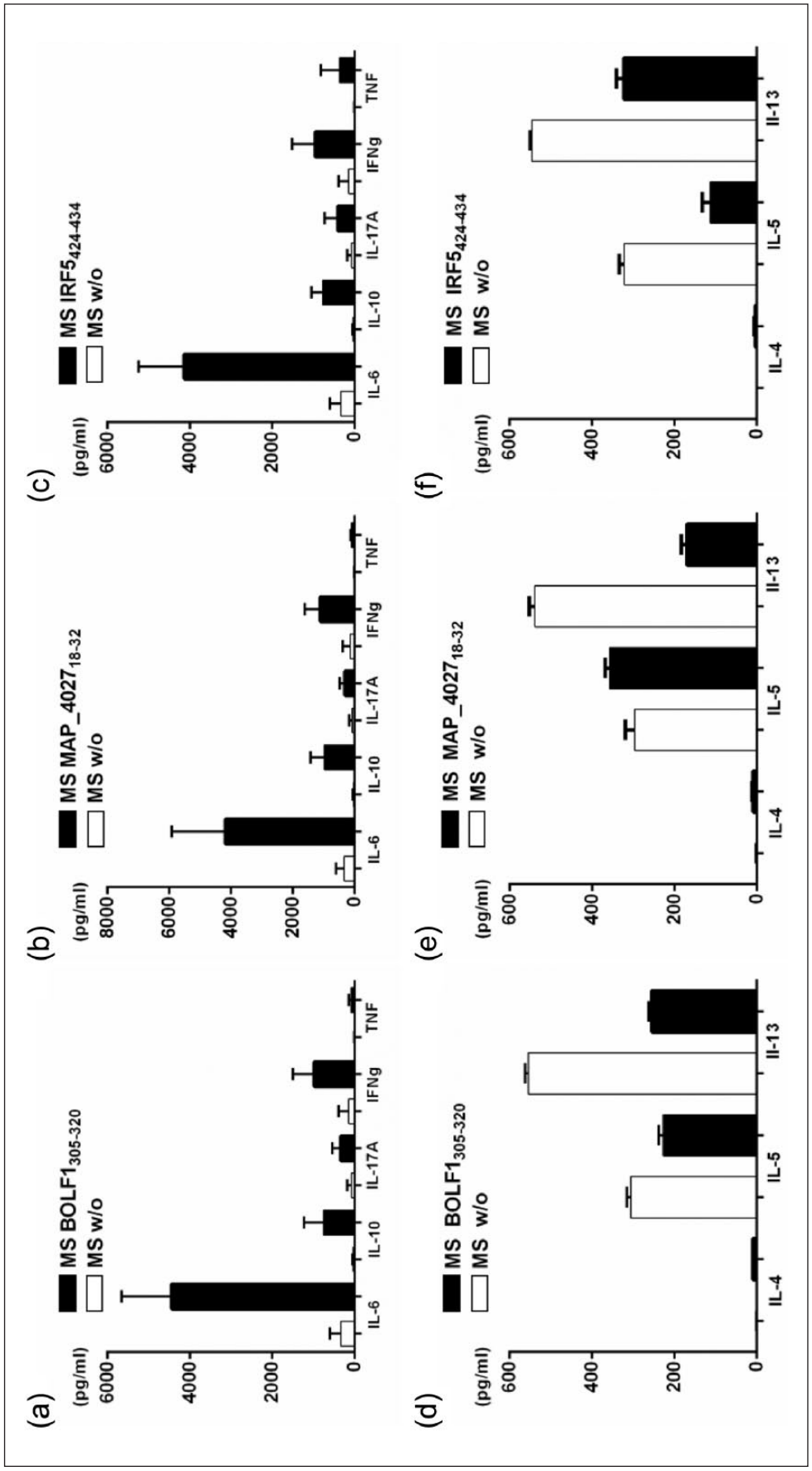

Figure 4. Bar graphs depicting secreted cytokine levels measured by a Multi-Analyte ELISArray Kit. PBMCs from four MS patients were stimulated with 25 μM of single peptides ($BOLF1_{305-320}$, MAP_4027 $_{18-32}$ and $IRF5_{424-434}$) for 48 hours; the experiment was carried out in duplicate. IL-6, IL-10, IL-17A, IFN-γ and TNF-α (a–c) and IL-4, IL-5 and IL-13 (d–f)) levels were measured out after peptide-stimulation in MS #1–4. White bars represent the controls without peptide stimulation. PBMCs: peripheral blood mononuclear cells; MS: multiple sclerosis; MAP: *Mycobacterium avium* subsp. *paratuberculosis*; IRF5: human interferon regulatory factor 5; IL: interleukin; IFN-γ: interferon gamma; TNF: tumor necrosis factor alpha.

