From the Rheumatology Unit, Department of Medicine,
Karolinska Institutet, Stockholm, Sweden

STUDIES ON MOLECULAR REGULATION OF INFLAMMATION IN CUTANEOUS LUPUS ERYTHEMATOSUS

Vilija Oke, MD

Karolinska
Institutet

Stockholm 2011

On the cover:

"YOU are the big drop of dew under the lotus leaf, I am the smaller one on its upper side,' said the dewdrop to the lake." *Rabindranath Tagore*

Illustration by the author.

All previously published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet.

© Vilija Oke, 2011

ISBN 978-91-7457-280-3

Printed by

www.reproprint.se

Gårdsvägen 4, 169 70 Solna

Humanitate et scientia!

(Dr. V. Nagevičius, 1908)

To my Family

ABSTRACT

Lupus erythematosus (LE) is an autoimmune disease with a wide range of clinical manifestations. This spectrum spans from limited cutaneous disease to life-threatening rheumatic disorder involving vital organs. Sun exposure is an evident exogenous trigger of both cutaneous (CLE) and systemic LE (SLE). CLE-resembling skin lesions can also be experimentally induced using artificial ultraviolet radiation (UVR). Skin is an organ which is physically available for clinical observation and biopsy acquisition. This provides a possibility to relate the clinical appearance of developing and healing lesions to the molecular and cellular events observed in the skin specimens.

In the studies included in this thesis we aimed to define the molecular regulation of cutaneous inflammation in CLE and to evaluate if a standardized photoprovocation is a suitable method to study CLE in a multicenter study. Firstly, we explored what cytokines are involved in the regulation of inflammation in UVR-induced CLE lesions; secondly, we investigated if the autoantigen Ro52 is expressed in UVR-induced and spontaneous CLE lesions and lastly, if UVR and reactive nitrogen species (NO) could modulate the expression of autoantigen Ro52 in the LE-target cell keratinocyte. We also wanted to examine which domains within the Ro52 protein that determine its subcellular localization, and if Ro52 can interact with ubiquitin conjugating enzymes residing in different cell compartments.

To achieve our goals we used skin biopsy material derived from spontaneously occurring CLE lesions, and also a longitudinal collection of cutaneous specimens acquired from experimentally UVR-induced LE-specific lesions. We established patient- and healthy control-derived primary keratinocyte cultures in order to investigate Ro52 expression under the influence of UVR and NO. Furthermore, by constructing green fluorescent protein-Ro52 (GFP-Ro52) mutants and transfecting HeLa cells with them, we investigated the sequences of importance for subcellular localization of this autoantigen.

In paper I we demonstrated that a standardized photoprovocation allows inducing CLE-resembling lesions in approximately half of the patients and is a safe and reproducible method suitable for multicenter studies.

In paper II we demonstrated that HMGB1, an alarmin with cytokine-like functions, is upregulated and translocated to the extracellular space in UVR-induced CLE lesions, and that its highest expression coincides with the peak of clinical activity of the lesions. Other investigated cytokines TNF-α and IL-1β seemed to be of less importance.

In paper III we showed that Ro52 is strongly expressed in the epidermis and dermis of spontaneous and UVR-induced CLE lesions and is predominantly located in the keratinocyte cytoplasm. Moreover, our results of *in vitro* experiments indicate that UVR can upregulate the expression of this autoantigen in the cytoplasm of keratinocytes.

In paper IV we determined that NO can modulate the subcellular localization of Ro52 in human keratinocytes and HeLa cells *in vitro*. We have also demonstrated that Ro52 is expressed in close proximity to iNOS and is located in both cytoplasmic and nuclear compartments of the cells present in CLE skin lesions. In addition, we proved that the sequence located within the leucine zipper/coiled coil domain of Ro52 is the one that retains the protein in the cell cytoplasm while the B30.2 domain is important for the nuclear translocalization of Ro52. We have also demonstrated that Ro52 can interact with both nuclear and cytoplasmic ubiquitin conjugating enzymes.

In conclusion, the studies presented in this thesis provide novel insights into the molecular events that occur in the skin of CLE patients during lesion development. Our findings indicate that UVR and NO can modulate the expression of the autoantigen Ro52 in keratinocytes, which are the target cells of autoimmunity in LE. We demonstrate that standardized photoprovocation is a safe and reproducible method to study UVR-induced CLE in multicenter studies.

LIST OF PUBLICATIONS

I. **Photoprovocation in Cutaneous Lupus Erythematosus: A Multicenter Study Evaluating a Standardized Protocol**
A Kuhn, A Wozniacka, JC Szepietowski, R Gläser, P Lehmann, M Haust, A Sysa-Jedrzejowska, A Reich, V OKE, R Hügel, C Calderon, DE. de Vries, F Nyberg
Journal of Investigative Dermatology. Accepted for publication, 2011.

II. **Translocation of the novel cytokine HMGB1 to the cytoplasm and extracellular space coincides with the peak of clinical activity in experimentally UV-induced lesions of cutaneous lupus erythematosus**
V BARKAUSKAITE, M Ek, K Popovic, HE Harris, M Wahren-Herlenius and F. Nyberg
Lupus, 2007, 16:794–802

III. **High Ro52 expression in spontaneous and UV-induced cutaneous inflammation**
V OKE, I Vassilaki, A Espinosa, L Strandberg, VK Kuchroo, F Nyberg and M Wahren-Herlenius
Journal of Investigative Dermatology, 2009, 129:2000-10

IV. **The autoantigen Ro52 is an E3 ligase resident in the cytoplasm but enters the nucleus upon cellular exposure to nitric oxide**
A Espinosa, V OKE, Å Elfving, F Nyberg, R Covacu, M Wahren-Herlenius
Experimental cell research, 2008, 314:3605-13

In the thesis, the papers are referred to by their Roman numerals.

CONTENTS

1 Background 1
1.1 Lupus erythematosus 1
1.1.1 Photosensitivity in LE 3
1.1.2 Autoantibodies in LE 4
1.2 Lupus erythematosus and the skin 5
1.2.1 Clinical manifestations and prognosis of CLE subtypes 6
1.2.2 Histo- and immunopathology of CLE 8
1.3 Genetic associations in lupus erythematosus 9
1.3.1 Genetics of CLE and anti-Ro/SSA positive disease 9
1.4 LE triggering factors 10
1.4.1 The sun and ultraviolet radiation (UVR) 11
1.4.2 Smoking 13
1.4.3 Gender and sex hormones 14
1.4.4 Viral infection 14
1.4.5 Drugs 14
2 Autoimmune inflammation in the target organ 16
2.1 HMGB1 17
2.1.1 HMGB1 in inflammation 18
2.1.2 HMGB1 in autoimmunity 18
2.2 Ro52 19
2.2.1 The functions of Ro52 19
2.2.2 The expression of Ro52 21
2.2.3 Ro52 and interferon (IFN) responses in LE 21
2.3 Reactive oxygen and nitrogen species (ROS) 22
3 Aspects of the systemic autoimmune inflammation in LE 24
3.1 Cell death 24
3.2 CRP and anti-CRP antibodies 24
3.3 Complement 25
3.4 Phagocytes and recognition of ICs 25
3.5 The adaptive immune system in LE 26
3.5.1 Dendritic cells (DCs) and LE 26
3.5.2 T cells and LE 27
3.5.3 B cells and LE 28
4 Aims of the thesis 30
5 Results and discussion 31
5.1 Photoprovocation in CLE: a multicenter study 31
5.1.1 Rationale, background and methodological considerations 31
5.1.2 Multicenter photoprovocation study 34
5.1.3 Discussion 35
5.1.4 Concluding remarks and future perspectives 38
5.2 The role of HMGB1 in UVR-induced CLE 38
5.2.1 Rationale, aims and methodological approach 38
5.2.2 Results 39
5.2.3 Discussion 42

5.2.4 Conclusion 44
5.3 Ro52 in LE 44
5.3.1 Rationale, aims and methodological approach 44
5.3.2 Results 45
5.3.3 Discussion 46
5.3.4 Conclusion 48
5.4 NO modulates the cellular localization of Ro52 49
5.4.1 Rationale and aims 49
5.4.2 Results 50
5.4.3 Discussion 51
5.4.4 Conclusion 52
6 A hypothesis on CLE/LE pathogenesis 53
7 Concluding remarks 58
8 Acknowledgements 60
9 References 63

LIST OF ABBREVIATIONS

Ab	Antibody
ACLE	Acute cutaneous lupus erythematosus
ACR	American College of Rheumatology
AE	Adverse event
ANA	Antinuclear antibodies
APC	Antigen presenting cell
AR	Antigen retrieval
BCR	B cell receptor
C	Complement component
CCLE	Chronic cutaneous lupus erythematosus
CLE	Cutaneous lupus erythematosus
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DEJ	Dermo-epidermal junction
ds	Double stranded
dsDNA	Double stranded DNA
EBV	Epstein Barr virus
HMGB1	High-mobility group box 1
IC	Immune-complex
ICAM	Intercellular adhesion molecule
ICLE	Intermittent lupus erythematosus
IFN	Interferon
Ig	Immunoglubulin
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
LBT	Lupus band test
LE	Lupus erythematosus

LET	Lupus erythematosus tumidus
LPS	Lipopolysaccharide
mAbs	Monoclonal antibodies
MED	Minimal erythema dose
MHC	Major histocompatibility complex
MTD	Minimal tanning dose
NK	Natural killer cell
NLE	Neonatal lupus erythematosus
NO	Nitric oxide
PAMP	Patogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cell
qPCR	Quantitative polymerase chain reaction
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen and nitrogen species
SCLE	Subacute cutaneous lupus erythematosus
SLE	Systemic lupus erythematosus
SS	Sjogren’s syndrome
ss	Single stranded
TCR	T cell receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRIM	Tripartite motif
UVA	Ultraviolet A radiation
UVB	Ultraviolet B radiation
UVR	Ultraviolet radiation

1 BACKGROUND

1.1 LUPUS ERYTHEMATOSUS

The term Lupus Erythematosus (LE) (in Latin – the red wolf) dates from 19th century when the French physician Cazenave described the facial changes resembling a wolf bite in their appearance [1]. Interestingely, already in the first descriptions of LE it was emphasized that weather factors could aggravate the disease.

Lupus erythematosus (LE) is a prototype systemic autoimmune disease in which the immune system is directed to ubiquitous intracellular molecules: nucleic acids and cellular proteins. LE has a range of clinical manifestations with varying severity and prognosis. LE can manifest solely as a dermatological disease and is then denoted cutaneous LE (CLE). Systemic LE (SLE) commonly involves skin, joints, the cardiovascular system, the CNS, serous cavities and is a potentially life-threatening disease. In a temporal perspective, demarcation between the limited cutaneous or life-threatening SLE is not completely clear as localized disease can progress into systemic disorder or systemic condition may remit. The hallmark of SLE is presence of autoantibodies against intracellular targets. Antinuclear autoantibodies (ANA) and anti-double stranded DNA antibodies (anti-dsDNA) are the most specific for SLE [2]. Anti-Ro/SSA autoantibodies is the third most common variety detected in SLE patients [3]. Not all patients carrying these autoantibodies necessarily fulfill the ACR criteria for SLE. Anti-Ro/SSA positivity is prevalent among photosensitive patients who have CLE diagnosis [4, 5].

Extensive clinical diversity in the disease symptoms emphasizes the challenge to the physician in establishing diagnosis and evaluating prognosis. These rely on the overall clinical picture together with laboratory and serological findings, possibly including skin or renal biopsies. In order to categorize the symptoms and objective findings into a diagnosis, the American College of Rheumatology (ACR) proposed SLE classification criteria in 1982 [6]. The latest update of the original criteria was proposed in 1997 (***Table 1***) [7].

Table 1

The Revised ACR Classification Criteria for Systemic Lupus Erythematosus

(Abbreviated from Tan *et al* [6] with the update by Hochberg [7]).-

Criterion		Definition
1	Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare nasolabial folds
2	Discoid rash	Erythematous raised patches with adherent keratotic scalling and follicular plugging; atrophic scarring may occur in older lesions
3	Photosensitivity	Skin rash as a result of unusual reaction to sunlight by patient history or a physician's observation
4	Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by a physician
5	Arthritis	Non-erosive arthritis involving 2 or more peripheral joints
6	Serositis	a) pleuritis or b) pericarditis
7	Renal disorder	Persistent proteinuria >0.5 g/24h or cellular casts
8	Neurologic disorder	a) seizures or b) psychosis
9	Hematological disorder	a) hemolytic anemia with reticulocytosis or b) leukopenia or c) lymphopenia or d) thrombocytopenia
10	Immunological disorder	a) anti-dsDNA or b) anti-Sm or c) positive finding of phospholipid antibodies or d) false positive test for syphilis
11	Antinuclear antibodies	An abnormal titer of antinuclear antibodies by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with the "drug-induced lupus " syndrome

The presence of four or more of the total 11 criteria is necessary for the diagnosis of SLE. The criteria provide high sensitivity (96%) and specificity (92%) for the diagnosis [6]. In clinical practice it is usually patients that have already developed moderate or

severe disease manifestations that fulfill the requirement of four criteria. For patients in the initial phase of SLE or with mild manifestations these criteria are less sensitive and it is up to each specialist to be alert and to not miss a diagnosis or discontinue follow-up [8, 9].

Notably, mucocutaneous involvement comprises a substantial part of the diagnostic criteria for SLE (4 out of 11), and the data from the SLE cohort at Karolinska University Hospital indicate that up to 87% of SLE patients have cutaneous manifestations [10].

1.1.1 Photosensitivity in LE

Already in the very first descriptions by Cazenave sun exposure has been associated with lupus erythematosus skin lesions [1]. Freund followed 507 LE patients during 1920-1927 and observed clustering of inductions and exacerbations of the disease within the spring and summer months. During the period when phototherapy was being introduced into dermatology, a few cases of patients with DLE who were treated with UV lamps were reported to develop systemic manifestations (reviewed by Lehman [11]). Case reports of SLE induced in previously healthy individuals following extensive sun exposure have also been described [12, 13]. CLE lesions have a predilection to appear in a photo-distributed pattern and are commonly localized to the head, décolleté, neck, upper back, extensor aspects of the arms and forearms and dorsal aspects of the hands [14-16]. It thus appears that in a susceptible individual sun exposure can induce exacerbation of LE, but importantly even systemic disease in a previously healthy individual.

Photosensitivity is commonly reported by LE patients and serves as one of the eleven ACR criteria for the diagnosis of SLE (***table 1***). The ACR definition for photosensitivity is relatively difficult to interpret in several aspects. Sun-induced CLE lesions typically develop with a certain delay of several days up to several weeks and therefore the patient may miss to relate and admit sun exposure. In addition, another photodermatosis, polymorphic light eruption, is common among CLE patients, and when described by a patient and not examined by a specialist could easily be confused with and regarded as being sun sensitivity [17, 18]. Several experts have criticized the ACR definition of photosensitivity and a more objective definition of photosensitivity is of interest [19].

1.1.2 Autoantibodies in LE

The ACR classification criteria for SLE include the presence of antinuclear (ANA) and/or anti-RNP and/or anti-Sm and anti-dsDNA autoantibodies [6]. Anti-dsDNA autoantibodies are highly specific for SLE and are detected in approximately 70% of patients, but in less than 0.5% of the healthy population or patients with other autoimmune diseases [20]. The spectrum of LE-associated antibodies is much broader than that included in the ACR classification criteria for SLE. Many of the targeted molecules are ubiquitous cellular proteins, but autoantibodies against cell surface and soluble molecules can also be identified in some patients [21]. In addition to the above mentioned specificities, other detectable varieties include anti-La/SSB, rheumatoid factor, anticardiolipin, anti-βGP1, anti-CRP and anti-HMGB1 [3, 22, 23].

Autoantibodies against Ro/SSA characterize a subgroup of lupus patients who have CLE, a history of photosensitivity and an increased risk for development of a neonatal lupus syndrome (NLE) in the fetus during pregnancy [15, 17, 24, 25]. About 30% - 60% of all SLE patients have anti-Ro/SSA autoantibodies [4, 10]. Those diagnosed with purely CLE exhibit anti-Ro/SSA in the following frequencies: 83% of SCLE and 26% of DLE patients [4, 15, 17]. In addition, these autoantibodies are associated with primary Sjögrens syndrome (SS) and dermatomyositis with anti-synthetase syndrome [2, 26]. It was reported that up to 0.2-0.44 % of healthy blood donors test positive for anti-Ro/SSA [27, 28]. Probably some of these individuals will progress into an autoimmune disease in the future, since anti-Ro/SSA autoantibodies are reported to be detected several years before the development of a systemic disease [9]. Notably, two sub-specificities under the common title Ro/SSA have been identified and are directed against autoantigens Ro52 and Ro60 [29]. CLE patients usually have higher levels of anti-Ro52 than anti-Ro60 [24]. In the literature many authors do not specify the two autoantibody specificities in more detail due to historical tradition and there is still no clear consensus among scientists and clinical practitioners if it is correct to do so. It seems that separate testing for both subspecificities is of major importance in the obstetric patients and [24, 30].

Why and how autoantibodies develop against certain intracellular molecules is still unknown. Whether the presence of autoantibodies in LE patients is an incidental finding or if they are indeed pathogenic and can impede the function of their

intracellular targets is a question to be answered in the future. NLE is the condition in which it is believed that autoantibodies of the IgG type that are transported through the placenta to the fetus and directly account for the disease manifestations [25].

1.2 LUPUS ERYTHEMATOSUS AND THE SKIN

The ACR classification criteria for SLE include only some of all possible cutaneous manifestations associated with LE. Skin involvement in LE is classified into two major groups according to the observed histopathology of a skin biopsy: LE-specific and LE non-specific disease. LE-specific disease is denoted cutaneous LE (CLE) and is further sub-classified into several categories which share several common histopathological patterns. The initial classification of CLE was suggested by Gilliam in 1977 and a modification was proposed by Kuhn in 2003, ***table 2*** [31, 32].

Table 2

Classification of Cutaneous Lupus Erythematosus (2003)

Classification of Cutaneous Lupus Erythematosus (2003)	
Acute cutaneous lupus erythematosus (ACLE)	
Subacute cutaneous lupus erythematosus (SCLE)	
Chronic cutaneous lupus erythematosus (CCLE)	
	Discoid lupus erythematosus (DLE)
	Lupus erythematosus profundus (LEP)
	Childblain lupus erythematosus (CHLE)
Intermittent cutaneous lupus erythematosus (CLE)	
	Lupus erythematosus tumidus (LET)

A diagnosis of CLE is a constellation of anamnestic, clinical, histological and serological findings. ACLE, SCLE and chronic CCLE can be present in a patient with SLE [32]. ICLE seems to be a purely dermatological disease [31].

LE non-specific cutaneous manifestations include dermatological disorders, but also systemic manifestations of life-threatening disease, for example vasculitis. An overview of the variety of LE-nonspecific cutaneous manifestations is presented in ***table 3*** [14, 19]. Occurrence of the skin lesions might reflect an underlying clinical activity or precede a flare of SLE [11].

Table 3

An overview of LE-nonspecific cutaneous manifestations [14]

Category	Manifestation	Details
Vascular manifestations:		
	Vasculitis	
		Palpable or unpalpable purpura
		Urticaria-like vasculitis
		Hypocomplementemic urticarial vasculitis
	Livedo reticularis	
	Raynaud's phenomenon	
	Cutaneous ulcers	
	Palmar erythema	
Other manifestations:		
	Alopecia	
		Scarring and non-scarring
		- patchy or diffuse
	Oral ulcers	
	Photosensitivity	
		- restricted to cutaneous symptoms
		- systemic symptoms, e.g. weakness, arthralgia, fever
	Others	

The studies included in this thesis were focused on CLE and the following discussion will be dedicated to this subject, excluding LE-nonspecific cutaneous manifestations. Samples which were investigated in the studies studies included in this thesis originated from patients diagnosed ACLE (SLE), SCLE and DLE (the most common variant of CCLE). In the following discussion we will therefore concentrate on these subtypes of CLE.

1.2.1 Clinical manifestations and prognosis of CLE subtypes

ACLE

ACLE is a CLE manifestation exclusively associated with SLE. The most typical appearance of the lesion is a facial erythema of 'butterfly' shape over malar eminences. Sometimes ACLE can be disseminated as a maculopapular eruption and usually heals without scarring [4, 11]. These lesions are often associated with previous sun exposure,

and might be mistaken by a patient to be a sunburn [2, 11]. Sun sensitivity is a common problem in these patients and is included in the ACR classification for SLE [6]. ACLE lesions can last from a few days up to a few weeks and often precede a multisystem disease by weeks or months [4, 11].

SCLE

SCLE usually manifests as annular or papulosquamous, psoriasis-like scaling erythematous plaques. Lesions usually heal without scarring but may leave hyper- and/or hypopigmentation. SCLE is associated with photosensitivity and presence of anti-Ro/SSA [15]. Patients often have a mild systemic disease activity manifesting in parallel to the development of cutaneous symptoms [16]. Systemic symptoms may include musculoskeletal complains, increased erythrocyte sedimentation rate (ESR), lymphopenia, anemia, leukopenia and low serum levels of complement factors 2 or 4 [4, 33]. SCLE can overlap with DLE or ACLE [11]. Severe CNS, progressive kidney disease or severe systemic vasculitis are all uncommon in association with SCLE, occurring in less than 10% of patients.

CCLE

CCLE includes several sub-variants (Table 2). DLE is the most classical and prevalent (98% of all CCLE cases) morphologic lesion in CCLE [5, 16]. DLE lesions are flat or slightly elevated, sharply demarcated, scaling erythematous macules or plaques. The scale usually extends into dilated follicles. Lesions usually remind coins or disks in shape and with time can grow, become confluent and disfiguring. The periphery of the plaque is often hyperpigmented secondary to inflammation. After a certain time, lesions become depressed with scarring, depigmentation and telangiectasia [11, 16]. DLE commonly localizes to the ears, scalp or face and may involve mucosa. DLE localized to the neck and above is subcategorized into localized DLE and spread below the neck is denoted generalized DLE. DLE lesions localized to the scalp may leave scarring alopecia [11]. The presence of DLE serves as one of the ACR criteria for classification of SLE [6]. Patients diagnosed with generalized DLE have a higher risk to progress into systemic disease and up to 20% of these patients will subsequently develop SLE. Progression of localized DLE to SLE is less common [14]. Some of DLE patients have also positive serology for anti-Ro/SSA autoantibodies [4, 11]. In DLE patients elevated

ESR and hematological abnormalities are observed at a lower frequency than in SCLE [4, 5].

