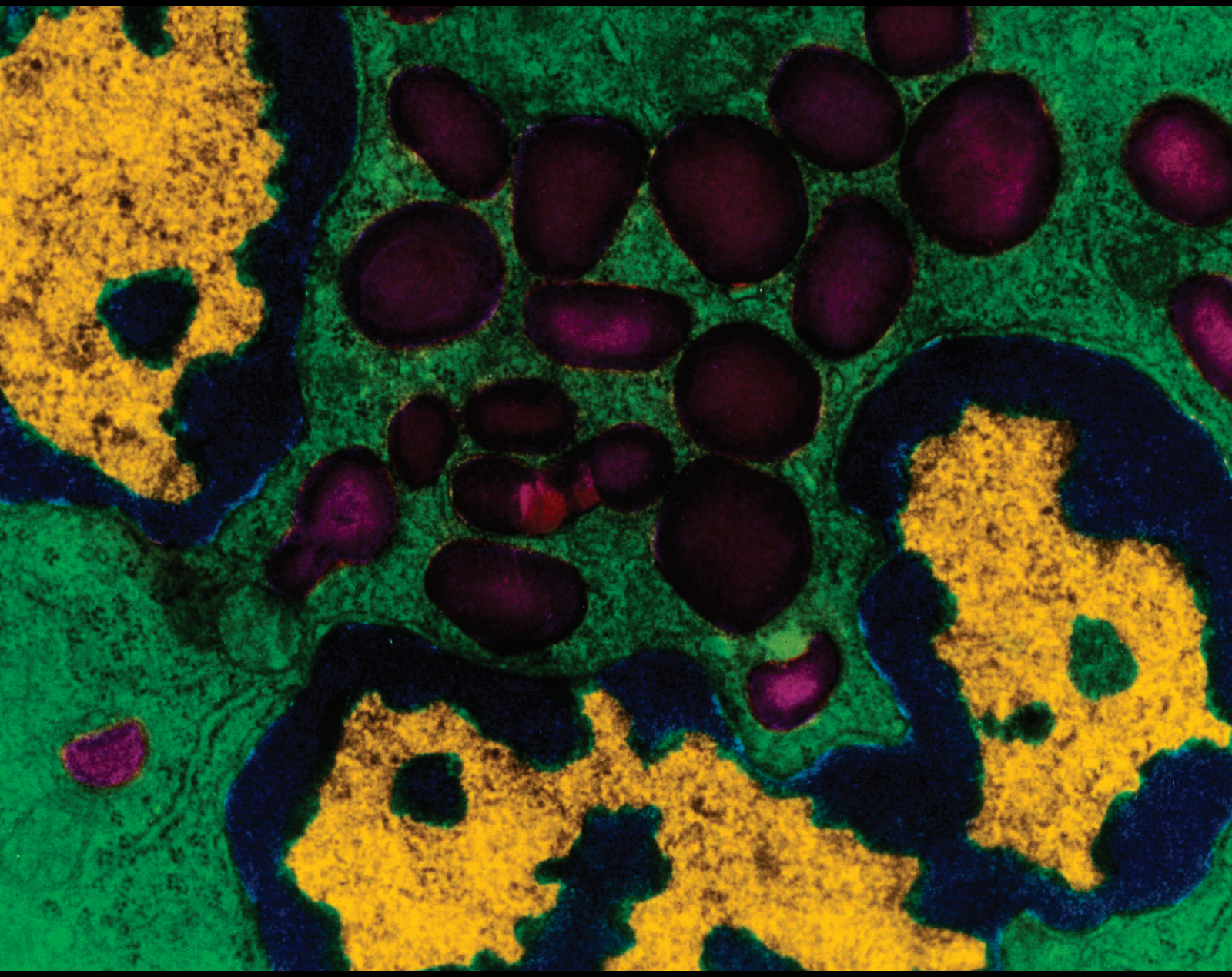
Mediators of Inflammation

Inflammatory Mediators in Autoimmunity and Systemic Autoimmune Diseases

Guest Editors: Britt Nakken, György Nagy, Peter C. Huszthy, Even Fossum, Yrjö Konttinen, and Peter Szodoray



