Interferon regulatory factor 5 is a potential target of autoimmune response triggered by Epstein-Barr virus and M*ycobacterium avium* subsp. *paratuberculosis* in rheumatoid arthritis: investigating a mechanism of molecular mimicry

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Abstract

Objective

Rheumatoid arthritis (RA) is a chronic disease characterised by a pro-inflammatory cytokines linked erosive joint damage and by humoral and cellular response against a broad range of self-peptides. Molecular mimicry between Epstein-Barr virus (EBV), Mycobacterium avium subsp. paratuberculosis (MAP) and host peptides has long been regarded as an RA pathogenetic mechanism. Using bioinformatic analysis we identified high sequence homology among interferon regulatory factor 5 (IRF5), EBV antigen BOLF1 and MAP antigen MAP_4027.

Our objective was to evaluate the presence in sera of RA patients of antibodies (Abs) directed against human homologous IRF5 cross-reacting with BOLF1 and MAP_4027.

Methods

Frequency of reactivity against $IRF5_{424-434}$, $BOLF1_{305-320}$ and MAP_4027_{18-32} was tested by indirect ELISA in sera from 71 RA patients and 60 healthy controls (HCs).

Results

RA sera show a remarkable high frequency of reactivity against $IRF5_{424-434}$ in comparison to HCs (69% vs. 8%; p<0.0001). Similarly, seroreactivity against $BOLF1_{305-320}$ was more frequently detected in RA sera than in HCs counterpart (58% vs. 8%; p<0.0001). Frequency of Abs against MAP_4027_{18-32} was 17% in RA sera vs. 5% in HCs with a p-value at the threshold level (p<0.051). Prevalence of Abs against at least one of the assessed epitopes reached 72% in RA patients and 15% among HCs. Levels of Abs in RA patients were significantly related to systemic inflammation.

Conclusion

IRF5 is a potential autoimmune target of RA. Our results support the hypothesis that EBV and MAP infections may be involved in the pathogenesis of RA, igniting a secondary immune response that cross-reacts against RA self-peptides.

Key words

Interferon regulatory factor 5 (IRF5), Epstein-Barr virus (EBV), *Mycobacterium avium* subsp. *paratuberculosis* (MAP), cross-reactivity, rheumatoid arthritis

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Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disease characterised by chronic erosive polyarthritis and by the presence of a specific immune response against a broad spectrum of auto-antigens including cartilage components, chaperones, enzymes, nuclear antigens and post-translational modified proteins (1-3). The aetiology of RA is hitherto unknown while its pathogenesis is ascribed to an interplay between genetic and environmental factors (4). However, only 50% of the risk for RA is attributable to genetic predisposition; the remaining 50% is due to environmental contributors such as microbial infections (1). Epstein-Barr virus (EBV) and *Mycobacterium avium* subsp. *paratuberculosis* (MAP) were among pathogens frequently reported to be involved in RA pathogenesis (2, 5-9).

In recent years the potential role of interferon regulatory factor 5 (IRF5) in the pathogenesis of RA has been frequently reported (5, 10, 11). IRF5 is a key transcription factor of the immune system, playing an important role in modulating inflammatory immune responses in many cell types including dendritic cells and macrophages by driving them toward a proinflammatory phenotype in concert with cytokines and chemokines expression, and by regulating B cell maturity and antibody production (12-17).

Recent *in vivo* studies identified the importance of IRF5 as a new link between the pathogenic activation of RNA-sensing Toll-like receptors and proinflammatory cytokine production in inflamed joints of arthritic mice (18). Intriguingly, we recently demonstrated the presence in various autoimmune diseases of a specific seroreactivity against EBV and MAP epitopes cross-reacting with homologous human antigens such as IRF5 (19, 20). We supposed that significantly increased antibody titers following infection with MAP and EBV may cross-react with homologous peptide of the transcription factor IRF5 leading to modulation of its expression.

Based on the above preliminary findings, our study aimed at evaluating the presence of a cross-reactivity between three homologous peptides derived from EBV tegument protein BOLF1 ($BOLF1_{305-320}$), the MAP_4027 antigen (MAP_4027_{18-32}) [21, 22] and the human interferon regulatory factor 5 ($IRF5_{424-434}$) in a group of RA patients and healthy controls (HCs).

