Polymorphisms of the ITGAM Gene Confer Higher Risk of Discoid Cutaneous Than of Systemic Lupus Erythematosus

Tiina M. Järvinen1,2,3,4, Anna Hellquist4, Sari Koskenmies2, Elisabet Einarsdottir2,5, Jaana Panelius1, Taina Hasan6, Heikki Julkunen7, Leonid Padyukov8, Marika Kvarnström8, Marie Wahren-Herlenius8, Filippa Nyberg9,10, Mauro D’Amato4, Juha Kere2,3,4, Ulpu Saarialho-Kere1,11

1 Departments of Dermatology, Allergology and Venereology, Institute of Clinical Medicine and Biomedicum Helsinki, University of Helsinki and Helsinki University Central Hospital, Helsinki, Finland, 2 Department of Medical Genetics, University of Helsinki, Helsinki, Finland, 3 Folkhälso Institute of Genetics, University, Finland, 4 Department of Biosciences and Nutrition, Karolinska Institute, Huddinge, Sweden, 5 Research Programme for Molecular Medicine, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland, 6 Department of Dermatology, University of Tampere and Tampere University Hospital, Tampere, Finland, 7 Peijas Hospital, Helsinki University Central Hospital, Vantaa, Finland, 8 Department of Medicine, Rheumatology Unit, Karolinska Institute, Solna, Sweden, 9 Department of Dermatology, Uppsala University, Akademiska University Hospital, Uppsala, Sweden, 10 Institute for Clinical Sciences, Karolinska Institutet at Danderyd Hospital, Stockholm, Sweden, 11 Department of Clinical Science and Education, and Section of Dermatology, Karolinska Institutet at Stockholm Soder Hospital, Stockholm, Sweden

Abstract

Background: Lupus erythematosus (LE) is a heterogeneous disease ranging from mainly skin-restricted manifestations (discoid LE [DLE] and subacute cutaneous LE) to a progressive multisystem disease (systemic LE [SLE]). Genetic association studies have recently identified several strong susceptibility genes for SLE, including integrin alpha M (ITGAM), also known as CD11b, whereas the genetic background of DLE is less clear.

Principal Findings: To specifically investigate whether ITGAM is a susceptibility gene not only for SLE, but also for cutaneous DLE, we genotyped 177 patients with DLE, 85 patients with sporadic SLE, 190 index cases from SLE families and 395 population control individuals from Finland for nine genetic markers at the ITGAM locus. SLE patients were further subdivided by the presence or absence of discoid rash and renal involvement. In addition, 235 Finnish and Swedish patients positive for Ro/SSA-autoantibodies were included in a subphenotype analysis. Analysis of the ITGAM coding variant rs1143679 showed highly significant association to DLE in patients without signs of systemic disease (P-value = 4.73 × 10−11, OR = 3.20, 95% CI = 2.23–4.57). Significant association was also detected to SLE patients (P-value = 8.29 × 10−5, OR = 2.14, 95% CI = 1.52–3.00), and even stronger association was found when stratifying SLE patients by presence of discoid rash (P-value = 3.59 × 10−6, OR = 3.76, 95% CI = 2.29–6.18).

Significance: We propose ITGAM as a novel susceptibility gene for cutaneous DLE. The risk effect is independent of systemic involvement and has an even stronger genetic influence on the risk of DLE than of SLE.

Introduction

Lupus erythematosus (LE) is a complex autoimmune disease with variable clinical course and manifestations. Cutaneous lupus erythematosus (CLE) is a heterogeneous disease entity with manifestations primarily confined to the skin. CLE can be subdivided into chronic cutaneous lupus erythematosus, of which discoid lupus erythematosus (DLE) is the most common form, subacute cutaneous lupus erythematosus (SCLE), and acute cutaneous lupus erythematosus (ACLE) [1]. ACLE is characteristic for systemic lupus erythematosus (SLE) that manifests with severe organ involvement and the presence of haematological and immunological abnormalities, whereas well demarcated, scarring plaques of the face, scalp and ears are often seen in DLE patients. Mild extracutaneous involvement may be present in 14–27% of patients with DLE [2], while only 5–10% progress to an overt SLE.
Lesions typical for DLE are seen in 15–20% of SLE patients [4]. Population-based studies on the prevalence of CLE are rare, but this condition is estimated to be 2–3 times more frequent than SLE [5]. Although the pathogenic mechanisms underlying LE are not yet entirely established, susceptibility is influenced by multiple genetic and environmental factors, including ultraviolet (UV) radiation, certain drugs and oestrogen [3].

A complex genetic component similar to that of SLE may also underlie CLE [3]. The genetic architecture of CLE is poorly understood, but polymorphisms in HLA genes, the TNF-2 promoter and complement molecules have been suggested as strong candidates for CLE susceptibility [6]. We have recently shown that the known SLE risk genes TYK2 and IRF5 also associate with CLE [7].

ITGAM, a member of the immune complex processing pathway, has been consistently replicated as an SLE susceptibility gene [8–11]. The coding variant rs1143679 (R77H) was recently shown to influence the risk of discoid rash in SLE patients [12]. ITGAM (CD11b, Mac-1) encodes the ζ-chain of the β2-integrin (CD11b/CD18 or CR3), a cell surface receptor for multiple ligands, such as complement 3 cleavage fragment and intercellular adhesion molecule-1 (ICAM-1) [13]. The receptor is expressed on neutrophils, macrophages and dendritic cells and is involved in leukocyte adhesion, phagocytosis and regulation of apoptosis [13]. The exact disease aggravating mechanism is not known, but altered interaction with ligands [10] as well as defects in leukocyte trafficking and uptake of apoptotic cells or immune complexes have been suggested [14]. As abnormal removal of apoptotic cells plays an important role in evolving discoid lesions [6], ITGAM is a plausible candidate gene not only for SLE, but also for cutaneous DLE.

In the present study, we investigated the role of ITGAM in a well-characterised cohort of DLE patients without signs of systemic disease. The disease risk was further compared between DLE and a cohort of SLE patients that was stratified for various systemic disease. The risk of discoid rash was 2–3 times more frequent than SLE [5]. Although the pathogenic mechanisms underlying LE are not yet entirely established, susceptibility is influenced by multiple genetic and environmental factors, including ultraviolet (UV) radiation, certain drugs and oestrogen [3].

