Toll-like receptor 3 increases antigen-presenting cell responses to a pro-apoptotic stimulus, yet does not contribute to systemic lupus erythematosus genetic susceptibility

A. De Groof[1], J. Ducreux[1], L.Vidal-Bralo[2], D. Tyteca[3], C. Galant[1,4], L. Marot[5], P.G. Coulie[3], B.J. Van den Eynde[3,6], L. Rodriguez-Martinez[2], M.J. Santos[7], A. Suarez[8], P. Carreira[9], M. Marchini[10], A. Gonzàlez[2], F.A. Houssiau[1,11], B.R. Lauwerys[1,11]

[1]Pôle de Pathologies Rhumatismales Systémiques et Inflammatoires, Institut de Recherche Expérimentale et Clinique, Université catholique de Louvain, Brussels, Belgium; [2]Instituto de Investigacion Sanitaria and Hospital Clinico Universitario de Santiago, Santiago de Compostela, Spain; [3]de Duve Institute, Université catholique de Louvain, Brussels, Belgium; [4]Pôle de Morphologie, Institut de Recherche Expérimentale et Clinique, Université catholique de Louvain, Brussels, Belgium; [5]Department of Dermatology, Cliniques Universitaires Saint-Luc, Brussels, Belgium; [6]Ludwig Institute for Cancer Research, Université catholique de Louvain, Brussels, Belgium; [7]Hospital Garcia de Orta, Almada, Portugal; [8]Department of Functional Biology, Hospital Universitario Central de Asturias, Universidad de Oviedo, Spain; [9] Department of Rheumatology Hospital 12 de Octubre, Madrid, Spain; [10]Center for Systemic Autoimmune Diseases, Fondazione IRCCS and Granda Ospedale Maggiore Policlinico and University of Milan, Italy; [11]Department of Rheumatology, Cliniques Universitaires Saint-Luc, Brussels, Belgium.

Abstract

Objective

TLR3 mediates skin solar injury by binding nuclear material released from apoptotic keratinocytes, resulting in the production of pro-inflammatory cytokines. Because the TLR3 gene is located in 4q35, a known systemic lupus erythematosus (SLE) susceptibility locus, we wondered whether TLR3 single nucleotide polymorphisms (SNPs) were associated with inflammatory mechanisms relevant to the development of SLE, and disease susceptibility.

Methods

Functional assays were carried out in TLR3-transfected HEK293 cells and in monocyte-derived dendritic cells (moDCs). TLR3 and IFNβ immunofluorescence studies were performed in skin samples from 7 SLE patients and 3 controls. We performed a SNP association study in a discovery cohort of 153 patients and 105 controls, followed by a confirmation study in an independent cohort of 1,380 patients and 2,104 controls.

Results

TLR3 and IFNβ are overexpressed in SLE skin lesions. TLR3 overexpression in HEK293 cells amplifies their sensitivity to a pro-apoptotic stimulus. Taking advantage of a naturally occurring polymorphic TLR3 variant (rs3775291) that weakly versus strongly responds to poly I:C stimulation, we found that TLR3 is associated with amplified apoptotic responses, production of the Ro/SSA autoantigen and increased maturation of myeloid-derived dendritic cells (moDC) after exposure to UV irradiation. However, TLR3 SNPs are not associated with susceptibility to SLE in a large population of patients and controls.

Conclusion

TLR3 is overexpressed in SLE skin lesions and amplifies apoptotic and inflammatory responses to UV-irradiation in antigen-presenting cells in vitro. However, TLR3 SNPs do not impact susceptibility to the development of the disease.

Key words

TLR3, systemic lupus erythematosus, apoptosis, antigen-presenting cell, Ro/SSA autoantigen

*Aurélie De Groof, PhD**
*Julie Ducreux, PhD**
Laura Vidal-Bralo, PhD
Donatienne Tyteca, PhD
Christine Galant, MD, PhD
Liliane Marot, MD, PhD
Pierre G. Coulie, MD, PhD
Benoît J. Van den Eynde, MD, PhD
Lorena Rodriguez-Martinez
Maria Jose Santos, MD, PhD
Ana Suarez, MD, PhD
Patricia Carreira, MD, PhD
Maurizio Marchini, MD, PhD
Antonio Gonzàlez, MD, PhD
Frédéric A. Houssiau, MD, PhD
Bernard R. Lauwerys, MD, PhD

**These first authors contributed equally to this manuscript.*

Please address correspondence and reprint requests to:
Bernard Lauwerys,
Pôle de Pathologies Rhumatismales,
Institut de Recherche Expérimentale et Clinique,
Université catholique de Louvain,
Avenue Mounier 53, boîte B2.5390,
1200 Brussels, Belgium.
E-mail: bernard.lauwerys@uclouvain.be

Received on July 15, 2019; accepted in revised form on September 30, 2019.

Funding: this work was supported by grants from the WELBIO, Fonds National de la Recherche Scientifique and Fonds Spéciaux pour la Recherche (Communauté française de Belgique), and by Instituto de Salud Carlos III (grants PI11/01048 and RD12/0009/0008 that are partially financed by the European Regional Development Fund of the European Union).
A. De Groof is funded by WELBIO.
J. Ducreux is partly funded by a UCB "Chaire en rhumatismes inflammatoires et systémiques" grant.
B. Lauwerys is partly funded by the Fonds National de la Recherche Scientifique.
Competing interests: none declared.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterised by a breakdown of tolerance to autoantigens, and the production of antibodies (Abs) directed against nuclear antigens, *i.e.* double- and single-stranded DNA, RNA-containing molecules such as Ro/SSA, and other targets such as histone proteins or nucleosomes. The aetiology of SLE is unknown, but evidence indicates that it develops as a multistep process in genetically susceptible individuals (1). Interactions between endogenous nucleic acids-containing material (generated by apoptosis, or by NETosis) and pattern recognition receptors (PRR), such as Toll-like receptors (TLR) or intracytoplasmic sensors play a central role in the pathogenesis of the disease (2-7).

TLRs bind multiple sorts of ligands, including bacterial lipoproteins, lipopolysaccharide, flagellin, single-stranded and double-stranded RNA, and unmethylated CpG DNA. While TLR1, -2, -4, -5, and -6 recognise bacterial products, TLR3, -7 and -9 recognise nucleic acids which can originate from both pathogenic microorganisms and host (8). TLR3, -7 and -9 are localised in intracellular compartments that are not accessible to endogenous ligands in normal conditions. However, in some conditions such as SLE, when clearance of apoptotic cells is defective, endogenous material from dying cells may become available for these TLRs, resulting in exacerbated inflammatory responses.

TLRs are suspected to play a role in the pathogenesis of SLE by inducing type I interferons (IFNs), and IFN-induced genes expression after ligation of nucleic acids contained in circulating immune complexes (9-11). In this respect, the role of TLR7 and TLR9 has been extensively documented whereas the involvement of TLR3 in the pathogenesis of SLE has not been thoroughly investigated.

TLR3 binds double-stranded (viral) RNA but also endogenous double-stranded RNA-like structures (12, 13). Several somatic cell populations express TLR3 in humans, including fibroblasts and keratinocytes. In haematopoïetic cells, TLR3 expression is restricted to myeloid-derived dendritic cells (moDC) (14). Recent work demonstrated that TLR3 mediates skin solar injury by binding UV-modified noncoding RNA released from apoptotic keratinocytes, resulting in the production of pro-inflammatory cytokines such as tumour necrosis factor (TNF)α or interleukin (IL)-6 (15).