Materials and methods

Subjects

Consecutive RA patients who met the criteria of the American College of Rheumatology (23, 24) were enrolled at the outpatient clinic of the Rheumatology Unit, Department of Clinical and Experimental Medicine, University Hospital of Sassari, Italy. Collected data included: duration of RA; steroid treatment; DMARDs and/or anti-tumor necrosis factor-alpha therapy, tocilizumab, rituximab and abatacept; levels of C-reactive protein (CRP), mg/dL; erythrocyte sedimentation rate (ESR) levels, mm/h; rheumatoid factor positivity; anti-cyclic citrullinated peptide positivity (anti-CCP); Disease Activity Score-28 (DAS-28) and Health Assessment Questionnaire (HAQ). HCs were recruited at the Blood Transfusion Centre of Sassari, Italy. Ethical clearance for all subjects was obtained from the Ethics Committee of ASL 1 Sassari (1134/L, 16/04/2013 and 1192/L-04/02/2014). All methods were carried out in accordance with the approved guidelines and informed written consent was obtained from all participants.

Homology search

The following peptides were included in the study: $BOLF1_{305-320}$ [AAVPV-LAFDAARLRLLE], MAP_4027_{18-32} [AVVPVLAYAAARL-LL], $IRF5_{424-434}$ [VVPV—AARL-LLE].

All peptides were synthesised commercially at >90% purity (LifeTein, South Plainfield, NJ 07080, USA). Peptides alignment was obtained using Clustal W 2 (Fig 1E).

ELISA assays

Blood samples were collected in Vacutainer tubes for separation of serum and further screening for antibodies (Abs) against BOLF, IRF5 and MAP by indirect ELISA.