In the present study, we investigated the role of ITGAM in a well-characterised cohort of DLE patients without signs of systemic disease. The disease risk was further compared between DLE and a cohort of SLE patients that was stratified for various clinical subtypes.

### Materials and Methods

#### Ethics statement

This study was conducted in accordance with the Declaration of Helsinki Principles. All subjects gave their written informed consent and the study was approved by the Ethical Review Boards of Helsinki and Tampere University Central Hospitals, Finland, and the Regional Human Ethics Committee at the Karolinska University Hospital, the Institutional Ethics Board and the Regional Ethics Board, Sweden.

#### Finnish patients and control individuals

Patients from two Finnish cohorts were included in the present study. The recruitment of the case-control cohort and its clinical characteristics have been described recently [15] (Table 1). Altogether 177 DLE (76% women) and 85 SLE (93% women) patients with LE-specific skin manifestations [16] diagnosed by a dermatologist were recruited. The SLE family cohort included 190 probands (94% women) together with their family members (total n = 236) [17]. The diagnosis of DLE was based on generally accepted clinical, histological and immunofluorescence findings [1] and exclusion of SCLE or SLE. In DLE patients without the characteristic discoid rash (n = 14/177 patients) the diagnosis was based on other phenotype-specific manifestations, such as absence of antinuclear and/or double-stranded DNA antibodies, no signs of extracutaneous disease and dermal perifollicular or perivascular mononuclear cell infiltrate [1] as well as careful clinical examination of patients and patient chart review. Furthermore, the histology and location of lesions usually in the head region, the absence of systemic manifestations and negative laboratory findings in these 14 cases supported the diagnosis of DLE rather than SCLE or SLE. All SLE patients fulfilled the revised 1982 ACR classification criteria by Tan et al. [18] for SLE as confirmed by a rheumatologist. A total of 356 anonymous Finnish controls (49% women) comprising of unaffected spouses or common-law spouses of patients and a collection of unrelated healthy individuals were included in the study.

#### Swedish patients and control individuals

Patients from two separate Swedish cohorts were also utilised in the present study. Swedish patients (n = 91) participating in a study assessing the incidence and prevalence of SCLE in Stockholm in 1996–2002 [19] (Table 2) were included. In brief, patients positive for Ro/SS-A-autoantibodies (Ro-positive patients without signs of systemic inflammation = 21, DLE = 2, SCLE = 8, SLE = 31, Sjögren’s syndrome (SS) = 23, undifferentiated connective tissue disease (UCTD) = 6) reported their occurrence of photosensitivity and skin symptoms in a questionnaire. Patients were examined clinically (KP and FN) at the Department of Dermatology, Danderyd Hospital, Stockholm, Sweden. Clinical data were completed based on patient history and medical records. The diagnoses of DLE and SCLE were based on the clinical and histopathological features [1]. The diagnoses of SLE and SS were based on the revised 1982 ACR [18] and the revised European criteria [20], respectively. A diagnosis was based on generally accepted clinical, histological and immunofluorescence findings [1].

### Table 1. Clinical and demographic characteristics of the sets of Finnish patient samples in this study.

| Characteristic                  | Finnish case-control sample | SLE families (n = 236 patients) |
|--------------------------------|-----------------------------|---------------------------------|
|                                | DLE (n = 177)               | SLE (n = 85)                    |
|                                | Female                      | Mean age at onset (range; yrs)  | Mean age at diagnosis (range; yrs) |
|                                | 76                          | 42 (15–77)                      | 45 (17–77)                       |
|                                | 94                          | 36 (8–85)                       | 40 (13–86)                       |
|                                |                             |                                | 29 (1–66)                       |
|                                |                             |                                | 33 (6–72)                       |
|                                | 11                          | 72                              | 51                              |
|                                | 72                          | 92                              | 44                              |
|                                | 65                          | 80                              | 69                              |
|                                | 4                           | 18                              | 18                              |
|                                | 2                           | 64                              | 83                              |
|                                | 1                           | 20                              | 30                              |
|                                | 7                           | 37                              | 68                              |
|                                | 5                           | 17                              | 16                              |
|                                | 28                          | 98                              | na                              |
|                                | 25                          | 66                              | na                              |
|                                | 4                           | 31                              | na                              |
|                                | 9                           | 37                              | na                              |

1Because of differences in laboratory methods and reference values, only patients from Helsinki were included. Na = not available. Percentage (%) of patients with each phenotype is shown except for mean age, which is shown in years (yrs). doi:10.1371/journal.pone.0014212.t001
of UCTD was based on signs and symptoms suggestive of a connective tissue disease and the presence of antinuclear antibodies at two different occasions [21]. Ro-autoantibodies were tested in accredited laboratories in Stockholm using enzyme-linked immunosorbent assay (ELISA) [19].

Another Swedish cohort consisted of patients with SS (n = 73) fulfilling the revised European criteria [20] and attending the Karolinska University Hospital, Stockholm, Sweden during 1998–2008 (Table 2). The diagnosis was verified by a combination of clinical examination by rheumatologists or medical doctors and a questionnaire. Ro/SSA-autoantibodies were detected by an ELISA using recombinant, purified Ro52 and Ro60 antigens [22].

A total of 164 Swedish patients with a diagnosis of a connective tissue disease from two distinct cohorts were thus included in the study. Out of these 164 genotyped patients, altogether 134 individuals were positive for Ro/SSA-autoantibodies and were further utilised to evaluate the role of ITGAM in the presence of Ro response. Swedish control samples (n = 295; 90.5% women) were population-based anonymous control individuals from the Epidemiological Investigation of Rheumatoid Arthritis (EIRA) study [23].

### Statistical analysis

Haplotype v. 4.0 [25] was used to study linkage disequilibrium (LD) patterns, estimate haplotypes (Figure 1A–C) and perform association analyses. To increase the statistical power of the association analysis, the sporadic SLE cases (n = 85) were analysed in combination with unrelated probands (n = 190) from the SLE family cohort, thus giving a total of 275 independent Finnish SLE patients and 356 controls for analysis. Allele and haplotype counts were compared between patients and controls by chi-square test, and permutation testing (10,000 iterations) as implemented into Haplovew was performed in order to obtain a measure of significance corrected for multiple testing bias. Due the strong LD (Figure 1A) throughout the ITGAM locus and thus dependence between markers, traditional Bonferroni correction would likely to be overly conservative. Two-tailed, uncorrected P-values are reported in the Results section, and odds ratios (OR) with their respective 95% confidence intervals (CI) were calculated using GraphPad Prism v.4.03 (GraphPad Software Inc., La Jolla, CA, USA).