1.2.2 Histo- and immunopathology of CLE

Histopathological findings of LE-specific lesions share a pattern of lichenoid tissue reaction [32]. The changes include atrophy of epidermis, hydropic degeneration of basal cell layer with presence of apoptotic keratinocytes, hyperkeratosis, follicular plugging, basement membrane thickening, dermal mononuclear cell infiltrate and dermal edema [14]. ACLE, SCLE and DLE subtypes share these histological findings, but some of them are more typical and pronounced than others [14, 32]. Differences in the opinions exist as to whether ACLE, SCLE and DLE lesions can be reliably distinguished by the histopathological appearances alone. Moreover, a similar picture of lichenoid tissue reaction/interface dermatitis is observed in dermatomyositis, lichen planus lesions and some immune reactions against drugs and viruses (reviewed in [34, 35]).

As the most common manifestation of ACLE is 'butterfly rash' involving the face and clinical diagnosis is relatively easy, skin biopsies are therefore seldom acquired for diagnostic purposes. Furthermore histopathological changes observed in ACLE, especially in the early lesions, are relatively modest and non-specific despite dramatic clinical manifestations. Well-established lesions have slightly more prominent changes that include a mild degree of vacuolar alteration in the basal keratinocytes, some extravasated erythrocytes, cell-poor interface dermatitis and a sparse perivascular infiltrate of mononuclear cells, accompanied with some neutrophils. Depositions of mucin are usually observed in the dermis. Basal membrane thickening, follicular plugging or alteration of epidermal thickness is uncommon [35].

SCLE usually presents with interface dermatitis with vacuolar degeneration of basal keratinocytes and the epidermis is usually atrophic with mild hyperkeratosis. Typical findings in the dermis include edema, mucin deposition, a sparse lymphocytic infiltrate in the upper dermis (interface), around blood vessels and periadnexal structures [35].

Classical DLE presents with hyperkeratosis, variable epidermal atrophy and/or parakeratosis and follicular plugging. Large numbers of lymphocytes invade the basal

epidermis and the follicular epithelium, where basal hydropic degeneration is evident. The epidermal basal membrane is markedly thickened. In the dermis dense, patchy, perivascular and perifollicular lymphocytic inflammatory infiltrates are accompanied by dermal mucin deposition. In the scarring DLE lesions the dense inflammatory cell infiltrate subsides and is replaced by dermal fibroplasia [35].

Immune complexes (ICs) deposited along the dermo-epidermal (DEJ) junction is a frequent finding in CLE patients and is detected by direct immunofluorescence [36]. This finding is denoted 'lupus band test' (LBT). Immunoglubulins (Ig) of IgM, IgG and complement components (C), particularly C3, are found most commonly. Positive LBT is found in CLE lesions in over 90% of cases. In SLE patients, a positive LBT can be detected in uninvolved sun-protected skin and was suggested to be specific for the condition [37].

1.3 GENETIC ASSOCIATIONS IN LUPUS ERYTHEMATOSUS

LE/SLE may cluster to certain families and the estimated concordance rate for lupus is 24-58% among monozygotic twins and approximately 2-5% in dizygotic twins [38]. Sequencing of the human genome and technological advances in genotyping tools has led to a revolution in the field of clinical genetics [39]. Genome-wide association studies assessing copy number variation and single nucleotide polymorphisms have revealed important genetic associations in LE/SLE (***table 4***). Data indicate that predisposition for LE/SLE is inherited in a complex polygenic manner [40]. Many of the associated loci encode molecules involved in the innate and adaptive immunity [41]. A list of LE/SLE-associated candidate genes and the major putative function affected by these is presented in the table 4.

1.3.1 Genetics of CLE and anti-Ro/SSA positive disease

Several studies have reported genetic variations specifically associated with CLE. [14, 42-44]. The reported candidate genes can be sub-classified into MHC and non-MHC genes. The associations observed within the MHC group embody variations in HLA-DRB*0301, HLA-DRB1*1501, HLA-DRB1*1302, HLA-DRB1*1601, HLDQA*010, and also C2, C4, or combined C2 and C4 complement deficiencies. The associations observed in non-MHC loci are TNF-α (-308A), TYK2, IRF5, ITGAM and TREX1.

Polymorphisms in the intron 1 and 3 of the Ro52 gene have been associated with and anti-Ro52-positive Sjögren's syndrome and a polymorphism upstream exon 2 has been associated with SLE [45-47].

Table 4

LE/SLE risk loci and associated biological pathways [40, 41, 44, 48, 49]

Function	Gene
Adaptive immunity	
Antigen presentation	HLA-DR2 (DRB1*0301) HLA-DR3 (DRB1*1501)
T and B cell signaling	PTPN22, CTLA4, PDHX/CD44 BANK1, BLK
T helper cell regulation	STAT4, TNFSF4
Innate immunity	
Interferon and TLR7/9 signaling	IRF5, TNFAIP3, IRAK1, MECP2, UBE2L3, IRF7, PHRF1, TYK2, IL8, IKBKE
Fc receptor	FCGR2A, FCGR3B
Phagocyte activity	ITGAM
Clearance of immune complex	C1q, C2, C4, CRP
Other	IL-10, TREX1

1.4 LE TRIGGERING FACTORS

It was suggested that genetic factors influence an individual's lifetime risk to develop LE and environmental factors are likely to provide a trigger for the onset of clinical manifestation(s) of disease [41]. This fact emphasizes the importance of gene-environment interaction in the induction of LE.

1.4.1 The sun and ultraviolet radiation (UVR)

Photosensitivity is a common feature of LE and understanding of the mechanisms underlying UV damage to human skin can therefore help in delineating pathological processes occurring in LE patients subsequent to the sun exposure.

Sun light

The electromagnetic radiation emitted by the sun that reaches the surface of the earth consists of infrared radiation, visible light and ultraviolet radiation (UVR). The visual and infrared radiation is important for every living organism on earth, providing light and warmth. The UVR spectrum is less beneficial. UV radiation is responsible for activation of vitamin D3, but also accounts for sun-induced skin aging, modulation of the immune system and photocarcinogenesis. UVR has the wavelength between 280 and 400 nm. UV radiation is sub-classified according to the wavelength into UVB (280 – 315 nm) and UVA (315-400 nm). More UVA reaches the surface of the earth than UVB. UVB has a shorter wavelength than UVA, but higher energy and it is therefore UVB that leads to sun-burn after a longer exposure outdoors on a sunny day [50]. Due to its longer wavelength UVA penetrates through the epidermis to the reticular dermis and mediates its effects at this depth, but the effects are relatively modest. Meanwhile UVB is more potent but does not penetrate that deeply into the dermis. UVB mainly affects the epidermis and papillary dermis (***figure 1***) [50]. Humans are exposed to different amounts of UVR depending upon the latitude they live at. The race-associated differences in the natural skin pigmentation are an important factor determining skin sensibility to the sun [35].

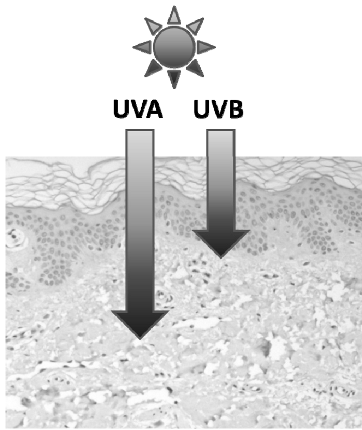

Figure 1. Penetrating abilities of UVA and UVB [50].

Immunological consequences of UVR

The sunlight can both suppress and activate the immune system of the human skin [50, 51]. A longer exposure to intensive sun induces sunburn, i.e. skin inflammation, while chronic or minimally erythematogenic doses have predominantly immunosuppressive effect [50]. Exposure to UVR induces DNA damage in keratinocytes. The cells are usually allowed to repair the damage. However, if the damage is too extensive or repair mechanisms fail, such cells undergo apoptosis and are consequently apparent in the skin biopsy as apoptotic 'sunburn' cells [52]. It is assumed that UVR-induced immunosuppression is biologically mediated by DNA damage and keratinocyte-secreted cytokine IL-10 and that it also depends on UVR-induced depletion of Langerhans cells and generation of regulatory T cells [53, 54]. The immunosuppressive effect is exploited in phototherapy of dermatological diseases such as psoriasis and atopic dermatitis, but may also lead to the development of dermatological neoplasms [55]. Proinflammatory effects of UVR are mediated by several mechanisms. Keratinocyte exposure to UVR results in increased expression of MyD88, activation of Nuclear Factor kappa B (NFkB) and inflammasome, subsequently leading to cytokine production and secretion (TNF-α , IL-1 (α and β), IL-6, IL-8 and IL-12). The secreted cytokines mediate multiple effects on the adjacent keratinocytes and cells in the dermis [50, 56]. The proinflammatory environment induces upregulation of adhesion molecules (ICAM1and E-selectin) in the adjacent blood vessels and keratinocytes and attracts leukocytes [57]. Production of NO is also induced and may contribute to keratinocyte apoptosis and other features of inflammation [58]. The recruited cells are

dedicated to clear the apoptotic keratinocytes and terminate the inflammation (reviewed by Maverakis *et al*.[50]).

Features of UVR-induced cutaneous inflammation in LE patients

Investigations including longitudinal skin biopsy material from experimentally UVR induced developing lesions have provided some insights into the biological processes occurring in the skin of CLE patients after UVR injury. It was demonstrated that CLE patients accumulate increasing numbers of apoptotic keratinocytes in their epidermis up to 72 h post-UVR exposure, whereas in healthy controls the increase of apoptotic cells is observed 24 h after UVR injury and unviable cells are completely cleared within 72 h [59]. It is assumed that accumulation of unviable cells is associated with clearance deficiencies and unremoved cells undergo secondary necrosis [60]. The released cell debris become opsonized by immunoglobulins and complement components as observed a few days later by positive 'lupus band' test [36, 61]. Prolonged and delayed induction of NO generation, as detected indirectly by the iNOS expression pattern, may contribute to an increased amount of apoptotic cells, but also enhance the secretion of proinflammatory cytokines and influx of leukocytes [50, 58, 62]. Expression of IFN-inducible protein MxA has been demonstrated to increase in parallel to the development of UVR-induced cellular infiltrates [63], and might therefore be related to the induction of IFNλ1 production by keratinocytes, as observed in spontaneous CLE lesions [64].

Several investigators attempted to explore if UVR could modulate the subcellular localization of Ro52 and Ro60 in human keratinocytes as anti-Ro/SSA autoantibodies are associated with photosensitivity. They demonstrated that Ro52, in parallel to Ro60, may translocate to apoptotic blebs after the cell exposure to UVR [65-68].

1.4.2 Smoking

Smoking is clearly associated with several autoimmune diseases: rheumatoid arthritis, pustulosis palmoplantaris and psoriasis [4, 69-71]. Smoking is also a risk factor for cutaneous manifestations of LE [4, 69, 70, 72], particularly in those with deficiency of complement factors C1q, C2, C4 or their combination [33]. Smokers usually have lower anti-Ro/SSA autoantibody levels [24]. CLE patients who smoke have less good

response to anti-malarial treatment, but cessation of smoking may re-establish sensitivity to anti-malarials [33, 73].

1.4.3 Gender and sex hormones

Nine out of 10 SLE patients are women, who usually are in their reproductive age at the onset of the disease. Among CLE patients the proportion of female patients varies 62-76% among CCLE and 69-89% among SCLE patients [4, 5]. Pregnancy can induce a flare of SLE [2]. Administration of exogenous estradiol can accelerate and exacerbate disease in several lupus-susceptible mouse models [74].

1.4.4 Viral infection

Several viral infections have been implicated in SLE induction, namely Epstein-Barr virus (EBV), parvovirus B19 and retroviruses [2, 75, 76]. This hypothesis might appear attractive as type I IFNs mediate antiviral response and 'interferon signature' is observed in a substantial proportion of SLE patients [77, 78]. It was described that one of the EBV proteins interacts with the LE-associated autoantigen La/SSB. This observation led to a hypothesis that the adaptive immune system might recognize a complex of viral particles and self-protein as a one antigen and thereby initiate autoimmunity. In addition, molecular mimicry between the autoantigen Ro60 and EBV viral proteins has been suggested (reviewed by James [75]). There is little evidence that viruses can directly induce LE. A possibility exists, that virus induced immune system activation may trigger LE in a genetically susceptible individual [79]. Importantly, symptoms of several viral infections may imitate SLE [2]. It is therefore recommendable to screen patients with SLE-like symptoms, for presence of certain viral infections [2].

1.4.5 Drugs

Several drugs have been associated with SLE and CLE induction. Treatment of viral infections with IFNα is associated with a risk of triggering SLE [80]. Development of cutaneous LE lesions at the site of imiquimod application (which is a TLR7 agonist and induces IFNα production by pDCs) has been reported [81, 82]. Interestingly, other classes of drugs that have been reported to induce SCLE lesions have little overlap with

those associated with drug-induced SLE (reviewed by Sontheimer in [83]). The list of SCLE-inducing drugs include calcium channel blockers, thiazide diuretics, antifungals, angiotensin converting enzyme inhibitors, β-blockers and proton pump inhibitors [8, 83]. Drug induced-SCLE cases are Ro/SSA positive at a similar rate as the idiopathic ones. It might be the case that a proportion of patients with drug-induced CLE had already had autoantibodies before the exposure to the medication, but the drug administration led to a clinical manifestation of the disease [8]. In contrast to the drug-induced SLE, positivity for anti-histone antibodies is detected only in less than half of drug-induced SCLE cases. Skin lesions usually resolve upon drug discontinuation, but not the anti-Ro/SSA autoantibodies [83].

2 AUTOIMMUNE INFLAMMATION IN THE TARGET ORGAN

Components of the innate and adaptive immune systems are involved in the disease processes of LE and those that are thought to be of major importance according to the currently available data will be overviewed below.

The skin damage observed in a histopathological picture of CLE is mediated by the autoreactive immune system. It is thought that the cascade of events leading to LE is usually initiated by an external factor that has cell-death inducing and/or proinflammatory nature (e.g. UVR, drugs). The observed changes in the CLE lesions include many signs of ongoing inflammation (***figure 2***) [14]. Apoptotic cells are present in the basal and the adjacent layers of epidermis [59]. Deposits of ICs are displayed at DEJ and some of them probably include cell debris opsonized by immunoglobulins and complement components, as detected by a positive 'lupus band' test in the majority of CLE lesions [14, 36]. Proinflammatory cytokines such as HMGB1, TNF, IL-1, IFNλ, IL-17 and IL-18 are expressed in the CLE lesions [64, 84-86]. They may further induce upregulation of chemokines and adhesion molecules at the site of injury. The upregulated adhesion molecules observed in the involved skin include ICAM1 and E-selectin and facilitate leukocyte influx [57, 63]. The Th1 type chemokines (CXCL9, CXCL10, CXCL11 and CXCL12) are the most strongly upregulated out of the whole chemokine family and are observed in those areas where epidermal and dermal injury is evident [87-90]. These chemokines may home lymphocytes bearing their ligands CXCR3 and CXCR4 and cells positive for these markers are detected within the lesions [89, 90]. Presence of CXCR4 expressing cells suggests accumulation of cutaneous DCs [91]. A substantial portion of the infiltrating cells are cytotoxic $CD8^+$ and effector $CD4^+$ T lymphocytes that might have been recruited via CXCR3 [34, 88]. Macrophages ($CD68^+$ cells) and pDCs are also present among dermis infiltrating cells [90, 92]. Notably, upregulation of IFN-inducible genes is evident in CLE lesions and includes IRF5, IRF7, MxA, CXCL9, CXCL10 and CXCL11 [42, 63, 89]. Evidently, keratinocytes seem to be poor producers of type I or II IFNs, but it has been recently demonstrated that they can produce IFNλ1 and express its receptor. Upregulation of this cytokine and its receptor in CLE lesions has been recently reported [64].

To conclude, the available knowledge suggests that CLE is driven by a Th1 immune response in parallel to the activated IFNs system. B cells secreting a special profile of autoantibodies are also typical for the condition.

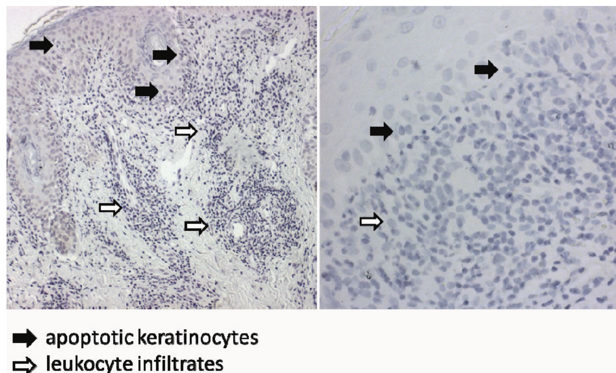

Figure 2. Histopathology of CLE lesion demonstrates inflammation.

The components of the immune system that are of specific interest in this thesis will be discussed in more detail below.

2.1 HMGB1

High mobility group box chromosomal protein 1 (HMGB1) or previously denoted amphoterin, is present in the nucleus of all nucleated cells and was known for many years as a protein with important nuclear functions [93]. HMGB1 contains two DNA-binding domains denoted box A and box B and is involved in transcription, replication and DNA repair [94, 95]. A decade ago novel qualities of extracellular HMGB1 were discovered. It was demonstrated that HMGB1 plays important roles in inflammation, regeneration and tumor-genesis [95-98]. The B box mediates the proinflammatory cytokine functions of the molecule, whereas the A box has an antagonistic anti-inflammatory effect [99]. HMGB1 can be passively released from the necrotic cells and alerts the body about injury [95]. It can also be actively secreted by activated cells by a non-classical pathway via secretory HMGB1-containing lysosomes [100].

2.1.1 HMGB1 in inflammation

Several types of leukocytes, such as monocytes, macrophages and dendritic cells, can be induced to actively secrete HMGB1. This usually occurs upon sensing conserved molecular motifs expressed on microbes (PAMPs, like lipopolysaccharide (LPS)) and/or pro-inflammatory cytokines (TNF-α, IL-1β and IFN-γ) [101]. The secreted HMGB1 functions as an alarmin with proinflammatory cytokine-like properties and enhances the immune response of the host. Extracellular HMGB1 can stimulate the surrounding cells inducing cytokine secretion via activation of NFκB, chemokine and adhesion molecule expression, as well as generation of ROS in the phagocytes [95, 102-104]. HMGB1, via interaction with CXCL12, guides chemotaxis of DCs and macrophages [105]. In addition, this protein is also important for maturation and activation of pDCs and T cells [106, 107]. HMGB1 functions via several receptors including RAGE, TLR2, TLR4 and TLR9 [99]. HMGB1 can act in solo or build complexes with other endogenous (IL-1β) and exogenous (LPS, nucleic acids) molecules to amplify its proinflammatory effect [108, 109].

HMGB1 is a mediator of both acute and chronic inflammation. High levels of HMGB1 in peripheral blood are associated with lethality in septic and hemorrhagic shock syndromes [110, 111]. The list of chronic inflammatory diseases in which HMGB1 is implicated as an important actor is constantly increasing [99, 112].

2.1.2 HMGB1 in autoimmunity

HMGB1 has been of great interest among immunologists during the last decade due to its role in the pathogenesis of inflammatory and autoimmune diseases. HMGB1 is detected in synovia and synovial fluid of patients with rheumatoid arthritis and HMGB1 blockade or inhibition alleviates the disease in animal models [99, 113]. Extracellular HMGB1 is detected in the muscle biopsies of patients diagnosed with inflammatory idiopathic myositis [114]. Patients with SLE have circulating HMGB1 and anti-HMGB1 autoantibodies in the peripheral blood [23, 99]. Intriguingly, HMGB1 complexed to nucleosomes has been demonstrated to induce IFNα production by pDCs and initiate autoimmune responses against dsDNA [115-117]. ICs containing HMGB1 might therefore be important players in the breakage of immunological tolerance to self-nucleic acids, a major target of autoantibodies in SLE. Importantly, our group has

recently demonstrated that HMGB1 was upregulated and translocated to the extracellular space in the spontaneous skin lesions of CLE patients [84].

2.2 Ro52

The Ro52 protein is one of the autoantibody targets in LE and Sjögrens syndrome patients [21, 118]. Ro52 was described in 1988 as one of the proteins targeted by Ro/SSA antibodies [29]. Historically, due to inaccuracies associated with methodological difficulties, Ro52 was thought to be associated with autoantigenic protein Ro60 into a complex denoted Ro/SSA [29]. Later, cloning and characterization of the Ro52 molecule allowed definition of the domains building the protein and indicated that there was no homology whatsoever with the Ro60 protein [119, 120]. Rather, Ro52 contains a RING and a B-box motifs, followed by a coiled-coil (CC) domain and a B30.2 (or PRYSPRY) region in the C-terminal end (***figure 3***) [121]. The RING, B-box and CC motif (RBCC) places Ro52 within the tripartite motif protein (TRIM) family [122]. Ro52 is thus also denoted TRIM21, and *Trim21* is the official name of the Ro52 gene [122].

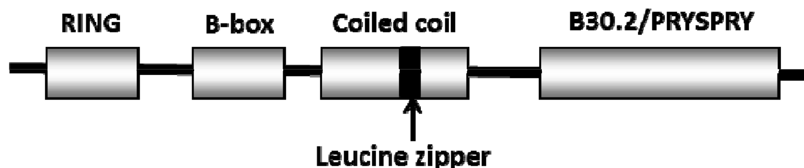

Figure 3. The structural domains of Ro52.