Since former reports indicated the presence of excessive amounts of non-engulfed apoptotic material in the SLE skin (16, 17), we wanted to investigate whether TLR3 might be involved in the inflammatory process characteristic of SLE skin lesions, and contribute to specific responses to an apoptotic challenge, resulting from, *e.g.* a physical stress (UV irradiation).

Material and methods

Patients and controls

All SLE patients met the American College of Rheumatology (ACR) revised criteria for the diagnosis of SLE (18). Clinical information and autoantibody profiles of the patients were retrieved from their medical records at baseline (see Supplementary Table S1). The immunohistochemistry, functional and initial genetic studies were approved by the ethics committee of Université catholique de Louvain. The confirmatory genetic study was approved by the ethics committee of Hospital Clinico Universitario de Santiago and by the local ethics review boards of all recruting centres. Informed consent was obtained from all patients and controls.

TLR3 and IFNβ immunostaining

Fresh biopsies from SLE skin lesions (n=7) and control skin (residual abdominoplasty material, n=3) were fixed overnight in 10% formalin buffer at pH 7.0, and embedded in paraffin. Immunolabelling was performed using a standard protocol, including removal of paraffin, and retrieval of antigenic sites in a 10 mM sodium citrate buffer (pH 5.8) at 97°C for 40 minutes. Tissue permeabilisation was performed in 0.3% Triton X-100 (v/v) in PBS, and non-specific binding was blocked prior to overnight incubation at 4°C with primary anti-IFNβ mouse monoclonal Ab,

and anti-TLR3 rabbit polyclonal Ab (Novus, dilution 1/50 for both antibodies). The sections were then incubated with the appropriate Alexa-labelled secondary antibodies (Invitrogen, dilution 1/200) and with DRAQ-5 DNA dye (Biolegend, dilution 1/500). The slides were finally examined with a LSM 510 META confocal microscope using a Plan-Apochromat 20X/0.8 objective (Zeiss, Jena, Germany).

TLR3-transfected HEK 293 cells

In some experiments, we used HEK-Blue hTLR3 cells, transfected with plasmids encoding both the human *TLR3* transcript, and a NF-κB-inducible *secreted embryonic alkaline phosphatase* (SEAP) reporter gene. In other experiments, we used stably TLR3-transfected HEK 293 cells generated by transfecting HEK 293 cells with different *TLR3 rs3775291* alleles, as follows. The pUNO *TLR3* plasmid, containing the *TLR3 rs3775291* G allele, was purchased from Invivogen. The pUNO *TLR3 rs3775291* A variant was constructed by 1) PCR amplification of *rs3775291* containing cDNA fragment from a *TLR3 rs3775291* A/A homozygous individual (see Suppl. Table S2 for primers), 2) digestion of the original plasmid and the PCR-amplified cDNA with SanDI and XbaI restriction enzymes, and 3) insertion of the digested PCR product into the digested plasmid. The control pUNO plasmid was generated by digestion of pUNO-*TLR3* with EcoRV and ligation of the free ends of the plasmid, which resulted in the removal of >1,500 bp in the sequence of the gene. The sequence of the insert was verified by sequencing in all plasmids before amplification.

HEK 293 cells were stably transfected with these plasmids, using blasticidin (50 μg/mL) as a selection agent, and clones were obtained by limiting dilutions. TLR3-transfected HEK 293 cell clones were tested by real-time PCR, in order to select clones containing similar amounts of transfected plasmids. For the experiments, selected clones were seeded in 24-well plates at 100,000 cells/well in DMEM, supplemented with 10% FCS and blasticidin 50 μg/mL.

TLR3 inhibition experiments

TLR3-HEK 293 cell clones were transfected with *TLR3*-specific (Hs_*TLR3*_8 siRNA, Qiagen) or control (Mm_*Lmna*_5 siRNA, Qiagen) siRNA, at a 50 nM concentration using the INTERFERin™ kit (Polyplus Transfection), and subjected to UV irradiation 3 days later. The efficiency of TLR3 inhibition was assessed by Western Blot, using TLR3 (BioLegend), and β-actin (Sigma-Aldrich) Abs.

Short-term peripheral blood mononuclear cells (PBMC) and monocyte-derived DC (moDC) cultures

PBMC were obtained from healthy individuals, and frozen. In some experiments, PBMC with known *TLR3 rs3775291* G/G, A/G, or A/A genotypes were stimulated with poly I:C (25 μg/mL) for 16h. For the generation of moDC, PBMC were thawed, and plastic-adherent cells were cultured for 6 days in medium supplemented with 10% FBS, GM-CSF (70 ng/mL) (Leucomax®, Novartis), and IL-4 (200 U/mL) (obtained from the laboratory of BVDE) at 37°C, 5% CO_2. On days 2 and 4, medium was refreshed with 35 ng/mL GM-CSF and 200 U/mL IL-4.

UV irradiation was performed in a Stratalinker™ 1800 oven (at 254 nanometer) (Stratagene). After overnight incubation, cell viability was assessed by Trypan blue exclusion on a Countess™ automated cell counter (Invitrogen), or using a MTS/PMS colorimetric assay (Promega). Apoptosis was evaluated by flow cytometry, using a CaspACE™ FITC-VAD-FMK (Promega) in situ marker. Cell surface expression of HLA-DR and CD86 (BD Pharmingen) were evaluated by flow cytometry. Ro/SSA and IL-6 secretion were evaluated by ELISA and bead-based immunoassay.

qRT-PCR experiments

Total RNA was extracted using a NucleoSpin RNA II extraction kit (Macherey-Nagel) and cDNA was synthesised using RevertAid™ Moloney Murine Leukemia Virus reverse transcriptase (Fermentas GmbH) and Oligo(dT) primers. qRT-PCR experiments were performed on a LightCycler 480® real-time PCR system (Roche Applied Science) using SYBR Green detection mix (1X). The melting curves obtained after each PCR amplification reaction confirmed the specificity of the SYBR Green assays. The data were normalised for *β-actin* gene expression, and normalised gene expression values were calculated using standard curves (see Suppl. Table S2 for primers).

Flow cytometry analyses

Cells were resuspended in 100 μL FACS buffer, and stained with the following mAbs: FITC conjugated anti-CD11c, PE conjugated anti-CD86, anti-HLA-DR, or FITC conjugated anti-CD4 mAbs, as well as with the appropriate isotype controls (BD Pharmingen) (dilution 1:20). After 30 minutes at 4°C, cell samples were washed and resuspended in FACS buffer containing 0.5% formaldehyde. Labelled cells were then analysed with a FACSCalibur flow cytometer (Becton Dickinson), and FlowJo software.

Apoptosis was also evaluated by flow cytometry, using a CaspACE™ FITC-VAD-FMK in situ marker (Promega). After incubation with the marker at a 5 μM final concentration at 37°C for 20 min, cells were resuspended in 300 μL FACS buffer, and immediately analysed on a FACSCalibur flow cytometer (Becton Dickinson), and FlowJo software.