### Patient stratification for subphenotype analysis

Finnish SLE patients were further stratified by presence of discoid rash (n = 55) and renal involvement (n = 76) defined as fulfilment of the ACR renal criteria [18]. Finnish (n = 101) and Swedish (n = 134) patients positive for Ro/SSA-autoantibodies were first analysed individually and further in combination (n = 235) as a pooled dataset in order to study the association between ITGAM and immunological factors [12,26]. Ro-autoantibodies were selected for detailed investigation, because they may have pathobiological importance in apoptosis and phagocytosis [27] similarly to ITGAM, and prognostic value for predicting systemic inflammatory disease [28]. Furthermore, several genetic regions harbouring genes for adhesion molecules have shown evidence of association to DLE in the presence of Ro-autoantibodies [6] and the disease progression may be highly dynamic in Ro-positive patients [29].

### Power calculations

The power to detect association of ITGAM to different forms of LE in our sample sets was estimated using the online Power for Association With Error program (http://linkage.rockefeller.edu/pave/). We assumed a risk allele frequency of 0.185, an OR of 1.70 [11] and alpha 0.05. The average power to detect association of ITGAM polymorphisms was 84% in DLE and 91% in SLE. In subphenotype analyses when using reported reference values [12], the power to detect association of rs1143679 was 77% in SLE with discoid rash and 92% in SLE with renal involvement. For polymorphisms (SNP) at the ITGAM locus showing replicated association to SLE in different populations were selected for genotyping: rs1143679, rs9936831, rs99357837, rs9888079, rs12928810, rs9886739, rs11860650, rs6565227, rs1143678, rs4548993 and rs11574637.

Genotyping was performed according to manufacturer’s instructions using Sequenom iPLEX Gold chemistry [24] (Sequenom Inc., San Diego, California, USA). Assay design and genotyping were performed in the genotyping core facility at Karolinska Institutet, Huddinge, Sweden. The average genotyping success rate of each marker was 94% for the Finnish case-control cohort, 96% for the Finnish SLE families and 94% for the Swedish case-control cohort; all markers were in Hardy-Weinberg equilibrium (P > 0.05) in controls. The markers rs12928810 (only in SLE families), rs6565227 and rs1143678 had success rates below our threshold (<85%) and were thus excluded.

### Table 2. Clinical and demographic characteristics of the Swedish sample sets in this study.

| Swedish case-control sample | Connective tissue disease patients (n = 91) | Sjögren’s syndrome patients (n = 73) |
|-----------------------------|------------------------------------------|--------------------------------------|
| Female                      | 91                                       | 95                                   |
| Mean age at onset (range; yrs) | 58 (18–83)                               | na                                   |
| Mean age at diagnosis (range; yrs) | na                                       | 52 (20–87)                           |
| Butterfly rash              | 13                                       | na                                   |
| Discoild rash                | 19                                       | na                                   |
| Annular SCLE lesions        | 14                                       | na                                   |
| Psoriasisform SCLE lesions  | 14                                       | na                                   |
| Photosensitivity            | 100                                      | na                                   |
| Mouth ulcers                | na                                       | na                                   |
| Arthritis                   | 5                                        | 23                                   |
| Renal involvement           | 5                                        | 4                                    |
| Leukopenia                  | na                                       | 18                                   |
| Thrombocytopenia            | na                                       | 3                                    |
| Elevated antinuclear antibodies | 74                                      | 55                                   |
| Ro/SSA antibody positivity  | 100                                      | 59                                   |
| La/SSB antibody positivity  | 38                                       | 38                                   |
| Double-stranded DNA antibody positivity | 13 | na |

1 Ro/SSA positive patients without signs of systemic inflammation = 21, DLE = 2, SCLE = 8, SLE = 31, SS = 23, UCTD = 6 from the study of Popovic et al. [19].

2 SS patients, data provided by Prof. Marie Wahren-Herlenius, MD, PhD, Dept. of Medicine, Rheumatology Unit, Karolinska Institutet, Solna, Sweden.

3 Mean age at initial testing for Ro/SSA-autoantibodies [19].

Na = data not available.

Percentage (%) of patients is shown except for mean age, which is shown in years (yrs).

doi:10.1371/journal.pone.0014212.t002

Genotyping

After review of the literature regarding ITGAM published prior to the initiation of this study [8–10], eleven single nucleotide polymorphisms (SNP) at the ITGAM locus showing replicated association to SLE in different populations were selected for genotyping: rs1143679, rs9936831, rs99357837, rs9888079, rs12928810, rs9886739, rs11860650, rs6565227, rs1143678, rs4548993 and rs11574637.

Genotyping was performed according to manufacturer’s instructions using Sequenom iPLEX Gold chemistry [24] (Sequenom Inc., San Diego, California, USA). Assay design and genotyping were performed in the genotyping core facility at Karolinska Institutet, Huddinge, Sweden. The average genotyping success rate of each marker was 94% for the Finnish case-control cohort, 96% for the Finnish SLE families and 94% for the Swedish case-control cohort; all markers were in Hardy-Weinberg equilibrium (P > 0.05) in controls. The markers rs12928810 (only in SLE families), rs6565227 and rs1143678 had success rates below our threshold (<85%) and were thus excluded.

### Statistical analysis

Haplotype v. 4.0 [25] was used to study linkage disequilibrium (LD) patterns, estimate haplotypes (Figure 1A–C) and perform association analyses. To increase the statistical power of the association analysis, the sporadic SLE cases (n = 85) were analysed in combination with unrelated probands (n = 190) from the SLE family cohort, thus giving a total of 275 independent Finnish SLE patients and 356 controls for analysis. Allele and haplotype counts were compared between patients and controls by chi-square test, and permutation testing (10,000 iterations) as implemented into Haplovew was performed in order to obtain a measure of significance corrected for multiple testing bias. Due the strong LD (Figure 1A) throughout the ITGAM locus and thus dependence between markers, traditional Bonferroni correction would likely to be overly conservative. Two-tailed, uncorrected P-values are reported in the Results section, and odds ratios (OR) with their respective 95% confidence intervals (CI) were calculated using GraphPad Prism v.4.03 (GraphPad Software Inc., La Jolla, CA, USA).

