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

M. Bo¹, G.L. Erre², M. Niegowska¹, M. Piras², L. Taras², M.G. Longu², G. Passiu², L.A. Sechi¹

¹Dipartimento di Scienze Biomediche, Sezione di Microbiologia e Virologia, Università di Sassari; ²Dipartimento di Medicina Clinica e Sperimentale, Azienda Ospedaliero-Universitaria di Sassari, Italy.

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
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}), MAP_Antigen MAP_4027 [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} [AAVPVLAFDAARLRLLE], MAP_Antigen MAP_4027 [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.
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 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.
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).

IRF5424-434 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 BOLF1305-320 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. 1A 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 BOLF1305-320 and IRF5424-434 peptides (\( R^2=0.68 \); Fig. 2A) and slightly lower for BOLF1305-320 and its MAP_4027 18-32 homolog (\( R^2=0.66 \); Fig. 2B).

**Fig. 2.** A-C) Scatter plot showing correlations between Abs titers recognising (A) BOLF1305-320 and IRF5424-434, (B) BOLF1305-320 and MAP_4027 18-32, (C) MAP_4027 18-32 and IRF5424-434 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.
Interferon regulatory factor 5 is a biomarker in RA / M. Bo et al.

...the two epitopes (R^2=0.61; Fig. 2C).

...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 double anti-MAP positivity, statistically significant, as assessed. Further analysis of T cell responses will provide additional indices assessing the functional capacity of these Abs to support the potential role of IRF5 as an important target in RA patients. Of the 28 responses induced in RA patients. Of the responses, 12 displayed a case of reactivity to the other two homologous epitopes, and 16 displayed a case of reactivity to the other two homologous epitopes, and 16 displayed a case of reactivity to the other two homologous epitopes. Of the 25 responses, 12 displayed a case of reactivity to the other two homologous epitopes, and 13 displayed a case of reactivity to the other two homologous epitopes. Of the 24 responses, 12 displayed a case of reactivity to the other two homologous epitopes, and 12 displayed a case of reactivity to the other two homologous epitopes. Of the 23 responses, 12 displayed a case of reactivity to the other two homologous epitopes, and 11 displayed a case of reactivity to the other two homologous epitopes. Of the 22 responses, 12 displayed a case of reactivity to the other two homologous epitopes, and 10 displayed a case of reactivity to the other two homologous epitopes. Of the 21 responses, 12 displayed a case of reactivity to the other two homologous epitopes, and 9 displayed a case of reactivity to the other two homologous epitopes. Of the 20 responses, 12 displayed a case of reactivity to the other two homologous epitopes, and 8 displayed a case of reactivity to the other two homologous epitopes. Of the 19 responses, 12 displayed a case of reactivity to the other two homologous epitopes, and 7 displayed a case of reactivity to the other two homologous epitopes. Of the 18 responses, 12 displayed a case of reactivity to the other two homologous epitopes, and 6 displayed a case of reactivity to the other two homologous epitopes. Of the 17 responses, 12 displayed a case of reactivity to the other two homologous epitopes, and 5 displayed a case of reactivity to the other two homologous epitopes. Of the 16 responses, 12 displayed a case of reactivity to the other two homologous epitopes, and 4 displayed a case of reactivity to the other two homologous epitopes. Of the 15 responses, 12 displayed a case of reactivity to the other two homologous epitopes, and 3 displayed a case of reactivity to the other two homologous epitopes. Of the 14 responses, 12 displayed a case of reactivity to the other two homologous epitopes, and 2 displayed a case of reactivity to the other two homologous epitopes. Of the 13 responses, 12 displayed a case of reactivity to the other two homologous epitopes, and 1 displayed a case of reactivity to the other two homologous epitopes. Of the 12 responses, 12 displayed a case of reactivity to the other two homologous epitopes.

In conclusion, our data suggest for the first time IRF5 as a target of immune reaction 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).

References

3. WANG D, LI Y, LIU Y, SHI G: The role of autoreactive T cell in the pathogenesis of rheumatoid arthritis and implications for T cell