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Editorial Board

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Editorial

Inflammatory Mediators in Autoimmunity and Systemic Autoimmune Diseases

Britt Nakken,[1] György Nagy,[2] Peter C. Huszthy,[1] Even Fossum,[3] Yrjö Konttinen,[4] and Peter Szodoray[1]

[1]*Department of Immunology, Oslo University Hospital Rikshospitalet and University of Oslo, Oslo, Norway*
[2]*Department of Genetics, Cell and Immunobiology, Semmelweis University, Budapest, Hungary*
[3]*K. G. Jebsen Center for Influenza Vaccine Research, Institute of Immunology, University of Oslo and Oslo University Hospital, Oslo, Norway*
[4]*Department of Medicine, Institute of Clinical Medicine, Helsinki University Central Hospital and ORTON Orthopaedic Hospital of the Invalid Foundation, Helsinki, Finland*

Correspondence should be addressed to Peter Szodoray; szodoray@gmail.com

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Autoimmune processes can be found physiologically, as a natural phenomenon in humans. A wide array of surveillance mechanisms, cells with regulatory properties, and tolerance mechanisms exists to control and decelerate autoimmunity and to avoid the development of autoimmune diseases. However, when not properly controlled, the intricate interplay of genetic, environmental, and immunological factors leads to the development of these debilitating diseases. The immune system has multiple levels of negative feedback mechanisms that dampen immune responses and counteract the establishment of chronic and destructive immunity. Exponentially growing knowledge on inflammatory mediators not only aids us in the follow-up of ongoing autoimmune processes, eventually full-blown autoimmune diseases, but also provides a tool to target and control these entities. Following inflammatory mediators reflecting autoimmunity helps us to intervene rapidly, before the eventual development of the disease and by doing so organ damage can be prevented. Knowledge and understanding of the pathogenic mechanisms that contribute to these conditions can lead to the development of novel diagnostic strategies and future effective therapies, providing better life expectancies to patients with autoimmune diseases.

In this special issue we present original research articles as well as review papers on the role of various inflammatory mediators in autoimmunity and autoimmune diseases.

The first review article from the editors gives an overview of the most important aspects in the pathogenesis of autoimmune diseases, with a special emphasis on the derailed balance between regulatory and Th17 cells. Additionally, we depict a cytokine imbalance, which gives rise to a biased T cell homeostasis in these patients. The review also portrays the multifaceted role of dendritic cells in the pathogenesis of autoimmunity; finally we describe the function and role of extracellular vesicles in particular autoimmune diseases.

The paper by N. Marton et al. describes the diverse roles of Src-like adaptor proteins (SLAP-1 and SLAP-2), their role in immunoregulation, and their effects on intracellular signaling pathways. The paper by C. Burbano et al. depicts the role of microparticles as modulatory structures in autoimmune processes and also introduces recent findings on microparticles in the pathogenesis in RA and SLE. The paper by S. G. Bourgoin et al. describes the effect of lysophosphatidic acid (LPA) on T cell recruitment via CXCL13 synthesis and its participation in inflammatory response regulation.

The development of lung fibrosis is a pathological process, characterized by abnormal accumulation of fibroblasts in the alveolar interstitium. The research article by P. Y. Cohen et al. depicts interesting aspects in the disease development in a mouse model by using gene chip analysis. The analysis concluded that lung myofibroblasts downregulated Thy1