### Patient stratification for subphenotype analysis

Finnish SLE patients were further stratified by presence of discoid rash (n = 55) and renal involvement (n = 76) defined as fulfilment of the ACR renal criteria [18]. Finnish (n = 101) and Swedish (n = 134) patients positive for Ro/SSA-autoantibodies were first analysed individually and further in combination (n = 235) as a pooled dataset in order to study the association between ITGAM and immunological factors [12,26]. Ro-autoantibodies were selected for detailed investigation, because they may have pathobiological importance in apoptosis and phagocytosis [27] similarly to ITGAM, and prognostic value for predicting systemic inflammatory disease [28]. Furthermore, several genetic regions harbouring genes for adhesion molecules have shown evidence of association to DLE in the presence of Ro-autoantibodies [6] and the disease progression may be highly dynamic in Ro-positive patients [29].

### Power calculations

The power to detect association of ITGAM to different forms of LE in our sample sets was estimated using the online Power for Association With Error program (http://linkage.rockefeller.edu/pave/). We assumed a risk allele frequency of 0.185, an OR of 1.70 [11] and alpha 0.05. The average power to detect association of ITGAM polymorphisms was 84% in DLE and 91% in SLE. In subphenotype analyses when using reported reference values [12], the power to detect association of rs1143679 was 77% in SLE with discoid rash and 92% in SLE with renal involvement. For
Ro-positive Finnish, Swedish and the pooled dataset a power of 70%, 75% and 95%, respectively, was obtained when previously reported risk estimates were assumed [11].

**Results**

Nine ITGAM SNPs were successfully genotyped in three datasets corresponding to two different LE subgroups (Finnish patients with DLE or SLE) and control individuals. Subphenotype analyses were further performed to investigate the role of ITGAM in specific clinical manifestations. These analyses showed that the magnitude of association was strongest in cutaneous DLE ($P$-value range from $4.7 \times 10^{-11}$ to $8.3 \times 10^{-8}$) (Table 3). The same marker was observed to reach a more humble, although statistically significant, effect in subphenotype analyses ($P$-value range from $6.4 \times 10^{-6}$ to 0.04) (Tables 4 and 5), which may mirror the impact of sample size. In addition to rs1143679, the eight other markers studied were observed to associate significantly in Finnish patients (Tables 3, 4, 5), whereas Swedish patients showed borderline association (Table 5). In general, the risk allele frequencies were somewhat higher in DLE, and SLE patients with discoid rash compared to unstratified SLE patients in our study (Table 3) or previous studies, whereas our control individuals had frequencies similar to other control populations of European origin [8,10,11]. A similar increase in ORs was also observed, the risk estimates being highest in SLE patients with discoid rash (range 2.46–3.95) and in DLE patients (range 1.65–3.20) followed by unstratified SLE patients (range 1.52–2.14) (Table 3), SLE patients with renal involvement (range 1.82–2.53) (Table 4) and Ro-positive patients (range 1.37–2.03 in the pooled dataset) (Table 5).

**Haplotype associations**

The haplotype structure of ITGAM was further investigated in each set of samples, but this neither improved statistical significance nor yielded any further information (Figure 1 B). One high risk marker was observed to reach a more humble, although statistically significant, effect in subphenotype analyses ($P$-value range from $6.4 \times 10^{-6}$ to 0.04) (Tables 4 and 5), which may mirror the impact of sample size. In addition to rs1143679, the eight other markers studied were observed to associate significantly in Finnish patients (Tables 3, 4, 5), whereas Swedish patients showed borderline association (Table 5). In general, the risk allele frequencies were somewhat higher in DLE, and SLE patients with discoid rash compared to unstratified SLE patients in our study (Table 3) or previous studies, whereas our control individuals had frequencies similar to other control populations of European origin [8,10,11]. A similar increase in ORs was also observed, the risk estimates being highest in SLE patients with discoid rash (range 2.46–3.95) and in DLE patients (range 1.65–3.20) followed by unstratified SLE patients (range 1.52–2.14) (Table 3), SLE patients with renal involvement (range 1.82–2.53) (Table 4) and Ro-positive patients (range 1.37–2.03 in the pooled dataset) (Table 5).
Table 3. Single marker association results in Finnish DLE, in SLE with discoid rash and in SLE patients.

| Marker | Alleles | Associated allele | Controls (n = 356) | DLE (n = 177) | SLE with discoid rash (n = 55) | SLE (n = 275) |
|--------|---------|-------------------|-------------------|--------------|-------------------|--------------|
|        |         |                   | Freq. | P-value (corrected) | OR (95% CI) | Freq. | P-value (corrected) | OR (95% CI) | Freq. | P-value (corrected) | OR (95% CI) |
| rs1143679 | A/G | A | 0.10 | 0.26 | 4.73 x 10^-11 (<0.0001) | 3.30 (2.23–4.57) | 0.29 | 3.59 x 10^-8 (<0.0001) | 3.76 (2.29–6.18) | 0.19 | 8.29 x 10^-6 (<0.0001) | 2.14 (1.52–3.00) |
| rs9936831 | T/A | T | 0.10 | 0.26 | 2.14 x 10^-10 (<0.0001) | 3.03 (2.13–4.31) | 0.31 | 3.99 x 10^-9 (<0.0001) | 3.95 (2.44–6.40) | 0.19 | 1.32 x 10^-5 (<0.0001) | 2.08 (1.49–2.90) |
| rs9937837 | G/T | G | 0.24 | 0.34 | 6.00 x 10^-4 (0.004) | 1.65 (1.24–2.20) | 0.43 | 1.97 x 10^-5 (5.00 x 10^-4) | 2.46 (1.61–3.76) | 0.32 | 1.20 x 10^-3 (0.01) | 1.52 (1.18–1.96) |
| rs9888779 | C/T | C | 0.10 | 0.26 | 7.57 x 10^-11 (<0.0001) | 3.06 (2.16–4.34) | 0.31 | 3.38 x 10^-9 (<0.0001) | 3.93 (2.43–6.34) | 0.19 | 1.10 x 10^-5 (<0.0001) | 2.06 (1.49–2.86) |
| rs12928810 | A/G | A | 0.10 | 0.23 | 8.03 x 10^-8 (<0.0001) | 2.77 (1.89–4.07) | na | na | na | na | na | na |
| rs9888739 | T/C | T | 0.10 | 0.25 | 2.91 x 10^-10 (<0.0001) | 3.01 (2.12–4.29) | 0.30 | 1.03 x 10^-8 (<0.0001) | 3.88 (2.38–6.33) | 0.18 | 7.52 x 10^-5 (7.00 x 10^-4) | 1.96 (1.40–2.74) |
| rs11860650 | T/C | T | 0.10 | 0.27 | 3.71 x 10^-11 (<0.0001) | 3.13 (2.21–4.43) | 0.31 | 1.10 x 10^-8 (<0.0001) | 3.81 (2.35–6.18) | 0.19 | 3.36 x 10^-5 (1.00 x 10^-4) | 1.99 (1.43–2.77) |
| rs4588893 | T/C | T | 0.15 | 0.32 | 1.10 x 10^-10 (<0.0001) | 2.74 (2.00–3.74) | 0.35 | 2.61 x 10^-7 (<0.0001) | 3.14 (2.49–4.94) | 0.22 | 9.00 x 10^-4 (0.008) | 1.64 (1.22–2.21) |
| rs11576437 | C/T | C | 0.15 | 0.30 | 2.37 x 10^-9 (<0.0001) | 2.57 (1.87–3.52) | 0.37 | 1.98 x 10^-8 (<0.0001) | 3.43 (2.19–5.37) | 0.22 | 5.00 x 10^-4 (0.004) | 1.69 (1.25–2.27) |