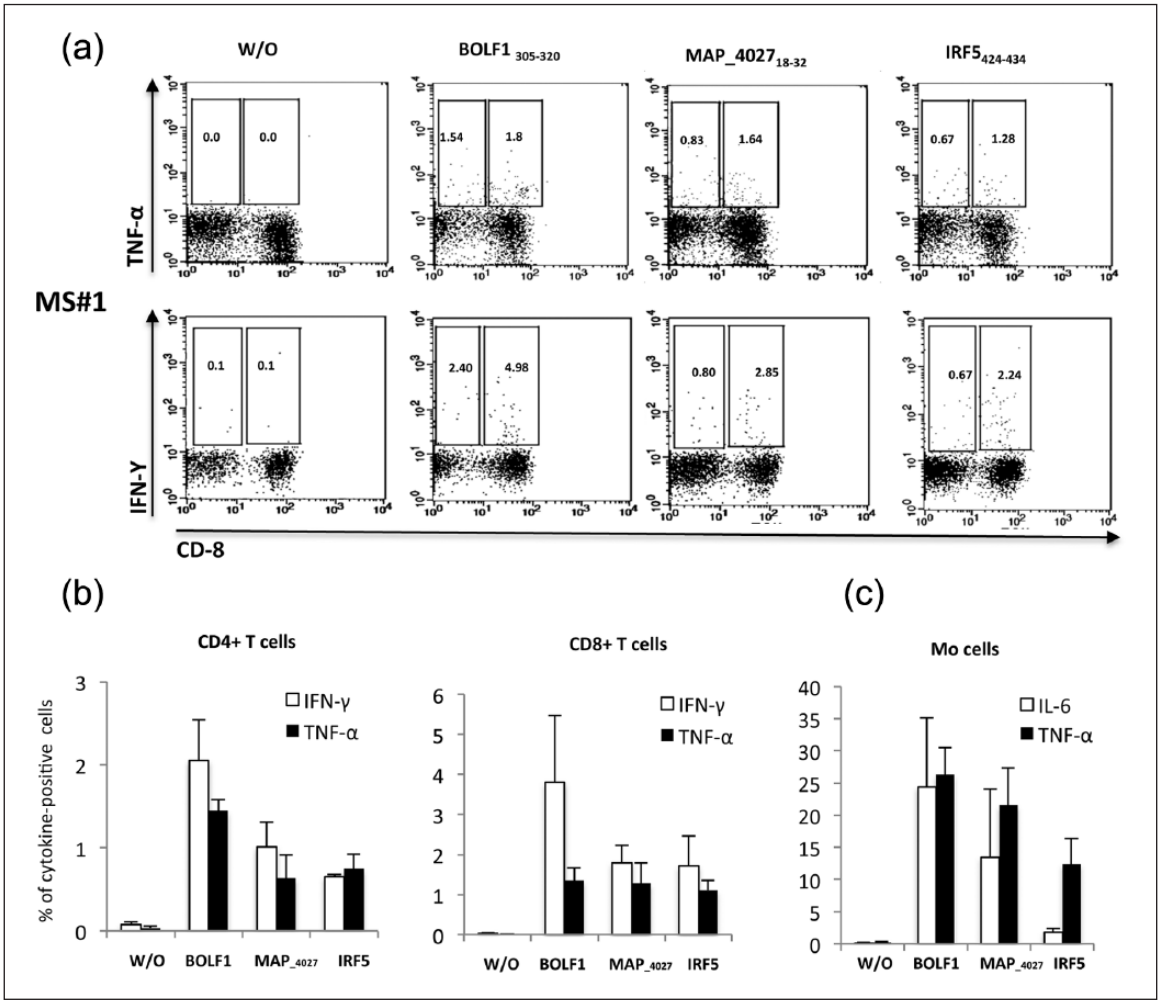

Figure 5. Detection of BOLF13$_{05–320}$-, MAP_4027$_{18–32}$- and IRF5$_{424-434}$-specific T-cells and monocytes by intracytoplasmic cytokine expression assay. Heparinized whole blood (0.5 ml) was stimulated for eight hours with the identified peptides in the presence of anti-CD28/CD49d antibody and brefeldin A. Gated to CD3+CD8+ cells, the expression of IFN-γ and TNF−α in CD4+ T cells (left) and CD8+ T cells (right) was measured in MS #1 (a). MS patients (MS #1–4) showed substantial expansions of CD8+ and CD4+ T-cells specific for all peptides; specifically BOLF1$_{305–320}$ induced a higher percentage of Th1 cytokine (IFN-γ and TNF−α)-positive cells (b). After peptide stimulation of the blood drawn from MS patients (MS #1–4), an increased percentage of IL-6 and TNF-α CD14+ positive cells (c) was detected in all of them. MAP: *Mycobacterium avium* subsp. *paratuberculosis*; IRF5: human interferon regulatory factor 5; PBMCs: peripheral blood mononuclear cells; MS: multiple sclerosis; IL: interleukin; IFN-γ: interferon gamma; TNF: tumor necrosis factor alpha.

activation of inflammatory cytokines, and functions downstream of the TLR-MyD88 pathway, where it is activated by both MyD88 and TNF receptor-associated factor 6 (TRAF6).[25]

IRF5 was also proven to be a downstream mediator of TLR7 signaling in EBV-infected B-cell lines with type III latency,[26] repressing the virus oncoprotein latent membrane protein 1 (LMP1).[27]

Previous studies of EBV- and MAP-derived peptides have been informative; however, the clinical parameters analyzed in relation to MS were restricted only to the humoral response (Ab positive/negative).[8,28] For this reason, we investigated the existence of a cell-mediated immune response in MS patients against EBV and MAP epitopes. To verify a possible link, we set up antigen-specific T-cell stimulation experiments in order to detect extracellular and intracellular cytokine levels.

The extracellular cytokine levels were measured by ELISA with the outcome demonstrating that the cellular immune response induced by these peptides was mainly ascribable to the Th1 subset, while Th2-related cytokines were downregulated.