2.2.1 The functions of Ro52

Like several other TRIM proteins, Ro52 has an E3 ligase activity and acts in the process of ubiquitination [123]. Ubiquitination is a mechanism of post-translational modification of proteins that allows eukaryotic cells to control biological processes such as protein degradation, trafficking and activation [124]. The process of ubiquitination is a complex three-step pathway requiring energy (ATP). The first step includes binding and activation of ubiquitin molecule by an ubiquitin activating enzyme (E1). The activated ubiquitin molecule is then transferred to an ubiquitin

conjugating enzyme (E2). In the last step, an ubiquitin ligase (E3) mediates the transfer of the ubiquitin to the target protein (***figure 4***)[125]. It has been demonstrated that Ro52 can interact with E2s UBE2D1 and UBE2E1 located in the cell cytoplasm and nucleus, respectively [123, 126, 127]. Described substrates for Ro52-mediated ubiquitination include interferon regulatory factors (IRF) IRF3, IRF5, IRF7 and IRF8 [128-131]. It was demonstrated that Ro52 mediates ubiquitination of IRF3, IRF5 and IRF7 and can target these transcription factors for degradation. Dual data was reported regarding IRF8 and IRF3 and it seems that Ro52 may sustain their activity in certain cell types and conditions and direct them to degradation under other conditions [128, 130, 132].

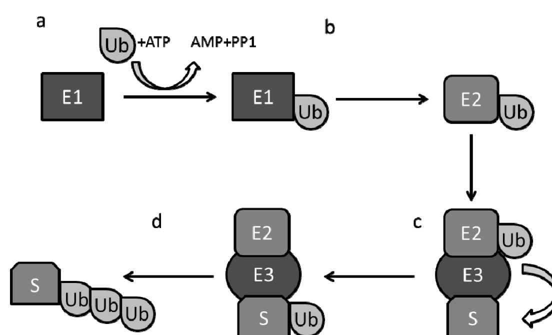

Figure 4. Post-translational modification with ubiquitin. A ubiquitin (Ub) molecule is activated and bound to the E1 (a), then transferred to the E2 (b) and finally E3 facilitates its attachment to the substrate (S) (c). Several ubiquitin molecules can be attached to the substrate if necessary (d).

It was observed that overexpression of Ro52 in a B cell line results in the increased cell sensitivity to the activation induced cell death [123]. In contrast to these findings Sabile *et al* reported that knock-down of Ro52 leads to an impaired progression of the cell cycle [133]. These observations are made in *in vitro* systems using different cells and experimental conditions. More investigations are needed to allow a better understanding what roles Ro52 has in the regulation of the cell cycle.

Several investigators have reported that Ro52 via its B30.2/PRYSPRY domain can bind to the Fc part of any IgG with unexpectedly high affinity, comparable to that of bacterial superantigen protein A [134, 135]. So far, it is not clear if this binding occurs *in vivo*.

2.2.2 The expression of Ro52

Ro52 is a predominantly cytoplasmic protein expressed in the cells of the immune system, as observed in a genetically modified animal [131, 136]. Ro52 becomes upregulated in a pro-inflammatory environment, such as exposure to type I and II IFNs [134, 137]. Cell stimulation with IFNα or H_2O_2 may lead to the accumulation of Ro52 in the cell nucleus [68, 137]. UVR has been demonstrated to induce Ro52 translocation to apoptotic blebs in keratinocytes and salivary ductal epithelia [66, 138].

SLE patients have higher numbers of Ro52 transcripts in the peripheral blood mononuclear cells (PBMC) as compared to healthy controls, although protein levels do not differ substantially [123].

2.2.3 Ro52 and interferon (IFN) responses in LE

A positive test for anti-Ro/SSA autoantibodies (and also ds-DNA) is associated with the presence of high IFNα activity in SLE patients [139]. Data reported by several groups indicate that Ro52 is an IFN-inducible protein and can regulate IFN responses. In evolutionary regard, type I IFN system evolved to protect the host against viral infections [77] and several members of TRIM protein family are important in the regulation of these responses [140].

Interferons (IFNs) and the regulatory role of Ro52

IFNs are subclassified into type I, II and III. Type I IFNs include several subtypes of IFNαs and IFNβ that are important in the antiviral response [77]. IFNγ belongs to type II IFNs and mediates host defense against intracellular microbes [77]. IFNλ1 is the most important member of type III IFNs and seems to have an important role in the antiviral and antitumor response in epithelial cells such as keratinocytes [141]. Plasmacytoid dendritic cells (pDCs) are the most potent producers of IFNα, but can also produce IFNλ [78, 142]. Transcription of type I IFNs is regulated by IRFs. IRFs are usually activated after the cell senses intracellular viral or bacterial nucleic acids via TLR-3, -7 or -9 or cytoplasmic sensors RIG1 or MDA5 [77]. IFNα has multiple effects on the host cells: can induce apoptosis of virus infected cells and stimulate the immune

response, including maturation and activation of APCs, activation of Th1 T cells, prolonging survival of cytotoxic T cells and enhancing antibody production in B cells [143-145]. Data indicate that Ro52 has a regulatory role in IFN responses and is involved in a regulatory loop: its expression can be upregulated by type I and II IFNs [134, 137] and Ro52 can subsequently inhibit IFNs and their inducible cytokines (IL-12/IL-23p40, IL-6, and TNF alpha) [130, 131].

IFNs in LE

Notably, a major part of LE patients display presence of 'IFN signature', i. e. upregulation of IFN-inducible genes is observed in their PBMCs and also CLE lesions [78, 89, 90]. A part of SLE patients also have increased IFNα concentrations in their peripheral blood [48, 78, 89, 90]. Interestingly, it was recently reported that a type III IFN (IFNλ1) and its receptor are highly expressed in CLE lesions and that serum levels of IFNλ1 correlate with the activity of CLE [64].

Genetic associations between LE and molecules involved in different pathways of interferon responses have been reported. These include molecules involved in sensing intracellular foreign nucleic acids (MDA5) and signal translation via the interferon receptor (TYK2). Additionally, polymorphisms in the transcription factors or other molecules involved in signal transduction downstream these receptors have also been reported (IRF5, IRF7, IRF8, IRAK1 and STAT4) (reviewed in [48]).

2.3 REACTIVE OXYGEN AND NITROGEN SPECIES (ROS)

ROS are a group of highly reactive oxygen derivatives. This group includes several gaseous molecules with a simple structure and a short half-life: nitric oxide (NO), hydrogen peroxide (H_20_2), peroxynitrite (ONOO). Activated leukocytes can produce ROS at the site of inflammation in order to kill invading microorganisms [77]. Skin exposure to UVR can induce keratinocytes and dermal endothelial cells to produce and release ROS, including NO [146, 147]. NO is an important mediator participating in vasodilation, signal transmission, apoptosis and wound healing [58, 148, 149]. It appears that the effects mediated by NO are determined by its concentration: low concentrations stimulate cell proliferation during wound healing, but high have cytostatic and proinflammatory effects [58, 150].

NO is a short-lived molecule and is therefore difficult to detect in a living organism. The track of NO production can be estimated by determining the presence of nitric oxide synthases (NOS) that are responsible for its production in the tissues [147, 150]. The synthases generate NO from L-arginine, producing equal amounts of citrulline (reviewed in [58]). Keratinocytes constitutively express nNOS (neuronal NOS) and dermal endothelial cells express eNOS (endothelial NOS). These enzymes are responsible for constant low production of NO and are often denoted by the common name constitutive NOS (cNOS). Constant NO production is necessary for maintenance of skin barrier function and blood circulation [58]. Inducible NOS (iNOS) is synthesized in response to various stimuli, including sun exposure or skin wounding [147]. UVR upregulates iNOS expression in the skin and sun-burn induced NO is one of the molecules responsible for the inflammatory skin reaction [58, 62].

Increased iNOS expression has been reported in the lupus nephritis kidney and UVR induced CLE skin lesions [62, 151]. Generation of NO and its derivate peroxynitrite (ONOO) (when NO encounters superoxide molecule) might modify autoantigens and form neo-epitopes [152]. It has been demonstrated that DNA modified with ONOO was more immunogenic than native DNA in an animal model of LE [153].

3 ASPECTS OF THE SYSTEMIC AUTOIMMUNE INFLAMMATION IN LE

3.1 CELL DEATH

Apoptosis is an essential phenomenon needed to ensure cellular homeostasis of multicellular organisms. It allows elimination of dangerous, damaged or unnecessary cells. This mechanism is highly controlled in order to insure removal of the right cells and to reduce the risk of damage to the surrounding tissues [154]. Apoptosis is usually a non-inflammatory process mediated by mononuclear phagocytes that secrete anti-inflammatory cytokines IL-10, TGFβ, and NO after the engulfment of unviable cells [155]. Efficient clearance of apoptotic cells is important to prevent exposure of self-antigens to the immune system. Conversely to apoptosis, necrosis is usually caused by external signals and subsequently molecules dedicated to alarming about the danger are released [95]. They are denoted alarmins and include HMGB1 and other molecules [95]. If apoptotic cells are not cleared within an appropriate time limit they undergo secondary necrosis and release intracellular constituents to the surrounding extracellular space [60]. HMGB1 tightly bound to chromatin is included in the debris released from necrotic cells and may elicit immune response against DNA [60, 115].

It has been demonstrated that lupus patients accumulate increased numbers of dying cells in the different tissues, including skin and lymph nodes [59, 156]. It is thought that it depends on clearance deficiency and/or on accelerated apoptosis.

3.2 CRP AND ANTI-CRP ANTIBODIES

CRP is an acute phase reactant and belongs to the pentraxin family [77]. It is produced by hepatocytes upon stimulation with IL-6 and IL-1. A distinct feature of CRP is a very prompt elevation (within first hours) following tissue injury. CRP, together with other opsonins such as C1q, IgG, is important in opsonization of the apoptotic cells and facilitation of phagocytosis [22, 157, 158]. SLE patients usually have low levels of CRP during flares, despite that they can produce large amounts of it upon infections [157]. The reason of low CRP levels during SLE exacerbations is not clear, but it was suggested that increased production of IFNα might have a role [157]. It is possible, that

lack of CRP contributes to impaired phagocytosis of apoptotic debris. Paradoxically, CRP levels are constantly at the upper level of the normal range in the majority of SLE patients. It seems that slight, but constant CRP elevation, is associated with increased incidence of cardiovascular disease in SLE patients [22, 157]. Moreover, many SLE patients have anti-CRP autoantibodies [22]. Anti-CRP Abs might contribute to LE pathogenesis by neutralizing CRP, increasing the numbers of circulating immune complexes and by atheroma plaque destabilization [157]. Polymorphism in the human CRP locus has been determined in SLE patients and was suggested to possibly influence basal CRP expression and as well as predispose to SLE development [158].

3.3 COMPLEMENT

The complement system is an important player in the immune response and, in particular, in clearance of apoptotic and necrotic cells [159]. Deficiencies of the complement components have been associated with LE, but interestingly, not with any other autoimmune disease [33].

The complement system is involved in elimination of immune complexes (ICs) and interaction of its components with CRP is of major importance in this process [158, 160]. CRP activates the complement cascade and upregulates complement receptors on phagocytes (reviewed in [158]). Low or immeasurable levels of certain complement components might be present due to genetic predisposition, impaired regulation of the complement system and/or consumption during inflammatory processes. Genetic deficiencies of complement components are associated with susceptibility to infectious diseases, but also SLE. Notably, SLE patients with total or partial C2, C4 or combined C2 and C4 deficiencies usually manifest with CLE lesions, photosensitivity and anti-Ro/SSA Abs, but seldom test positive for anti-dsDNA [33].

3.4 PHAGOCYTES AND RECOGNITION OF ICs

The phagocytes of the innate immune system include macrophages and monocytes, denoted mononuclear phagocytes, and neutrophils, termed polymorphonuclear phagocytes [77]. These cells are responsible for the recognition of antigen via pattern recognition receptors or FcγRs (opsonized antigens). Antigen recognition is usually associated with its engulfment with the purpose to eliminate it. Phagocytes are recruited

to the site of inflammation via chemokine gradient and interactions with cell adhesion molecules [77]. The removal of unviable cells and ICs is impaired in a substantial proportion of LE patients [60]. The available data indicate that phagocytes have reduced abilities to recognize cell debris and ICs most probably due to genetic polymorphisms of the genes encoding their receptors, such as complement receptor 3/integrin-α_M, FcγR2A and FcγRIIIb [41, 161].

3.5 THE ADAPTIVE IMMUNE SYSTEM IN LE

Multiple abnormalities in the activation and function of the cells of the adaptive immune system have been described in LE patients. In particular, it seems that a type 1 (Th1) $CD4^+$ T cell response with over-activation of pDCs that secrete high amounts of IFNα and B cells that secret a variety of autoantibodies have major roles in the pathogenesis of autoimmunity in LE. During recent years an important role of Th17 response has been also implicated in LE and these cells seems to have an interplay with Th1 cells.

3.5.1 Dendritic cells (DCs) and LE

The conventional DCs (cDCs) are termed professional antigen presenting cells (APCs) and their major function is antigen recognition, processing and presentation to T cells. Thus cDCs bridge innate and adaptive immunity [77]. It is believed that DCs are involved in the presentation of autoantigens to the adaptive immune system in autoimmune diseases [77]. It has been demonstrated that ICs containing autoantigens, such as HMGB1-nucleosome complexes, induce DC maturation and activation [115]. DCs derived from LE patients respond to such stimulation by secretion of much higher amounts of IL-12 as compared to the controls [162]. Intriguingly, several reports demonstrated that C1q deficiency results in the impaired recognition and engulfment of ICs by macrophages and therefore such ICs could be phagocytosed by DCs [163-165]. This could increase the chances for autoantigen presentation to T and/or B cells.

Plasmacytoid DCs (pDCs) are specialized for recognition of viral and bacterial nucleic acids through their endosomal receptors TLR-3, -7 and -9 or cytoplasmic sensors (RIG1 and MDA5). They can also recognize ICs via FcγRIIa [166, 167]. Activated pDCs upregulate the CXCR3 receptor, which directs them to the target tissue

expressing its ligands (CXCL-9, -10 and -11) [168, 169]. pDCs are detected in CLE skin lesions and are potent producers of type I IFNs [78, 92, 170]. Immune complexes can activate pDCs and induce IFNα production and C1q was demonstrated to be an important signal suppressing this activation [171]. It may be the case that the C1q deficiency observed in some of SLE patients is one of the important factors whereby IFNα production is not terminated in time.

3.5.2 T cells and LE

The available data demonstrate the involvement of Th1 and Th17 effector cells in the autoimmunity of LE [172-174]. Increased levels of circulating Th1 profile cytokines, IFNγ, IL-12 and IL-18 have been observed in SLE patients, especially in those with involvement of vital organs, such as kidneys [175]. Production of Th1 cytokines occurs in LE target organs, including kidneys and skin [85, 176]. In parallel, increased levels of circulating $CD4^+$ and $CD4^-CD8^-$ double negative T cells of Th17 subtype have also been reported in SLE patients [177]. IL-17 producing cells were demonstrated in lupus nephritis kidney and CLE lesions [86, 172, 178].

It is probable that the adaptive autoimmune response in LE patients is initiated when DCs, matured in the proinflammatory environment, take up the autoantigen released from secondary necrotic cells (as it is or opsonized) and present it to the naive (probably low self-reactive) T cells. Antigen recognition, accompanied by the costimulatory signal, usually leads to $CD4^+$ T cell activation and clonal expansion of effector T cells. DCs could also activate $CD8^+$ T cytotoxic T (CTLs) cells via cross-presentation [77]. Self-reactive Th1 cells might have multiple roles in LE: provide help to autoantibody secreting B cells, assist in recruitment of other cells to the site of inflammation, enhance the killing properties of phagocytes and CTLs [174]. Presence of $CD4^+$, $CD8^+$ and granzyme B or Tia1 positive cells was demonstrated in the CLE lesions [90].

Importantly, pDCs may divert the differentiation of $CD4^+$ cells to the Th17 phenotype in the presence of nucleic acids containing ICs *in vitro* [179]. Activated T cells of Th17 phenotype might contribute to the injury of the target organ by attracting and activating neutrophils. Th17 cells can stimulate B cells to produce more autoantibodies that will form immune-complexes with the cell constituents released from the dead cells and

may thus amplify the vicious proinflammatory circle [172]. Th17 axis seems to be of major importance in an animal model of lupus-like disease that develops in a genetically modified mouse lacking Ro52. Disruption of the Th17 pathway provided a complete protection from systemic autoimmunity, otherwise developing after minor skin injury in this model [131].

The immune system has developed mechanisms to downregulate the immune response when the pathogen is destroyed. Activated T cells can be suppressed by regulatory T cells (Tregs) that are defined by a $CD4^{+}CD25^{high}$ phenotype and expression of transcription factor FoxP3 [77]. These cells are induced by TGFβ and IL-2 and secrete the signature cytokines IL-10 and TGFβ [77]. Reduced numbers of Treg cells is the characteristic feature of the most autoimmune diseases. Decreased Treg numbers correlate negatively with disease activity in SLE patients [180]. Moreover, functions of these cells are impaired during SLE flares due to so far unknown reasons, but are restored during SLE remission [181]. Numbers of Tregs are reduced in the skin lesions of CLE when compared to other inflammatory skin diseases [182]. Aberrant function and/or low numbers of Tregs could partly explain hyperactivity of other types of T cells in LE.

3.5.3 B cells and LE

Presence of autoantibodies is the hallmark of SLE and this fact emphasizes the importance of B cells in pathogenesis of this disease [2]. B cell-produced autoantibodies lead to formation of ICs that are deposited in the target organs and induce inflammation and tissue damage [183]. B cell homeostasis seems to be aberrant in SLE patients: the circulating B cells are generally more activated as they express more costimulatory markers. The typical B cell profile in SLE is expansion of immature and memory B cells and plasma cells [183]. B cell activation factor BAFF/BLyS is important for B cell maturation, activation and differentiation and is produced by phagocytes or stromal cells of secondary lymphoid organs. Increased levels of BAFF are detected in the sera of lupus patients [184]. In an animal model, mouse transgenic for BAFF, but lacking functional T cells, still develop lupus-like disease due to the activation of B cells [185].

The important role of B cells is confirmed by using a B cell-depleting agent, Rituximab that can successfully induce remission in a substantial proportion of SLE patients with vital organ involvement [186]. In addition, a new compound affecting B cell activation via blockade of BAFF/BlyS has been recently approved for treatment of SLE patients and is on its way to the market [187].

In conclusion, this short overview of the major players of the immune system demonstrates the complexity of the autoimmunity in lupus. More research is still needed to understand why and how attack against self occurs.

4 AIMS OF THE THESIS

Lupus erythematosus (LE) is a prototype systemic autoimmune disease defined by abnormal activation of the immune system against ubiquitously expressed intracellular proteins and production of autoantibodies directed against them. Skin is an organ commonly targeted by the autoimmune inflammation in LE. Many of LE patients are photosensitive and exposure to the sun can trigger development of CLE lesions. A proportion these patients display anti-Ro52 autoantibodies. CLE lesions can also be induced using artificial sources of UVR. Skin is an easily available organ for biopsy acquisition and combined with experimental photoprovocation provides a valuable opportunity to investigate the cellular and molecular events occurring during CLE lesion development.

This thesis aimed to define molecular events occurring in the skin during development and healing of UVR-induced CLE lesions, and also to investigate if the autoantigen Ro52 is expressed in the CLE target organ and to define what molecules modulate its expression.

The specific aims were:

- To assess if a standardized photoprovocation protocol is a suitable and reproducible method for multicenter phototesting studies of CLE patients

- To investigate the dynamics of cytokine expression in UVR-induced developing and healing CLE lesions

- To investigate if the autoantigen Ro52 is expressed in the CLE target organ and to explore the dynamics of its expression in UV-induced developing and healing lesions

- To investigate what biological factors could modulate the expression and cellular localization of Ro52

5 RESULTS AND DISCUSSION

5.1 PHOTOPROVOCATION IN CLE: A MULTICENTER STUDY

5.1.1 Rationale, background and methodological considerations

Sun exposure can trigger CLE and artificial UVR sources can be utilized for experimental induction of such lesions. Experimental photoprovocation is a valuable scientific resource allowing study of the pathogenesis of UVR-induced CLE [17, 188]. The investigator can follow the clinical symptoms of the developing lesions and acquire skin biopsies for the exploration of the ongoing cellular and molecular processes. In clinical practice, experimental photoprovocation might be utilized for verification of the reported or suspected photosensitivity and in order to differentiate CLE from other photodermatoses [189].

The very first attempts to artificially induce CLE lesions using UVR were described in 1929 by Fuhs (reviewed in [11]). Photoprovocative studies were performed and described by several research groups [188, 190]. Their results allowed important advances in understanding the prevalence of photosensitivity among different CLE subtypes and importantly, cellular and molecular aspects of UVR role in the pathogenesis of this disease. Photoprovocation is a complex procedure. Several methodological aspects must be taken into account when planning a study to allow maximal benefit of this rewarding, but resources-demanding procedure [18, 188, 190-192].

The photoprovocation procedure

Photoprovocation is a time-consuming procedure and requires multiple visits to the unit. The obligatory points of a common protocol include: recording patient's history and general clinical assessment followed by threshold phototesting with UVB and/or UVA on day 1; readout of MED and MTD (*please see below for more details*), and estimation of the UVR dose to be used, as well as the first photoprovocation on day 2; on days 3 and 4 subsequent photoprovocations are performed, with the same or different UVR dose, as initially decided (***figure 5***). Furthermore, the irradiated area is

assessed clinically on every other-to every fourth day, up to 30 days. Importantly, the follow-up must be longer than 3 weeks since CLE lesion development usually occurs with a certain delay [188].

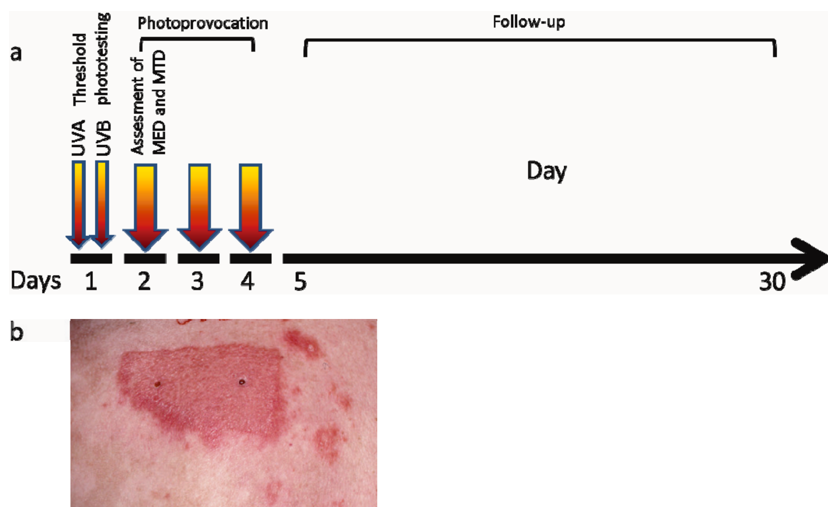

Figure 5. (a) An example of a schedule for a photoprovocation procedure. (b) An example of an experimentally UVR-induced CLE lesion (from paper II, figure 3).