The frequency of the associated allele in controls and cases is shown, as well as its uncorrected P-value and P-value corrected for multiple testing (in parentheses) as well as odds ratio (OR) with 95% confidence interval (CI).

Abbreviations: DLE, discoid lupus erythematosus; SLE, systemic lupus erythematosus.

The marker has success below the study threshold (<85%) and was excluded from analysis in SLE.

doi:10.1371/journal.pone.0014212.e003

haplotype carrying the minor allele A of rs1143679 (AGTGCCCT; P-value = 7.4 x 10^-12, OR = 3.33, 95% CI = 2.33–4.75) and two haplotypes with a protective effect (GAGTGCCCT; P-value = 7.6 x 10^-5, OR = 0.50, 95% CI = 0.44–0.76, and GAGTGCCCT; P-value = 1.7 x 10^-2, OR = 0.57, 95% CI = 0.36–0.92), reaching statistical significance, were found in DLE (Figure 1B). The same risk haplotype with equally strong effect on risk was observed in SLE (OR = 2.20, 95% CI = 1.57–3.09), in SLE patients with discoid rash (OR = 4.03, 95% CI = 2.47–6.59) (Figure 1B) and in SLE patients with renal involvement (OR = 2.59, 95% CI = 1.60–4.21) (data not shown). Finnish and Swedish patients positive for Ro(SSA)-autoantibodies analysed either separately or pooled together showed the same haplotype patterns as DLE and SLE patients (data not shown). In line with other studied patient groups, the risk conferring ATGCATTTC haplotype showed evidence of strong association (P = 3.0 x 10^-6, OR = 2.08, 95% CI = 1.52–2.63) within Ro-positive patients when Finnish and Swedish subjects were analysed in combination.

Table 4. Single marker association results in Finnish SLE patients with renal involvement.

| Marker | Alleles | Associated allele | Controls (n = 356) | Patients (n = 76) |
|--------|---------|-------------------|-------------------|-------------------|
|        |         |                   | Freq. | P-value (corrected) | OR (95% CI) | Freq. | P-value (corrected) | OR (95% CI) |
| rs1143679 | A/G | A | 0.10 | 0.21 | 1.00 x 10^-4 (0.003) | 2.49 (1.54–4.01) | 0.22 | 1.00 x 10^-4 (0.003) | 2.43 (1.51–3.89) |
| rs9936831 | T/A | T | 0.10 | 0.22 | 0.005 (0.03) | 1.74 (1.18–2.57) | 0.23 | 5.83 x 10^-3 (0.002) | 2.53 (1.59–4.02) |
| rs9937837 | G/T | G | 0.24 | 0.35 | 0.001 (0.01) | 2.22 (1.35–3.65) | 0.23 | 9.71 x 10^-3 (0.002) | 2.49 (1.55–3.97) |
| rs9888779 | C/T | C | 0.10 | 0.22 | 0.002 (0.02) | 1.99 (1.29–3.07) | 0.24 | 0.008 (0.05) | 1.82 (1.16–2.83) |
| rs11860650 | T/C | T | 0.15 | 0.25 | 0.001 (0.01) | 2.33 (1.50–3.66) | 0.22 | 0.002 (0.02) | 1.99 (1.29–3.07) |
| rs4588893 | T/C | T | 0.15 | 0.24 | 1.00 x 10^-4 (0.003) | 2.48 (1.54–4.01) | 0.22 | 1.00 x 10^-4 (0.003) | 2.43 (1.51–3.89) |

The frequency of the associated allele in controls and cases is shown, as well as its uncorrected P-value and P-value corrected for multiple testing (in parentheses) as well as odds ratio (OR) with 95% confidence interval (CI). The marker rs12928810 has success below the study threshold (<85%) and was excluded from analysis. Renal involvement is defined as fulfilment of the ACR renal criteria [18].