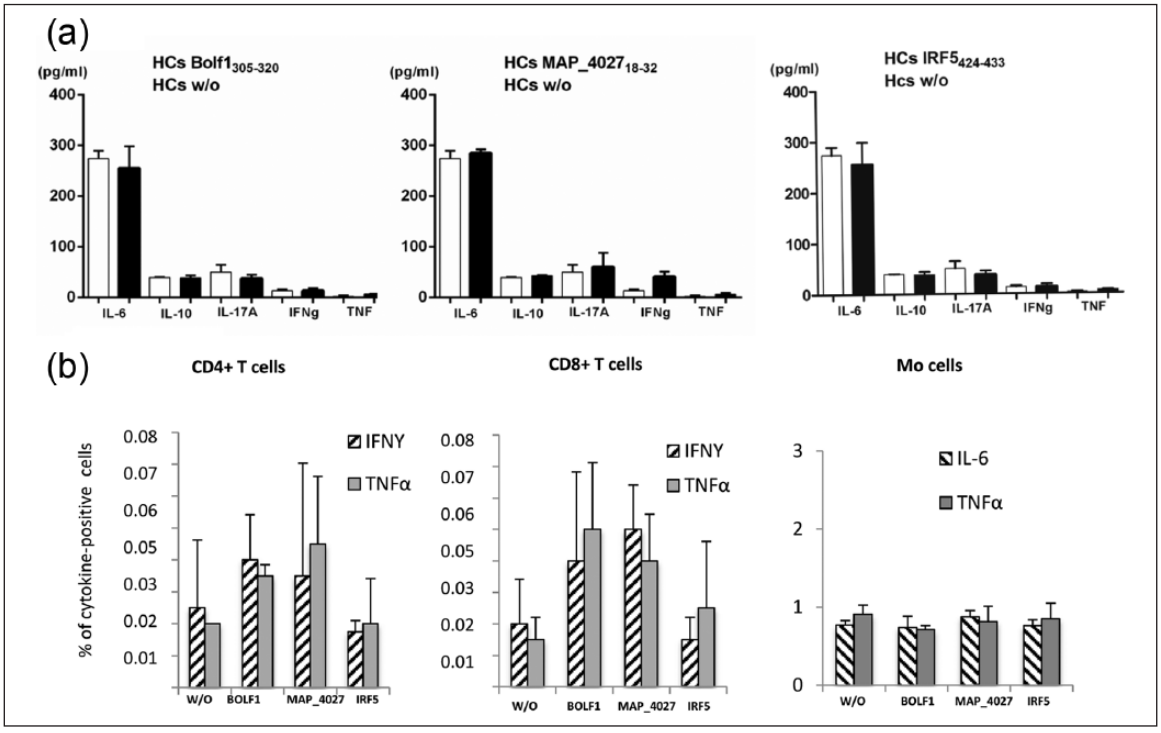

Figure 6. Detection of extracellular cytokine levels, and quantitation of intracytoplasmic cytokine expression produced by peptide-stimulated T lymphocytes/monocytes in healthy controls (HCs). PBMCs from two HCs (HC #1–2) were stimulated for 48 hours with 25 μM of single peptides ($BOLF1_{305–320}$, $MAP\_4027_{18–32}$ and $IRF5_{424–434}$), and IL-6, IL-10, IL-17A, IFN-γ and TNF-α expression levels were measured out by a Multi-Analyte ELISArray Kit (a). Flow cytometric analysis performed after peptide stimulation of whole blood samples from HC #1–2 highlighted the lack of expression of TNF-α and IFN-γ by CD4+/CD8+ T cells and IL-6/TNFα by CD14+ cells (b). PBMCs: peripheral blood mononuclear cells; MS: multiple sclerosis; MAP: *Mycobacterium avium* subsp. *paratuberculosis*; IRF5: human interferon regulatory factor 5; IL: interleukin; IFN-γ: interferon gamma; TNF: tumor necrosis factor alpha.

The levels of IL-6, IL-10 and IFN-γ were upregulated upon stimulation with all the selected peptides. While IL-6 and IFN-γ are the clear signature of a Th1 response, IL-10 is a regulatory pleiotropic cytokine. Although IL-10 plays an important role in the onset and development of autoimmune diseases such as MS,[29] the exact mechanisms by which this cytokine acts remain uncertain.