expression and diminished the *in vivo* inflammatory milieu, indicating that inflammation is not essential for evolution of fibrosis. The paper by C. B. Holt el al. describes the role of Ficolin B in diabetic kidney diseases in a mouse model of type I diabetes and concludes that the molecule has no effect on diabetes-induced changes on the kidneys, opposed to the negative role of mannan-binding lectin in the pathogenesis. The next chapter of the special issue including 5 papers introduces various pathogenic aspects in systemic autoimmune and rheumatic diseases. In this unit, the articles introduce the clinical and immunoserological characteristics of rheumatic diseases with coinciding psoriasis, the role of progranulin in association with disease activity in RA patients, and a review article summarizes the role of poly (ADP-ribose) polymerase 1 in RA. A. A. S. S. K. Dharmapatni et al. in their paper describe the beneficial effects of Embelin, a XIAP inhibitor in a mouse model of collagen-induced arthritis, indicated by suppressed inflammation and reduced levels of the systemic bone resorption marker, CTX-1. Finally, in this section patients with ankylosing spondylitis were assessed and found that serum IL-6 correlated with ESR and CRP levels, making IL-6 a potentially important biomarker in the disease.

In this special issue we also depict various aspects in the pathogenesis of inflammatory bowel diseases (IBD). The paper by B. R. R. de Mattos et al. summarizes the latest findings in the immunological pathomechanism of IBDs and depicts the role of biologicals in the treatment while the paper by L. M. Medrano et al. describes that the analysis of a particular set of genes is a useful tool in the assessment of response to infliximab in Crohn's disease.

Follicular helper T cells (TFH) have been described to play a pivotal role in the initiation and perturbation of autoimmune processes leading to the development of autoimmune diseases. The paper by K. Szabo et al. suggests that in Sjögren's syndrome the presence of TFH cells in labial salivary gland biopsies at the disease onset is a good biomarker and may predict a more pronounced clinical course of the disease. The paper by X. Fan et al. describes the pathogenic role of TFH cells in human neuroautoimmune diseases and portrays their animal models.

This issue also illustrates the usefulness of inflammatory mediators in ocular manifestations of autoimmune diseases. The paper by A. Rentka et al. describes decreased levels of vascular endothelial growth factor (VEGF) in tear samples of patients with systemic sclerosis, while the paper by S. Sharma et al. reports from a study using a multiplex immunoassay that elevated serum levels of soluble TNF receptors and adhesion molecules are associated with diabetic retinopathy in patients with type-1 diabetes.

This special issue encompasses basic, molecular mechanisms of the pathogenesis in connection with autoimmune processes and autoimmune diseases, illustrating useful cellular and molecular mediators of inflammation. We believe that these data may contribute to improved tests for diagnosis and improve our knowledge of the underlying disturbances in the immune system in these hitherto unexplained disorders and hopefully these mediators of inflammation will be useful therapeutic targets in the future management of autoimmune diseases.

Britt Nakken
György Nagy
Peter C. Huszthy
Even Fossum
Yrjö Konttinen
Peter Szodoray

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Research Article

The X-Linked Inhibitor of Apoptosis Protein Inhibitor Embelin Suppresses Inflammation and Bone Erosion in Collagen Antibody Induced Arthritis Mice

Anak A. S. S. K. Dharmapatni,[1] Melissa D. Cantley,[1] Victor Marino,[2] Egon Perilli,[3] Tania N. Crotti,[1] Malcolm D. Smith,[4,5] and David R. Haynes[1]

[1] *Discipline of Anatomy and Pathology, School of Medical Sciences, University of Adelaide, Adelaide, SA 5005, Australia*
[2] *School of Dentistry, University of Adelaide, Adelaide, SA 5005, Australia*
[3] *Medical Device Research Institute, School of Computer Science, Engineering and Mathematics, Flinders University, Bedford Park, SA 5042, Australia*
[4] *Department of Rheumatology, Flinders Medical Centre and Flinders University, Bedford Park, SA 5042, Australia*
[5] *Rheumatology Research Unit, Repatriation Hospital, Daw Park, SA 5041, Australia*

Correspondence should be addressed to Anak A. S. S. K. Dharmapatni; a.dharmapatni@adelaide.edu.au