doi:10.1371/journal.pone.0014212.e004
Table 5. Single marker association results in individual Finnish and Swedish patients positive for Ro/SSA-autoantibody as well as in a combined dataset.1

| Marker Alleles | Associated allele | Finnish | | Swedish | | Combined dataset | |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | Controls (n = 356) | Patients (n = 101) | Controls (n = 295) | Patients (n = 134) | Controls (n = 651) | Patients (n = 235) | |
| rs1143679 A/G | A | 0.10 0.22 | 6.61 x 10^-6 (0.0001) | 2.65 (1.71-4.10) | 0.10 0.15 | 0.04 (0.26) | 1.62 (1.03-2.54) | 0.10 0.18 | 6.38 x 10^-6 (2.00 x 10^-3) | 2.03 (1.49-2.78) |
| rs9936831 T/A | T | 0.10 0.23 | 1.35 x 10^-3 (0.0001) | 2.54 (1.65-3.90) | 0.11 0.16 | 0.05 (0.36) | 1.54 (0.99-2.39) | 0.11 0.19 | 1.54 x 10^-3 (2.00 x 10^-3) | 1.95 (1.43-2.65) |
| rs9937837 G/T | G | 0.24 0.30 | 0.06 (0.33) | 1.40 (0.99-2.00) | 0.25 0.31 | 0.1 (0.53) | 1.32 (0.95-1.83) | 0.24 0.31 | 0.01 (0.08) | 1.37 (1.07-1.73) |
| rs988879 C/T | C | 0.10 0.23 | 6.31 x 10^-6 (0.0001) | 2.55 (1.68-3.87) | 0.11 0.15 | 0.05 (0.31) | 1.55 (1.00-2.40) | 0.10 0.19 | 7.80 x 10^-6 (2.00 x 10^-3) | 1.97 (1.46-2.66) |
| rs12928810 A/G | A | 0.10 0.18 | 0.006 (0.0443) | 1.96 (1.21-3.19) | 0.10 0.16 | 0.01 (0.07) | 1.80 (1.16-2.82) | 0.10 0.17 | 2.00 x 10^-4 (0.002) | 1.86 (1.34-2.58) |
| rs988739 T/C | T | 0.10 0.22 | 1.47 x 10^-5 (0.0001) | 2.51 (1.64-3.85) | 0.11 0.15 | 0.08 (0.46) | 1.48 (0.95-2.31) | 0.10 0.18 | 3.00 x 10^-5 (4.00 x 10^-4) | 1.91 (1.40-2.59) |
| rs11860650 T/C | T | 0.10 0.23 | 9.86 x 10^-6 (0.0001) | 2.54 (1.66-3.87) | 0.11 0.14 | 0.17 (0.69) | 1.37 (0.87-2.16) | 0.11 0.18 | 8.04 x 10^-5 (8.00 x 10^-4) | 1.84 (1.36-2.51) |
| rs4548993 T/C | T | 0.15 0.27 | 6.96 x 10^-5 (0.0009) | 2.15 (1.46-3.14) | 0.19 0.21 | 0.43 (0.99) | 1.16 (0.80-1.68) | 0.17 0.24 | 8.00 x 10^-4 (0.008) | 1.57 (1.20-2.04) |
| rs11574637 C/T | C | 0.15 0.25 | 4.00 x 10^-4 (0.0048) | 1.99 (1.35-2.93) | 0.16 0.21 | 0.08 (0.48) | 1.39 (0.95-2.04) | 0.15 0.23 | 2.00 x 10^-4 (0.003) | 1.65 (1.26-2.16) |

The frequency of the associated allele in controls and cases is shown, as well as its uncorrected P-value, and P-value corrected for multiple testing (in parentheses) as well as odds ratio (OR) with 95% confidence interval (CI). 1The Finnish sample set consists of sporadic patients with DLE (n = 36), SCLE (n = 25) and SLE (n = 40) [15]. Ro/SSA information was not available for SLE family probands. The Swedish sample set consists of 21 Ro-positive patients without signs of systemic inflammation, 6 patients with UCTD, 2 DLE, 8 SCLE, 31 SLE, and 66 SS patients.

doi:10.1371/journal.pone.0014212.t005
Discussion

In the present study we have shown that polymorphisms at the ITGAM locus increase the risk of cutaneous DLE, three-fold compared to healthy control individuals, and the magnitude of the association is five times higher in DLE than in SLE patients. To our knowledge, the current study is the first to address the role of ITGAM in a clinically verified cohort of cutaneous DLE patients without signs of systemic disease. We furthermore replicated in our series of SLE patients the recent observation of Kim-Howard et al. [12] that the coding variant rs1143679 (R77H) may influence the risk of discoid rash in SLE.

The marker rs1143679 showed strong association in DLE, in SLE patients with discoid rash and in all SLE patients together. In patient subgroups stratified for renal involvement or Ro/SSA-antibody positivity more modest association was observed. The ITGAM exon 3 variant rs1143679 was originally identified and replicated in three different populations [10] parallel with GWA studies [8,9]. To date, its robust association with SLE has been confirmed in a meta-analysis encompassing populations across the world [11], but it has not previously been studied in the cutaneous forms of LE.

Several variants within ITGAM and the adjacent ITGAX gene (Table 3) were found to associate strongly to DLE and also to SLE with discoid rash. It has, however, been speculated that these multiple association signals are mainly reflecting the strong LD between rs1143679 and nearby markers [11] (Figure 1A), as any haplotype carrying the risk allele A at rs1143679 within ITGAM confers risk for SLE [10]. Our haplotype analysis is in line with this (Figure 1B).

Two previous studies have systematically screened several clinical subphenotypes of SLE, including patients with renal and immunological disorders, for association to ITGAM [12,26]. In line with these reports we observed increased risk in SLE patients with renal involvement. We further investigated the relationship between ITGAM and immunological disorders in a group of patients positive for Ro/SSA-antibodies and found significant association in accordance with findings from a previous study [26]. Possible pathomechanisms underlying these associations have been proposed [12,26], but not yet established. Interestingly, Ro-antibodies and the product of the ITGAM gene function on the same biological pathways of apoptosis and phagocytosis. It has recently been shown that high Ro32 expression induces apoptosis, and it has been postulated that apoptotic cell opsonisation with Ro-positive sera might rather prevent that than facilitate phagocytosis [27]. However, as Ro-antibodies are not exclusive to LE, it is difficult to draw definitive conclusions about this undoubtedly complex relationship between ITGAM, Ro-positivity and LE based on observations by us and others [26].

The only ITGAM variant for which a functional role has been proposed to date is the previously mentioned rs1143679. This polymorphism results in an amino acid change (R77H) that may alter the structure and function of the receptor protein and further affect its ligand binding activities [10] thus influencing, for instance, ICAM-1 mediated leukocyte recruitment [10], essential in the formation of the inflammatory lesions seen in DLE [6,14,18]. Interestingly, ICAM-1 has been shown to be involved in the pathogenesis of various types of skin disease [30] especially in the presence of Ro/SSA-response [3] and it is upregulated in skin lesions of patients with DLE [30].