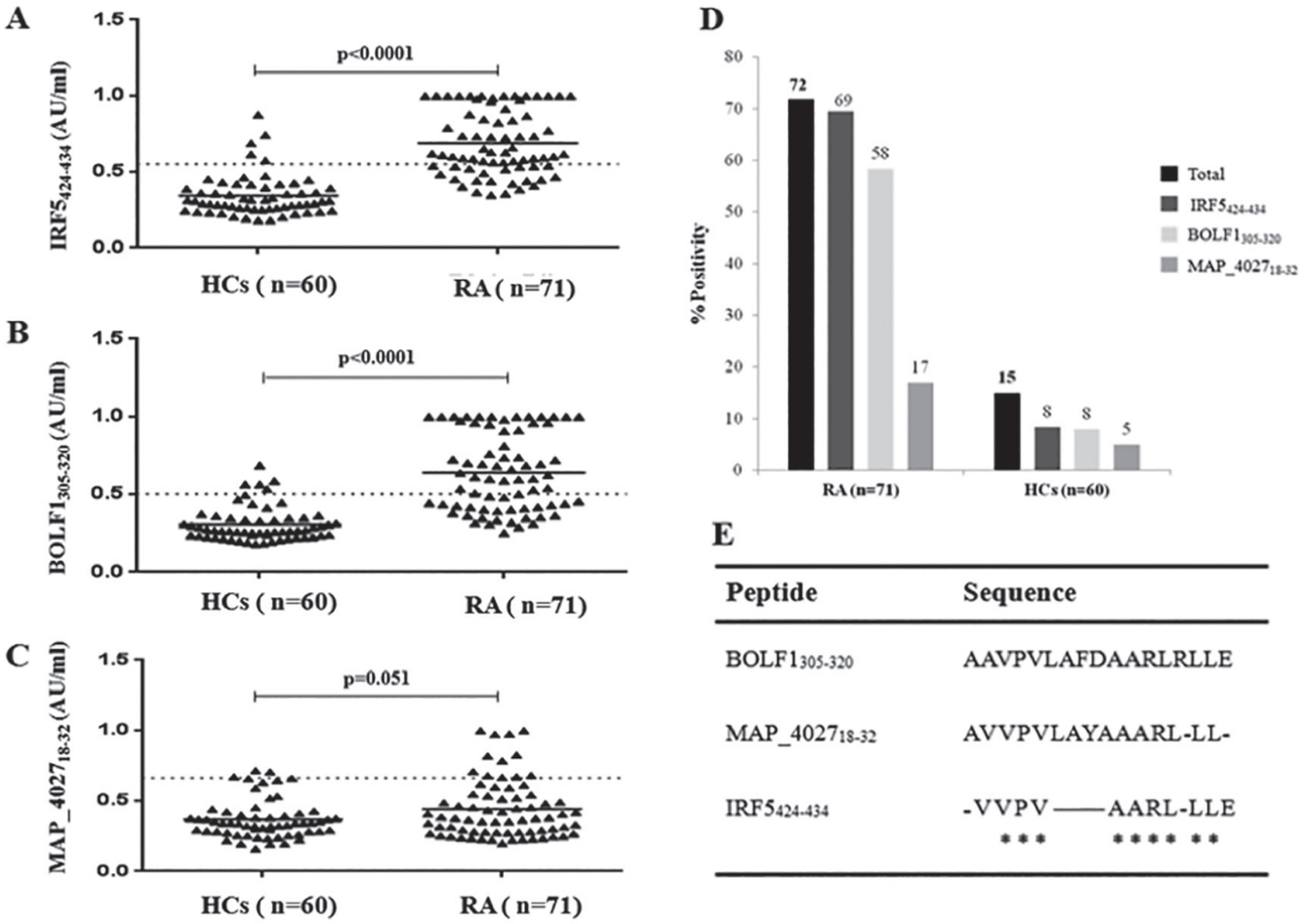


Fig. 1. A-C) ELISA-based analysis of Abs reactivity against the homologous epitopes derived from human, EBV and MAP in RA patients and HCs. The sera were tested against plate-coated $IRF5_{424-434}$ (A), $BOLF1_{305-320}$ (B) and MAP_4027_{18-32} (C) peptides. Bars represent the median ± interquartile range: Cutoff values for Abs positivity are indicated by dashed lines. P-values, respective to each epitope showed a statistical difference between RA patients and HCs and are reported above the distributions. **D**) Prevalence of Abs directed against $IRF5_{424-434,}$ $BOLF1_{305-320}$ and MAP_4027_{18-32} epitopes in RA patients and HCs. Total percentage of Abs positivity to at least one peptide is represented by the first bar in each group. Other bars correspond to a single-peptide positivity relative to each epitope. **E**) Peptides alignment obtained using Clustal W2. The results show the region of identity with a star (*), strong similar amino acid with a dot (.) and a missing region in dashes (-).