Ro/SSA, IFNα, IFNβ and IL-6 determinations

Ro/SSA concentrations in culture supernatants of UV-irradiated moDC were assessed by ELISA. Briefly, 96 well microlon ELISA-plates (Greiner Bio-One) were coated overnight at 4°C with a 100 μL rabbit anti-human SSA1 pAb (Epitomics) solution, diluted at 5 μg/mL in a 50 mM sodium carbonate (stock at 1 M, pH 10) coating buffer. Plates were blocked with milk for 2h at 37°C, before being incubated with 100 μL undiluted supernatants overnight. For the standard curve, we used serial dilutions of a recombinant Ro/SSA protein (USBiological). Finally, the wells were incubated for 2h at 37°C with a mouse anti-human SSA1 mAb (Abnova), diluted at 1 μg/mL in PBS 0.5%

BSA. The third antibody was a peroxidase-labelled rat anti-mouse IgG1 kappa mAb (LO-MK1, Unit of Experimental Immunology, Université catholique de Louvain, Brussels, Belgium), diluted at 0.5 μg/mL in PBS 0.5% BSA, for 2h at 37°C. Plates were washed 5 times with PBS Tween 20 (Sigma-Aldrich) 1/1,000 between each step. The reactions were revealed with 1-Step Ultra-TMB (Thermo Fisher Scientific), and stopped by the addition of 2M H_2SO_4, before reading the OD at 450 nm with a microplate reader.

IFNα and IFNβ concentrations in sera (n=173) of SLE patients were quantified using the Verikine IFNα multi subtype and High Sensitivity Human IFNβ ELISA kit (PBL IFN Source) respectively, according to manufacturer's instructions. Patients were followed-up at a single centre (Brussels), and renal / mucocutaneous BILAG scores were determined at the time of the sampling based on a review of the medical charts of the patients.

IL-6 secretion in cell supernatants were assessed using a Bio-Plex Pro™ assay (Bio-Rad). Undiluted supernatants were mixed with capture antibodies coated on magnetic beads for 45 minutes at room temperature. Next, detection antibodies were added for another 60 minutes, followed by a last 15 minutes incubation step with streptavidin-PE, according to manufacturer's instructions. The samples were analysed on a Luminex 100/200 station (Millipore).

Genotyping studies

Our initial exploratory *TLR3* gene association study was performed on a Belgian population of SLE patients (n=153) and age- and gender-matched controls (n=105), using 8 Tag SNP selected by the Tagger-MultiMarkerTagging algorithm in the HapMap Data Rel24/phase II database (r^2 cut-off > 0.8; minor allele frequency > 0.2; CEU population): *rs4862632*, *rs4862633*, *rs5743305*, *rs10025405*, *rs6857595*, *rs1519309*, *rs3775292* and *rs4608848*. The genotypes of these Tag SNP were assessed on PCR amplified genomic DNA fragments with the the primers listed in Supplementary Table S2, using the ABI Prism® SnaPshot™ Multiplex Kit (Applied Biosystems). The single base elongation reactions were carried out using primers of different lengths (Suppl. Table S3), enabling to pool several SNP analyses in the same reaction (Mix 1: *rs4608848*; *rs6857595*; Mix 2: *rs10025405*, *rs4862633*, *rs4862632*; *rs3775291*, *rs1519309* and *rs5743305* were analysed separately). The results of the single base elongation reactions were loaded on an ABI Prism 3130xl Genetic Analyzer according to the manufacturers' instructions. Analyses of the electropherograms were performed on GeneMapper 4.0. Absent calls in mixed or single reactions were re-run separately. The accuracy of the SnapShot readings was checked by comparison with sequencing data, obtained in parallel on at least 10 amplified genomic DNA fragments for each SNP.

Genotyping of the *TLR3 rs3775291* was performed in an independent population of 1,380 SLE patients and 2,104 healthy controls recruited in 7 European countries. Patients and controls from each location were matched according to self-reported ancestry of the same country and with 16 top Ancestry Informative Markers (AIMs) for European subpopulations (19). Genotyping of the *TLR3 rs10025405* SNP was performed in a subset of 351 SLE patients and 697 controls, recruited in 3 locations in Spain. Both, the *TLR3 rs3775291* and the *rs10025405* SNPs were genotyped in multiplex PCR reactions. PCR was done with the KAPA2G fast HotStart (Kapa Biosystems, Woburn MA) on a final volume of 10 μL, using 3 mM $MgCl_2$ and 0.2 μM of each primer. PCR products were purified by Exo-SAP digestion with Exonuclease I (Epicentre, Madison, WI) and FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific, Madrid, Spain) and a single-base extension reaction with the SNaPshot Multiplex kit was done. After a post-extension treatment with SAP, genotypes were loaded in ABI Prism 3130xl Genetic Analyzer and analyses were performed on GeneMapper 4.0. Results from the 7 countries were combined either by simply pooling or with the Mantel-Haenszel test to account for heterogeneity between collections. Call rates were higher than 95% for all SNPs and subanalyses. In all control groups, distribution of the *TLR3 rs3775291* and *rs10025405* genotypes was in Hardy-Weinberg equilibrium (p>0.01).

Statistical analyses

Statiscal analyses were performed using GraphPad Prism software (v. 5.0), using paired or unpaired, parametric or non-parametric tests according to the distribution of the data, as indicated in the figure legends.

Results

TLR3 and IFNβ are expressed in SLE skin lesions

We first investigated the presence of TLR3 in cutaneous lesions of patients with SLE *versus* controls biopsies. Compared to control cutaneous lesions (n=3), we found a positive TLR3 staining in mononuclear cells infiltrates present in skin lesions from SLE patients (n=7) (Fig. 1A). Not surprisingly, cells that stained positive for TLR3 also stained for IFNβ. In addition, IFNβ and IFNα were found at significantly higher concentration in sera of SLE patients with active mucocutaneous lesions, compared to patients with other manifestations of the disease (Fig. 1B). When patients with simultaneous renal involvement were excluded, only IFNβ was significantly elevated in sera of SLE patients with mucocutaneous lesions (Fig. 1C).

TLR3 increases susceptibility to apoptosis

HEK 293 cells are poorly sensitive to UV-induced apoptosis and cell death. However, we found that TLR3-transfected HEK 293 cells are susceptible to apoptosis in response to UV irradiation, compared to sham-transfected cells (Fig. 2A). Transfection of a *TLR3* siRNA into TLR3-expressing HEK 293 cells significantly reversed this effect, confirming that TLR3 amplifies the pro-apoptotic effects of UV irradiation (Fig. 2B).