ITGAM is also interesting in the context of the photosensitivity generally observed in DLE patients [6]. UV-B irradiation initiates a cascade of proinflammatory events in the skin [5,31]. Upon UV exposure, macrophages expressing ITGAM invade the dermis and epidermis [32,33], leading to a depletion of antigen presenting cells [32,34]. There is a delay in dendritic cell differentiation in UV-exposed skin [34] and antigenic tolerance is induced [31]. Furthermore, IL-10, a cytokine known to trigger DLE, and discoid rash in SLE [35], is upregulated upon UV exposure. Genetic alterations of ITGAM function(s) along this pathway may therefore lead to defects in the suppression of dendritic cell differentiation in combination with high IL-10 production and induce inadvertent immune reactions in DLE patients. Furthermore, UV induces also the production of TNF-α [36], a proinflammatory cytokine that has been, in combination with pronounced IL-10 production, suggested to increase DLE risk [35]. Moreover, TNF-α primes neutrophils to migrate into inflammatory sites through the upregulation of CD11b/CD18 (ť2β3-integrin) [37].

In the present study, a panel of ITGAM polymorphisms was specifically tested for association to cutaneous DLE in a well-described cohort of patients that is one of the largest reported. Our study has contributed with ITGAM to the short list of genes shown to be associated with DLE. The genetic background of CLE, and that of DLE in particular, is poorly characterised. Furthermore, studies on this disease have been difficult to evaluate and replicate due to low sample sizes non-reporting of risk effect estimates. There are only a few confirmed risk loci, including HLA genes, the TNF-α promoter and complement factors [6]. However, TNF-α shows only modest association [30,38] and the association may be merely due to the strong LD across the HLA region. These sparse data indicate that the genetic background of DLE and other forms of CLE may be very complex and require further studies. Only a few studies have addressed the gender differences in CLE pathogenesis [39] and it is still unclear, what the effect of sex in this disorder may be. Women are known to respond to infection, vaccination and trauma with increased antibody production and a more T helper cell 2 predominant immune response, whereas a T helper cell 1 response and inflammation are usually more severe in men [40]. Thus, it is reasonable to assume that, especially in the function of above mentioned immune system related genes, there would be alterations in the disease mechanisms between female and male subjects. However, investigating this in future studies is a challenging task given the female preponderance of the disease, and carefully matched control individuals are needed as well.

In conclusion, we have demonstrated here a strong novel association between ITGAM variants and cutaneous DLE without signs of systemic disease. These variants function independently of the systemic disease and increase substantially the risk of DLE with an effect even higher than in SLE. Our results further strengthen the hypothesis that different forms of LE are not genetically distinct entities even though their clinical course varies. Based on its function(s), ITGAM may predispose to DLE through impaired phagocytosis, leukocyte trafficking or immune suppression in UV-exposed skin. The exact mechanisms by which ITGAM contributes to the disease pathogenesis and how its expression may be altered in affected DLE skin, warrant further studies.

Acknowledgments

This study was completed before the untimely death of Professor Ulpu Saarialho-Kere (1960–2009) to whose memory this report is dedicated. We thank Professors Anna-Elina Lehesjoki and Albert de la Chapelle for providing control samples, Dr. Karin Popovic for clinical investigation and PhD Valma Harjutsalo for advice in the statistical analysis.

Author Contributions

Conceived and designed the experiments: TMJ. Performed the experiments: AH. Analyzed the data: TMJ. Contributed reagents/materials/analysis tools: SK EE JP TH HJ JP MK MWH FN JK USK. Wrote the paper: TMJ SK EE MD USK. Group leader: JK. Project supervision: JK USK. Critical review of the manuscript: EE MR.
1. Gilliam JN, Sontheimer RD (1981) Distinctive cutaneous subsets in the spectrum of lupus erythematosus. J Am Acad Dermatol 4: 471–475.

2. Kuhn A, Sontheimer RD (2005) Cutaneous lupus erythematosus: molecular and cellular basis of clinical findings. Curr Dir Autoimmun 10: 119–140.

3. Lee HJ, Sinha AA (2006) Cutaneous lupus erythematosus: understanding of clinical features, genetic basis, and pathobiology of disease guides therapeutic strategies. Autoimmunity 39: 433–444.

4. Yell JA, Mbaagbah J, Burge SM (1996) Cutaneous manifestations of systemic lupus erythematosus. Br J Dermatol 135: 355–362.

5. Werth VP (2007) Cutaneous lupus: insights into pathogenesis and disease classification. Bull NYU Hosp Jt Dis 65: 200–204.

6. Kuhn A, Rondinone R, Doria A, Shoenfeld Y (2005) 1st International Conference on Cutaneous Lupus Erythematosus Dusseldorf, Germany, September 1-5, 2004. Autoimmun Rev 4: 66–78.

7. Jarvinen TM, Hellquist A, Koskenius E, Einarsdottir E, Koskinen LLE, et al. (2010) Tyrosine kinase 2 and interferon regulatory factor 5 polymorphisms are associated with discoid and subacute cutaneous lupus erythematosus. Exp Dermatol 19: 123–131.

8. Harley JB, Alarcon-Riquelme ME, Criswell LA, Kimberly RP, et al. (2008) Association of Systemic Lupus Erythematosus with C8orf13-BLK and ITGAM-ITGAX. N Engl J Med 358: 956–961.

9. Ninh SK, Han S, Kim-Howard X, Kelly JA, Viewanathan P, et al. (2008) A nonsynonymous functional variant in integrin-alpha(M) (encoded by ITGAM) is associated with systemic lupus erythematosus. Nat Genet 40: 152–154.

10. Han S, Kim-Howard X, Deshmukh H, Kamatani Y, Viewanathan P, et al. (2009) Evaluation of imputation-based association in and around the integrin-alpha-M (ITGAM) gene and replication of robust association between a non-synonymous functional variant within ITGAM and systemic lupus erythematosus (SLE). Hum Mol Genet 18: 1171–1180.

11. Kim-Howard X, Maiti AK, Anaya JM, Bruner GR, Browen E, et al. (2009) ITGAM coding variant rs1143676 influences the risk of renal disease, discoid rash, and immunologic manifestations in lupus patients with European ancestry. Ann Rheum Dis 68: 1329–1332.

