Expression of ERAP2 and LST1 is increased before start of therapy in rheumatoid arthritis patients with good clinical response to glucocorticoids

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Abstract

Objective

Glucocorticoids (GC) remain a cornerstone of rheumatoid arthritis (RA) therapy, although a third of patients do not respond adequately. In order to find potential predictors for clinical response, the gene expression profile of CD4+T-cells as important players in the pathogenesis of RA was analysed before pulse therapy with 1000 mg methylprednisolone.

Methods

Patients were treated with 3x1000 mg methylprednisolone in 5 days; hereafter response was determined by the European League Against Rheumatism (EULAR) response criteria. Before start of treatment, $CD4^+$T-cells (and CD14+monocytes) were separated by MACS sorting. Labelled cRNA from CD4+T-cells from 5 responders and 5 non-responders was hybridised to Agilent 4x44K microarray chips and differentially expressed genes were identified via mixed-model analysis of variance based on permutation-based false discovery rates. Selected genes were validated by quantitative real-time PCR (qPCR).

Results

Four genes were significantly increased in $CD4^+$T-cells of GC-responders; expression of ERAP2 (endoplasmic reticulum aminopeptidase 2), LST1 (leucocyte-specific transcript 1) and FAM26F (Family With Sequence Similarity 26, Member F) was confirmed by quantitative PCR (qPCR); their expression was inversely correlated with DAS28 at day 5 (LST1 and FAM26F $p<0.05$; ERAP2: $p=0.07$). Elevated expression of ERAP2 was also detected by qPCR in CD14+monocytes and after 24 hours in both cell types (all $p<0.02$).

Conclusion

The increased expression of ERAP2, LST1 and FAM26F in GC-responders before therapy warrants further investigation into their role as potential predictors for the response to GC, and in the inflammatory process of RA.

Key words

glucocorticosteroids, rheumatoid arthritis, CD4 positive T-lymphocyte, gene expression profiling

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease, affecting 0.5 –1% of the general population. Several observations, such as the occurrence of class switched auto-antibodies, the characterisation of auto-reactive T-cells and the genetic association with certain HLA-proteins indicate a prominent role for T-cells (1). Additionally, macrophages are central mediators of synovitis, and are abundant in RA-synovial tissue. They secrete inflammatory cytokines and interact with T-cells, thereby propagating the inflammatory process (1).

Due to the inhibition of radiographic damage (2), Glucocorticoid (GC)-therapy has gained recognition as a disease modifying drug (DMARD). The rapid anti-inflammatory effect is used in pulse therapy (≥250mg prednisone equivalent), which is given to patients with high disease burden as bridging until the effect of a conventional DMARD becomes apparent. Pulse treatment results in rapid amelioration clinically and serologically, lasting for a mean of 10 weeks (3).

About one third of RA patients do not respond to GC adequately, a phenomenon which is also seen in other inflammatory diseases. Although several mechanisms underlying GC-resistance have been described, no predictors of GC-sensitivity in RA-patients have been established (4). During the last decade gene expression profiling has been used to analyse and predict the response to (mainly biological) therapy (5). Furthermore, a recent study analysed the changes in gene expression in peripheral blood mononuclear cells (PBMC) after administration of Leflunomide and low dose glucocorticoid and found several genes implicated in RA pathogenesis, which were downregulated in GC users (*e.g.* MIF, IL4R, NFKB1 and STAT6) (6). In contrast, the aim of our study was to find possible biomarkers for the *response* to GC in RA-patients using gene expression profiling of $CD4^+$T-cells, as main players of RA-pathogenesis.

Materials and methods

Patients

Patients fulfilling the revised American College of Rheumatology criteria for RA and scheduled by their treating rheumatologist for GC-pulse therapy, on stable medication and without GC-treatment during the previous 4 weeks, were recruited (7).

The study was approved by the medical ethics committee of the Utrecht University Medical Center and patients signed informed consent for the study protocol.

Treatment and clinical measurements

Patients were given three doses of 1000 mg methylprednisolone on alternate days. Disease activity was assessed by DAS28 before start and at the end of treatment (12–24 hours after the third dose = T5). Treatment response was defined according to the European league against rheumatism response criteria. Good responders were classified by a DAS28<3.2 and a ΔDAS28 of ≥1.2. Considering the intensity of treatment, patients not fulfilling the criteria for good response were considered non-responders. The timepoint T5 was chosen to measure the effect of a very high dosage of GC only given in severe clinical situations, in which the effect is needed (and seen) rapidly after initiation of therapy.