In order to confirm these observations, we also performed similar experiments using two naturally occurring variants of TLR3 (weak *vs*. strong), distinguished by a single base substitution in the coding region of the gene (*TLR3*

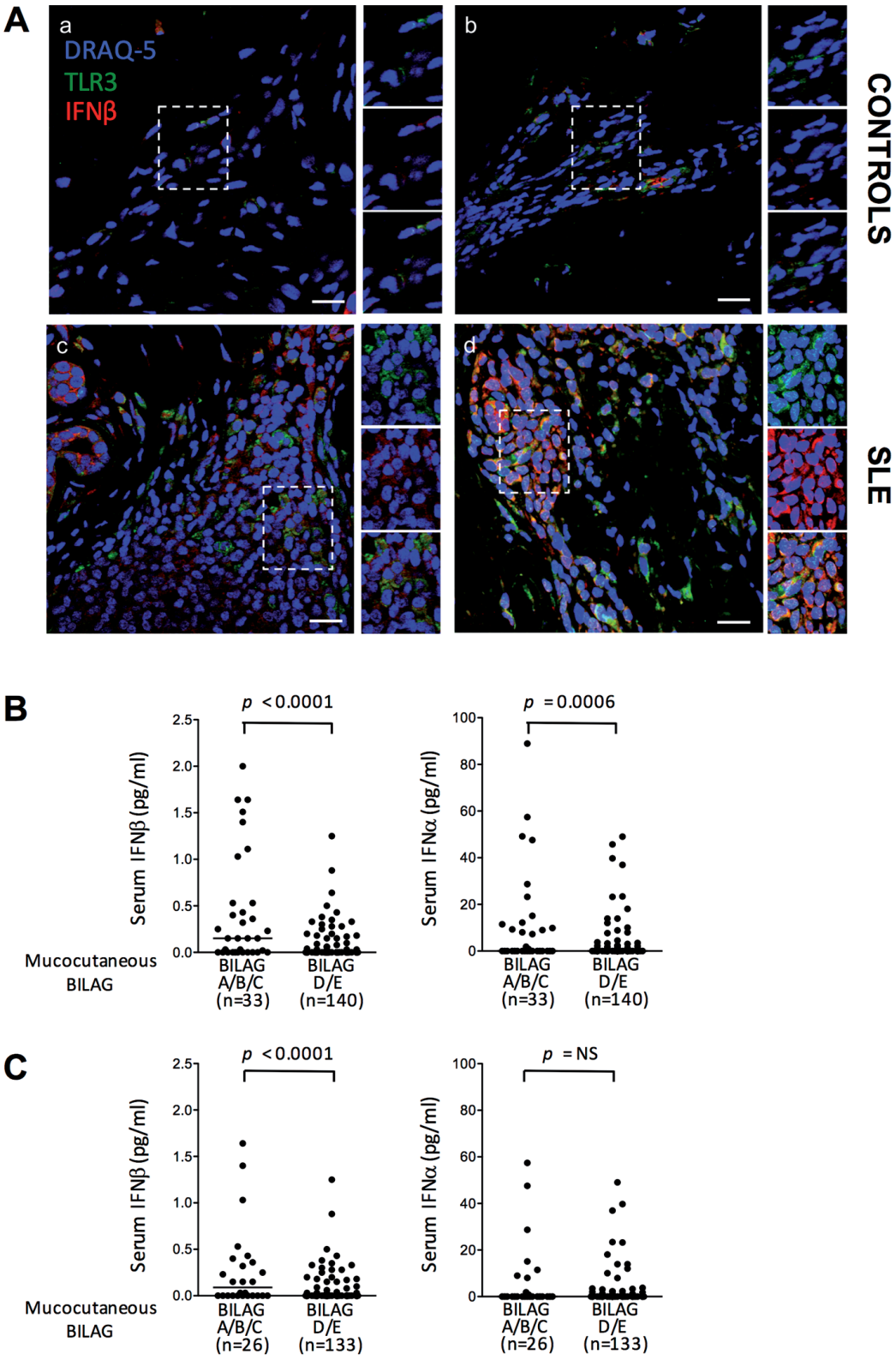

Fig. 1. Increased TLR3 and IFNβ production in SLE skin lesions.
A: TLR3 and IFNβ immunostaining in skin biopsies of SLE patients *vs*. control patients. Biopsies were obtained from sub-acute skin lesions of 7 SLE patients *vs*. 3 controls. Sections were double-stained with anti-TLR3 (green) and anti-IFNβ (red) antibodies. DRAQ-5 (DNA intercalating dye) was used to stain nuclei in blue. Characteristic images of 2 SLE patients and 2 controls are shown (original magnification x 20).
B: IFNβ and IFNα concentrations in sera from SLE patients with *vs*. without mucocutaneous manifestations. BILAG scores A, B and C = active mucocutaneous lesions; BILAG Scores D and E = mucocutaneous lesions are not present or were never present.
C: IFNβ and IFNα concentrations in sera from SLE patients with *vs*. without mucocutaneous manifestations after exclusion of patients with active renal involvement (renal BILAG A, B and C). The horizontal bars represent the median value. *p*-values are calculated using Mann-Whitney U-test.

rs3775291). *TLR3 rs3775291* is the only frequent single nucleotide polymorphism (SNP) in the coding region of *TLR3* with a significant impact on the function of the molecule (20). The Leu to Phe amino-acid substitution encoded by the SNP in the ligand-binding pocket of TLR3, results in increased responses of TLR3 to stimulation, in particular increased expression of IFN-induced genes (Fig. 2C) in the presence of the G (stronger) allele. Using these two variants, we demonstrated that HEK 293 cells expressing the *TLR3 rs3775291* G allele were more susceptible to UV-induced apoptosis and cell death, compared to those expressing the A allele (Fig. 2D-E).