12. Fagerhol MS, Varis M, Stefanidakis M, Hilden TJ, Gahmberg CG (2006) Alpha-Chain phosphorylation of the human leukocyte CD11b/CD18 (Mac-1) integrin is pivotal for integrin activation to bind ICAMs and leukocyte extravasation. Blood 108: 3379–3386.

13. Rhodes B, Vye TJ (2008) The genetics of SLE: an update in the light of genome-wide association studies. Rheumatology (Oxford) 47: 1600–1611.

14. Koskenius S, Jarvinen TM, Oskamo P, Panaclo P, Tuovinen U, et al. (2008) Clinical and laboratory characteristics of Finnish lupus erythematosus patients with cutaneous manifestations. Lupus 17: 337–347.

15. Lin JH, Dutz JP, Sontheimer RD, Werth VP (2007) Pathophysiology of cutaneous lupus erythematosus. Clin Rev Allergy Immunol 33: 85–106.

16. Koskenius S, Widen E, Kere J, Julkunen H (2001) Familial systemic lupus erythematosus in Finland. J Rheumatol 28: 739–760.

17. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, et al. (1982) The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 25: 1271–1277.

18. Popovic K, Nyberg F, Wahren-Herlenius M, Nyberg F (2007) 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 56: 253–264.

19. Vitali C, Bombardieri S, Jonsson R, Mountopoulos HM, Alexander EL, et al. (2002) Classification criteria for Sjögren’s syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 61: 544–550.

20. Mosca M, Neri R, Bombardieri S (1999) Undifferentiated connective tissue diseases (UCTD): a review of the literature and a proposal for preliminary classification criteria. Clin Exp Rheumatol 17: 615–620.

21. Tengner P, Halse AK, Haga HJ, Jonsson R, Wahren-Herlenius M (1998) Detection of anti-Ro/SSA and anti-La/SSB autoantibody-producing cells in salivary glands from patients with Sjögren’s syndrome. Arthritis Rheum 41: 2238–2246.

22. Stolt P, Bengtsson C, Nordmark B, Lundsblad S, Lundberg I, et al. (2003) Quantification of the influence of cigarette smoking on rheumatoid arthritis: results from a population based case-control study, using incident cases. Ann Rheum Dis 62: 833–841.

23. Jurinke C, van den Boom D, Cantor CR, Koster H (2002) Automated genotyping using the DNA MassArray technology. Methods Mol Biol 187: 179–192.

24. Barrett JC, Fry B, Maller J, Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21: 263–265.

25. Yang W, Zhao M, Hiraoka N, Lau CS, Mok CC, et al. (2009) ITGAM is associated with disease susceptibility and renal nephritis of systemic lupus erythematosus in Hong Kong Chinese and Thai. Hum Mol Genet 18: 2063–2070.

26. Oke V, Vassilikis I, Espinosa A, Straulberg L, Kuchroo VK, et al. (2009) High Re/Sc Expression in Spontaneous and UV-Induced Cutaneous Inflammation. J Invest Dermatol 129: 2000–2010.

27. Popovic K, Brauner S, Ek M, Wahren-Herlenius M, Nyberg F (2007) 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 16: 10–17.

28. Popovic K, Wahren-Herlenius M, Nyberg F (2008) Clinical follow-up of 102 anti-Ro/SSA-positive patients with dermatological manifestations. Acta Derm Venereol 88: 379–375.

29. Kuhn A, Sonntag M, Sunderkotter C, Lehmann P, Vestweber D, et al. (2002) Upregulation of epidermal surface molecule expression in primary and ultraviolet-induced lesions of lupus erythematosus tumidus. Br J Dermatol 146: 801–809.

30. Hammerberg C, Kattay SK, Carroll MC, Cooper KD (1998) Activated complement component 3 (C3) is required for ultraviolet induction of immunosuppression and antigen tolerance. J Exp Med 187: 1133–1138.

31. Kuhn A, Gilliam AC, Chen C, Tootell E, Cooper KD (1998) In human skin, UVB initiates early induction of IL-10 over IL-12 preferentially in the expanding dermal mononuclear/macrophagic population. J Invest Dermatol 111: 31–38.

32. Kolgen W, Van Weelden H, Den Hengst S, Guikers KL, Kiekens RC, et al. (1999) CD11b+ cells and ultraviolet-B-resistant CD1a+ cells in skin of patients with polymorphous light eruption. J Invest Dermatol 113: 4–10.

33. Takahara M, Kang K, Liu L, Yoshida Y, McCormick TS, et al. (2003) cCb3 arrests monocyte cell differentiation into CD1c-expressing dendritic cell precursors: a mechanism for transiently decreased dendritic cells in vivo after human skin injury by ultraviolet B. J Invest Dermatol 120: 802–809.

34. Suarez A, Lopez P, Mozlo L, Gutierrez C (2005) Differential effect of IL-10 and TNF(α)/alpha) genotypes on determining susceptibility to discoid and systemic lupus erythematosus. Ann Rheum Dis 64: 1605–1610.

35. Werth VP, Zhang W, Dzembruk K, Sullivan K (2000) Association of a promoter polymorphism of tumor necrosis factor-alpha with subacute cutaneous lupus erythematosus and distinct photoregulation of transcription. J Invest Dermatol 115: 726–730.

36. Montecucco F, Steffens S, Burger F, Da Costa A, Bianchi G, et al. (2008) Tumor necrosis factor-alpha (TNF-alpha) induces integrin CD11b/CD18 (Mac-1) up-regulation and migration to the CC chemokine CCL3 (MIP-1alpha) on human neutrophils through defined signalling pathways. Cell Signal 20: 557–568.

37. Billard TP, Kondeatis E, Cox A, Wilson AG, Grabczynska SA, et al. (2001) A candidate gene analysis of three related photosensitivity disorders: cutaneous lupus erythematosus, polymorphic light eruption and actinic prurigo. Br J Dermatol 145: 223–226.

38. Vera-Recabarren MA, Garcia-Carrasco M, Ramos-Casals M, Herrero C (2010) Cutaneous lupus erythematosus: clinical and immunological study of 301 patients stratified by gender. Clin Exp Dermatol 35: 729–735.

39. Fairweather D, Friasanco-Kiss S, Rose NR (2008) Sex differences in autoimmune disease from a pathological perspective. Am J Pathol 173: 600–609.