MED and MTD

Sensitivity to UVR is a complex feature and differs between individuals, partly due to the skin phototype [193]. Individual sensitivity to UVR can be assessed by estimating minimal erythema dose (MED) and/or minimal tanning dose (MTD). MED is actually the minimal UVB dose inducing sunburn. MED is defined as the lowest dose of UVB that induces barely perceptible erythema with well-distinguished edges [193]. In practice, MED is assessed by irradiating small skin areas with increasing doses of UVB and determined after 24 hours according to the above described definition. MTD (sometimes denoted minimal persistent pigment darkening (MPPD)) dose is determined accordingly, but for UVA. MTD is defined as the UVA dose that induces skin pigmentation with clear demarcation [188, 190]. MED and MTD are usually estimated before the start of experimental photoprovocation. The determined MED and MTD are used to calculate the UVA and UVB doses to be applied in the study.

Dosing of UVR

CLE-resembling lesions can be reproduced using UVB, UVA or a combination of both [188, 191]. The UVR dose to be used should be calculated by the estimated MED and/or MTD, and usually 2- or 3- fold MED, and/or 1 MTD that is applied [188, 190, 191]. Doses of UVA needed to induce lesions are much higher than those of UVB since UVA has less energy. The majority of published reports indicate that in order to induce a CLE-resembling lesion photoprovocation must be performed on two or three subsequent days, since single exposure seldom leads to lesion development [188, 194, 195]. It has been argued that provocation with a combination of UVA and UVB is the most relevant to use as it resembles the spectrum of radiation emitted by the sun.

Table 5

A proposal on a standardized protocol for UVR photoprovocation

Criteria	Proposal and motivation
Choice of subject	SLE patient preferentially in remission or low disease activity No need to involve only patients with a history of photosensitivity Preferentially no systemic medication for the cutaneous disease during the whole photoprovocation and follow-up
Season	Preferentially not in summer, due to a risk for influence of outdoors sun
UVR source	Combined UVB and UVA lamp that emits radiation close to that emitted by the sun on a sunny summer day
Pretesting	Determination of MED and MTD
Dosage	Calculated as assessed by MTD and MED. Usually 75-100 J/cm^2 of UVA and 1.5 MED of UVB on 3 consecutive days
Anatomical location	Preferentially upper back, since this is the location where spontaneous lesions frequently occur but also upper back has lower risk for daily sun exposure and is associated with less cosmetic discomfort after the provocation is over
Size of test area	At least 4x5 cm, as photoprovocation on smaller fields is often associated with negative results
Evaluation and follow-up	Daily on day 1, 2, 3 and then every 3-4 days up to 4 weeks after the last irradiation
Criteria for a positive test	Skin lesions resembling CLE in their clinical appearance and histopathological changes

Anatomical localization and size of the area

Spontaneous lesions predominantly occur in the skin areas frequently exposed to the sun such as face, ears, neck, décolleté, upper back, extensor aspects of the arms and forearms as well as dorsal aspects of the hands. Most investigators choose the upper back or extensor aspects of overarms for provocative irradiation due to practical and cosmetic reasons [17, 188]. Investigators who performed photoprovocations on other anatomical locations could hardly induce CLE lesions [194, 195]. The size of the photoprovoked skin area must be at least 4-6 cm x 5-10 cm in order to induce CLE lesions [18, 188, 190, 191, 194, 196].

The most important aspects of the photoprovocation procedure are summarized in ***table 5*** [17, 63, 188, 191, 194, 197].

5.1.2 Multicenter photoprovocation study

In paper I we aimed to evaluate if photoprovocation is a reproducible method to assess photosensitivity in CLE patients. In study I we included 47 CLE patients (14 with SCLE, 20 with DLE, 13 with LET) and 13 healthy volunteers. None of the patients filled the ACR criteria for SLE. All subjects underwent photoprovocation at 7 European sites (Sweden, Germany, Scotland and Poland). On day 1 all subjects underwent threshold testing with UVA and UVB irradiation. On day 2 MTD for UVA and MED for UVB were estimated for each subject. Thereafter, each subject was irradiated daily on uninvolved skin on the upper back with their MTD (UVA) on day 1 followed by 1.5 MEDs (UVB) on day 2 and 3. UVR exposed areas were assessed for LE-specific skin lesions on days 2-32. Twenty-two (47%) CLE subjects (57% of SCLE, 35% of DLE and 54% of LET patients) and none of the healthy volunteers developed clinically CLE resembling lesions (paper I, figure 2). Nineteen (86%) of these lesions had histopathological changes compatible with CLE. Fitzpatrick's phototype I or II were more common (86% vs. 52%) among CLE subjects who developed lesions and they also had significantly lower mean MED ($p=0.004$) (paper I, figure 1, table 1 and 2). The majority (93%) of all included patients were positive for ANA. Prevalence of anti-dsDNA was low (14%). Anti-Ro/SSA positivity was detected in 71% of SCLE patients, 21% of DLE patients, but none of LET patients had these autoantibodies. CLE subjects who developed lesions,

had twice as long time from their last CLE flare when compared to those who were non-responders (9.5 vs. 5 months) (paper I, table 2). Eight CLE patients were treated with antimalarials and continued the treatment during the study. Only 2 (25%) of these subjects developed lesions, in comparison to 20 (51%) responders in the systemic immunomodulatory medication-free group. Nineteen of those 20 subjects who were not exposed to anti-malarials and developed lesions were smokers (95%). In the non-responder group smoking was less prevalent (68%) (paper I, table 1).

The number of participants reporting adverse events (AE) were highest among CLE lesion-positive subjects (59%), followed by lesion-negative patients (36%) and controls (31%) (paper I, table 3). Most of the AE were negligible. Seven CLE patients developed CLE-associated AE. Four of those were photoprovocation-positive and 3 photoprovocation-negative subjects. In 4 patients changes in their pre-existing lesions were observed. New CLE lesions, developing outside the irradiated area, were noted in 3 study subjects.

There was no significant difference in photoprovocation results between the study sites and no clinically significant differences in safety were observed between CLE subjects and healthy volunteers following the photoprovocation.

5.1.3 Discussion

In study I we present results of a multicenter photoprovocation study in which 22 CLE patients (47%) developed UVR-induced skin lesions clinically resembling CLE. The patients with the diagnosis of SCLE had the highest rate of positive result (57%), followed by LET (54%) and DLE (35%). The previously reported rate of overall positive results (all CLE subtypes as a group) varied between 25-93%. Among the subtypes of CLE, these proportions ranged following: 25-85% for SLE/ACLE, 50-100% for SCLE, 10-64% for DLE/CCLE and 50-76% for LET/ICLE [17, 188, 191, 196, 197]. The variations in the results reported by these studies most probably depend on the natural photosensitivity of the included subjects but also some methodological differences. Investigators who used 'high intensity' protocols (e.g. increased the UVR doses during the ongoing study and performed irradiations on more than 3 following occasions) reported the highest rates of positive results 83-93% [191, 196]. It is of importance that prevalence of the photosensitivity varies

among CLE subtypes and the numbers of the included patients with different CLE subtypes must be taken into account when comparing these results in general. SCLE and LET are the most photosensitive entities as previously reported and confirmed by our results [17, 31, 189].

The observation that those CLE subjects who had a relatively recent flare less often developed a positive phototest result is interesting and has not been reported before. It could be the case that those who had a recent flare and had been on systemic medication that was discontinued before the study start, still had a suppressed immune system and therefore were protected from the effects of UVR. This explanation is very probable, since the half-life of hydroxychloroquine, which had been used by some patients before the study start, is more than 40 days [198].

Several investigators reported that CLE patients are more susceptible to sunlight in general as assessed by MED. Our results confirm this finding, especially in the phototest-positive DLE and LET patients (paper I, table I) [17, 195, 197]. SCLE is the most photosensitive subtype according to our data, but interestingly these patients had higher MED than the two other groups (paper I, table 2). Evidently, there are some other factors of importance that determine the lesion development after exposure to UVR in this subgroup of patients. The most distinct feature of SCLE is the high prevalence of anti-Ro/SSA autoantibodies (71% in our group). The available data indicate that these autoantibodies are indeed pathogenic in the induction of clinical manifestations of NLE, including SCLE-resembling lesions [199, 200]. The risk of NLE manifestations wanes after 6 months of age as maternal IgG, and also anti-Ro/SSA autoantibodies, are cleared from circulation. These facts strongly suggest that these autoantibodies could also be associated with the UVR induced SCLE lesions in adult patients. Several studies reported that patients who carry anti-Ro/SSA autoantibodies develop a pathological reaction to photoprovocation more often [4, 5, 17, 188]. We could however not confirm this association in our study group at a statistically significant level, most probably due to a smaller sample size.

The history of smoking was associated with a positive phototest result in the patient group who were not on any immunomodulatory systemic medication (95% smokers in the phototest positive group, vs. 68% smokers in non-responders group) (paper I, table I). It was previously reported that smoking is associated with CLE and

especially in those with complement deficiencies [33, 72]. Interestingly, smokers usually have lower anti-Ro/SSA autoantibody levels. Tobacco smoke could therefore act as an independent trigger of CLE without the necessity of autoantibodies [24].

Our study included 8 individuals who were treated with antimalarials and 2 of them (25%) developed CLE lesions after photoprovocation. The rate of positive phototest was 51% in the group of CLE subjects without any systemic medication. These findings suggest that antimalarials could be protective in this context (25% of vs. 51%), but does not supress this risk completely. There were two CLE subjects who developed lesions despite treatment with antimalarials and they both were smokers. Our sample size was small, but it was previously reported that CLE diagnosed smokers have less good responses to anti-malarial treatment and maybe, also less protection from photosensitivity [73]. Importantly cessation of smoking may re-establishe sensitivity to anti-malarials and counseling on smoking cessation is therefore of major importance [33, 73].

Our study included only CLE subjects with a history of photosensitivity, but previuos reports indicate that CLE lesions can be induced in 58% of patients who negate history of photosensitivity [188]. Investigators should therefore not be discouraged from inclusion of such patients in the experimental phototesting studies.

Approximately half of the included subjects reported AE, but most of them were negligible (paper I, table III). Seven patients developed CLE-associated AE, 4 of them were photoprovocation-positive and 3 non-responders. In 3 of them these included small clinical changes in the preexisting lesions. In one subject the changes in the previous lesion required topical therapy. Three patients developed new lesions outside the irradiated area. Low rate of AE with minor or moderate severity and similar rates between responders and non-responders to the phototest (4 vs. 3) indicates that CLE-associated AE were most probably due to the natural course of the disease. None of the subjects developed symptoms compatible with SLE diagnosis in our study, in which only CLE patients were included. No cases of exacerbations of systemic disease were reported in those photoprovocation studies that included subjects with SLE [17, 190, 196, 197]. One negative aspect of the photoprovocation is that the induced lesion may heal leaving temporary hypo- or hyperpigmentation associated with certain cosmetic discomfort [188].

5.1.4 Concluding remarks and future perspectives

Our results indicate that photoprovocation is a reproducible and safe method with overall positive results in approximately 50% of the participating patients and is a suitable tool for utilization in multicenter studies and clinical trials.

Much of the available knowledge about the pathogenesis of sun-induced CLE comes from such studies ([59, 63, 201] and paper II). To understand the pathogenesis of CLE, it is of major interest to delineate the primary and subsequent biological events occurring in the skin post-UVR exposure. An additional method that could provide direct insight into the biological processes occurring in the skin following UVR is microdialysis, which is already used in the field of dermatology and is less invasive than skin biopsy acquisition [202, 203]. Sample acquisition via a microdialysis catheter inserted in the skin prior, during and directly after UV irradiation could allow investigation of the biological processes taking place in real time. Recognition of these processes would provide a better insight into the primary events in the irradiated skin and could guide the development of future sun-screens and medications.

5.2 THE ROLE OF HMGB1 IN UVR-INDUCED CLE

5.2.1 Rationale, aims and methodological approach

Our group has previously demonstrated that HMGB1 is expressed at the site of local inflammation in spontaneous lesions of CLE patients, and that it is accompanied by strong upregulation of other cytokines such as TNF-α and IL-1β [84].

In study II we therefore aimed to define the relation between the expression of HMGB1, TNF-α and IL-1β and the clinical activity of developing and healing CLE lesions induced experimentally by UVR. For this study we used a collection of sequential skin biopsies obtained during a photoprovocation study described previously by Nyberg *et al*. [17, 18]. For the current study sequential biopsies from 9 CLE patients and 2 healthy controls were available. Skin specimens were sectioned and stained using immunohistochemistry (IHC) with rabbit polyclonal anti-HMGB1, mouse monoclonal anti-TNF-α and anti-IL-1β antibodies (Abs). The expression of cytokines was assessed

by two investigators in a blinded semi-quantitative manner validated previously [84]. During assessment the skin section was divided into two different observational areas: epidermis and dermis. The distribution of HMGB1 was assessed in three different compartments: the cell nucleus, cytoplasm, and extracellular space. Slides stained for TNF-α and IL-1β were assessed for the proportion of stained cells within the dermal infiltrate, since no or very little expression in the epidermis was noted.

We chose to perform a manual analysis of the immunohistochemical stainings and did not use a computerized image analysis, even though it might be a less biased method for some purposes. The aim of our study was to analyze the subcellular localization of the protein of interest (HMGB1 in study I and Ro52 in study II), and there was no available software that could differentiate between the nuclear, cytoplasmic and extracellular staining. Therefore we chose a manual method and slides were assessed in a blinded manner by two investigators. The assessment results of each section were compared between the observers and if the difference was greater than 20% such slides were discussed and reassessed until the values were agreed upon within the variation of 20%. This method was utilized in previous studies published by our group [84]. The staining of TNF-α and IL-1β was assessed by manually estimating the proportion of the stained cells. Other investigators reported that such manual assessments provide a satisfactory correlation with the results of computerized image analysis [204].

5.2.2 Results

In paper II we demonstrate that HMGB1 was expressed weakly in unaffected skin with predominant nuclear localization in the basal and adjacent layers of epidermis and skin appendages, and in the endothelia within the dermis. In the active CLE lesions up to 50% of epidermal cells and more than half of the cells comprising dermal infiltrates stained positively for HMGB1. Furthermore, an increase in the dermis infiltrating cells positive for cytoplasmic HMGB1 was observed in the active lesions. These numbers decreased as lesions faded (coefficient of concordance (c.c.) = 0.75, $p<0.05$). Translocated extracellular HMGB1 was detected in both dermis and epidermis in the active CLE lesions and cleared in healing lesions (c.c. = 0.80, $p<0.05$ and c.c. = 0.48, $p<0.005$, respectively) (***figure 6*** and paper II, figure 2 and 3 and table 1).

Interestingly, in a few patients for whom early biopsies were available, an increase in HMGB1 expression and transfer from cell nucleus was noted early in the course of lesion development, while the highest expression and extracellular deposition corresponded to the clinically most active inflammation. Additionally, some extracellular HMGB1 was still observed in the healing lesions (***figure 6*** and paper II, figure 2).

The proportion of IL-1β positive cells detected in the most active lesions varied substantially among individuals. Unexpectedly, the highest expression of IL-1β was observed in the healing lesions. TNF-α expression was upregulated in the active lesions of 6 CLE patients, but the other 3 out of 9 investigated (33%) patients displayed almost no TNF-α in the biopsies acquired from active lesions. In the healing lesions, we did not observe any particular pattern of TNF-α expression, since individual variation of expression was substantial (***figure 6*** and paper II, figure 4).

In healthy individuals up to 30% of all epidermal cells stained positively for nuclear HMGB1. The nuclear staining decreased in abundance and a weak cytoplasmic staining was observed in the epidermal cells, but no or little extracellular HMGB1 was detected in the biopsies acquired 3 days after the last exposure to UVR. Photoprovocation resulted in the appearance of some IL-1β positive cells in the dermis, but presence of TNF-α was not detectable (paper II, figure 5).

We also tested whether HMGB1 complexed to LPS could induce Ro52 upregulation or translocation in primary human keratinocytes *in vitro*, but no such changes were observed (unpublished preliminary results) using the conditions described by others [109].

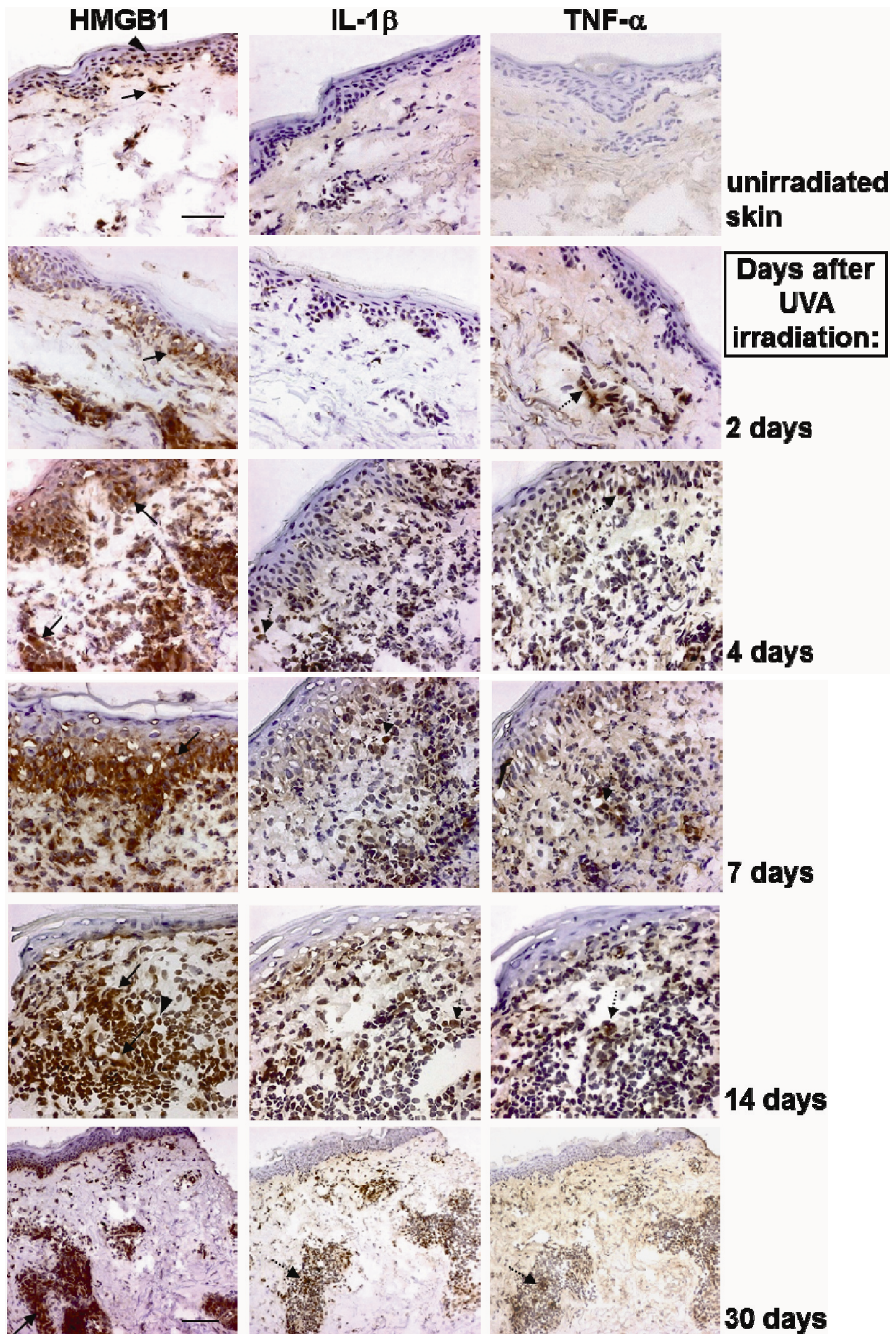

Figure 6. HMGB1, IL-1β and TNFα expression patterns in unaffected and photoprovoked skin.

5.2.3 Discussion

In paper II we demonstrated that increased numbers of keratinocytes express HMGB1 in the cell cytoplasm in the clinically most active CLE lesions. It might be the case that UVR itself or cytokines induced by it caused this induction [102]. It was previously reported that it is mainly activated cells that increase synthesis of HMGB1 and translocate it to the cell cytoplasm, which subsequently leads to HMGB1 secretion [99, 205]. Our results suggest that keratinocytes can upregulate HMGB1 in response to a proinflammatory stimulus. The observed increase in extracellular HMGB1 within the epidermis invites a hypothesis that epidermal keratinocytes might be able to actively secrete HMGB1. In addition, extracellular HMGB1 observed in the basal and adjacent layers of the lesional epidermis, might have been passively released. UVR induces apoptosis of keratinocytes and clearance of these cells is impaired in a substantial proportion of LE patients [59, 60] and remarkably, extracellular HMGB1 may also contribute to impaired removal of apoptotic cells via binding to phosphatidylserine and impeding recognition of apoptotic cells [206]. Non-removed apoptotic keratinocytes most probably undergo secondary necrosis and passively release HMGB1 bound to chromatin [60, 95, 115]. Consequently, the extracellular HMGB1 observed in the epidermis of CLE lesions might be a product of activated keratinocytes and secreted as cytokine and/or passively released from secondary necrotic cells.

An increase of cells with cytoplasmic HMGB1 was observed in the dermis of the most active CLE lesions. In parallel, staining for extracellular HMGB1 expanded. Previous studies indicated that activated macrophages/monocytes are the cells producing this cytokine in rheumatoid arthritis synovia [207]. $CD68^{+}$ macrophages are present among other dermis infiltrating cells in CLE lesions [90] and it is therefore probable that the cells with strong cytoplasmic HMGB1 expression are activated mononuclear phagocytes readily secreting HMGB1.

In summary, we suggest that the extracellular HMGB1 observed in CLE lesions might have been actively secreted by keratinocytes or mononuclear phagocytes. In addition, a portion of the observed HMGB1 might have been released from secondary necrotic keratinocytes.