RNA of CD4+T-cells and $CD14^+$ monocytes

PBMC from patients were obtained from Li-heparinised blood by Ficoll-Paque™ Plus density gradient centrifugation (GE-Healthcare, Bio-Sciences AB, Uppsala, Sweden). $CD14^+$monocytes were isolated by positive and $CD4^+$T-cells by negative selection by AutoMACS Pro Separator (Miltenyi Biotec, Bergisch Gladbach, Germany; isolation kits: Miltenyi Biotec). Purity of the viable population assessed by flow cytometry (BD FACSCalibur, Franklin Lakes, New Jersey, USA) was generally >90%. RNA was isolated using the RNeasyMini Kit from QIAGEN (Venlo, The Netherlands). Total RNA concentration was measured by Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA purity and integrity were verified by lab-on-chip technology (Agilent 2100 Bioanalyzer, Santa Clara, CA, USA). All procedures were performed according to the manufacturers' instructions.

Microarray RNA profiling

From the first 5 GC-responders and 5 non-responders meeting the quality requirements RNA of $CD4^+$T-cells was evaluated on human whole genome gene expression microarrays v1 (Agilent Technologies, Belgium) with 41000 homo sapiens 60-mer probes in a 4x44K layout. Universal Human Reference RNA (Stratagene) was used as a common reference.

RNA amplification and labelling were performed on an automated system (Caliper Life Sciences NV/SA, Belgium) with 2 µg total RNA. Hybridisations were done on a HS4800PRO system with QuadChambers (Tecan Benelux B.V.B.A.) using 1000 ng labelled cRNA per channel (8). All patient samples were labelled with cy5, reference cRNA was labelled with cy3. Hybridised slides were scanned on an Agilent scanner (G2565BA) at 100% laser power, 30% PMT.

Quantitative real-time PCR (qPCR)

Quantitative PCR was performed using a BioRad MyiQ real time PCR Detection System (BioRad, Hercules, CA, USA). RNA was reverse transcribed into cDNA using a BioRad iScript cDNA synthesis kit (BioRad). For the reaction mixture, iQ SYBR Green Supermix kit (BioRad) was used, with 5 µM of each primer and 20 ng of the cDNA sample. For the target genes endoplasmic reticulum aminopeptidase 2 (ERAP2), leucocyte-specific transcript 1 (LST1) and Family With Sequence Similarity 26, Member F (FAM26F) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) the PCR primers are shown in Table I. Expression levels of target genes were calculated as ΔCT relative to GAPDH ($\Delta CT = CT^{target\ gene} - CT^{GAPDH}$) with a higher ΔCT corresponding to a lower quantity of mRNA.

Statistical analysis

• *Microarray analysis*

After automated data extraction using Imagene 8.0 (BioDiscovery), Loess normalisation was performed on mean spot-intensities. Data were analysed using MAANOVA (9). *P*-values were determined by a permutation F_2-test, in which residuals were shuffled 10000 times across probes. Genes with $p<0.05$ after FDR-based correction (False discovery rate; Benjamini-Hochberg) were considered significantly changed. For other statistical analyses SPSS v. 20.0 (SPSS, Chicago, IL, USA) and Graph Pad Prism (v. 5) were used. *P*-values were calculated using unpaired Students *t*-tests and χ^2 tests, as appropriate; reported correlations are Pearson's. *P*-values <0.05 are considered significant.

Table I. Primer sequences.

Sequence (5' - 3')	
Forward	Reverse
ERAP2 F' TGGATGGGACCAACTCATTACA	ERAP2 R' TGCACCAACTAGCTGAAACAC
FAM26F F' CACCCGATGCCTATCTCCAG	FAM26F R' TTTGCTGCCACTCTTTCATGC
LST1 F' AGGAACTTGAGGCAAGTCACC	LST1 R' CAGCCTCTGCAGAGATGCATAGT
GAPDH F' AGAAGGCTGGGGCTCATTT	GAPDH R' GAGGCATTGCTGATGATCTTG

Sequences of forward and reverse primers for genes of interest and the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are given.