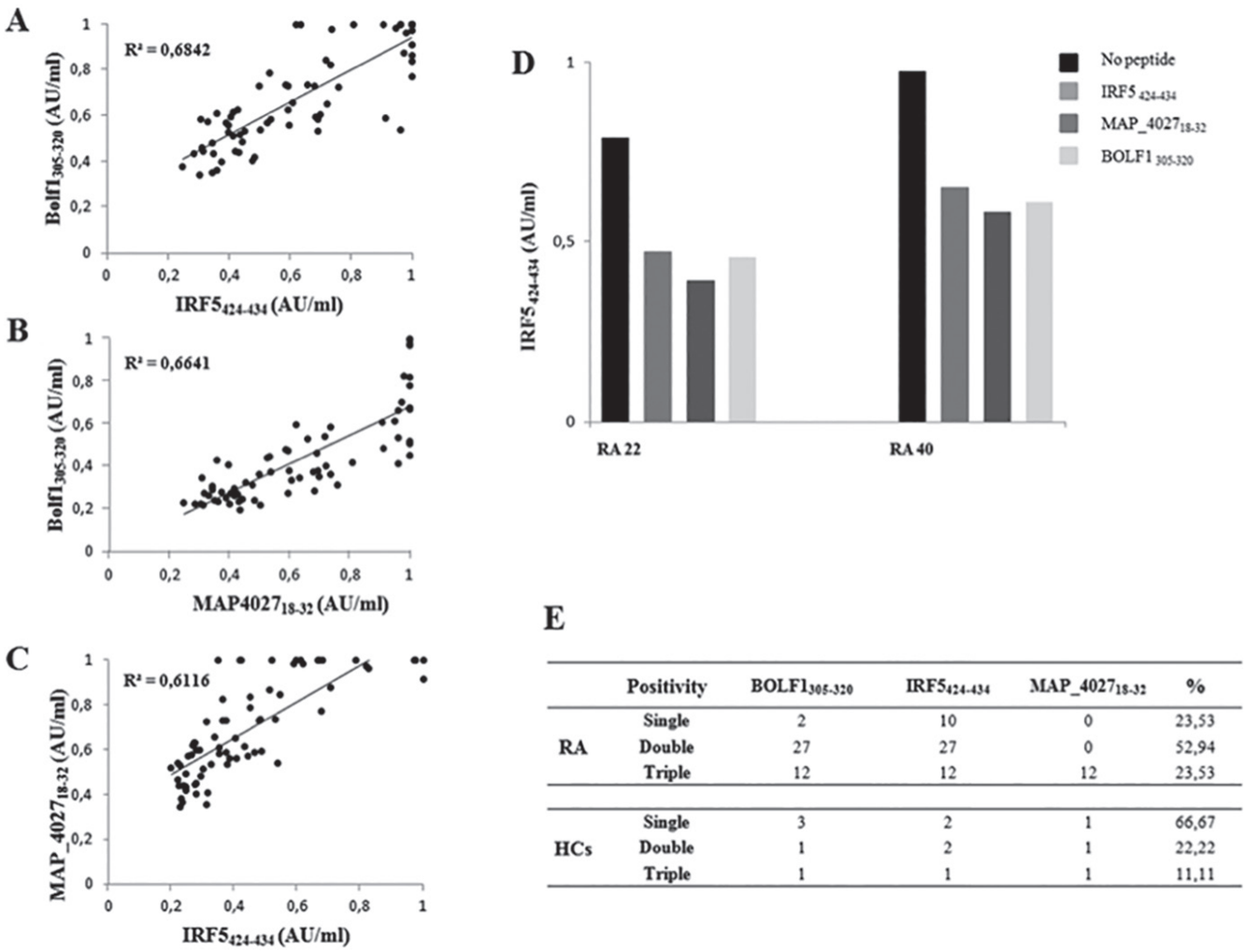
96-well plates were coated with the selected peptides dissolved in carbonate/bicarbonate buffer at 10mM concentration and incubated overnight at 4°C. The next day the wells were saturated with 200 µl of blocking solution (1xPBS + 0.05% Tween20 containing 5% skim milk) for 1 h at room temperature. Afterwards the plates were washed twice times with 200µl PBS-Tween, diluted sera (1:100) were added in duplicate and incubated for 2 h. The plates were subjected to 5 washes with 200µl PBS-Tween with subsequent addition of the alkaline phosphatase-conjugated goat anti-human IgG polyclonal Abs at a dilution of 1:1000, and left to incubate for 1 h at room temperature. Finally, upon another five washes with 200µl PBS-Tween, para-nitrophenyl phosphate substrate solution was added to each well and the plates were incubated at room temperature in the dark for 10 min. The optical density (OD) was read at a wavelength of 405 nm using SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA). Data was normalised to a positive control serum included in all experiments, the reactivity of which was fixed to 1.0 arbitrary units (AU)/ml.

Competitive inhibition assay

Sera of two RA patients diluted 1:100 in PBS were pre-incubated for 2h at room temperature with MAP_4027, BOLF1 or IRF5 peptide at saturating concentrations of 5µg/ml, 10µg/ml or 50µg/ml for each epitope. The same samples without peptide were treated accordingly as references of seroreactivity. An ELISA was then performed on a plate coated with $IRF5_{424-434}$ following the protocol described in the previous section.

Statistical analysis

The results were expressed as a mean of three separate experiments and the statistical significance of the data was determined using Graphpad Prism 6.0 software (San Diego, CA, USA). Continuous data are expressed as median (IQR) and comparison was made using Mann-Whitney *U*-test. Comparison of positivity to the assessed peptides between RA patients and HCs was performed through Fisher's exact test with both Tukey's and Yate's corrections.