Extracellular HMGB1 may act as a proinflammatory cytokine and further amplify the production of other cytokines, such as TNF-α, IL-1β and IL-6, by mononuclear phagocytes [102]. In addition, cells respond to the presence of these mediators by further enhancing HMGB1 synthesis and secretion [101]. This self-escalating proinflammatory loop of the cytokine induction might be consistent with the extended pro-inflammatory response to UVR which is evident in CLE patients. Interestingly, IL-1β expression was not that extensive in the most active UVR-induced lesions as previously observed in the spontaneous CLE [84]. This could reflect a biological difference in the nature of UVR-induced transient and the longer-lasting spontaneous lesions, but might also indicate that HMGB1 is a more important factor in the development of CLE lesions. Subsequently to our publication it was discovered that HMGB1 builds complexes with IL-1β [109]. It is possible that we have underestimated detection of this cytokine by IHC using monoclonal antibodies since the specific epitope might have been hidden in the IL-1β-HMGB1 complexes. In our study, TNF-α expression was increased in more than half (66%) of patients with active lesions, but almost totally absent in the additional three. This finding is not consistent with previously reported TNF-α upregulation in all spontaneous CLE lesions. Our finding that HMGB1, but not TNF-α or IL-1β upregulation mirrors the clinical activity of the UVR-induced lesions suggests that HMGB1 might really be an important mediator of inflammation in CLE [84].

HMGB1 released from secondary necrotic cells is highly immunogenic and was demonstrated to incite autoimmunity in experimental settings [95, 115]. HMGB1-nucleosome complexes released from secondary necrotic cells might induce maturation of antigen presenting cells [115] and, when opsonized by circulating immunoglobulins, stimulate IFNα secretion in pDC [208]. Moreover, such ICs were demonstrated to induce autoreactive B cell activation and anti-dsDNA autoantibody production in a mouse model [115, 116]. As deposition of ICs is a common finding in CLE at the dermo-epidermal junction (DEJ) it is therefore possible that extracellular HMGB1 observed in this zone forms such complexes: HMGB1-nucleosomes-Ig.

We have also observed the presence of extracellular HMGB1 in the late CLE lesions. HMGB1 has important functions in cell migration and chemotaxis and might therefore be involved in the healing processes [102]. The functional HMGB1 receptors RAGE, TLR4, TLR5 and TLR9 are expressed on keratinocytes and phagocytes within the skin,

and HMGB1 can be expected to exert its chemotactic and pro-inflammatory functions locally [209, 210].

5.2.4 Conclusion

To conclude, in paper II we demonstrate that HMGB1 is strongly upregulated and translocated in UVR-induced CLE lesions, and that the highest expression coincides with the peak of the clinically activity of these lesions. High proinflammatory activity of HMGB1 alone or in complexes with other molecules, might play an important role in the induction and amplification of the inflammatory processes taking place in the UVR injured skin of CLE patients. In addition, HMGB1 observed in the late lesions might have a role in the process of healing. The role of TNF-α and IL-1β seems to be of less importance in this setting.

5.3 Ro52 IN LE

5.3.1 Rationale, aims and methodological approach

Since Ro52 is a common target of circulating autoantibodies in lupus it is of major interest to understand which tissues and cells within them express this protein and under what conditions, as well as what cellular function the protein has and how it can become an antigenic target.

Attempts to investigate the expression of Ro52 in different organs have been made, but these studies have relied on the detection of mRNA or use of human autoimmune sera [122, 138, 211-213]. Monoclonal autoantibodies (mAbs) to Ro52 were lacking and specific anti-sera raised by immunization with the antigen have been difficult to obtain due to the folding properties of Ro52 [121, 214]. Even if sera is claimed to be monospecific there is an obvious risk of contaminating specificities. Attempts to affinity purify against Ro52 have proven difficult due to the folding properties of the protein and may also be associated with loss of antigenic reactivity [121]. After careful biophysical characterization of Ro52 [121, 214], our group was successful in generating mouse hybridomas producing a panel of anti-human Ro52 mAbs [137]. In paper III we aimed to confirm the specificity of the selected hybridomas and to investigate Ro52 expression both in spontaneously occurring inflammatory skin

diseases as well as UVR-induced CLE. As a tool to detect Ro52 we utilized a panel of four selected mAbs: one mAb recognizing a Zinc finger motif and three mAbs detecting different peptides within the CC domain [137].

First we confirmed the specificity of the mAbs by Western blot and ELISA (paper III, figure 1). We preferentially intended to use formalin-fixed paraffin embedded skin biopsy material, since this method is superior in preserving morphology. A problem associated with this fixation method is the crosslinking of antigenic epitopes by hydroxy-methylene bridges [215]. Consequently, antigenic epitopes become inaccessible to mAbs. A solution for this problem is antigen retrieval (AR). We tested several AR protocols. The AR was performed in citrate buffer at pH 6 in all experiments. The explored AR conditions included heating in the microwave for 5-10 min three times and changing buffer between the heating procedures. This method gave inconsistent results, probably due to the fact that microwave heats the fluid in a non-homogenous fashion. Heating on the stove provided easier controllable conditions and AR was performed at 95-100 °C. Different durations of heating were tested: 10 min, 20 min and 40 min. Active cooling by immersing the specimens in PBS at 24°C was tested against passive cooling in the buffer of retrieval, until 24° C was attained. Cooking in citrate buffer at pH 6 at 95-100° C for 40 min followed by passive cooling provided the best and the most consistent results and was utilized for AR of the whole sample of paraffin embedded skin specimens. After the AR method was established, the suitability of four mAbs for immunohistochemistry was determined using different methods for tissue preservation and fixation. As indicated in figure 2 of paper III all four tested mAbs provided similar staining patterns in paraffin embedded, as well as in fresh-frozen, acetone fixed samples. Pre-incubation of autoantibody with the full length Ro52 overnight resulted in loss of the staining in IHC and detection in ELISA (paper III, figure 1).

5.3.2 Results

After we had developed the IHC protocol and confirmed that the staining pattern was the same with all four anti-Ro52 mAbs, we utilized one anti-Ro52 mAb recognizing a sequence within the CC domain of Ro52 to investigate the expression of this protein in CLE and other inflammatory skin diseases. We also aimed to determine whether UVR could modulate expression of this autoantigen.

Ro52 was upregulated in all CLE lesions (paper III, figure 2 and 3). Ro52 expression was increased in the epidermis of spontaneous CLE lesions as compared to control biopsies from unaffected skin of the same individual or healthy controls (paper II, figure 3). About 80% of the cells in the dermal infiltrates were also positive for Ro52. In uninvolved skin of patients and healthy controls Ro52 was mainly expressed in the epidermal cell nuclei, cytoplasmic expression being weak (paper III, figure 3). Upregulation of cytoplasmic Ro52 was observed in the epidermal cells of both spontaneous and UVR-induced CLE lesions (paper III, figure 3 and 5). Interestingly, in a proportion of patients we could observe an intense staining for Ro52 in the basal cell layer (paper III, figure 2 and 3). Moreover, UVR exposure induced Ro52 upregulation in human keratinocytes *in vitro* within 24 h (paper III, figure 6) as demonstrated by immunocytochemistry and qPCR. The staining revealed that Ro52 was predominantely located to the cytoplasm of the cultured cells and UVR did not influence its subcellular localization.

Importantly, strong Ro52 expression was observed in all CLE lesions investigated (ACLE, SCLE, DLE) independently of CLE subtype and presence or absence of anti-Ro/SSA autoantibodies. Unexpectedly, relatively similar upregulation of Ro52 was detected in the epidermis and dermal infiltrates of LE-nonrelated inflammatory skin diseases such as psoriasis, atopic eczema and lichen planus (paper III, figure 4).

5.3.3 Discussion

We observed a strong upregulation of Ro52 in the cytoplasm of epidermal cells in both spontaneous and UVR-induced CLE skin lesions. Intensive staining for Ro52 was observed in the basal and adjacent keratinocytes in a proportion of patients. This zone is a common target of autoimmune attack and that is where the most of apoptotic keratinocytes reside [14]. We also demonstrated that keratinocyte exposure to UVR upregulate Ro52 expression *in vitro*. The biological mechanism underlying this event is not known. Since Ro52 is an IFN-inducible protein and UVR exposure has been demonstrated to activate IRF7 in other cell types, it is therefore possible that a temporary IFNα production occurs, which could subsequently lead to the induction of Ro52 transcription [134, 137, 216]. Increased levels of Ro52 might be needed to

terminate the UVR-induced cutaneous inflammation, as Ro52 was demonstrated to act as a negative regulator of inflammation [129, 131].

The expression of Ro52 is upregulated in CLE lesions and, in parallel, many signs of ongoing cutaneous inflammation are present in parallel, including upregulation of IFN-inducible proteins [63, 64]. One could speculate that Ro52 is incapable to act as a negative feedback regulator in CLE/LE patients. Genetic polymorphisms in the Ro52 gene have been associated with SLE and anti-Ro52 autoantibody production in SS, although it is not known whether these polymorphisms affect the quality or levels of Ro52 expression [45, 46]. Interestingly, our group has recently demonstrated that patient-derived anti-Ro52 autoantibodies directed against the RING domain of Ro52 inhibit its E3 ligase activity *in vitro.* The inhibition occurs through steric hinderance by blocking the access of the E2 to its binding site in the RING domain (Espinosa *et al*, submitted). It is therefore possible that Ro52 autoantibodies do interfere with the function of their target *in vivo*. Alternatively, another molecule involved in the Ro52 ubiquitination pathway could be aberrantly expressed in lupus patients and impede Ro52 function (for example IRF5 and polymorphisms in this gene have been identified in SLE and CLE patients) [42, 217].

UVR-induced upregulation of Ro52 might also contribute to the anti-Ro52-directed autoimmunity. Forced expression of Ro52 in a lymphoma-derived B cell line leads to a decreased rate of proliferation and increased susceptibility to activation-induced cell death [123]. The increased Ro52 expression in the basal keratinocytes of CLE lesions could therefore, at least partly, account for the high numbers of apoptotic cells typically found by histopathology [14]. Several investigators have reported that keratinocytes translocate Ro52 (accompanied by other common LE autoantigens) to the apoptotic blebs after exposure to UVR *in vitro* [65-67]. A hypothesis was therefore suggested that this is how the autoantigen Ro52 could be exposed to the immune system. As a substantial part of LE patients have defects in removal of unviable cells [60, 156], we assume that Ro52 might be also released by secondary necrotic cells and importantly, the increased Ro52 production in the basal keratinocytes just before the cell death could contribute to an overwhelming load of this autoantigen. Presence of Ro52, together with other highly proinflammatory molecules (eg HMGB1-nucleosome complexes, cytokines ([115] and paper II)) in the surrounding, could therefore facilitate maturation of the professional APCs, uptake of the autoantigen Ro52 and its presentation to the

anergic or low-reactive T and/or B cells. Subsequently, this mechanism could lead to the induction of the adaptive immunity against Ro52 in a genetically susceptible individual.

Interestingly, James *et al*. have proposed a hypothesis on how anti-Ro52 autoantibodies might contribute to the deposition of ICs in the target organs and impede their clearance [135]. They suggested that extracellular Ro52, for example released from secondary necrotic cells, could bind Fc part of any locally available IgG. In patients with anti-Ro52-positive disease such Ro52-IgG complex could be additionally bound by autoantibodies against other epitopes of Ro52 and thus lead to formation of huge ICs [135]. Authors suggest that such ICs could be difficult to recognize via FcγR or complement receptors and they could account for ICs deposits observed in CLE skin lesions and lupus nephritis [36, 218].

We found equivalently strong Ro52 expression in the skin lesions of all CLE subtypes (ACLE, SCLE and DLE) investigated independently to their anti-Ro52 autoantibody status. Moreover, corresponding Ro52 upregulation was also observed in the skin lesions of other LE-unrelated inflammatory skin diseases. These findings prompt a hypothesis that Ro52 has a more general regulatory role in the inflammation, but function of this protein is probably impaired in at least a subgroup of LE patients. This assumption is further supported by the recent report on an animal model in which lack of Ro52 leads to an uncontrollable inflammatory response to a minor skin injury that subsequently advances to the development of lupus-like disease [131].

Approximately 80% of the dermal infiltrates comprising cells stained positive for Ro52 [219]. It is known that a substantial part of these cells are activated $CD4^+$ and $CD8^+$ T cells and macrophages [90]. All these cells are able to express Ro52 [123, 131]. High Ro52 expression in macrophages and T cells has been demonstrated have dual effects: could enhance their proinflammatory properties, such as production of IL-12p40 and IL-2 respectively, but also negatively regulate their activation [128, 220].

5.3.4 Conclusion

To conclude, our findings suggest that UVR can upregulate Ro52 expression in the CLE target cell keratinocyte. High Ro52 expression might sensitize keratinocytes for

cell-death inducing impulses and subsequently lead to an overwhelming load of this autoantigen upon secondary necrosis. Equivalent upregulation of the Ro52, lupus autoantigen, in LE-nonassociated inflammatory skin diseases indicates that Ro52 has a more general role in the regulation of cutaneous inflammation and high Ro52 expression is not specifically associated with the presence of anti-Ro52 autoantibodies. Despite high expression levels, Ro52 may have impaired capability to act as a negative feedback regulator in LE patients. Increased IFNα production observed in many LE patients could be one of the negative consequences of the non-functional Ro52.

5.4 NO MODULATES THE CELLULAR LOCALIZATION OF Ro52

5.4.1 Rationale and aims

Nitric oxide (NO) is a small and short-lived gaseous molecule belonging to a class of molecules termed reactive oxygen/nitrogene species (ROS) [58]. Keratinocytes constantly produce low levels of NO, but production can be enhanced by exposure to stress factors such as UVR [58]. This occurs via induction of the generating enzyme inducible nitric oxide synthase (iNOS) [62]. CLE patients have aberrant timing in iNOS expression in response to UVR [62]. Interestingly, it was previously demonstrated that exposure of cells to another ROS, hydrogen peroxide (H_2O_2) or activating cytokines (IFNα) results in the nuclear enrichment of Ro52 [68, 137]. Ro52 has an E3 ubiquitin ligase activity and interacts with two ubiquitin conjugating enzymes located in the different cellular compartments: UBE2D1 (cytoplasmic) and UBE2E1 (nuclear), as it was demonstrated previously [123, 126]. Little is known about what intrinsic factors influence the cellular localization Ro52. Cytoplasmic localization of several other TRIM family proteins depends on their CC domains and some of them (e.g. TRIM27) are actively exported from the nucleus by Exportin-1 [221, 222].

In study IV we therefore aimed to define what the natural factors that determine subcellular localization of Ro52 are and if NO could modulate subcellular localization of Ro52.

5.4.2 Results

First we confirmed that both ubiquitin conjugating enzymes UBE2D1 and UBE2E1 support Ro52-mediated ubiquitination (paper III, figure 1). Furthermore, we transfected HeLa cells with fluorescent reporter fused Ro52, UBE2D1 and UBE2E1. In these cells Ro52 and UBE2D1 were located in the cell cytoplasm under steady state conditions. UBE2E1 resided strictly in the cell nucleus and was enriched in the nucleoli (paper IV, figure 2). We proceeded by constructing GFP-Ro52 and several deletion mutants of GFP-Ro52 and expressed them in HeLa cells. GFP-Ro52 was located in the cell cytoplasm, while deletion of the CC or leucine zipper domains resulted in the nuclear accumulation (paper IV, figure 3). Deletion of the B30.2 domain in the leucine zipper lacking constructs abolished the nuclear localization. As Ro52 contains a hypothetical leucine rich exportation signal (NES) within the CC domain we therefore wanted to determine if Ro52 is exported from the nucleus via Exportin-1 mediated pathway that utilizes this sequence. Neither addition of leptomicin B, an inhibitor of the latter pathway, nor mutation of the putative NES had any influence on the typically predominant cytoplasmic localization of Ro52 (paper IV, figure 4).

We further explored if NO could modulate subcellular localization of Ro52. Exposure to NO donor DETA-NANOate resulted in a relatively prompt (within 6 h) nuclear accumulation of Ro52 in both primary human keratinocytes and GFP-Ro52 transfected HeLa cells (paper IV, figure 5 and 6a). Importantly, cells expressing GFP-Ro52 with the mutated B30.2 domain retained the protein in the cytoplasm despite cell stimulation with NO. Our results indicate that the CC domain is important for the cytoplasmic retention of Ro52 and B30.3 for its ability to stay in the nucleus.

Since NO displayed the ability to modulate subcellular localization of Ro52 *in vitro,* we proceeded to investigate the expression of Ro52 and iNOS in the skin biopsy material derived from CLE patients lesions. We detected iNOS positive cells in the epidermis and dermal infiltrates (paper IV, figure 6a). In adjacent skin sections cells with nuclear, but also cytoplasmic, Ro52 were present. It is thus possible that NO generated at the site of inflammation can modulate subcellular localization of Ro52 and influence in which cellular compartment Ro52 is operative as E3 ligase.

5.4.3 Discussion

We determined that an intact CC domain, and in particular the leucine zipper within it, is important for the cytoplasmic localization of Ro52. When this region was deleted, Ro52 was translocated into the nucleus. Interestingly, an alternatively spliced transcript, Ro52β that lacks the leucine zipper domain, was reported [223]. The reported genetic polymorphism in the intron 3 within the Ro52 gene is positioned close to one of the splicing sites used for generating Ro52β [45]. This polymorphism is associated with anti-Ro52-positive SS, but has not been investigated in anti-Ro52 associated LE so far. Such a polymorphism could influence the amount of Ro52β produced. If this would be the case, the amount of cytoplasmic/nuclear Ro52 could be expected to shift. As a consequence some of the Ro52 functions might be affected since it operates as E3 ligase both in the cell cytoplasm and nucleus, according to our and other investigators results (paper IV, figure 1 and 2) [123, 129-131].

UVR induces iNOS, leading to NO production in human keratinocytes [52, 147]. NO, generated following skin exposure to UVR, contributes to the sunburn-induced inflammation including keratinocyte death [50, 52, 58]. In UVR-induced, developing CLE lesions the dynamics of iNOS expression has been demonstrated to be completely opposite to that observed in healthy controls: iNOS upregulation is delayed and observed only 2-3 days post-UVR, whereas in healthy individuals induction occurs within 24h and is resolved within 48h post-UVR. Concomitantly to increasing iNOS expression in the skin, numbers of apoptotic keratinocytes increases up to 72h post-UVR [59, 62]. Induced NO might therefore contribute to the increased numbers of apoptotic keratinocytes observed in the CLE lesions. In papers III (figure 3 and 5) and IV (figure 6) we demonstrated nuclear and cytoplasmic staining of Ro52 in the spontaneous and UVR-induced CLE lesions. NO is commonly generated at the site of inflammation and Ro52 is upregulated at the active CLE lesions (paper III, figure 3 and 5 and paper IV). These findings suggest that NO could be an important factor determining subcellular localization of Ro52 and consequently governing in what cell compartment Ro52 is operating as an E3 ligase.

NO is an important mediator of inflammation. It is believed that NO has anti-inflammatory properties at lower concentrations and is one of the essential molecules that can dull the responsiveness of the immune system to apoptotic cells [224]. Pro-

inflammatory cytokines can induce substantial iNOS expression and subsequent NO synthesis, that may have the potential to further amplify inflammation [225]. Macrophages, when exposed to NO, initiate secretion of pro-inflammatory cytokines including HMGB1, TNF-α and IL-1β [226]. Moreover, these cytokines can further fuel inflammation by inducing iNOS and NO production [58, 148, 227, 228]. NO might thus contribute to ignition of a self-fueling pro-inflammatory loop observed in CLE [225] and, at high concentrations could contribute to the increased rate of keratinocyte apoptosis [229].

5.4.4 Conclusion

In paper IV we confirmed that Ro52 is predominately cell cytoplasm-located protein, but can fluctuate between the cell compartments. We demonstrated that the CC domain is important for the cytoplasmic accumulation and B30.2 for nuclear retention. The ability to shuttle between the nucleus and cytoplasm allows Ro52 to operate as E3 ligase and interact with E2s present in both cellular compartments. Our findings indicate that NO is able to modulate the cellular localization of Ro52. Under pro-inflammatory conditions Ro52 may need to convey both nuclear and cytoplasmic functions. According to our results, Ro52 is expressed in close proximity to iNOS and is located in the respective cellular compartments of the cells present in CLE skin lesions.

6 A HYPOTHESIS ON CLE/LE PATHOGENESIS

The results of the studies included in this thesis contribute to a better understanding of the biological events occurring at the site of UVR-induced skin injury during CLE lesion development and healing. Taken together with the results of other investigators my data allows the suggestion of a hypothesis regarding the pathogenesis of LE (***figure*** *7*).