Table II. Patient characteristics of glucocorticoid (GC)-responders and non-responders

	GC-responders	non-responders
Female (%)	3/5 (60)	3/5 (60)
Age (mean±SEM)	55.6 ± 8.1	49 ± 9.6
Concomitant MTX (%)	3/5 (60)	2/5 (40)
Disease duration (mean ± SEM)	12.8 ± 6	8.6 ± 3.9
Erosive disease (%)	2/5 (40)	4/5 (80)
RF positive (%)	4/5 (80)	5/5 (100)
ACPA positive (%)	3/5 (60)	3/5 (60)
Inflammation (ESR; mean ± SEM)	34.6 ± 20.6	68.2 ± 27
Current smoking (%)	**0/5 (0)**	**3/5 (60)**
DAS28 day 0 (mean ± SEM)	6.6 ± 1.1	6.3 ± 1.5
DAS28 day 5 (mean ± SEM)	**2.6 ± 0.3**	**4.0 ± 0.4**
ΔDAS (mean ± SEM)	4.1 ± 0.8	2.3 ± 1.3

Except for smoking, and DAS28 at day 5 no statistically significant differences were seen in the two RA patient groups. Patients' characteristics for gender, concomitant methotrexate medication, erosive disease, antibody positivity and smoking are given as percentage. Age, disease duration, ESR and disease activity at baseline and after therapy are given as mean with standard error. For smoking, antibody status, erosive disease and medication χ^2 test was used, Student's t-test was employed for the remaining items. MTX: Methotrexate; RF: rheumatoid factor; ACPA: Anti-citrullinated-protein-antibodies; DAS28: disease activity score. Significant differences were only seen in the percentage of active smokers ($p<0.05$) and the DAS28 at day 5 ($p<0.001$), both indicated in bold letters. The *p*-value for ΔDAS was $p=0.054$.

Results

Patients

Patient characteristics are given in Table II. The groups did not differ in age, gender, concomitant methotrexate-, disease duration, or disease activity at the start of therapy. Likewise seropositivity for anti-citrullinated-protein-antibodies or rheumatoid factor or inflammation (ESR) were similar in both groups. Interestingly, there were significantly more active smokers in the non-responders ($p<0.05$). In response to treatment, the DAS28 at day 5 (DAS28 T5) and ΔDAS differed between GC-responders and non-responders ($p<0.001$ and $p=0.054$ resp.).

Microarray RNA profiling

Four known genes were differentially expressed in $CD4^+$T-cells; all were increased in GC-responders. Differences of gene expression were consistent between the different probes for each transcript of respective genes. A heat map displaying the expression level of the respective probes in individual patients is shown in Fig. 1A. All microarray gene expression data have been deposited in the public data repository of GEO (http://www.ncbi.nlm.nih.gov/geo/) with accession nr GSE48823.