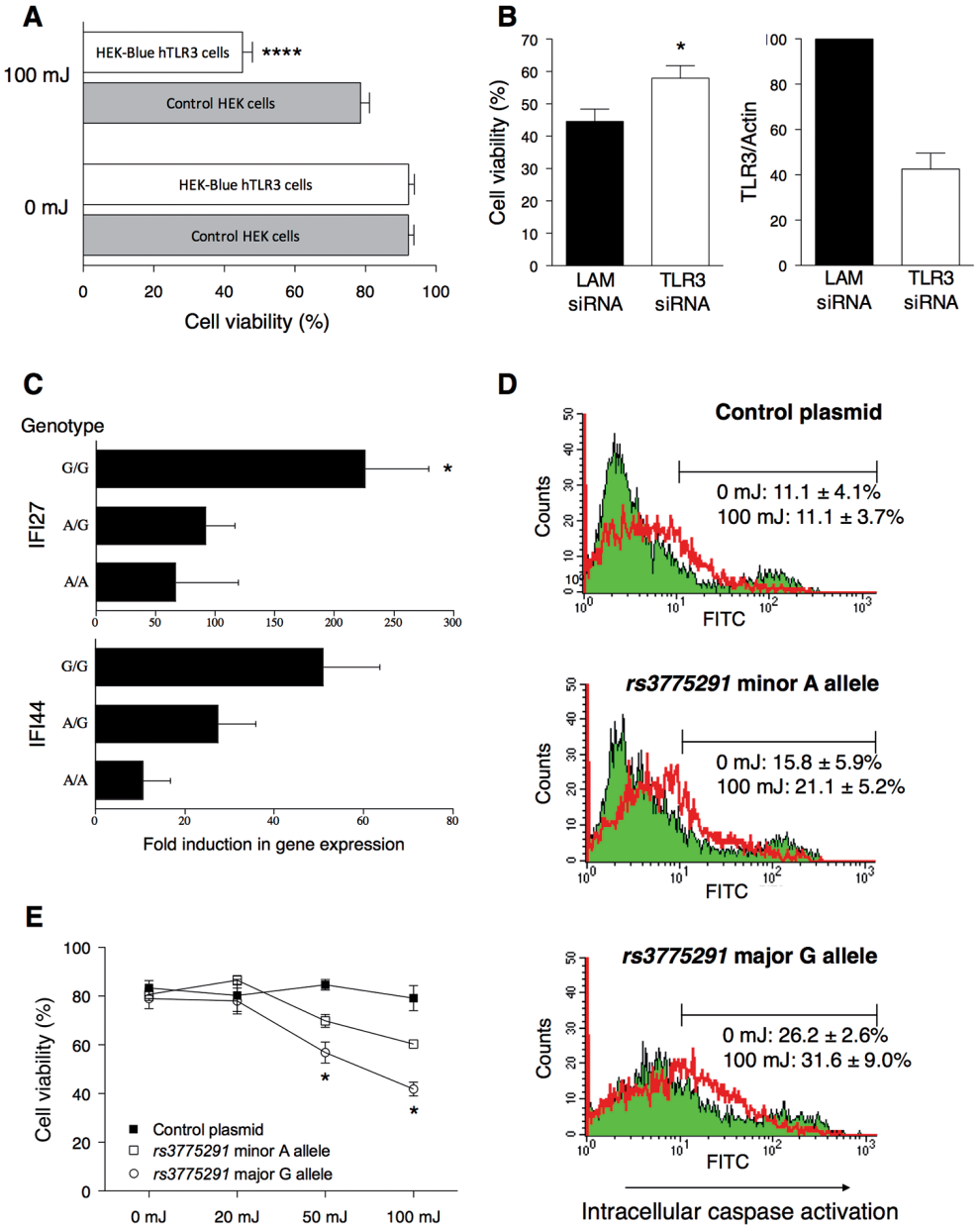

Fig. 2. TLR3 amplifies apoptotic responses induced by UV irradiation.
A: Cell viability (MTS/PMS assay) of HEK-blue hTLR3 cells (white bars) or control pUNO plasmid-transfected HEK 293 cells (grey bars) in response to UV-irradiation (n=3 in sextuplicates).
B: TLR3-expressing HEK 293 cells were transfected with a TLR3-specific (TLR3 siRNA), or a control (LAM siRNA) siRNA, and submitted to 100 mJ UV-irradiation (n=4 in triplicates). Left panel: cell viability (automated reading after Trypan blue exclusion) ; right panel : quantification of TLR3 down-regulation (Western Blot).
C: Role of *TLR3 rs3775291* genotypes on TLR3 responses to Poly I: C stimulation. qRT-PCR evaluation of *IFI27* and *IFI44* expression in healthy *rs3775291* G/G (n=15), A/G (n=16), and A/A (n=3) PBMC in response to poly I:C stimulation. Results are represented as the mean fold induction of the gene (± SEM) as compared to baseline.
D. Effect of *TLR3 rs3775291* genotypes on UV-induced apoptosis. Apoptosis was assessed by flow cytometry (CaspACE™ FITC-VAD-FMK staining). The graphs show representative results obtained in one experiment (green: 0 mJ, red: 100 mJ) and the numbers are the mean (± SEM) values obtained from 3 independent experiments (in duplicates).
E: Effect of *TLR3 rs3775291* genotypes on UV-induced cell death. Transfected HEK 293 cells (filled squares: control plasmid, open squares: *rs3775291* A allele, open circles: *rs3775291* G allele) were irradiated with the indicated doses of UV light (3 experiments in triplicates). Cell viability readings (automated assessment after Trypan blue exclusion) are expressed as the mean (± SEM) values. * $p<0.05$ using Student's *t*-tests (in C : G/G *vs*. A/G; in E : G *vs*. A allele).

TLR3 increases monocyte-derived dendritic cells (moDC) maturation in response to a pro-apoptotic challenge

In order to explore the role of TLR3 in DC phenotype and function, we used moDC from healthy individuals and we took again advantage of the natural occurrence of weak *versus* strong *TLR3* variants based on their *TLR3 rs3775291* genotype. We first showed that moDC from *TLR3 rs3775291* G/G individuals were more susceptible to UV-induced apoptosis than moDC from A/G or A/A individuals (Fig. 3A). In addition, we demonstrated that UV exposure induced higher release of the Ro/SSA autoantigen (Fig. 3B), and higher cell surface expression of MHC II and CD86 molecules (Fig. 3C) in *TLR3 rs3775291* G/G compared to A/G or A/A moDC. TLR3 stimulation triggers interleukin IL-6 production through NF-κB activation. Accordingly, we found that IL-6 production by *TLR3 rs3775291* G/G moDC after UV exposure was higher than in A/G or A/A moDC (Fig. 3D).

Distribution of TLR3 rs3775291 alleles and genotypes in SLE patients and controls

Finally, we wondered whether the distribution of *TLR3* variants was skewed in SLE patients, compared to controls. We first studied the distribution of 8 *TLR3* Tag SNP (designed in order to cover the majority of the polymorphic changes in the sequence of the gene) alleles and genotypes in a small cohort of 153 SLE patients *versus* 105 controls. As displayed in Supplementary Table S4, we found a significant association with SLE for *TLR3 rs5743305* and with anti-Ro/SSA positive SLE cases for *TLR3 rs5743305* and *rs10025405*. *TLR3 rs5743305* is in the promoter region of the gene, and *TLR3 rs10025405* is an intronic SNP in linkage disequilibrium with the functional *TLR3 rs3775291* SNP, described in the previous paragraphs of this manuscript (21). We therefore studied *TLR3 rs3775291*