Importantly, susceptibility to LE is inherited by multiple genes involved in regulation of the innate and adaptive immune responses. An external trigger of proinflammatory and/or cell-death inducing nature (e.g.UVR) is usually needed to induce clinical manifestation of the disease. The process of apoptosis and/or the response of the immune system to the presence of apoptotic cells is abnormal in at least a proportion of lupus-susceptible individuals [60]. Apoptotic cells are not cleared in the appropriate period of time and therefore accumulate [59]. Non-removed apoptotic cells usually undergo secondary necrosis and cannot longer withhold intracellular constituents, which are passively released to the surrounding [60]. An external trigger of a pro-inflammatory nature (e.g. UVR) that induces a local transient inflammatory response in a non-susceptible individual, may induce a stronger inflammatory reaction in an LE-susceptible person. Through activation of MyD88, NFκB and inflammasome, UVR stimulates keratinocytes to produce and secrete proinflammatory cytokines such as TNFα, IL-1β, IL-6, IL-8 and probably HMGB1 ([50, 230] and paper II) (***figure*** *7*, **I**). Furthermore, synthesis of IFNs could also be initiated, but remains to be proved [64, 216]. In addition, our data indicate that UVR induces upregulation of the autoantigen Ro52 in keratinocytes (paper III). High Ro52 expression might additionally increase keratinocyte sensitivity to the cell death-inducing stimuli [123]. Dying keratinocytes that had upregulated Ro52 just before the death might release Ro52 and other autoantigens into the extracellular space together with highly pro-inflammatory and immunogenic nuclear material such as HMGB1 tightly complexed with nucleosomes [115]. Circulating Igs and complement components opsonize the cell debris and build ICs that further promote inflammation, as in LE-susceptible individuals they cannot be efficiently removed due to the lack of complement components or phagocyte inability to recognize and engulf them [41, 163, 165]. In such a proinflammatory environment chemokines (CXCL-9, -10, -11

and -12) and adhesion molecules (ICAM1 and E-selectin) are upregulated and mediate leukocyte influx from the circulation [63]. These chemokines are expressed in the CLE lesions and home CXCR3 expressing cells that probably are activated effector T cells and pDCs and CXCR4 positive cutaneous DCs [89-91, 231]. The inflammatory infiltrates were demonstrated to be composed of $CD4^+$ and $CD8^+$ T cells, $CD68^+$ macrophages and the majority of infiltrating cells express CXCR3. Granzyme B and Tia1 positive CTLs are also present in the lesions [90]. The presence of proinflammatory cytokines and ICs induces maturation of APCs. The ICs containing nuclear constituents might be recognized by pDCs and induce secretion of type I IFN, in particular IFNα [208, 232]. This cytokine has multiple effects on the immune system and is evolutionary directed to kill virus-infected cells; it is also activated in the major proportion of LE patients [63, 78, 143]. Importantly, Ro52 is an interferon-inducible protein and its upregulation, as observed in CLE lesions, might reflect the effect of presence of IFNs in the surrounding environment. In a mouse model with genetically disrupted Ro52, its absence, but not overexpression, leads to an uncontrollable inflammatory response to minor skin injury, which advances into lupus-like autoimmunity [131]. It is therefore possible that at least in some of LE patients Ro52, despite obvious upregulation, cannot convey its functions due to genetic polymorphisms in the Ro52 gene or that the present autoantibodies impede its function.

Ro52, when released from secondary necrotic cells in a highly proinflammatory environment, in which matured APCs are present, might be taken up by DCs or recognized by activated B cells, which are otherwise anergic or low self-reactive. Subsequently the adaptive immune response against Ro52 could be initiated and autoantibodies generated (***figure** 7,* **II**). The attack of the adaptive immune system on the target tissue via produced autoantibodies and activated effector T cells might further promote the vicious circle inducing cell death (***figure** 7,* **III**). Escalating inflammation in a genetically susceptible individual might lead to cutaneous or even systemic manifestations of LE.

To date, very little is known about factors predisposing to the development of one or another CLE subtype in a specific individual. The available data suggest some potential pathways. For example, the onset of SLE is usually preceded by arising titers of anti-dsDNA autoantibodies, which are strongly associated with lupus nephritis (LN). The

pathogenesis of LN is associated with the deposition of ICs in the target tissue [9]. Accordingly, positive LBT, reflecting the deposition of ICs, can be detected in both ACLE involved and non-involved sun-protected skin of SLE patients and it therefore seems that ICs are randomly deposited in the skin [37]. Manifestation of facial ACLE is associated with sun exposure. It could be the case that a combination of sun-induced inflammation with simultaneous presence of the skin deposited ICs results in ACLE lesion development.

SCLE is strongly associated with anti-Ro/SSA autoantibodies. Levels of anti-Ro52 antibodies are usually higher than anti-Ro60 in these patients [15, 24]. Anti-Ro52 autoantibodies are also related to NLE manifestations, such as congenital heart block and SCLE-like sun-induced/aggravated skin lesions. Importantly, the risk of NLE manifestations wanes after 6 months of age as maternal IgG, and anti-Ro/SSA, are clearing from the infant circulation [200]. It is therefore possible that anti-Ro/SSA mediate SCLE development after sun exposure even in adults.

ITGAM gene has been demonstrated to confer higher risk to DLE rather than SLE [161]. This gene encodes a subunit of integrin-α_M which is a subunit of type 3 complement receptor (CR3) and could therefore influence leukocyte trafficking via ICAM-1. Integrin-α_M could also account for the presence of dense cell infiltrates observed in DLE and could also affect the recognition and uptake of unviable cells and ICs via CR3 [41].

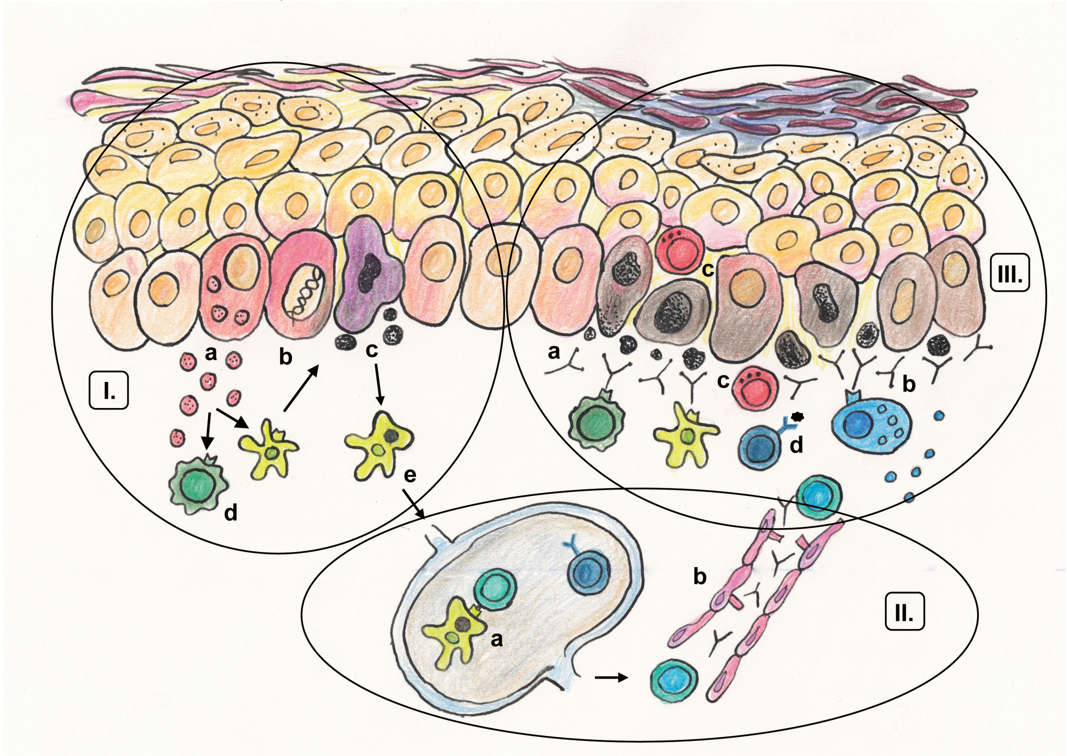

Figure 7. A hypothesis on CLE/LE pathogenesis

***Figure** 7*. A hypothesis on CLE/LE pathogenesis.

I. UVR-induced skin injury: a) activated keratinocytes secrete proinflammatory cytokines, express chemokines and upregulate Ro52 expression; b) cell cycle arrest due to UVR-induced DNA damage, possibility for DNA repair, but cell undergoes apoptosis if the damage is too extensive or the repair mechanisms fail; c) unremoved apoptotic keratinocytes undergo secondary necrosis and passively release intracellular constituents to the surrounding; d) macrophages and DCs are activated due to the presence of proinflammatory cytokines and unviable cells; e) DCs ingest cell debris and, probably, autoantigens.

II. The initiation of the adaptive immune response against self: a) DCs present autoantigens to the cells of the adaptive immune system in the lymph node; b) cell influx to the site of UVR injury via upregulated adhesion molecules and chemokine gradient.

III. Autoimmunity induced skin injury:

a) unviable cell debris is opsonized by Igs and complement components. ICs are formed, maybe also Ro52-anti-Ro52; b) HMGB1-DNA-Ig activate pDCs and initiate IFNα secretion; c) cytotoxic and $CD4^+$ effector T cells; d) HMGB1-DNA-Igs stimulate B cells.

7 CONCLUDING REMARKS

The studies presented in this thesis provide new insights into which molecules are involved in the regulation of cutaneous inflammation in lupus erythematosus, and show that experimental photoprovocation is a reproducible, safe and valuable method to verify photosensitivity and study the pathogenesis of UVR-induced CLE.

More specifically, we demonstrated that HMGB1 is upregulated and translocated to the extracellular space in UVR-induced CLE skin lesions, and that the highest expression of HMGB1 coincides with the peak of the clinical activity of the lesions. Expression patterns of TNF-α and IL-1β were not consistent, and in some patients expression was barely detectable. This fact emphasizes the importance of our findings regarding HMGB1 and prompts an idea that it may indeed be of major importance in the pathogenesis of CLE. Subsequent to publication of our study other investigators demonstrated that HMGB1 attached to nucleosomes is released from secondary necrotic cells. Our finding of extracellular HMGB1 within the epidermis and especially at the basal cell layer, where the majority of unviable cells are localized, might correspond to passively released HMGB1 from secondary necrotic cells. HMGB1 bound to chromatin is highly proinflammatory and also autoantigenic. Such complexes, when distributed extracellularly in a proinflammatory environment, might activate cells of the immune system and contribute to breakage of immunological tolerance in a genetically susceptible individual.

In papers III and IV we focused on the autoantigen Ro52. We determined its expression in the skin – a target organ of LE autoimmunity and defined factors that can modulate the cellular expression and localization of this autoantigen. Ro52 was strongly expressed in CLE lesions, in both keratinocytes and dermal-infiltrating cells, and in both keratinocyte cytoplasm and nucleus. Notably, similarly high Ro52 expression was observed in other inflammatory skin diseases not related to LE. Moreover, keratinocyte exposure to UVR upregulated its expression in the cytoplasm and NO induced its accumulation in the cell nucleus. We have also demonstrated that Ro52 in its capacity as an E3 ligase has both cytoplasmic and nuclear interaction partners, ubiquitin conjugating enzymes. In parallel to the studies included in the thesis, it was demonstrated that Ro52 interacts with IRFs and regulates inflammatory response.

Moreover, our group has developed an animal model with genetically disrupted Ro52. Intriguingly, loss of Ro52 resulted in uncontrollable systemic inflammation after minor skin injury that led to the development of lupus-like disease.

A combination of findings that: 1*) UVR induces upregulation of Ro52 in the keratinocytes that subsequently will die, but will not be removed efficiently; 2) Ro52 is upregulated in all CLE patients despite their subtype and anti-Ro52 autoantibody status; 3) similar strong Ro52 expression is observed in LE-nonrelated inflammatory skin diseases; 4) loss of Ro52, rather than its overexpression, leads to uncontrolled inflammation advancing to autoimmunity in an animal model*; allows me to propose the following hypothesis: Ro52 is expressed in patients with CLE but cannot convey its function as a negative feedback regulator of inflammation. Ro52 is probably released by secondary necrotic keratinocytes in a proinflammatory environment and maybe that is how it is recognized by the adaptive immune system that subsequently initiates an autoimmune response against it in a genetically susceptible individual. More research is needed to investigate if this is indeed the case, and if so, identify the factors interfering with Ro52 and furthermore, determine how they can be defeated.

8 ACKNOWLEDGEMENTS

I am very happy that I had a possibility to carry out my PhD studies at Karolinska Institutet, Unit of Rheumatology. My years as a PhD student have been very interesting and prosperous in all possible meanings. Actually, I had luck to work at three places: Rheumatology lab and Rheumatology Clinic at Karolinska University Hospital and also at Dermatology Unit at Danderyds Hospital. Everywhere I met so many nice and giving people. I am so grateful to all of YOU!

I would like to express my special gratitude to:

Marie Wahren-Herlenius, my main supervisor. Thank you so much for giving me the possibility to do my PhD in your group and allowing me study the subject I am really interested in. I admire your broad knowledge in medicine, immunology and molecular biology, communication skills and humanism. Thank you for sharing all this with me. I am also very grateful for your support in my aspiration to work as a clinician.

Filippa Nyberg, my co-supervisor. Thank you very much for introducing me to the dermatology and the world of cutaneous lupus and UVR. It is such an interesting subject to study! Thank you so much for this possibility.

Lars Klareskog, the chief of Rheumatology Unit at KI, Solna. Thank you very much for open and stimulating environment at CMM!

Johan Bratt, the former head of Rheumatology Clinic, thank you for giving me the possibility to join the Rheumatology Clinic and for promoting research among clinicians. **Cecilia Carlens**, the current chief, thank you for assuring positive atmosphere at the clinic.

I would like to thank all the members of Marie Wahren-Herlenius group: **Monica Ek** for your patient introduction into the practical labwork and even into the Swedish language! **Karin Popovic**, thank you for sharing interest in the same subject and giving me strategic advices. **Ismini Vassilaki**, a temporarily adjunct to the group, thank you for teaching me CLE histopathology and helping me with interpretation of my data. **Marika Kvarnström**, thank you for sharing your interest in the clinically related Ro52 research; good luck with all your projects! **Nånnis**, you are a passionate scientist who still knows (almost) everything outside the lab-walls; I really adore your interest in so many things! **Vijole**, thank you for the chats in Lithuanian and all the culinary miracles you invited me for. **Åse**, thank you for teaching me many practical things about Sweden and all the sincere talks. **Aurelie**, thank you for inviting scientific discussions about your data and French breakfast at the lab meetings, and big thank to **Erwan** for helping my brother. **Alex**, you are the know-how and know-why guy, I hope you will enjoy the US. **Amanda**, I appreciate your interest in biology and economics! **Joanna, Sabrina, Maria** and **Bala** you are the promising future, I am sure that more hot news about the importance of Ro52 will be coming soon! Good luck! Thanks to all previous and new members of the group and their contribution to the better understanding of Ro52 and it related autoimmunity: **Stina, Linn, Therese, Wei, Elisabeth** and **Robert**.

I would also like to express my gratitude to all personnel at the Rheumatology Clinic. Particularly **Brigitte Dupre**, being my supervisor and mentor, thank you for your

sincere advices and help! Many thanks to the senior physicians and scientists **Elisabeth Svenungsson** and **Iva Gunnarsson** for sharing your experience in lupus. I would also like to express my gratitude to **Maryam Dastmalchi** for balancing the schema and finding possibilities for my scientific leave. Thanks to all the colleagues at the Rheumatology clinic for sharing your experience and knowledge with me and for contributing to a warm climate: **Ingrid Lundberg, Lena Björnådal, Anders Harju, Ralph Nisell, Bo Ringertz, Ronald van Vollenhoven, Tomas Zweig, Birgitta Nordmark, Staffan Lindblad, Jon Lampa, Anca Catrina, Esbjörn Larsson, Lara Dani, Erik af Klint, Ola Börjesson, Johanna Gustafsson, Annika Nordin, Johan Askling, Petra Neregård, Thorunn Jonsdottir, Per-Johan Jakobsson, Christina Stranger, Saedis Saevarsdottir, Christina Dorph, Hamed Rezaei, Sara Wedren, Louise Ekholm, Karatina Chatzidionysiou, Dimitris Makrygiannakis, Gudrun Björksdottir , Erik Hellbacher and Aase Hensvold.**

Anna Vikerfors, thank you for being a wonderful colleague and friend!

Many thanks to **Gunnel Bemerfeldt, Susanne Karlfeldt, Christina Ingemarsson** and **Anette Hjärne** for kind help with all the practicalities along the way.

I greatly appreciated all the scientific input from all the group leaders at Rheumatology Unit: **Helena Erlandsson Harris, Vivi Malmström, Leonid Padyukov** and **Gustavo Nader**.

I am grateful for the personnel at Dermatology Department, Danderyd Hospital: the doctors for welcoming me to their unit and nurses for demonstrating me the practicalities about the photoprovocation.

Annegret Kuhn, thank you for initiating European Society for CLE and devoted research in this area.

Bob Harris, thank you for correcting my English.

I have met so many nice people at the CMM floor 4. Thanks to ALL of you!
I just would like to express my personal thanks to **Lasse** and **Marcus**, for always being there and kindly helping with the computer problems! Thanks to **Marianne E**. and **Eva L**., for all advices regarding immunohistochemistry. **Mohsen K**, for always being so kind and helping with the PCR. **Andreas F,** for nice company in the office and the input in my Swedish! and thanks to **Emelie** who took over and made it as good. **Marina K,** for always positive talks**. Karina G,** for sharing interest in the clinical rheumatology. **Hulda**, for your company in the hard work to the very FINAL. **Karin P**, for sharing experiences in antigen retrieval and interest in HMGB1. **Ingela**, for nice talks helping to reload the energy in difficult moments during the thesis writing. **Rux**, I will miss your smile, but I wish you all the best! **Heidi,** for always being so positive! **Paulius,** for increasing prevalence of Lithuanian scientists in Stockholm! **Shankar, Jayesh** and **Namrata,** for inviting me for Indian desserts and the baby talks! **Melanie**, for the OBAND music! **Peter L,** for the cell room chats**.** Other nice people: **Hanna S, Patrick L, Lotta, Lena, Sevim BH, Omri S, Hiba, Maria S, Charlotta S, Anna T, Louise B, Dannika S, Margarita D, Nada, Magnus L, Magda L, Eva J and Gull-Britt A.**

When starting my life in Sweden more than 6 years ago I had only a few Lithuanian friends here. Happily, since then I have met many nice people whom I shared my life

after the work with. I would like to thank: **Cynthia**, my wonderful companion at work and after-work, thank you for your creative personality and introducing us to **Andreas**! **Helen** and **Claes,** thank you for all cozy fikas and interesting books "про восток ☺"; **Johanna** and **Daniel,** for brunches and diners, and thank you all for the time we share with the babies! **Anna** and **Kasper,** for all the good time, interest in Lindy-hop and nature. **Andre** and **Johan,** thank you for being friends that do always listen, and I promise we will invite you for Lithuanian borsch one day! **Denise** and **Tommy**, thank you for the friendship, unexpectedly Brazilians and Lithuanians have much in common! **Sabine,** thank you for being a caring friend and giving me much practical input at the emergency unit! **Nele**, for the lady-doctors talks and all the good time! **Markus,** for being my first Swedish friend and all the help during my very first days in Linköping and all the joyful moments later on.

Our Lithuanian friends in Sweden: **Laura** and **Audas**, for being also from Kaunas, LT© and your fantastic sense of humor and optimism. **Rolandas,** for your friendship with our family and being the first visitor of baby Herkus. **Agnė** and **Tomas**, for the realistic attitude to the reality, loving science and outdoor activities. **Aurelija** and **Valentinas,** for all the good moments we had when you lived in Stockholm. **Jurga** and **Evaldas**, for sharing the passion for dance.

My Lithuanian friends in Lithuania: **Jovita**, thank you for always being with me when it is needed! **Inga** and **Renata**, you were my friends as long as I remember myself and it is a very special feeling, thanks. **Gedas**, thank you for visiting us and bringing Lithunian goods every time you are in Sweden and **Dainius** for keeping us amazed with your virtuosity in cooking.

Thanks to my teachers at Kaunas University of Medicine, Lithuania, especially professor **Apolinaras Zaborskis**, whom I started my first research project with.

I also want to express my gratitude to the medical student corporation **"*Fraternitas Lituanica*"** for my years with it. My membership in the corporation helped me to form my professional goals and initiated my interest in research. *Vivat, creascat et floreat korp!"Fraternitas Lituanica" in aeternum!*

I will thank my family in Lithuanian:
Noriu išreikšti didelį dėkingumą **Mamai** ir **Tėčiui**, už vertybes kurias manyje ugdėte ir leidote užaugti tuo kuo esu. Didelis dėkui Jums. **Vytautai**, ačiū kad tu su mumis!

The true love is almighty and endless. **Mantas** and **Herkus,** thank you for being with me!

9 REFERENCES

1. Cazenave, *Manual of the disease of the skin*. 1851: London: Henry Renshawm (Translated with notes and additions by Burgess).
2. Klippel, *Primer on The Rheumatic Diseases*. Thirteenth Edition ed, ed. J.H. Klippel. 2008: Springer.
3. Jimenez, Cervera, Font and Ingelmo, *The epidemiology of systemic lupus erythematosus.* Clin Rev Allergy Immunol, 2003. **25**(1): p. 3-12.
4. Koskenmies, Jarvinen*, et al.*, *Clinical and laboratory characteristics of Finnish lupus erythematosus patients with cutaneous manifestations.* Lupus, 2008. **17**(4): p. 337-47.
5. Vera-Recabarren, Garcia-Carrasco, Ramos-Casals and Herrero, *Comparative analysis of subacute cutaneous lupus erythematosus and chronic cutaneous lupus erythematosus: clinical and immunological study of 270 patients.* Br J Dermatol, 2010. **162**(1): p. 91-101.
6. Tan, Cohen*, et al.*, *The 1982 revised criteria for the classification of systemic lupus erythematosus.* Arthritis Rheum, 1982. **25**(11): p. 1271-7.
7. Hochberg, *Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus.* Arthritis Rheum, 1997. **40**(9): p. 1725.
8. Popovic, Wahren-Herlenius and Nyberg, *Clinical follow-up of 102 anti-Ro/SSA-positive patients with dermatological manifestations.* Acta Derm Venereol, 2008. **88**(4): p. 370-5.
9. Arbuckle, McClain*, et al.*, *Development of autoantibodies before the clinical onset of systemic lupus erythematosus.* N Engl J Med, 2003. **349**(16): p. 1526-33.
10. Gronhagen, Gunnarsson, Svenungsson and Nyberg, *Cutaneous manifestations and serological findings in 260 patients with systemic lupus erythematosus.* Lupus, 2010. **19**(10): p. 1187-94.
11. Kuhn A., *Cutaneous Lupus Erythematosus*. Springer-Verlag Berlin. 2005, Heidelberg.
12. Pace, Erb and Kitas, *Sunlight-induced recurrent acute confusion as the initial presentation of systemic lupus erythematosus.* Rheumatology (Oxford), 2001. **40**(1): p. 108-9.
13. Fruchter and Edoute, *First presentation of systemic lupus erythematosus following ultraviolet radiation exposure in an artificial tanning device.* Rheumatology (Oxford), 2005. **44**(4): p. 558-9.
14. Lee and Sinha, *Cutaneous lupus erythematosus: understanding of clinical features, genetic basis, and pathobiology of disease guides therapeutic strategies.* Autoimmunity, 2006. **39**(6): p. 433-44.
15. Sontheimer, Maddison*, et al.*, *Serologic and HLA associations in subacute cutaneous lupus erythematosus, a clinical subset of lupus erythematosus.* Ann Intern Med, 1982. **97**(5): p. 664-71.
16. Rothfield, Sontheimer and Bernstein, *Lupus erythematosus: systemic and cutaneous manifestations.* Clin Dermatol, 2006. **24**(5): p. 348-62.
17. Nyberg, Hasan*, et al.*, *Occurrence of polymorphous light eruption in lupus erythematosus.* Br J Dermatol, 1997. **136**(2): p. 217-21.
18. Nyberg, *Photosensitivity in Cutaneous Lupus Erythematosus clinical and experimental studies*, in *Department of Dermatology*. 1998, Karolinska Institutet: Stockholm.