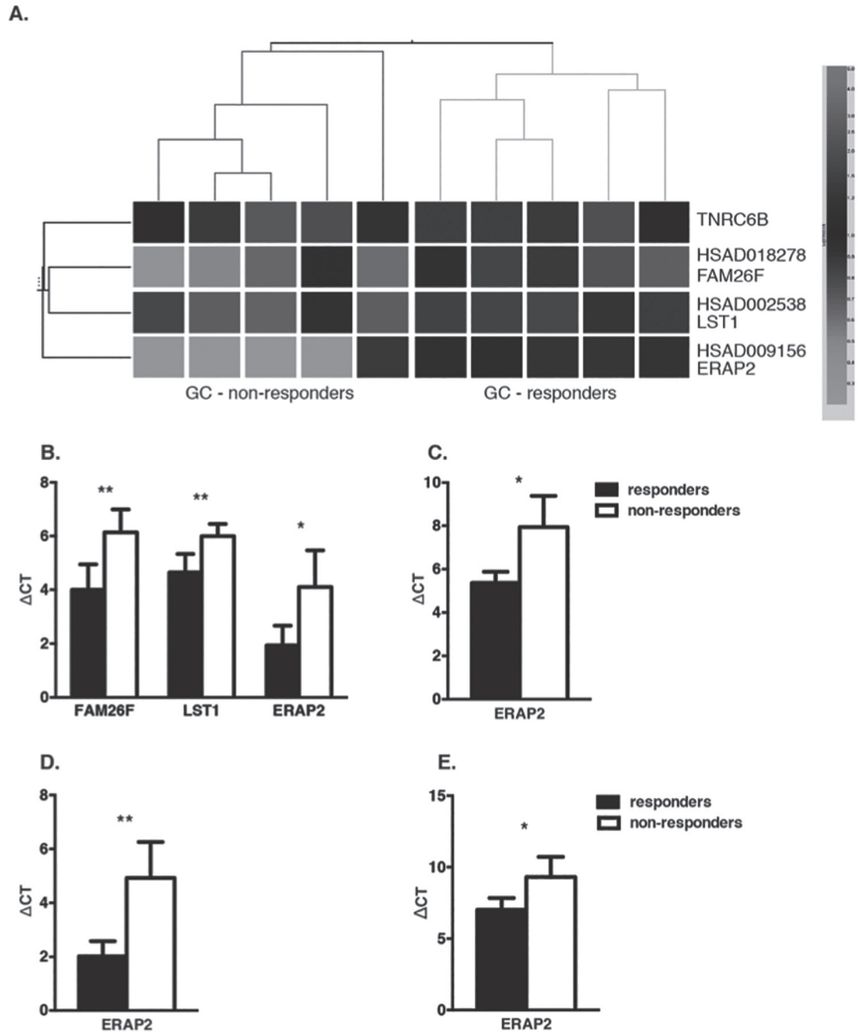

Fig. 1. Differentially expressed genes in glucocorticoid (GC)-responders *vs*. non-responders:
1**A**: Heat map of CD4⁺T-cell gene-expression in individual patients. Transcript levels are expressed as median centered ratios across all samples. Scale bar (log2 ratio): increased (red), decreased (green) or identical (black).
1**B**: Quantitative PCR results of selected genes in CD4⁺T-cells of GC-responders and non-responders: Higher ΔCT ($CT^{\text{gene of interest}} - CT^{GAPDH}$) correspond to lower gene expression. $p<0.05$ (unpaired Students t-test) was considered statistically significant. *: $p<0.05$; **: $p<0.01$.
1**C**, **D**, **E**: ERAP2 gene-expression in CD14⁺monocytes before therapy (1C); in CD4⁺T-cells and CD14⁺monocytes 24 hours after therapy-start (1D, 1E): ERAP2 expression was significantly higher (ΔCT lower) in GC-responders in all instances.

qPCR

Three genes were tested by qPCR (Fig. 1B). The difference between responders and non-responders was 4.4-fold ($p<0.006$) for FAM26F; 2.5-fold ($p<0.009$) for LST1 and 4.5-fold ($p<0.02$) for ERAP2, remaining increased 24 hours after therapy start. (fold change: FC=7.5, $p<0.006$; Fig. 1D) Again the expression of these genes was increased in GC-responders.

The correlation of gene expression before therapy with DAS28T5 was analysed considering the greater clinical difference and relevance between responders and non-responders in absolute DAS28T5 than ΔDAS28. The correlation proved significant for FAM26F ($p<0.02$) and LST1 ($p<0.05$), showing the same trend for ERAP2 ($p=0.072$). (Fig. 2A-C).

ERAP2 gene expression was also found upregulated in CD14⁺ cells and could be analysed by qPCR in CD14⁺monocytes revealing significantly increased expression in responders ($p<0.02$; FC=8.6; Fig. 1C) before and 24 hours after therapy ($p<0.02$, 4.9 fold change, Fig. 1E). In CD14⁺monocytes a significant correlation of gene expression of ERAP2 before start of therapy with DAS28T5 was observed ($p<0.05$; Fig. 2D).

There was no correlation of gene expression with separate parameters of DAS28. A possible confounding role for smoking on the expression of ERAP2, FAM26 and LST was addressed by analysing the correlation between these genes and pack years as surrogate for smoking history. This was not found.

Discussion

In this pilot-study, gene expression profiling of CD4⁺T-cells was used to identify potential biomarkers for the clinical response to GC.

Three genes were increased in CD4⁺T-cells from GC-responders more than 2-fold as confirmed by qPCR. The most striking gene was ERAP2, which was increased in CD4⁺T-cells of GC-responders, and was likewise found to be increased in monocytes, which are also vital players in RA-pathogenesis. (Fig. 1A-E). ERAP2 is a gene duplication of ERAP1and both genes have been discovered as susceptibility loci for autoimmune diseases (10).