	Positivity	BOLF1$_{305-320}$	IRF5$_{424-434}$	MAP_4027$_{18-32}$	%
RA	Single	2	10	0	23,53
	Double	27	27	0	52,94
	Triple	12	12	12	23,53
HCs	Single	3	2	1	66,67
	Double	1	2	1	22,22
	Triple	1	1	1	11,11

Fig. 2. A-C) Scatter plot showing correlations between Abs titers recognising (A) BOLF1$_{305-320}$ and IRF5$_{424-434}$, (B) BOLF1$_{305-320}$ and MAP_4027$_{18-32}$ (C) MAP_4027$_{18-32}$ and IRF5$_{424-434}$ in 71 RA patients. Person's correlation was calculated through Graphpad Prism 6.0 software. **D**) Competitive inhibition assay in IRF5-coated ELISA plate. Sera of two RA patients were selected randomly among subjects highly positive for Abs against the three homologous epitopes. Bars indicate AU/ml levels relative to the sera pre-incubated with single peptides or with no peptide. **E**) Coincidence of seroreactivity to the homologous peptides among Abs-positive RA and HCs subjects.

The cut-off for positivity was established in the range of 0.53-0.66 (AU)/ml respectively to each peptide based on the receiver operating characteristic (ROC) curve at ≥94% specificity and the corresponding sensitivity. Correlation analysis between Abs and RA features, RA activity (DAS-28), systemic inflammation (ESR, CRP) and type of immunosuppressive treatment was explored by bivariate correlation analysis, univariate and multivariate regression analysis. Probability values lower than 0.05 were considered statistically significant.

Results

We enrolled in the study 71 consecutive RA patients (11 males, 60 females; median age 56.18) and 60 HCs (23 males, 37 females; median age 46.1; demographic and clinical features of RA patients are summarised in the Supplementary file, Table I).

IRF5$_{424-434}$ was found to have the highest Abs seroreactivity among the selected antigens being positive in 49 out of 71 RA patients (69%) and only in 5 out of 60 HCs (8%; AUC=0.9; p<0.0001; Fig. 1A and 1D).

Abs against BOLF1$_{305-320}$ were observed in 41 out of 71 RA patients (58%) and in 5 out of 60 HCs (8%; AUC=0.9; p<0.0001; Fig. 1B and 1D). Humoral responses against MAP_4027$_{18-32}$ was detectable in 12 out of 71 RA patients (17%), whereas HCs showed Abs prevalence accounting for only 5% giving a p-value close to the threshold of statistical significance (AUC= 0.6; p=0.051; Fig. 1C and Fig. 1D).

Globally, RA subjects displayed much more increased positivity to at least one of the assessed epitopes compared to HCs with a high degree of statistical significance (71% *vs*. 15%, respectively; p<0.0001; Fig. 1D).

A good correlation was found for all the homologous pairs with the highest coefficient observed for BOLF1$_{305-320}$ and IRF5$_{424-434}$ peptides (R^2=0.68; Fig. 2A) and slightly lower for BOLF1$_{305-320}$ and its MAP_4027$_{18-32}$ homolog (R^2=0.66; Fig. 2B). MAP_4027$_{18-32}$ and the human-derived IRF5$_{424-434}$ peptides correlated to a somewhat lower extent that however still permitted us to hy-

pothesise a cross-reactivity between the two epitopes (R^2=0.61; Fig. 2C).

To further demonstrate the presence of a cross-reactivity between MAP/EBV and self epitopes, we performed ELISA-based competitive inhibition assays selecting $IRF5_{424–434}$ as antigen representative of the highest Abs responses induced in RA patients. Of the three inhibiting concentrations tested, incubation with 10μg/ml peptide solution yielded the most accurate results with lowest variability among replicates. The low OD values obtained for sera of two RA patients (RA22 and RA40) pre-incubated with BOLF1 and MAP_4027 peptides indicate that binding of Abs specific for the plate-coated $IRF5_{424–434}$ was efficiently inhibited by its homologs (Fig. 2D). Most likely anti-BOLF1 and anti-MAP_4027 Abs are cross-reactive and target the same IRF5 conformational epitope.

Moreover, the double IRF5-BOLF1 positivity pattern clearly visible among RA patients was changed in HCs that displayed a case of reactivity to IRF5-MAP_4027 and a single Abs response against MAP (Fig. 2E).