ELISA revealed that IL-6 is the most secreted cytokine, and cytometry analysis showed as well a significant percentage of monocytes positive for this cytokine. IL-6 and TNF-α are pleitropic cytokines able to regulate both inflammatory and immunological responses, and are also capable of stimulating the terminal differentiation of activated B-cells into Ig-secreting plasma cells.[30] IL-6 secretion could interfere with the suppressive activity of T-reg cells thus facilitating the priming of autoreactive T-cells.[31] Cytometric analyses showed a significant increase of TNF-α $CD4^+$ and $CD8^+$ double-positive cells after stimulation with all peptides, while 25% of $CD14^+$ monocytes were also positive for TNF-α after $BOLF1_{305–320}$- and $MAP\_4027_{18–32}$-specific stimulation. Altogether, the results produced by flow cytometry highlighted that the selected peptides are able to induce a TNF-α-mediated T-cell polarization.

TNF-α is the principal proinflammatory cytokine in MS and an important mediator of immunological and inflammatory responses; it can lead to the damage of myelin and oligodendrocytes in the central nervous system (CNS).[32]

Both cytometry and ELISA revealed significant IFN-γ production on PBMCs stimulation with the three selected epitopes. The IFN-γ response was mainly ascribable to the $CD8^+$ T-cell, and in particular the main cytokine inducer was $BOLF1_{305–320}$. Different studies support a prominent role of IFN-γ in driving MS disease. Indeed it was reported that an increase of IFN-γ production precedes relapse phases in MS

patients.[33,34] Moreover, IFN-γ along with other type 1 cytokines, was proven to be augmented in $CD4^+$, $CD8^+$ and $CD14^+$ cells in MS patients undergoing disease reactivation, while they were normalized in stable patients or individuals undergoing IFN treatment.[35] In addition, it was also demonstrated that IFN-γ levels are upregulated during RR exacerbation phases, while IL-17 levels increase only in clinically isolated syndrome (CIS) patients.[35] These observations are in line with our results as the investigated peptides present a strong INF-γ-inducing ability, thus suggesting that IFN-γ plays a more important role than IL-17 does in this subset of RRMS patients.

A possible explanation for the obtained data could be the following: In MS, a T-cell attack might be directed to self-components, even if the primary target is yet to be discovered. Indeed, it is acknowledged that the autoimmune attack is directed against multiple targets.[36] When Abs against self-epitopes are produced, the epitope-spreading phenomenon may contribute to tissue destruction and to the production of Abs against a previously "sequestered" antigen, leading in turn to a secondary autoimmune response against the newly released antigen. Therefore, the specific response mounted against the non-self-epitopes ($BOLF1_{305–320}$ and $MAP\_4027_{18–32}$) might be enhanced by the release of $IRF5_{424–434}$ from the intracellular compartment. We think that Abs production against $IRF5_{424–434}$, $BOLF1_{305–320}$ and $MAP\_4027_{18–32}$ does contribute to the MS pathogenesis but solely a small part (e.g. Abs may act as ligands activating macrophages contributing to the tissue inflammation). Conversely, recent findings support the hypothesis that Abs have the ability to enter neurons in an epitope-specific manner.[37,38] The constant presence of cross-reactive Abs against $IRF5_{424–434}$, $BOLF1_{305–320}$ and $MAP\_4027_{18–32}$ may favor the entrance of this Abs into the cytosol, possibly via endocytosis. Therefore, Abs targeting these three homologous epitopes may alter the function of the IRF5 protein, modulating or inhibiting its activity.

In conclusion, the results of our experiments support the assumption that antibody-mediated cross-recognition of non-self-epitopes ($BOLF1_{305–320}$, $MAP\_4027_{18–32}$) and self-epitope ($IRF5_{424–434}$), may mediate/modulate the immune dysregulation responsible in turn for favoring MS development. Moreover, the same peptides are able to induce a Th1-immune response in MS patients, highlighting the relevant role of IL-6, TNF-α and IFN-γ in this process observed on PBMCs stimulation with these three epitopes.

The main drawback of the study is the small sample size cohort in which to assess the intracytoplasmic cytokine expression levels. This made it difficult to carry out statistical significant analyses. Moreover, it would be interesting to compare more MS patients' cytokine levels with the ones displayed by the HCs.