Their function is the enzymatic removal of amino acids from N-termini of peptides, by which they interfere in several biological processes among which antigen presentation (11). ERAP2 preferably targets Arginine or Lysine, possibly resulting in altered presentation of arginine residues (becoming citrulline after deimination) by the MHC-I by RA-monocytes or T-cells. Only recently an effect on antigen-presentation of ERAP1 has been suggested in HLA-B27 positive patients with Spondylarthritis (12). A role for ERAP1 in the cleavage of cytokine receptors TNFRI, IL6Rα and IL1RII has been demonstrated, which act as decoy for their respective inflammatory cytokines (10, 13). Although ERAP2's functional similarity to ERAP1 might imply cytokine receptor shedding the serum level of TNFRI, IL6Rα and IL1RII showed no correlation with ERAP2 gene expression at baseline (data not shown). Nevertheless, this does not preclude an influence of ERAP2 in receptor shedding on a cellular level.

LST1 constitutes a susceptibility locus

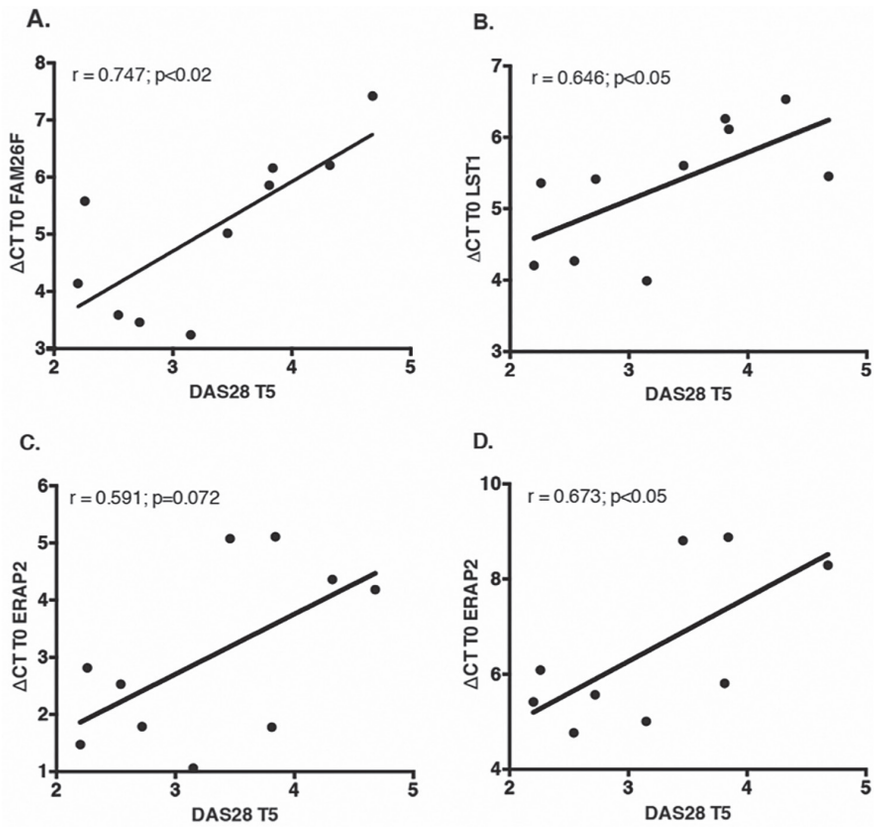

Fig. 2. Differentially expressed genes in Glucocorticoid (GC)-responders *vs*. non-responders: 2**A**-**C**: FAM26F (2A), LST1 (2B) and ERAP2 (2C) gene expression in $CD4^+$T-cells was correlated (Pearson's correlation) with the DAS28 measured after therapy (DAS T5) as clinically relevant surrogate for response is depicted. A higher ΔCT, signifying a lower gene expression, at baseline is correlated with a higher DAS28 at the end of therapy for LST1 and FAM26F. $p<0.05$ was considered statistically significant. 2**D**: The correlation of ERAP2 expression was also tested in CD14+monocytes and was statistically significant.

in RA (14). It regulates T-cell proliferation and is expressed in mononuclear cells with increased expression in blood and synovial tissue from RA-patients (15). In our study we found a 2.5-fold increased expression of LST1 in GC-responders, implicating a hitherto uncharacterised mechanistic connection.

The more than 4-fold increased gene expression of FAM26F in GC-responders warrants further research into this protein, the function of which has not been characterised to date.