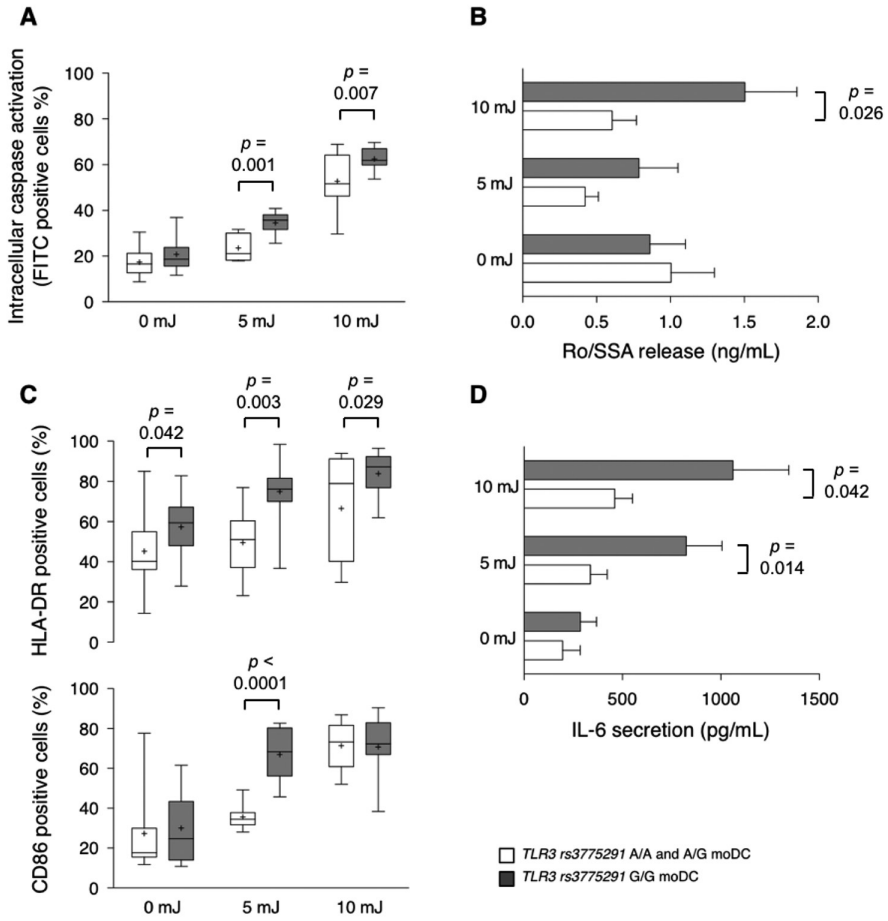

Fig. 3. Effect of *TLR3 rs3775291* genotypes on moDC responses to UV irradiation. *rs3775291* G/G (grey bars) or *rs3775291* A/A or A/G (white bars) moDC were seeded in triplicates. The effects of 0, 5 and 10 mJ UV irradiation were evaluated on
A: apoptosis (CaspACE™ FITC-VAD-FMK staining),
B: release of Ro/SSA auto-antigen (ELISA),
C: HLA-DR and CD86 expression (flow cytometry),
D: IL-6 secretion (Bio-Plex assay). In A and C, results are represented as the median (10-90 percentile) and mean (depicted as a "+") values (10 to 14 experiments). In B and D, data are represented as mean (± SEM) values (8 to 12 experiments). *p<0.05, **p<0.005, ***p<0.0005 using Student's t-tests.

Table I. *TLR3 rs3775291* and *rs5743305* genotypes in two independent collections of SLE patients and controls.

SNP	% Controls	% SLE	% SLE anti-Ro/SSA+	OR (CI)
rs3775291	**GG**	**GG**	**GG**	
Spain (1,006; 596; 155)	48.4	41.3	47.1	0.95 (0.68 – 1.33)
Greece (379; 194; 43)	58.8	55.2	51.2	0.73 (0.39 – 1.38)
Milan (227; 208; 60)	48.0	51.9	51.7	1.16 (0.65 – 2.05)
Netherlands (160; 97; 43)	46.5	49.5	58.1	1.60 (0.81 – 3.15)
Portugal (138; 116; 30)	54.0	57.8	53.3	0.97 (0.44 – 2.15)
Hungary (99; 90; 35)	49.4	51.1	45.7	0.86 (0.40 – 1.86)
Germany (95; 79; 22)	42.1	45.6	54.5	1.65 (0.65 – 4.19)
Combined (2,104; 1,380; 388)	50.2	47.7	50.3	1.00 (0.81 – 1.24)
rs5743305	**TT**	**TT**	**TT**	
Spain (697 ; 351 ; 102)	36.3	35.9	32.3	0.84 (0.54 – 1.31)

Population (n controls; n SLE patients, n anti-Ro/SSA positive SLE patients). OR (C.I.): Odds ratios (confidence interval) for the comparison of anti-Ro/SSA+ with healthy controls.

alleles and genotypes distribution in a large independent population of SLE patients (n=1,380) and controls (n=2,104), but found no significant association in either all or anti-Ro/SSA positive cases. Similarly, no significant association was found for *TLR3 rs10024405* alleles and genotypes, upon testing in a subset of this independent population (Table I).

Discussion

In a small exploratory study performed on skin biopsies, we found that TLR3 and IFNβ are over-expressed in inflammatory infiltrates in SLE skin lesions. Serum IFNβ concentrations are significantly elevated in sera from SLE patients with active mucocutaneous involvement. We demonstrated that TLR3 amplifies in vitro not only apoptotic responses, but also Ro/SSA autoantigen production, and maturation of moDC exposed to a pro-apoptotic stress (UV irradiation). These observations suggest that TLR3 contributes to the inflammatory processes involved in SLE skin lesions. However, distribution of TLR3 SNPs is not biased in SLE patients, with or without anti-Ro/SSA autoantibodies.

Involvement of TLR7 and TLR9 in the break of tolerance to RNA- and DNA-containing autoantigens is well documented in the context of SLE. Both TLR7 and TLR9 have the ability to bind endogenous mammalian nucleic acids (*e.g.* released during apoptosis or NETosis), thereby resulting in the activation of innate immune cells and production of IFNα (9, 22, 23). In genetically susceptible individuals, production of or response to IFNα is increased at a level where autoimmunity may occur, due to the stimulatory effects of the cytokine on antigen-presenting abilities of dendritic cells, and sustained activation of autoreactive T and B cells (24). Noteworthy, TLR7 and TLR9 also contribute to the pathogenesis of the disease through IFNα-independent mechanisms, either in immune cells or in the kidney (25-27).

By opposition to these TLRs, the involvement of TLR3 in the pathogenesis of SLE has not been thoroughly investigated. This is because excessive

production of IFNα, and not IFNβ, is purportedly associated with the disease (28). Yet, recent (29) and our own data indicate that IFNβ is also part of the type I interferon bias observed in SLE patients. As such, the role of TLR3 in the pathogenesis of SLE was mainly investigated by Christensen *et al*., who generated TLR3-deficient MRL/*lpr* mice and compared production of autoantibody production and onset of nephritis in wild-type, TLR3-deficient and TLR9-deficient mice (30). In this specific model, they demonstrated that TLR9 deficiency resulted in suppression of anti-dsDNA and anti-chromatin antibodies, while TLR3 deficiency had no effect on autoantibody profiles. The prevalence of immune-complex mediated glomerulonephritis was similar in all strains of mice. The authors did not investigate whether TLR3 deletion influenced other manifestations of the disease in different target organ. While TLR3 does not impact production of autoantibodies in lupus nephritis, it may have an impact on end-organ responses. Thus, human and mouse mesangial cells respond to TLR3 stimulation by producing chemokines such as CXCL1 (31), and in vivo poly I:C administration to MRL/*lpr* mice resulted in more severe lupus nephritis (32). In a model of mixed connective tissue disease induced after immunisation with U1-70-kd small nuclear RNP using U1 RNA as an adjuvant, Greidinger *et al*. reported that lung disease did not develop in TLR3-deficient mice. Instead, immunised TLR3-deficient animals developed nephritis, by opposition to TLR3 wild-type animals. The authors hypothesised that in the absence of TLR3-mediated moDC stimulation, U1RNA in TLR3-deficient animals were able to activate plasmacytoid DC through TLR7 and TLR8 resulting in the development of lupus nephritis (33).

In haematopoietic cells, TLR3 expression is restricted to moDC. TLR3 stimulation leads to recruitment of Toll-IL-1 receptor domain-containing adaptor molecule-1 (TICAM-1) that activates the NF-κB and interferon regulatory factor-3, resulting in preferential expression of IFNβ (34, 35). The main function of TLR3 is to bind double-stranded RNA viruses, but the molecule also has the ability to ligate endogenous nucleic acids. TLR3 stimulation by RNA originated from necrotic cells is long known (12, 13), but TLR3 ligation by molecules that contain loop structures mimicking double-stranded RNA also occurs in more physiological conditions. Thus, RNP-associated RNA molecules with regions of self complementarity (such as Ro-associated Y5, or U1) stimulate the proliferation of AM14 B cells when they are part of immune complexes, and this effect is TLR3-dependent (36). More recently, Bernard *et al*. found that UV irradiation affects the secondary structure of several small non-coding RNA (in particular U1) by increasing the size of their loop domains, thereby also increasing their ability to bind to TLR3 (15). The authors demonstrated that *in vitro* (HEK293 cells) and *in vivo* (skin) induction of pro-inflammatory cytokines such as IL-6 and TNFα after UV irradiation is TLR3-dependent. Accordingly, their study shows that TLR3 mediates solar injury and suggests that this receptor might play a major role in other light-sensitive disorders, such as SLE.