Conflict of interest

None declared

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References

1. Venkatesan A and Johnson RT. Infections and multiple sclerosis. *Handb Clin Neurol* 2014; 122: 151–171.
2. Libbey JE, Cusick MF and Fujinami RS. Role of pathogens in multiple sclerosis. *Int Rev Immunol* 2014; 33: 266–283.
3. Sotgiu S, Mameli G, Serra C, et al. Multiple sclerosis-associated retrovirus and progressive disability of multiple sclerosis. *Mult Scler* 2010; 16: 1248–1251.
4. Sospedra M and Martin R. Molecular mimicry in multiple sclerosis. *Autoimmunity* 2006; 39: 3–8.
5. Mameli G, Cossu D, Cocco E, et al. EBNA-1 IgG titers in Sardinian multiple sclerosis patients and controls. *J Neuroimmunol* 2013; 264: 120–122.
6. Cossu D, Masala S, Cocco E, et al. Are *Mycobacterium avium* subsp. *paratuberculosis* and Epstein-Barr virus triggers of multiple sclerosis in Sardinia? *Mult Scler* 2012; 18: 1181–1184.
7. Cossu D, Masala S and Sechi LA. A Sardinian map for multiple sclerosis. *Future Microbiol* 2013; 8: 223–232.
8. Mameli G, Cossu D, Cocco E, et al. *Epstein-Barr* virus and *Mycobacterium avium* subsp. *paratuberculosis* peptides are cross recognized by anti-myelin basic protein antibodies in multiple sclerosis patients. *J Neuroimmunol* 2014; 270: 51–55.
9. Esposito M, Venkatesh V, Otvos L, et al. Human transaldolase and cross-reactive viral epitopes identified by autoantibodies of multiple sclerosis patients. *J Immunol* 1999; 163: 4027–4032.
10. Adhikary D, Behrends U, Boerschmann H, et al. Immunodominance of lytic cycle antigens in *Epstein-Barr* virus-specific CD4+ T cell preparations for therapy. *PLoS One* 2007; 2: e583.