When Fisher's exact test was repeated excluding the two HCs with single and double anti-MAP positivity, statistical significance relative to MAP_4027 greatly improved (p<0.0061). This picture may indicate the presence of Abs specific for the extracellular MAP phenotype that entered in contact with the HC not MAP susceptible host conferring a natural protection against mycobacterial infection. On the other hand, a higher efficiency of the MAP-derived antigen to inhibit binding of anti-IRF5 Abs coupled with a simultaneous reactivity to the other two homologous epitopes in cases with the triple-peptide positivity may be indicative of the synergistic role attributed to EBV and MAP in RA.

Analysis of correlation between RA features and positivity of Abs demonstrated a close link between Abs and the burden of systemic inflammation (Supplementary Table II). In particular multivariate regression analysis show that levels of CRP are the main predictors of high levels of Abs against $BOLF1_{305-320}$ and $IRF5_{424-434}$ (Supplementary Table III).

Discussion

RA is a multifactorial autoimmune disorder with an unknown aetiology. Given that the genetic component *per se* is not sufficient to be considered as the only explanation of RA onset, a possible role played by the environment needs to be taken into account. The currently accepted theory of RA pathogenesis assigns the main role in autoimmune reactions supposedly induced by different viral or bacterial infections leading to joint inflammation and chronic anatomical damage (25). Although activation of the T-cell response has a crucial role in RA pathogenesis, the B-cell response is equally responsible for the development of the disease due to the enhanced synthesis of immunoglobulins, usually IgGs (26).

Within pathogenetic hypothesis, the theory of the "molecular mimicry" is based on amino acid sequence or structural motif homology between microbial epitopes and self proteins: due to this homology the immune response against microbial epitopes could also induce undesired humoral and/or cellular immunity against host proteins.

Data from our study demonstrated a remarkable reactivity of RA sera against IRF5 supporting the potential role of IRF5 as an important target in RA specific autoimmunity. Moreover, we also demonstrated a high grade of cross-reactivity between IRF5, EBV and MAP epitopes.

A possible explanation for these results could be that in RA patients past EBV and/or MAP infection may induce specific humoral immunity reacting against IRF5 host protein. This secondary response may contribute, through the epitope-spreading phenomenon, to synovial tissue destruction with the production of Abs against previously "sequestered" antigens and amplification of autoimmune cascade.

Study of genome-wide analysis demonstrated association between IRF5 polymorphisms and anti-CCP negative RA (27, 28) suggesting that RA associated IRF5 polymorphism may lead to higher level of IRF5 expression by promoting stability of IRF5 messenger RNA. In addition, it has been recently demonstrated that IRF5 may act as a crucial mediator of joint inflammation, through Toll-like receptors signals activation in synovial macrophages cells promoting a vicious loop of proinflammatory cytokine and chemokine production and release in articular microenvironment (29).

In this scenario the cross-reactivity mounted against $IRF5_{424–434}$, $BOLF1_{305–320}$ and $MAP\_4027_{18–32}$ may alter the function of the IRF5 protein, modulating or inhibiting its activity. Penetration of Abs into cell cytosol by endocytosis, a mechanism that has been demonstrated in some cells (30, 31) may "regulate" IRF5 expression in a pro-inflammatory manner.

The presence of a strong correlation between CRP levels and Abs titer in our series of patients further supports the hypothesis that autoantibodies against IRF5 may increase TLR-regulated production of pro-inflammatory cytokine boosting systemic and local inflammation in RA.

Shortcomings of our study were mainly related to small sample size and a cross-sectional design including RA patients under immunosuppressive and anti-inflammatory treatment at the moment of study enrolment.

In conclusion, our data suggest for the first time IRF5 as a target of immune response in RA. Moreover, we propose molecular mimicry involving EBV and MAP as a potential mechanism of anti-IRF5 autoimmune response. While association between anti-IRF5 autoantibodies and RA is strongly significant, the functional capacity of these Abs to modulate IRF-5 activity remains to be assessed. Further analysis of T cell responses will provide additional indices on cross-reactivity between BOLF1, MAP and IRF5 epitopes in a similar fashion to previously described EBV gp110 and *E. coli* dnaJ (32, 33).

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