By demonstrating the presence of a TLR3 allele dose-effect on IL-6 production and expression of HLA class II and CD86 molecules by UV-irradiated moDC, our results indicate that TLR3 has a role in the moDC maturation process in specific situations. This observation might support the hypothesis that TLR3 is part of the inflammatory process in SLE skin lesions. TLR3 is probably not exclusively involved in mediating responses to apoptotic material in the SLE skin, but the presence of IFNβ in SLE cutaneous lesions, and significantly higher serum IFNβ concentrations in patients with mucocutaneous disease activity are compelling arguments in favour of a role for TLR3 in the pathogenic chain of events.

Ligation of TLR3 induces pro-inflammatory signals, but is also an additional pro-apoptotic trigger, resulting in the sustained production of (lupus-related) autoantigens. The mechanisms linking TLR3 to induction of apoptosis are still incompletely mapped. In tumour cells, TLR3 stimulation interferes with activation of both the intrinsic and extrinsic pathways, resulting in the activation of caspase 9 and 8 (37), respectively, and parts of the effects of the molecule are also mediated through inhibition of the PI3K/Akt pathway (38). It is unclear how these mechanisms translate in the present situation, but our data evidence a synergy between TLR3 over-expression and UV irradiation on apoptosis in HEK293 cells. In moDC homozygous for a functionally more active allele of TLR3, UV irradiation results in increased apoptosis and production of Ro/SSA, a known autoantigen in SLE. Taken together, these results demonstrate that TLR3 is part of an amplification loop that increases apoptotic responses after a physical stress (UV irradiation), and facilitates presentation of autoantigens.

TLR3 is located in 4q35, a locus associated with susceptibility to SLE in patients with anti-Ro/SSA antibodies (39). According to online repositories, the frequency of the major, functionally more active, *TLR3 rs3775291* allele in people of African descent is >95%, an observation of particular interest in view of the severity of SLE manifestations (including mucocutaneous symptoms) in this population. Based on our initial results in a small SLE and control population using TagSNPs across the gene, we analysed the association between *TLR3 rs3775291* (the only frequent SNP in the coding region of the gene with functional consequences), and *rs5743305* (a SNP located in the promoter region of the *TLR3* gene), and disease susceptibility in a large cohort of patients and controls. At this stage, however, our results are not in favor of a link between *TLR3 rs3775291* and SLE, either in the whole population of patients, or in the subgroup of patients with anti-Ro/SSA autoantibodies. Similarly, we did not find confirmatory evidence that *TLR3 rs5743305* is associated with susceptibility to SLE or development of anti-Ro/SSA antibodies. It is possible that additional analyses, using other component phenotypes (such as photosensitivity) rather than autoantibody profiles, would yield other insights about a potential genetic contribution of TLR3 to the pathogen-

esis of the disease, but our data do not enable to perform such analyses at the present time.

Despite the absence of genetic association between *TLR3* SNPs and SLE, our data indicate that TLR3 activation could play a role in the pathogenesis of SLE skin lesions by increasing autoantigen production and antigen-presenting abilities of moDC, thereby contributing to the break of tolerance to Ro/SSA. While it is recognised that TLR9- and TLR7-mediated mechanisms are involved in many manifestations of SLE through plasmacytoid DC activation, exploration of additional pathways involved in the pathogenesis of the disease is of importance, in particular in view of the development of IFN-blocking agents as a therapeutic strategy in SLE (7, 40-43).

Acknowledgements

The authors are grateful to Anne-Lise Maudoux and Carmen Pena, for excellent technical assistance, Drs M. De Meyer, M. Jadoul, E. Goffin, Y. Pirson (Kidney Transplantation Unit and Department of Nephrology, Université catholique de Louvain, Brussels, Belgium), Dr. Marc Bijl (Martini Hospital, Groningen, The Netherlands), Dr Cees G Kallenberg (University Medical Center Groningen, Groningen, The Netherlands), Drs Torsten Witte and Reinhold E. Schmidt (Hannover Medical School, Germany), Dr Attila Kovacs (Hospital of Hungarian State Railways, Szolnok, Hungary), Dr Emöke Endreffy (Albert Szent-Györgyi Medical Centre, Szeged, Hungary), Drs Francisco J Blanco and Ignacio Rego (INIBIC-Complexo Hospitalario Universitario A Coruña, Spain), Dr Carmen Gutierrez (Hospital Universitario Central de Asturias, Oviedo, Spain), Drs Josep Ordi-Ros and Eva Balada (Hospital Val d'Hebron of Barcelona, Spain), Dr Lorenzo Beretta (University of Milan and Fondazione IRCCS Ospedale Maggiore Policlinico from Milan, Italy), Dr Fotini N Skopouli (Euroclinic of Athens, Athens, Greece), Dr Maria Mavromati (Athens University Medical School, Athens, Greece), Drs Chryssa Papasteriades and Alexandra Tsirogianni (Evangelismos Hospital, Athens, Greece) and Drs Eva Perez-Pampin and Juan J Gomez-Reino (Hospital Clinico Universitario of Santiago, Santiago de Compostela, Spain) for inclusion of patients in this study.