11. Handel AE, Williamson AJ, Disanto G, et al. An updated meta-analysis of risk of multiple sclerosis following infectious mononucleosis. *PLoS One* 2010; 5: e12496.
12. Ascherio A, Munger KL, Lennette ET, et al. *Epstein-Barr* virus antibodies and risk of multiple sclerosis: A prospective study. *JAMA* 2001; 286: 3083–3088.
13. Thacker EL, Mirzaei F and Ascherio A. Infectious mononucleosis and risk for multiple sclerosis: A meta-analysis. *Ann Neurol* 2006; 59: 499–503.
14. Masuda T, Iwamoto S, Yoshinaga R, et al. Transcription factor IRF5 drives P2X4R+-reactive microglia gating neuropathic pain. *Nat Commun* 2014; 5: 3771.
15. Krausgruber T, Blazek K, Smallie T, et al. IRF5 promotes inflammatory macrophage polarization and TH1–TH17 responses. *Nat Immunol* 2011; 12: 231–238.
16. Vandenbroeck K, Alloza I, Swaminathan B, et al. Validation of *IRF5* as multiple sclerosis risk gene: Putative role in interferon beta therapy and human herpes virus-6 infection. *Genes Immun* 2011; 12: 40–45.
17. Clark DN, Read RD, Mayhew V, et al. Four promoters of IRF5 respond distinctly to stimuli and are affected by autoimmune-risk polymorphisms. *Front Immunol* 2013; 4: 360.
18. Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 revision to the McDonald criteria. *Ann Neurol* 2011; 69: 292.
19. Suni MA, Picker LJ and Maino VC. Detection of antigen-specific T cell cytokine expression in whole blood by flow cytometry. *J Immunol Methods* 1998; 212: 89–98.
20. Meydan C, Otu HH and Sezerman OU. Prediction of peptides binding to MHC class I and II alleles by temporal motif mining. *BMC Bioinformatics* 2013; 14 (Suppl 2): S13.
21. Ramagopalan SV and Ebers GC. Multiple sclerosis: Major histocompatibility complexity and antigen presentation. *Genome Med* 2009; 1: 105.
22. Friese MA and Fugger L. Pathogenic CD8(+) T cells in multiple sclerosis. *Ann Neurol* 2009; 66: 132–141.
23. Disanto G, Morahan JM, Barnett MH, et al. The evidence for a role of B cells in multiple sclerosis. *Neurology* 2012; 78: 823–832.
24. Qiu W, Pham K, James I, et al. The influence of non-HLA gene polymorphisms and interactions on disease risk in a Western Australian multiple sclerosis cohort. *J Neuroimmunol* 2013; 261: 92–97.
25. Takaoka A, Yanai H, Kondo S, et al. Integral role of *IRF-5* in the gene induction programme activated by Toll-like receptors. *Nature* 2005; 434: 243–249.
26. Martin HJ, Lee JM, Walls D, et al. Manipulation of the toll-like receptor 7 signaling pathway by *Epstein-Barr* virus. *J Virol* 2007; 81: 9748–9758.
27. Ning S, Huye LE and Pagano JS. Interferon regulatory factor 5 represses expression of the *Epstein-Barr* virus oncoprotein LMP1: Braking of the IRF7/LMP1 regulatory circuit. *J Virol* 2005; 79: 11671–11676.
28. Cossu D, Masala S, Frau J, et al. Antigenic epitopes of MAP2694 homologous to T-cell receptor gamma-chain are highly recognize in multiple sclerosis Sardinia patients. *Mol Immunol* 2014; 57: 138–140.
29. Ouyang W, Rutz S, Crellin NK, et al. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu Rev Immunol* 2011; 29: 71–109.
30. Erta M, Quintana A and Hidalgo J. Interleukin-6, a major cytokine in the central nervous system. *Int J Biol Sci* 2012; 8: 1254–1266.
31. Schneider A, Long SA, Cerosaletti K, et al. In active relapsing–remitting multiple sclerosis, effector T cell resistance to adaptive T(regs) involves IL-6-mediated signaling. *Sci Transl Med* 2013; 5: 170ra15.
32. Rossi S, Motta C, Studer V, et al. Tumor necrosis factor is elevated in progressive multiple sclerosis and causes excitotoxic neurodegeneration. *Mult Scler* 2014; 20: 304–312.
33. Hidaka Y, Inaba Y, Matsuda K, et al. Cytokine production profiles in chronic relapsing–remitting experimental autoimmune encephalomyelitis: IFN-γ and TNF-α are important participants in the first attack but not in the relapse. *J Neurol Sci* 2014; 340: 117–122.
34. Brambilla R, Ashbaugh JJ, Magliozzi R, et al. Inhibition of soluble tumour necrosis factor is therapeutic in experimental autoimmune encephalomyelitis and promotes axon preservation and remyelination. *Brain* 2011; 134: 2736–2754.
35. Lindquist S, Hassinger S, Lindquist JA, et al. The balance of pro-inflammatory and trophic factors in multiple sclerosis patients: Effects of acute relapse and immunomodulatory treatment. *Mult Scler* 2011; 17: 851–866.
36. Fraussen J, Claes N, de Bock L, et al. Targets of the humoral autoimmune response in multiple sclerosis. *Autoimmun Rev*. Epub ahead of print 6 August 2014. DOI: 10.1016/j.autrev.2014.07.002.
37. Geis C, Weishaupt A, Hallermann S, et al. Stiff person syndrome-associated autoantibodies to amphiphysin mediate reduced GABAergic inhibition. *Brain* 2010; 133: 3166–3180.
38. Magrys A, Anekonda T, Ren G, et al. The role of anti-alpha-enolase autoantibodies in pathogenicity of autoimmune-mediated retinopathy. *J Clin Immunol* 2007; 27: 181–192.