19. Albrecht, Berlin, *et al.*, *Dermatology position paper on the revision of the 1982 ACR criteria for systemic lupus erythematosus.* Lupus, 2004. **13**(11): p. 839-49.
20. Smith and Gordon, *Systemic lupus erythematosus: clinical presentations.* Autoimmun Rev, 2010. **10**(1): p. 43-5.
21. Hoffman, Peene, *et al.*, *Specific antinuclear antibodies are associated with clinical features in systemic lupus erythematosus.* Ann Rheum Dis, 2004. **63**(9): p. 1155-8.
22. Sjowall, Zickert, *et al.*, *Serum levels of autoantibodies against C-reactive protein correlate with renal disease activity and response to therapy in lupus nephritis.* Arthritis Res Ther, 2009. **11**(6): p. R188.
23. Jiang and Pisetsky, *Expression of high mobility group protein 1 in the sera of patients and mice with systemic lupus erythematosus.* Ann Rheum Dis, 2008. **67**(5): p. 727-8.
24. Popovic, Brauner, *et al.*, *Fine specificity of the Ro/SSA autoantibody response in relation to serological and clinical findings in 96 patients with self-reported cutaneous symptoms induced by the sun.* Lupus, 2007. **16**(1): p. 10-7.
25. Wahren-Herlenius and Sonesson, *Specificity and effector mechanisms of autoantibodies in congenital heart block.* Curr Opin Immunol, 2006. **18**(6): p. 690-6.
26. Peene, Meheus, Veys and De Keyser, *Diagnostic associations in a large and consecutively identified population positive for anti-SSA and/or anti-SSB: the range of associated diseases differs according to the detailed serotype.* Ann Rheum Dis, 2002. **61**(12): p. 1090-4.
27. Fritzler, Pauls, Kinsella and Bowen, *Antinuclear, anticytoplasmic, and anti-Sjogren's syndrome antigen A (SS-A/Ro) antibodies in female blood donors.* Clin Immunol Immunopathol, 1985. **36**(1): p. 120-8.
28. de Vlam, De Keyser, *et al.*, *Detection and identification of antinuclear autoantibodies in the serum of normal blood donors.* Clin Exp Rheumatol, 1993. **11**(4): p. 393-7.
29. Ben-Chetrit, Chan, Sullivan and Tan, *A 52-kD protein is a novel component of the SS-A/Ro antigenic particle.* J Exp Med, 1988. **167**(5): p. 1560-71.
30. Strandberg, Winqvist, *et al.*, *Antibodies to amino acid 200-239 (p200) of Ro52 as serological markers for the risk of developing congenital heart block.* Clin Exp Immunol, 2008. **154**(1): p. 30-7.
31. Kuhn, Bein and Bonsmann, *The 100th anniversary of lupus erythematosus tumidus.* Autoimmun Rev, 2009. **8**(6): p. 441-8.
32. Gilliam and Sontheimer, *Distinctive cutaneous subsets in the spectrum of lupus erythematosus.* J Am Acad Dermatol, 1981. **4**(4): p. 471-5.
33. Lipsker and Hauptmann, *Cutaneous manifestations of complement deficiencies.* Lupus, 2010. **19**(9): p. 1096-106.
34. Wenzel and Tuting, *An IFN-associated cytotoxic cellular immune response against viral, self-, or tumor antigens is a common pathogenetic feature in "interface dermatitis".* J Invest Dermatol, 2008. **128**(10): p. 2392-402.
35. Klaus Wolff, *Fitzpatrick's Dermatology in General Medicine*, The McGraw-Hill Companies.
36. Beutner, Holborow and Johnson, *A new fluorescent antibody method: mixed antiglobulin immunofluorescence or labelled antigen indirect immunofluorescence staining.* Nature, 1965. **208**(5008): p. 353-5.
37. Dahl, *Usefulness of direct immunofluorescence in patients with lupus erythematosus.* Arch Dermatol, 1983. **119**(12): p. 1010-7.

38. Block, Winfield, et al., *Studies of twins with systemic lupus erythematosus. A review of the literature and presentation of 12 additional sets.* Am J Med, 1975. **59**(4): p. 533-52.
39. Lander, Linton, et al., *Initial sequencing and analysis of the human genome.* Nature, 2001. **409**(6822): p. 860-921.
40. Graham, Hom, Ortmann and Behrens, *Review of recent genome-wide association scans in lupus.* J Intern Med, 2009. **265**(6): p. 680-8.
41. Rhodes and Vyse, *The genetics of SLE: an update in the light of genome-wide association studies.* Rheumatology (Oxford), 2008. **47**(11): p. 1603-11.
42. Jarvinen, Hellquist, et al., *Tyrosine kinase 2 and interferon regulatory factor 5 polymorphisms are associated with discoid and subacute cutaneous lupus erythematosus.* Exp Dermatol, 2010. **19**(2): p. 123-31.
43. Rosenbaum ML et Werth VP, *Cutaneous Lupus Erythematosus*. Springer, ed. L.P. Kuhn A., Ruzicka T. . 2005, Heidelberg. Chapter 18.
44. Namjou, Kothari, et al., *Evaluation of the TREX1 gene in a large multi-ancestral lupus cohort.* Genes Immun, 2011.
45. Nakken, Jonsson and Bolstad, *Polymorphisms of the Ro52 gene associated with anti-Ro 52-kd autoantibodies in patients with primary Sjogren's syndrome.* Arthritis Rheum, 2001. **44**(3): p. 638-46.
46. Frank, Itoh, et al., *The mapping of the human 52-kD Ro/SSA autoantigen gene to human chromosome 11, and its polymorphisms.* Am J Hum Genet, 1993. **52**(1): p. 183-91.
47. Imanishi, Morinobu, et al., *A novel polymorphism of the SSA1 gene is associated with anti-SS-A/Ro52 autoantibody in Japanese patients with primary Sjogren's syndrome.* Clin Exp Rheumatol, 2005. **23**(4): p. 521-4.
48. Sandling, Garnier, et al., *A candidate gene study of the type I interferon pathway implicates IKBKE and IL8 as risk loci for SLE.* Eur J Hum Genet, 2010.
49. Lessard, Adrianto, et al., *Identification of a systemic lupus erythematosus susceptibility locus at 11p13 between PDHX and CD44 in a multiethnic study.* Am J Hum Genet, 2011. **88**(1): p. 83-91.
50. Maverakis, Miyamura, et al., *Light, including ultraviolet.* J Autoimmun, 2010. **34**(3): p. J247-57.
51. Schwarz and Schwarz, *Molecular mechanisms of ultraviolet radiation-induced immunosuppression.* Eur J Cell Biol, 2010.
52. Kulms and Schwarz, *Independent contribution of three different pathways to ultraviolet-B-induced apoptosis.* Biochem Pharmacol, 2002. **64**(5-6): p. 837-41.
53. Bohm, Luger, et al., *New insight into immunosuppression and treatment of autoimmune diseases.* Clin Exp Rheumatol, 2006. **24**(1 Suppl 40): p. S67-71.
54. Schwarz, Maeda, et al., *Prevention of UV radiation-induced immunosuppression by IL-12 is dependent on DNA repair.* J Exp Med, 2005. **201**(2): p. 173-9.
55. Grundmann and Beissert, *Regulation of cellular immunity by Photo(chemo)therapy.* Front Biosci, 2009. **14**: p. 4326-36.
56. Nestle, Di Meglio, Qin and Nickoloff, *Skin immune sentinels in health and disease.* Nat Rev Immunol, 2009. **9**(10): p. 679-91.
57. Bennion and Norris, *Ultraviolet light modulation of autoantigens, epidermal cytokines and adhesion molecules as contributing factors of the pathogenesis of cutaneous LE.* Lupus, 1997. **6**(2): p. 181-92.
58. Cals-Grierson and Ormerod, *Nitric oxide function in the skin.* Nitric Oxide, 2004. **10**(4): p. 179-93.

59. Kuhn, Herrmann*, et al.*, *Accumulation of apoptotic cells in the epidermis of patients with cutaneous lupus erythematosus after ultraviolet irradiation.* Arthritis Rheum, 2006. **54**(3): p. 939-50.
60. Munoz, Lauber*, et al.*, *The role of defective clearance of apoptotic cells in systemic autoimmunity.* Nat Rev Rheumatol, 2010. **6**(5): p. 280-9.
61. Nyberg, Skoglund and Stephansson, *Early detection of epidermal dust-like particles in experimentally UV-induced lesions in patients with photosensitivity and lupus erythematosus.* Acta Derm Venereol, 1998. **78**(3): p. 177-9.
62. Kuhn, Fehsel*, et al.*, *Aberrant timing in epidermal expression of inducible nitric oxide synthase after UV irradiation in cutaneous lupus erythematosus.* J Invest Dermatol, 1998. **111**(1): p. 149-53.
63. Reefman, Kuiper*, et al.*, *Type I interferons are involved in the development of ultraviolet B-induced inflammatory skin lesions in systemic lupus erythaematosus patients.* Ann Rheum Dis, 2008. **67**(1): p. 11-18.
64. Zahn, Rehkamper*, et al.*, *Evidence for a pathophysiological role of keratinocyte-derived type III interferon (IFNlambda) in cutaneous lupus erythematosus.* J Invest Dermatol, 2011. **131**(1): p. 133-40.
65. Casciola-Rosen and Rosen, *Ultraviolet light-induced keratinocyte apoptosis: a potential mechanism for the induction of skin lesions and autoantibody production in LE.* Lupus, 1997. **6**(2): p. 175-80.
66. Saegusa, Kawano*, et al.*, *Oxidative stress mediates cell surface expression of SS-A/Ro antigen on keratinocytes.* Free Radic Biol Med, 2002. **32**(10): p. 1006-16.
67. Lawley, Doherty*, et al.*, *Rapid lupus autoantigen relocalization and reactive oxygen species accumulation following ultraviolet irradiation of human keratinocytes.* Rheumatology (Oxford), 2000. **39**(3): p. 253-61.
68. Nobuhara, Kawano*, et al.*, *Is SS-A/Ro52 a hydrogen peroxide-sensitive signaling molecule?* Antioxid Redox Signal, 2007. **9**(3): p. 385-91.
69. Metelitsa and Lauzon, *Tobacco and the skin.* Clin Dermatol, 2010. **28**(4): p. 384-90.
70. Popovic, Nyberg and Wahren-Herlenius, *A serology-based approach combined with clinical examination of 125 Ro/SSA-positive patients to define incidence and prevalence of subacute cutaneous lupus erythematosus.* Arthritis Rheum, 2007. **56**(1): p. 255-64.
71. Padyukov, Silva*, et al.*, *A gene-environment interaction between smoking and shared epitope genes in HLA-DR provides a high risk of seropositive rheumatoid arthritis.* Arthritis Rheum, 2004. **50**(10): p. 3085-92.
72. Boeckler, Milea*, et al.*, *The combination of complement deficiency and cigarette smoking as risk factor for cutaneous lupus erythematosus in men; a focus on combined C2/C4 deficiency.* Br J Dermatol, 2005. **152**(2): p. 265-70.
73. Jewell and McCauliffe, *Patients with cutaneous lupus erythematosus who smoke are less responsive to antimalarial treatment.* J Am Acad Dermatol, 2000. **42**(6): p. 983-7.
74. Roubinian, Talal*, et al.*, *Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F1 mice.* J Exp Med, 1978. **147**(6): p. 1568-83.
75. Harley and James, *Everyone comes from somewhere: systemic lupus erythematosus and Epstein-Barr virus induction of host interferon and humoral anti-Epstein-Barr nuclear antigen 1 immunity.* Arthritis Rheum, 2010. **62**(6): p. 1571-5.
76. Pavlovic, Kats, Cavallo and Shoenfeld, *Clinical and molecular evidence for association of SLE with parvovirus B19.* Lupus, 2010. **19**(7): p. 783-92.

77. Abbas, *Cellular and Molecular Immunology*. &th edition ed. 2007: Saunders Elsevier.
78. Ronnblom, Alm and Eloranta, *Type I interferon and lupus.* Curr Opin Rheumatol, 2009. **21**(5): p. 471-7.
79. Bengtsson, Widell, Elmstahl and Sturfelt, *No serological indications that systemic lupus erythematosus is linked with exposure to human parvovirus B19.* Ann Rheum Dis, 2000. **59**(1): p. 64-6.
80. Yilmaz and Cimen, *Pegylated interferon alpha-2B induced lupus in a patient with chronic hepatitis B virus infection: case report.* Clin Rheumatol, 2009. **28**(10): p. 1241-3.
81. Burnett, English and Ferris, *Development of subacute cutaneous lupus erythematosus associated with the use of imiquimod to treat actinic keratoses.* J Drugs Dermatol, 2010. **9**(8): p. 1022-4.
82. Ronnblom, Alm and Oberg, *Possible induction of systemic lupus erythematosus by interferon-alpha treatment in a patient with a malignant carcinoid tumour.* J Intern Med, 1990. **227**(3): p. 207-10.
83. Sontheimer, Henderson and Grau, *Drug-induced subacute cutaneous lupus erythematosus: a paradigm for bedside-to-bench patient-oriented translational clinical investigation.* Arch Dermatol Res, 2009. **301**(1): p. 65-70.
84. Popovic, Ek*, et al.*, *Increased expression of the novel proinflammatory cytokine high mobility group box chromosomal protein 1 in skin lesions of patients with lupus erythematosus.* Arthritis Rheum, 2005. **52**(11): p. 3639-45.
85. Wittmann, Macdonald and Renne, *IL-18 and skin inflammation.* Autoimmun Rev, 2009. **9**(1): p. 45-8.
86. Tanasescu, Balanescu*, et al.*, *IL-17 in cutaneous lupus erythematosus.* Eur J Intern Med, 2010. **21**(3): p. 202-7.
87. Loetscher, Gerber*, et al.*, *Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes.* J Exp Med, 1996. **184**(3): p. 963-9.
88. Flier, Boorsma*, et al.*, *Differential expression of CXCR3 targeting chemokines CXCL10, CXCL9, and CXCL11 in different types of skin inflammation.* J Pathol, 2001. **194**(4): p. 398-405.
89. Meller, Winterberg*, et al.*, *Ultraviolet radiation-induced injury, chemokines, and leukocyte recruitment: An amplification cycle triggering cutaneous lupus erythematosus.* Arthritis Rheum, 2005. **52**(5): p. 1504-16.
90. Wenzel, Zahn*, et al.*, *The expression pattern of interferon-inducible proteins reflects the characteristic histological distribution of infiltrating immune cells in different cutaneous lupus erythematosus subsets.* Br J Dermatol, 2007. **157**(4): p. 752-7.
91. Kabashima, Shiraishi*, et al.*, *CXCL12-CXCR4 engagement is required for migration of cutaneous dendritic cells.* Am J Pathol, 2007. **171**(4): p. 1249-57.
92. Blomberg, Eloranta*, et al.*, *Presence of cutaneous interferon-alpha producing cells in patients with systemic lupus erythematosus.* Lupus, 2001. **10**(7): p. 484-90.
93. Goodwin, Sanders and Johns, *A new group of chromatin-associated proteins with a high content of acidic and basic amino acids.* Eur J Biochem, 1973. **38**(1): p. 14-9.
94. Bustin, *Regulation of DNA-dependent activities by the functional motifs of the high-mobility-group chromosomal proteins.* Mol Cell Biol, 1999. **19**(8): p. 5237-46.
95. Scaffidi, Misteli and Bianchi, *Release of chromatin protein HMGB1 by necrotic cells triggers inflammation.* Nature, 2002. **418**(6894): p. 191-5.

96. Vezzoli, Castellani, *et al.*, *HMGB1 release and redox regulation accompany regeneration and remodeling of skeletal muscle.* Antioxid Redox Signal, 2011.
97. Zhang, Shu, Qi and Li, *Inhibition of tumor angiogenesis by HMGB1 A box peptide.* Med Hypotheses, 2008. **70**(2): p. 343-5.
98. Taguchi, Blood, *et al.*, *Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases.* Nature, 2000. **405**(6784): p. 354-60.
99. Andersson and Harris, *The role of HMGB1 in the pathogenesis of rheumatic disease.* Biochim Biophys Acta, 2010. **1799**(1-2): p. 141-8.
100. Gardella, Andrei, *et al.*, *The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway.* EMBO Rep, 2002. **3**(10): p. 995-1001.
101. Yang, Wang, Czura and Tracey, *The cytokine activity of HMGB1.* J Leukoc Biol, 2005. **78**(1): p. 1-8.
102. Andersson, Wang, *et al.*, *High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes.* J Exp Med, 2000. **192**(4): p. 565-70.
103. Li, Kokkola, *et al.*, *Structural basis for the proinflammatory cytokine activity of high mobility group box 1.* Mol Med, 2003. **9**(1-2): p. 37-45.
104. Fan, Li, *et al.*, *Hemorrhagic shock induces NAD(P)H oxidase activation in neutrophils: role of HMGB1-TLR4 signaling.* J Immunol, 2007. **178**(10): p. 6573-80.
105. Campana, Bosurgi, *et al.*, *Requirement of HMGB1 for stromal cell-derived factor-1/CXCL12-dependent migration of macrophages and dendritic cells.* J Leukoc Biol, 2009. **86**(3): p. 609-15.
106. Dumitriu, Baruah, *et al.*, *Requirement of HMGB1 and RAGE for the maturation of human plasmacytoid dendritic cells.* Eur J Immunol, 2005. **35**(7): p. 2184-90.
107. Dumitriu, Baruah, *et al.*, *Release of high mobility group box 1 by dendritic cells controls T cell activation via the receptor for advanced glycation end products.* J Immunol, 2005. **174**(12): p. 7506-15.
108. Bianchi, *HMGB1 loves company.* J Leukoc Biol, 2009. **86**(3): p. 573-6.
109. Hreggvidsdottir, Ostberg, *et al.*, *The alarmin HMGB1 acts in synergy with endogenous and exogenous danger signals to promote inflammation.* J Leukoc Biol, 2009. **86**(3): p. 655-62.
110. Wang, Bloom, *et al.*, *HMG-1 as a late mediator of endotoxin lethality in mice.* Science, 1999. **285**(5425): p. 248-51.
111. Ombrellino, Wang, *et al.*, *Increased serum concentrations of high-mobility-group protein 1 in haemorrhagic shock.* Lancet, 1999. **354**(9188): p. 1446-7.
112. Volz, Seidel, *et al.*, *HMGB1: the missing link between diabetes mellitus and heart failure.* Basic Res Cardiol, 2010. **105**(6): p. 805-20.
113. Kokkola, Li, *et al.*, *Successful treatment of collagen-induced arthritis in mice and rats by targeting extracellular high mobility group box chromosomal protein 1 activity.* Arthritis Rheum, 2003. **48**(7): p. 2052-8.
114. Ulfgren, Grundtman, *et al.*, *Down-regulation of the aberrant expression of the inflammation mediator high mobility group box chromosomal protein 1 in muscle tissue of patients with polymyositis and dermatomyositis treated with corticosteroids.* Arthritis Rheum, 2004. **50**(5): p. 1586-94.
115. Urbonaviciute, Furnrohr, *et al.*, *Induction of inflammatory and immune responses by HMGB1-nucleosome complexes: implications for the pathogenesis of SLE.* J Exp Med, 2008. **205**(13): p. 3007-18.
116. Avalos, Kiefer, *et al.*, *RAGE-independent autoreactive B cell activation in response to chromatin and HMGB1/DNA immune complexes.* Autoimmunity, 2010. **43**(1): p. 103-10.

117. Tian, Avalos, *et al.*, *Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE.* Nat Immunol, 2007. **8**(5): p. 487-96.
118. Venables, *Sjogren's syndrome.* Best Pract Res Clin Rheumatol, 2004. **18**(3): p. 313-29.
119. Chan, Hamel, Buyon and Tan, *Molecular definition and sequence motifs of the 52-kD component of human SS-A/Ro autoantigen.* J Clin Invest, 1991. **87**(1): p. 68-76.
120. Itoh, Itoh and Frank, *Protein heterogeneity in the human Ro/SSA ribonucleoproteins. The 52- and 60-kD Ro/SSA autoantigens are encoded by separate genes.* J Clin Invest, 1991. **87**(1): p. 177-86.
121. Ottosson, Hennig, *et al.*, *Structural, functional and immunologic characterization of folded subdomains in the Ro52 protein targeted in Sjogren's syndrome.* Mol Immunol, 2006. **43**(6): p. 588-98.
122. Reymond, Meroni, *et al.*, *The tripartite motif family identifies cell compartments.* EMBO J, 2001. **20**(9): p. 2140-51.
123. Espinosa, Zhou, *et al.*, *The Sjogren's syndrome-associated autoantigen Ro52 is an E3 ligase that regulates proliferation and cell death.* J Immunol, 2006. **176**(10): p. 6277-85.
124. Hershko, Heller, Elias and Ciechanover, *Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown.* J Biol Chem, 1983. **258**(13): p. 8206-14.
125. Clague and Urbe, *Ubiquitin: same molecule, different degradation pathways.* Cell, 2010. **143**(5): p. 682-5.
126. Plafker, Plafker, Weissman and Macara, *Ubiquitin charging of human class III ubiquitin-conjugating enzymes triggers their nuclear import.* J Cell Biol, 2004. **167**(4): p. 649-59.
127. Wada and Kamitani, *Autoantigen Ro52 is an E3 ubiquitin ligase.* Biochem Biophys Res Commun, 2006. **339**(1): p. 415-21.
128. Kong, Anderson, *et al.*, *Cutting edge: autoantigen Ro52 is an interferon inducible E3 ligase that ubiquitinates IRF-8 and enhances cytokine expression in macrophages.* J Immunol, 2007. **179**(1): p. 26-30.
129. Higgs, Lazzari, *et al.*, *Self protection from anti-viral responses--Ro52 promotes degradation of the transcription factor IRF7 downstream of the viral Toll-Like receptors.* PLoS One, 2010. **5**(7): p. e11776.
130. Higgs, Ni Gabhann, *et al.*, *The E3 ubiquitin ligase Ro52 negatively regulates IFN-beta production post-pathogen recognition by polyubiquitin-mediated degradation of IRF3.* J Immunol, 2008. **181**(3): p. 1780-6.
131. Espinosa, Dardalhon, *et al.*, *Loss of the lupus autoantigen Ro52/Trim21 induces tissue inflammation and systemic autoimmunity by disregulating the IL-23-Th17 pathway.* J Exp Med, 2009. **206**(8): p. 1661-71.
132. Yang, Shi, *et al.*, *TRIM21 is essential to sustain IFN regulatory factor 3 activation during antiviral response.* J Immunol, 2009. **182**(6): p. 3782-92.
133. Sabile, Meyer, *et al.*, *Regulation of p27 degradation and S-phase progression by Ro52 RING finger protein.* Mol Cell Biol, 2006. **26**(16): p. 5994-6004.
134. Rhodes, Ihrke, *et al.*, *The 52 000 MW Ro/SS-A autoantigen in Sjogren's syndrome/systemic lupus erythematosus (Ro52) is an interferon-gamma inducible tripartite motif protein associated with membrane proximal structures.* Immunology, 2002. **106**(2): p. 246-56.
135. Keeble, Khan, Forster and James, *TRIM21 is an IgG receptor that is structurally, thermodynamically, and kinetically conserved.* Proc Natl Acad Sci U S A, 2008. **105**(16): p. 6045-50.