Clearly this study has shortcomings. In order to define these genes as predictors of GC-response, they will have to be tested in a separate, bigger cohort and the mechanism of how these genes possibly influence the GC-response or RA-pathogenesis needs to be elucidated. Also, we cannot exclude that the non-genomic effects of high dose Glucocorticoids may have influenced our study, although appropriate regulation of known GC-response genes (*e.g.* IL1R2 and CXCL10) after 24 hour in CD4 T-cells of these patients suggest a strong genomic effect (data not shown). In this respect, also the relation between GC-response with ERAP2 expression might (at least partly) be based on non-genomic mechanisms, considering a reported localisation of ERAP2 in the plasma membrane, where it could interact with a membrane glucocorticoid receptor.

Altogether we identified 3 genes with a possible role in RA-pathogenesis and GC-response, of which ERAP2 and LST1 have been implicated previously in autoimmune disease. Our findings warrant further investigations into these genes.

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References

1. McINNES IB, SCHETT G: The pathogenesis of rheumatoid arthritis. *N Engl J Med* 2011; 365: 2205-19.
2. HOES JN, JACOBS JW, BUTTGEREIT F, BIJLSMA JW: Current view of glucocorticoid co-therapy with DMARDs in rheumatoid arthritis. *Nat Rev Rheumatol* 2010; 6: 693-702.
3. FORSTER PJ, GRINDULIS KA, NEUMANN V, HUBBALLL S, McCONKEY B: High-dose intravenous methylprednisolone in rheumatoid arthritis. *Ann Rheum Dis* 1982; 41: 444-6.
4. BARNES PJ, ADCOCK IM: Glucocorticoid resistance in inflammatory diseases. *Lancet* 2009; 373: 1905-17.
5. SMITH SL, PLANT D, EYRE S, BARTON A: The potential use of expression profiling: implications for predicting treatment response in rheumatoid arthritis. *Ann Rheum Dis* 2013; 72: 1118-24.
6. CUTOLO M. VILLAGGIO B., BRIZZOLARA R *et al*.: Identification and quantification of selected inflammatory genes modulated by leflunomide and prednisone treatment in early rheumatoid arthritis patients. *Clin Exp Rheumatol* 2011; 29: 72-9.
7. ARNETT FC, EDWORTHY SM, BLOCH DA *et al*.: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
8. ROEPMAN P, WESSELS LF, KETTEALRIJ N, SLOOTWEGG PJ, HOLSTEGE FC: An expression profile for diagnosis of lymph node metastases from primary head and neck squamous cell carcinomas. *Nat Genet* 2005; 37: 182-6
9. WU H, KERR MK *et al*.: 2003. MAANOVA: A software package for the analysis of spotted cDNA microarray experiments, *In* PARMIGIANI G, GARRETT ES, IRIZARRY RA, ZEGER SL (Eds.), The Analysis of Gene Expression Data: Methods and Software, Springer, New York.
10. FIERABRACCI A, MILILLO A, LOCATELLI F, FRUCI D: The putative role of endoplasmic reticulum aminopeptidases in autoimmunity: insights from genomic-wide association studies. *Autoimmun Rev* 2012; 12: 281-8.
11. SAVEANU L, CARROLL O, LINDO V *et al*.: Concerted peptide trimming by human ERAP1 and ERAP2 aminopeptidase complexes in the endoplasmic reticulum. *Nat Immunol* 2005; 6: 689-97.
12. MARTIN-ESTEBAN A, GOMEZ-MOLINA P, SANZ-BRAVO A, LOPEZ DE CASTRO JA: Combined effects of ankylosing spondylitis-associated ERAP1 polymorphisms outside the catalytic and peptide-binding sites on the processing of natural HLA-B27 ligands. *J Biol Chem* 2014; 289: 3978-90.
13. CUI X, ROUHANI FN, HAWARI F, LEVINE SJ: Shedding of the type II IL-1 decoy receptor requires a multifunctional aminopeptidase, aminopeptidase regulator of TNF receptor type 1 shedding. *J Immunol* 2003; 171: 6814-9.
14. KILDING RR, ILES MM, TIMMS JM, WORTHINGTON J, WILSON AG: Additional genetic susceptibility for rheumatoid arthritis telomeric of the DRB1 locus. *Arthritis Rheum* 2004; 50: 763-9.
15. MULCAHY H, O'ROURKE KP, ADAMS C, MOLLOY MG, O'GARA F: LST1 and NCR3 expression in autoimmune inflammation and in response to IFN-gamma, LPS and microbial infection. I*mmunogenetics* 2006; 57: 893-903.