References

1. FAIRHURST AM, WANDSTRAT AE, WAKELAND EK: Systemic lupus erythematosus: multiple immunological phenotypes in a complex genetic disease. *Adv Immunol* 2006; 92: 1-69.
2. BOUTS YM, WOLTHUIS DF, DIRKX MF *et al*.: Apoptosis and NET formation in the pathogenesis of SLE. *Autoimmunity* 2012; 45: 597-601.
3. HURST J, VON LANDENBERG P: Toll-like receptors and autoimmunity. *Autoimmun Rev* 2008; 7: 204-8.
4. KATO H, FUJITA T: RIG-I-like receptors and autoimmune diseases. *Curr Opin Immunol* 2015; 37: 40-45.
5. SHARMA S, CAMPBELL AM, CHAN J *et al*.: Suppression of systemic autoimmunity by the innate immune adaptor STING. *Proc Natl Acad Sci USA* 2015; 112: 710-17.
6. ZHANG W, CAI Y, XU W, YIN Z, GAO X, XIONG S: AIM2 facilitates the apoptotic DNA-induced systemic lupus erythematosus via arbitrating macrophage functional maturation. *J Clin Immunol* 2013; 33: 925-37.
7. LAUWERYS BR, DUCREUX J, HOUSSIAU FA: Type I interferon blockade in systemic lupus erythematosus: where do we stand ? *Rheumatology* (Oxford) 2014; 53: 1369-76.
8. IWASAKI A, MEDZHITOV R: Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 2004; 5: 987-95.
9. BARRAT FJ, MEEKER T, GREGORIO J *et al*.: Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. *J Exp Med* 2005; 202: 1131-39.
10. MEANS TK, LATZ E, HAYASHI F, MURALI MR, GOLENBOCK DT, LUSTER AD: Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. *J Clin Invest* 2005; 115: 407-17.
11. VOLLMER J, TLUK S, SCHMITZ C *et al*.: Immune stimulation mediated by autoantigen binding sites within small nuclear RNAs involves Toll-like receptors 7 and 8. *J Exp Med* 2005; 202: 1575-85.
12. BRENTANO F, SCHORR O, GAY RE, GAY S, KYBURZ D: RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via Toll-like receptor 3. *Arthritis Rheum* 2005; 52: 2656-65.
13. CAVASSANI KA, ISHII M, WEN H *et al*.: TLR3 is an endogenous sensor of tissue necrosis during acute inflammatory events. *J Exp Med* 2008; 205: 2609-21.
14. MATSUMOTO M, FUNAMI K, TANABE M *et al*.: Subcellular localization of Toll-like receptor 3 in human dendritic cells. *J Immunol* 2003; 171: 3154-62.
15. BERNARD JJ, COWING-ZITRON C, NAKATSUJI T *et al*.: Ultraviolet radiation damages self noncoding RNA and is detected by TLR3. *Nat Med* 2012; 18: 1286-90.
16. KUHN A, HERRMANN M, KLEBER S *et al*.: Accumulation of apoptotic cells in the epidermis of patients with cutaneous lupus erythematosus after ultraviolet irradiation. *Arthritis Rheum* 2006 54: 939-50.
17. REEFMAN E, DE JONG MC, KUIPER H *et al*.: Is disturbed clearance of apoptotic keratinocytes responsible for UVB-induced inflammatory skin lesions in systemic lupus erythematosus? *Arthritis Res Ther* 2006; 8: R156.
18. TAN EM, COHEN AS, FRIES JF *et al*.: The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; 25: 1271-77.
19. ALONSO-PEREZ E, SUAREZ-GESTAL M, CALAZA M *et al*.: Association of systemic lupus erythematosus clinical features with European population genetic substructure. *PLoS One* 2011; 6: e29033.
20. RANJITH-KUMAR CT, MILLER W, SUN J *et al*.: Effects of single nucleotide polymorphisms on Toll-like receptor 3 activity and expression in cultured cells. *J Biol Chem* 2007; 282: 17696-705.
21. ASKAR E, BREGADZE R, MERTENS J *et al*.: TLR3 gene polymorphisms and liver disease manifestations in chronic hepatitis C. *J Med Virol* 2009; 81: 1204-11.
22. BARRAT FJ, MEEKER T, CHAN JH, GUIDUCCI C, COFFMAN RL: Treatment of lupus-prone mice with a dual inhibitor of TLR7 and TLR9 leads to reduction of autoantibody production and amelioration of disease symptoms. *Eur J Immunol* 2007; 37: 3582-6.
23. SAVARESE E, CHAE OW, TROWITZSCH S *et al*.: U1 small nuclear ribonucleoprotein immune complexes induce type I interferon in plasmacytoid dendritic cells through TLR7. *Blood* 2006; 107: 3229-34.
24. BLANCO P, PALUCKA AK, GILL M, PASCUAL V, BANCHEREAU J: Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. *Science* 2001; 294: 1540-3.
25. WOLF SJ, THEROS J, REED TJ *et al*.: TLR7-mediated lupus nephritis is independent of type I IFN signaling. *J Immunol* 2018; 202: 393-405.
26. JENKS SA, CASHMAN KS, ZUMAQUERO E *et al*.: Distinct effector B cells induced by unregulated Toll-Like Receptor 7 contribute to pathogenic responses in systemic lupus erythematosus. *Immunity* 2018; 49: 725-39.
27. YUANG Y, YANG M, WANG K *et al*.: Excessive activation of the TLR9/TGF-β1/PDGF-B pathway in the peripheral blood of patients with systemic lupus erythematosus. *Arthritis Res Ther* 2017; 19: 70.
28. CROW MK: Type I interferon in the pathogenesis of lupus. J Immunol 2014. 192: 5459-68.
29. HAMILTON JA, WU Q, YANG PA *et al*.: Intracellular IFN-β and distinct type I IFN expression patterns in circulating systemic lupus erythematosus B cells. *J Immunol* 2018; 201: 2203-8.
30. CHRISTENSEN SR, KASHGARIAN M, ALEXOPOULOU L, FLAVELL RA, AKIRA S, SHLOMCHIK MJ: Toll-like receptor 9 controls anti-DNA autoantibody production in murine lupus. *J Exp Med* 2005; 202: 321-31.
31. IMAIZUMI T, AIZAWA T, SEGAWA C *et al*.: Toll-like Receptor 3 signaling contributes to the expression of a neutrophil chemoattractant, CXCL1 in human mesangial cells. *Clin Exp Nephrol* 2015; 19: 761-70.

32. PATOLE PS, GRÖNE HJ, SEGERER S *et al*.: Viral double-stranded RNA aggravates lupus nephritis through Toll-like Receptor 3 on glomerular mesangial cells and antigen-presenting cells. *J Am Soc Nephrol* 2005; 16: 1326-38.
33. GREIDINGER EL, ZANG Y, JAIMES K *et al*.: A murine model of mixed connective tissue disease induced with U1 small nuclear RNP autoantigen. *Arthritis Rheum* 2006; 54: 661-69.
34. MATSUMOTO M, KIKKAWA S, KOHASE M, MIYAKE K, SEYA T: Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. *Biochem Biophys Res Commun* 2002; 293: 1364-69.
35. OSHIUMI H, MATSUMOTO M, FUNAMI K, AKAZAWA T, SEYA T: TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat Immunol* 2003; 4: 161-67.
36. GREEN NM, MOODY KS, DEBATIS M, MARSHAK-ROTHSTEIN A: Activation of autoreactive B cells by endogenous TLR7 and TLR3 RNA ligands. *J Biol Chem* 2012; 287: 39789-99.
37. SUN R, ZHANG Y, LV Q *et al*.: Toll-like receptor 3 (TLR3) induces apoptosis via death receptors and mitochondria by up-regulating the transactivating p63 isoform alpha (TAP63alpha). *J Biol Chem* 2011; 286: 15918-28.
38. NUMATA T, ARAYA J, FUJII S *et al*.: Insulin-dependent phosphatidylinositol 3-kinase/Akt and ERK signaling pathways inhibit TLR3-mediated human bronchial epithelial cell apoptosis. *J Immunol* 2011; 187: 510-19.
39. RAMOS PS, KELLY JA, GRAY-MCGUIRE C *et al*.: Familial aggregation and linkage analysis of autoantibody traits in pedigrees multiplex for systemic lupus erythematosus. *Genes Immun* 2006; 7: 417-32.
40. LAUWERYS BR, HACHULLA E, SPERTINI F *et al*.: Down-regulation of interferon signature in systemic lupus erythematosus patients by active immunization with interferon alpha-kinoid. *Arthritis Rheum* 2013; 65: 447-56.
41. FURIE R, KHAMASHTA M, MERRILL JT *et al*.: Anifrolumab, an anti-interferon-α receptor monoclonal antibody, in moderate-to-severe systemic lupus erythematosus. *Arthritis Rheumatol* 2017; 69: 376-86.
42. MORAND EF, TRASIEVA T, BERGLIND A, ILLEI GG, TUMMALA R: Lupus Low Disease Activity State (LLDAS) attainment discriminates responders in a systemic lupus erythematosus trial: post-hoc analysis of the Phase IIb MUSE trial of anifrolumab. *Ann Rheum Dis* 2018; 77: 706-13.
43. DI BATTISTA M, MARCUCCI E, ELEFANTE E *et al*.: One year in review 2018: systemic lupus erythematosus. *Clin Exp Rheumatol* 2018; 36: 763-77.