136. Pourmand, Blange, Ringertz and Pettersson, *Intracellular localisation of the Ro 52kD auto-antigen in HeLa cells visualised with green fluorescent protein chimeras.* Autoimmunity, 1998. **28**(4): p. 225-33.
137. Strandberg, Ambrosi*, et al.*, *Interferon-alpha induces up-regulation and nuclear translocation of the Ro52 autoantigen as detected by a panel of novel Ro52-specific monoclonal antibodies.* J Clin Immunol, 2008. **28**(3): p. 220-31.
138. Ohlsson, Jonsson and Brokstad, *Subcellular redistribution and surface exposure of the Ro52, Ro60 and La48 autoantigens during apoptosis in human ductal epithelial cells: a possible mechanism in the pathogenesis of Sjogren's syndrome.* Scand J Immunol, 2002. **56**(5): p. 456-69.
139. Weckerle, Franek*, et al.*, *Network analysis of associations between serum interferon alpha activity, autoantibodies, and clinical features in systemic lupus erythematosus.* Arthritis Rheum, 2010.
140. Munir, *TRIM proteins: another class of viral victims.* Sci Signal, 2010. **3**(118): p. jc2.
141. Witte, Gruetz*, et al.*, *Despite IFN-lambda receptor expression, blood immune cells, but not keratinocytes or melanocytes, have an impaired response to type III interferons: implications for therapeutic applications of these cytokines.* Genes Immun, 2009. **10**(8): p. 702-14.
142. Coccia, Severa*, et al.*, *Viral infection and Toll-like receptor agonists induce a differential expression of type I and lambda interferons in human plasmacytoid and monocyte-derived dendritic cells.* Eur J Immunol, 2004. **34**(3): p. 796-805.
143. Nordmark, Alm and Ronnblom, *Mechanisms of Disease: primary Sjogren's syndrome and the type I interferon system.* Nat Clin Pract Rheumatol, 2006. **2**(5): p. 262-9.
144. Theofilopoulos, Baccala, Beutler and Kono, *Type I interferons (alpha/beta) in immunity and autoimmunity.* Annu Rev Immunol, 2005. **23**: p. 307-36.
145. Thibault, Chu*, et al.*, *IRF9 and STAT1 are required for IgG autoantibody production and B cell expression of TLR7 in mice.* J Clin Invest, 2008. **118**(4): p. 1417-26.
146. Hanson, Gratton and Bardeen, *Sunscreen enhancement of UV-induced reactive oxygen species in the skin.* Free Radic Biol Med, 2006. **41**(8): p. 1205-12.
147. Chang, Tsao, Wang and Yu, *Expression of nitric oxide synthases in keratinocytes after UVB irradiation.* Arch Dermatol Res, 2003. **295**(7): p. 293-6.
148. Yamaoka, Kawana and Miyachi, *Nitric oxide inhibits ultraviolet B-induced murine keratinocyte apoptosis by regulating apoptotic signaling cascades.* Free Radic Res, 2004. **38**(9): p. 943-50.
149. Stallmeyer, Kampfer*, et al.*, *The function of nitric oxide in wound repair: inhibition of inducible nitric oxide-synthase severely impairs wound reepithelialization.* J Invest Dermatol, 1999. **113**(6): p. 1090-8.
150. Liu and Wu, *Differential roles of nitric oxide synthases in regulation of ultraviolet B light-induced apoptosis.* Nitric Oxide, 2010. **23**(3): p. 199-205.
151. Wang, Tseng*, et al.*, *Expression of inducible nitric oxide synthase and apoptosis in human lupus nephritis.* Nephron, 1997. **77**(4): p. 404-11.
152. Oates and Gilkeson, *The biology of nitric oxide and other reactive intermediates in systemic lupus erythematosus.* Clin Immunol, 2006. **121**(3): p. 243-50.
153. Habib, Moinuddin and Ali, *Peroxynitrite-modified DNA: a better antigen for systemic lupus erythematosus anti-DNA autoantibodies.* Biotechnol Appl Biochem, 2006. **43**(Pt 2): p. 65-70.

154. Reefman, Limburg, Kallenberg and Bijl, *Apoptosis in human skin: role in pathogenesis of various diseases and relevance for therapy.* Ann N Y Acad Sci, 2005. **1051**: p. 52-63.
155. Voll, Herrmann*, et al.*, *Immunosuppressive effects of apoptotic cells.* Nature, 1997. **390**(6658): p. 350-1.
156. Baumann, Kolowos*, et al.*, *Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus.* Arthritis Rheum, 2002. **46**(1): p. 191-201.
157. Meyer, *Anti-CRP antibodies in systemic lupus erythematosus.* Joint Bone Spine, 2010. **77**(5): p. 384-9.
158. Sjowall and Wettero, *Pathogenic implications for autoantibodies against C-reactive protein and other acute phase proteins.* Clin Chim Acta, 2007. **378**(1-2): p. 13-23.
159. Truedsson, Bengtsson and Sturfelt, *Complement deficiencies and systemic lupus erythematosus.* Autoimmunity, 2007. **40**(8): p. 560-6.
160. Gullstrand, Martensson*, et al.*, *Complement classical pathway components are all important in clearance of apoptotic and secondary necrotic cells.* Clin Exp Immunol, 2009. **156**(2): p. 303-11.
161. Jarvinen, Hellquist*, et al.*, *Polymorphisms of the ITGAM gene confer higher risk of discoid cutaneous than of systemic lupus erythematosus.* PLoS One, 2010. **5**(12): p. e14212.
162. Berkun, Verbovetski*, et al.*, *Altered dendritic cells with tolerizing phenotype in patients with systemic lupus erythematosus.* Eur J Immunol, 2008. **38**(10): p. 2896-904.
163. Santer, Hall*, et al.*, *C1q deficiency leads to the defective suppression of IFN-alpha in response to nucleoprotein containing immune complexes.* J Immunol, 2010. **185**(8): p. 4738-49.
164. Frisoni, McPhie*, et al.*, *Nuclear autoantigen translocation and autoantibody opsonization lead to increased dendritic cell phagocytosis and presentation of nuclear antigens: a novel pathogenic pathway for autoimmunity?* J Immunol, 2005. **175**(4): p. 2692-701.
165. Reefman, Horst*, et al.*, *Opsonization of late apoptotic cells by systemic lupus erythematosus autoantibodies inhibits their uptake via an Fcgamma receptor-dependent mechanism.* Arthritis Rheum, 2007. **56**(10): p. 3399-411.
166. Bave, Magnusson*, et al.*, *Fc gamma RIIa is expressed on natural IFN-alpha-producing cells (plasmacytoid dendritic cells) and is required for the IFN-alpha production induced by apoptotic cells combined with lupus IgG.* J Immunol, 2003. **171**(6): p. 3296-302.
167. Seitz and Matsushima, *Dendritic cells in systemic lupus erythematosus.* Int Rev Immunol, 2010. **29**(2): p. 184-209.
168. Krug, Uppaluri*, et al.*, *IFN-producing cells respond to CXCR3 ligands in the presence of CXCL12 and secrete inflammatory chemokines upon activation.* J Immunol, 2002. **169**(11): p. 6079-83.
169. Vanbervliet, Bendriss-Vermare*, et al.*, *The inducible CXCR3 ligands control plasmacytoid dendritic cell responsiveness to the constitutive chemokine stromal cell-derived factor 1 (SDF-1)/CXCL12.* J Exp Med, 2003. **198**(5): p. 823-30.
170. Farkas, Beiske*, et al.*, *Plasmacytoid dendritic cells (natural interferon-alpha/beta-producing cells) accumulate in cutaneous lupus erythematosus lesions.* Am J Pathol, 2001. **159**(1): p. 237-43.
171. Lood, Gullstrand*, et al.*, *C1q inhibits immune complex-induced interferon-alpha production in plasmacytoid dendritic cells: a novel link between C1q deficiency*

and systemic lupus erythematosus pathogenesis. Arthritis Rheum, 2009. **60**(10): p. 3081-90.
172. Crispin and Tsokos, *Interleukin-17-producing T cells in lupus.* Curr Opin Rheumatol, 2010. **22**(5): p. 499-503.
173. Crispin, Kyttaris, Terhorst and Tsokos, *T cells as therapeutic targets in SLE.* Nat Rev Rheumatol, 2010. **6**(6): p. 317-25.
174. Hoffman, *T cells in the pathogenesis of systemic lupus erythematosus.* Clin Immunol, 2004. **113**(1): p. 4-13.
175. Liu, Bao and Hu, *Elevated interleukin-18 and skewed Th1:Th2 immune response in lupus nephritis.* Rheumatol Int, 2010.
176. Calvani, Tucci*, et al.*, *Th1 cytokines in the pathogenesis of lupus nephritis: the role of IL-18.* Autoimmun Rev, 2005. **4**(8): p. 542-8.
177. Garrett-Sinha, John and Gaffen, *IL-17 and the Th17 lineage in systemic lupus erythematosus.* Curr Opin Rheumatol, 2008. **20**(5): p. 519-25.
178. Yang, Chu*, et al.*, *Th17 and natural Treg cell population dynamics in systemic lupus erythematosus.* Arthritis Rheum, 2009. **60**(5): p. 1472-83.
179. Lombardi, Van Overtvelt, Horiot and Moingeon, *Human dendritic cells stimulated via TLR7 and/or TLR8 induce the sequential production of Il-10, IFN-gamma, and IL-17A by naive CD4+ T cells.* J Immunol, 2009. **182**(6): p. 3372-9.
180. Barath, Soltesz*, et al.*, *The severity of systemic lupus erythematosus negatively correlates with the increasing number of CD4+CD25(high)FoxP3+ regulatory T cells during repeated plasmapheresis treatments of patients.* Autoimmunity, 2007. **40**(7): p. 521-8.
181. Valencia, Yarboro, Illei and Lipsky, *Deficient CD4+CD25high T regulatory cell function in patients with active systemic lupus erythematosus.* J Immunol, 2007. **178**(4): p. 2579-88.
182. Franz, Fritzsching*, et al.*, *Low number of regulatory T cells in skin lesions of patients with cutaneous lupus erythematosus.* Arthritis Rheum, 2007. **56**(6): p. 1910-20.
183. Mok, *The immunological basis of B-cell therapy in systemic lupus erythematosus.* Int J Rheum Dis, 2010. **13**(1): p. 3-11.
184. Vallerskog, Heimburger*, et al.*, *Differential effects on BAFF and APRIL levels in rituximab-treated patients with systemic lupus erythematosus and rheumatoid arthritis.* Arthritis Res Ther, 2006. **8**(6): p. R167.
185. Groom, Fletcher*, et al.*, *BAFF and MyD88 signals promote a lupuslike disease independent of T cells.* J Exp Med, 2007. **204**(8): p. 1959-71.
186. Jonsdottir, Gunnarsson*, et al.*, *Treatment of refractory SLE with rituximab plus cyclophosphamide: clinical effects, serological changes, and predictors of response.* Ann Rheum Dis, 2008. **67**(3): p. 330-4.
187. Dall'Era and Wofsy, *Connective tissue diseases: Belimumab for systemic lupus erythematosus: breaking through?* Nat Rev Rheumatol, 2010. **6**(3): p. 124-5.
188. Kuhn, Sonntag*, et al.*, *Phototesting in lupus erythematosus: a 15-year experience.* J Am Acad Dermatol, 2001. **45**(1): p. 86-95.
189. Millard, Hawk and McGregor, *Photosensitivity in lupus.* Lupus, 2000. **9**(1): p. 3-10.
190. Lehmann, Holzle*, et al.*, *Experimental reproduction of skin lesions in lupus erythematosus by UVA and UVB radiation.* J Am Acad Dermatol, 1990. **22**(2 Pt 1): p. 181-7.
191. Sanders, Van Weelden*, et al.*, *Photosensitivity in patients with lupus erythematosus: a clinical and photobiological study of 100 patients using a prolonged phototest protocol.* Br J Dermatol, 2003. **149**(1): p. 131-7.

192. Reefman, Kuiper, et al., *Skin sensitivity to UVB irradiation in systemic lupus erythematosus is not related to the level of apoptosis induction in keratinocytes.* Rheumatology (Oxford), 2005.
193. Wulf, Philipsen and Ravnbak, *Minimal erythema dose and minimal melanogenesis dose relate better to objectively measured skin type than to Fitzpatricks skin type.* Photodermatol Photoimmunol Photomed, 2010. **26**(6): p. 280-4.
194. Lokitz, Billet, et al., *Failure of physiologic doses of pure UVA or UVB to induce lesions in photosensitive cutaneous lupus erythematosus: implications for phototesting.* Photodermatol Photoimmunol Photomed, 2006. **22**(6): p. 290-6.
195. Reefman, Kuiper, et al., *Skin sensitivity to UVB irradiation in systemic lupus erythematosus is not related to the level of apoptosis induction in keratinocytes.* Rheumatology (Oxford), 2006. **45**(5): p. 538-44.
196. van Weelden, Velthuis and Baart de la Faille, *Light-induced skin lesions in lupus erythematosus: photobiological studies.* Arch Dermatol Res, 1989. **281**(7): p. 470-4.
197. Wolska, Blaszczyk and Jablonska, *Phototests in patients with various forms of lupus erythematosus.* Int J Dermatol, 1989. **28**(2): p. 98-103.
198. Tett, Cutler, Day and Brown, *Bioavailability of hydroxychloroquine tablets in healthy volunteers.* Br J Clin Pharmacol, 1989. **27**(6): p. 771-9.
199. Salomonsson, Sonesson, et al., *Ro/SSA autoantibodies directly bind cardiomyocytes, disturb calcium homeostasis, and mediate congenital heart block.* J Exp Med, 2005. **201**(1): p. 11-7.
200. O'Neill and Grimwood, *Neonatal lupus erythematosus syndrome.* Arch Dermatol, 1988. **124**(12): p. 1869-70.
201. Barkauskaite, Ek, et al., *Translocation of the novel cytokine HMGB1 to the cytoplasm and extracellular space coincides with the peak of clinical activity in experimentally UV-induced lesions of cutaneous lupus erythematosus.* Lupus, 2007. **16**(10): p. 794-802.
202. Mowbray, McLintock, et al., *Enzyme-independent NO stores in human skin: quantification and influence of UV radiation.* J Invest Dermatol, 2009. **129**(4): p. 834-42.
203. Salgo, Thaci, et al., *Microdialysis documents changes in the micromilieu of psoriatic plaques under continuous systemic therapy.* Exp Dermatol, 2011. **20**(2): p. 130-3.
204. Eloranta, Barbasso Helmers, et al., *A possible mechanism for endogenous activation of the type I interferon system in myositis patients with anti-Jo-1 or anti-Ro 52/anti-Ro 60 autoantibodies.* Arthritis Rheum, 2007. **56**(9): p. 3112-24.
205. Schierbeck, Wahamaa, Andersson and Harris, *Immunomodulatory drugs regulate HMGB1 release from activated human monocytes.* Mol Med, 2010. **16**(9-10): p. 343-51.
206. Liu, Wang, et al., *High mobility group protein-1 inhibits phagocytosis of apoptotic neutrophils through binding to phosphatidylserine.* J Immunol, 2008. **181**(6): p. 4240-6.
207. Kokkola, Sundberg, et al., *High mobility group box chromosomal protein 1: a novel proinflammatory mediator in synovitis.* Arthritis Rheum, 2002. **46**(10): p. 2598-603.
208. Lovgren, Eloranta, et al., *Induction of interferon-alpha production in plasmacytoid dendritic cells by immune complexes containing nucleic acid*

released by necrotic or late apoptotic cells and lupus IgG. Arthritis Rheum, 2004. **50**(6): p. 1861-72.
209. Lebre, van der Aar*, et al.*, *Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9.* J Invest Dermatol, 2007. **127**(2): p. 331-41.
210. Lohwasser, Neureiter*, et al.*, *The receptor for advanced glycation end products is highly expressed in the skin and upregulated by advanced glycation end products and tumor necrosis factor-alpha.* J Invest Dermatol, 2006. **126**(2): p. 291-9.
211. Itoh, Kriet and Reichlin, *Organ distribution of the Ro (SS-A) antigen in the guinea pig.* Arthritis Rheum, 1990. **33**(12): p. 1815-21.
212. Ioannides, Golden, Buyon and Bystryn, *Expression of SS-A/Ro and SS-B/La antigens in skin biopsy specimens of patients with photosensitive forms of lupus erythematosus.* Arch Dermatol, 2000. **136**(3): p. 340-6.
213. Bolstad, Eiken*, et al.*, *Increased salivary gland tissue expression of Fas, Fas ligand, cytotoxic T lymphocyte-associated antigen 4, and programmed cell death 1 in primary Sjogren's syndrome.* Arthritis Rheum, 2003. **48**(1): p. 174-85.
214. Hennig, Ottosson*, et al.*, *Structural organization and Zn2+-dependent subdomain interactions involving autoantigenic epitopes in the Ring-B-box-coiled-coil (RBCC) region of Ro52.* J Biol Chem, 2005. **280**(39): p. 33250-61.
215. Sompuram, Vani, Messana and Bogen, *A molecular mechanism of formalin fixation and antigen retrieval.* Am J Clin Pathol, 2004. **121**(2): p. 190-9.
216. Kim, Kim*, et al.*, *Chemotherapeutic DNA-damaging drugs activate interferon regulatory factor-7 by the mitogen-activated protein kinase kinase-4-cJun NH2-terminal kinase pathway.* Cancer Res, 2000. **60**(5): p. 1153-6.
217. Graham, Kozyrev*, et al.*, *A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus.* Nat Genet, 2006. **38**(5): p. 550-5.
218. Koffler, Agnello, Thoburn and Kunkel, *Systemic lupus erythematosus: prototype of immune complex nephritis in man.* J Exp Med, 1971. **134**(3): p. 169-79.
219. Oke, Vassilaki*, et al.*, *High Ro52 expression in spontaneous and UV-induced cutaneous inflammation.* J Invest Dermatol, 2009. **129**(8): p. 2000-10.
220. Ishii, Ohnuma*, et al.*, *SS-A/Ro52, an autoantigen involved in CD28-mediated IL-2 production.* J Immunol, 2003. **170**(7): p. 3653-61.
221. Harbers, Nomura, Ohno and Ishii, *Intracellular localization of the Ret finger protein depends on a functional nuclear export signal and protein kinase C activation.* J Biol Chem, 2001. **276**(51): p. 48596-607.
222. Wen, Meinkoth, Tsien and Taylor, *Identification of a signal for rapid export of proteins from the nucleus.* Cell, 1995. **82**(3): p. 463-73.
223. Chan, Di Donato*, et al.*, *52-kD SS-A/Ro: genomic structure and identification of an alternatively spliced transcript encoding a novel leucine zipper-minus autoantigen expressed in fetal and adult heart.* J Exp Med, 1995. **182**(4): p. 983-92.
224. Shibata, Nagata and Kobayashi, *Cutting edge: a critical role of nitric [corrected] oxide in preventing inflammation upon apoptotic cell clearance.* J Immunol, 2007. **179**(6): p. 3407-11.
225. Kobayashi, *The regulatory role of nitric oxide in proinflammatory cytokine expression during the induction and resolution of inflammation.* J Leukoc Biol, 2010. **88**(6): p. 1157-62.

226. Gwinn and Vallyathan, *Respiratory burst: role in signal transduction in alveolar macrophages.* J Toxicol Environ Health B Crit Rev, 2006. **9**(1): p. 27-39.
227. Jiang and Pisetsky, *The role of IFN-alpha and nitric oxide in the release of HMGB1 by RAW 264.7 cells stimulated with polyinosinic-polycytidylic acid or lipopolysaccharide.* J Immunol, 2006. **177**(5): p. 3337-43.
228. Kokkola, Andersson*, et al.*, *RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages.* Scand J Immunol, 2005. **61**(1): p. 1-9.
229. Wu, Wang*, et al.*, *Ultraviolet B light-induced nitric oxide/peroxynitrite imbalance in keratinocytes--implications for apoptosis and necrosis.* Photochem Photobiol, 2010. **86**(2): p. 389-96.
230. Lee, Kim*, et al.*, *Myeloid differentiation factor 88 regulates basal and UV-induced expressions of IL-6 and MMP-1 in human epidermal keratinocytes.* J Invest Dermatol, 2009. **129**(2): p. 460-7.
231. Lacotte, Brun, Muller and Dumortier, *CXCR3, inflammation, and autoimmune diseases.* Ann N Y Acad Sci, 2009. **1173**: p. 310-7.
232. Vollmer, Tluk*, 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**(11): p. 1575-85.