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Objective. To investigate the effect of Embelin, an inhibitor of X-Linked Inhibitor of Apoptosis Protein (XIAP), on inflammation and bone erosion in a collagen antibody induced arthritis (CAIA) in mice. *Methods*. Four groups of mice ($n = 6$ per group) were allocated: CAIA untreated mice, CAIA treated with Prednisolone (10 mg/kg/day), CAIA treated with low dose Embelin (30 mg/kg/day), and CAIA treated with high dose Embelin (50 mg/kg/day). Joint inflammation was evaluated using clinical paw score and histological assessments. Bone erosion was assessed using micro-CT, tartrate resistant acid phosphatase (TRAP) staining, and serum carboxy-terminal collagen crosslinks (CTX-1) ELISA. Immunohistochemistry was used to detect XIAP protein. TUNEL was performed to identify apoptotic cells. *Results*. Low dose, but not high dose Embelin, suppressed inflammation as reflected by lower paw scores ($P < 0.05$) and lower histological scores for inflammation. Low dose Embelin reduced serum CTX-1 ($P < 0.05$) and demonstrated lower histological score and TRAP counting, and slightly higher bone volume as compared to CAIA untreated mice. XIAP expression was not reduced but TUNEL positive cells were more abundant in Embelin treated CAIA mice. *Conclusion*. Low dose Embelin suppressed inflammation and serum CTX-1 in CAIA mice, indicating a potential use for Embelin to treat pathological bone loss.

1. Introduction

Rheumatoid arthritis (RA) involves changes in the synovial membrane, including thickening of the synovial lining, persistent infiltration of inflammatory cells, angiogenesis, and production of inflammatory cytokines leading to subsequent cartilage and bone degradation. The joint erosion in RA is associated with increased numbers of bone resorbing osteoclasts [1]. The proliferation of synovial cells and the persistence of inflammatory cells in the RA synovium are associated with a marked reduction in apoptosis [2]. Inducing apoptosis by targeting the upstream pathway with anti-death receptor 5 (DR5) [3] and soluble Fas monoclonal antibody [4] was beneficial in an adjuvant induced arthritis rat model and in a severe combined immunodeficient-HuRAg mouse model, respectively.

Apoptosis is activated through the extrinsic and/or intrinsic pathways. Both pathways converge downstream at the level of executor caspases such as caspases 3, 6, and 7 [5], which are directly responsible for morphological changes in apoptotic cells. The inhibitory apoptotic proteins family (IAPs) regulate both upstream (caspase-9) and downstream

caspases (3, 6, and 7) [6] via their baculovirus IAP repeat (BIR) domain. IAP members are also involved in other processes such as protein ubiquitination [7], cell cycle regulation [8, 9], immune function regulation via regulation of cell survival [10], and activation of immune cells. Cellular inhibitors of apoptosis 1 and 2 (cIAP1 and cIAP2) regulate cytokine and chemokine production by macrophages via regulating nucleotide-binding oligomerization domain 2 (NOD2) [11]. Additionally, XIAP regulates innate immunity to *Listeria* infection [12] and is associated with transforming growth factor beta (TGFβ) [13, 14] and interferes with nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and mitogen-activated protein kinases (MAPK) signalling via its BIR1 domain [15].

We have previously reported an increased expression of two IAP family members, XIAP and survivin, in synovial tissues from patients with RA [16] and subsequently demonstrated their modulation by disease modifying antirheumatic drugs (DMARDs) [17], indicating that inhibiting XIAP and/or survivin may be a strategy to treat RA. Supporting this, studies have demonstrated an association between serum survivin and joint erosion [18, 19] and the use of serum survivin levels as an indicator of patient response to infliximab treatment [20]. Suppression of inflammation in an antigen induced arthritis mouse by a Smac mimetic A-4.10099.1 (ABT), an IAP antagonist, supports IAP suppression as a promising target to treat RA [21].

Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) is a bioactive compound derived from a natural plant, *Embelia ribes*. Its pharmacological actions include antibiotic, antitumour, analgesic, and anti-inflammatory effects [22, 23]. Embelin has been used in acute and chronic models of skin inflammation in mice [24]. It is a cell permeable, nonpeptide small molecule inhibitor of XIAP that can be administered orally; hence it has potential to be an oral therapy for RA [25–27]. Importantly, Embelin can suppress receptor activator of nuclear factor kappa-B ligand (RANKL) induced osteoclastogenesis *in vitro* in RAW 264.7 cells and in myeloma and breast cancer cells [28]; however no studies have assessed the effect of Embelin on bone erosion *in vivo*.

This study investigated the effect of Embelin on inflammation and bone loss in a mouse model of inflammatory arthritis, the collagen antibody induced arthritis (CAIA).

2. Methods

2.1. CAIA Mice and Treatment Protocol. Experiments were performed in accordance with the Australian Code of Practice for the care and use of animals for scientific purposes (National Health and Medical Research Council) and approved by the University of Adelaide and Institute of medical Sciences Ethics Committees (ethics numbers M-2009-167 and IMVS 75/09, resp.). Induction of arthritis was performed as previously published [29]. Four groups of mice ($n = 6$ per group) consisted of the following: group 1: CAIA without treatment, group 2: CAIA treated with Prednisolone (10 mg/kg/day) [30], group 3: CAIA treated with low dose Embelin (30 mg/kg/day), and group 4: CAIA treated with high dose Embelin (50 mg/kg/day). The number of mice used in each group was kept to a minimum of 6 as the range 6–8 has been suggested for other RA animal models [31]. At day 0, all mice were injected with a 150 μL (1.5 mg total) cocktail of collagen type 2 antibodies (Chondrex kit, Redwood, WA, USA) via the tail vein. On day 3, mice were injected intraperitoneally (i.p.) with 20 μL (10 μg) of lipopolysaccharides (LPS). Embelin in PBS/10% EtOH was administered daily via oral gavage for 7 days (day 4 to day 10). The selected doses administered were in the range reported to give a similar reduction in serum sTNF-α to that seen with 30 mg/kg Prednisolone in acute and chronic models of skin inflammation in mice [24]. CAIA mice with no treatment were given the vehicle (PBS/10% EtOH) only. Mice were humanely killed on day 10 and paws were fixed in 10% normal buffer formalin overnight and then were washed with PBS and scanned with microcomputed tomography (micro-CT) before being decalcified and processed for histological evaluation. Serum was collected via cardiac puncture and analysed for CTX-1 using an enzyme-linked immunosorbent assay (CTX-1 ELISA, Ratlaps).

2.2. Micro-CT Scans and Image Analysis. To measure bone erosion, micro-CT scans of the paws were performed (SkyScan 1076, Kontich, Belgium) *ex vivo*. The scanning parameters used were as follows: tube voltage 74 kV, tube current 136 μA, isotropic pixel size 17.4 μm, 1.0 mm aluminium filter, and one frame averaging [29].

Cross-sectional images of the front right paws were reconstructed (NRecon, SkyScan) and aligned using the long axis of the paw as reference (Dataviewer, SkyScan). The right radiocarpal joint was chosen for quantitation of the bone volume (BV) [29]. The volume of interest included 40 cross-sections (=0.7 mm) below the epiphyseal growth plate (EGP) to 100 cross-sections (1.7 mm) above the EGP (CTAn V.1.12.04, SkyScan). Grey level images were binarized into bone and nonbone using a uniform threshold (CTAn, SkyScan) [29, 32]. BV (measured in mm^3) was calculated as the volume occupied by the voxels segmented as bone (CTAn, SkyScan) [29, 32].

2.3. Clinical Paw Scoring. Paw scoring was performed daily from day 0 to day 10, by two observers given to each group using a system previously described [33]. In addition, each group was presented randomly for scoring. For each paw a score of 1 was given for each single digit involved; a range of 0–5 was given for swelling of carpal/tarsal and 0–5 for the wrist/ankle [33]. Therefore, the maximal score for each paw could be 15 and the maximal score for each mouse could be 60. Other parameters measured included body weight and daily clinical observations of general health.

2.4. Histological Evaluation

2.4.1. Inflammation Score, Cartilage and Bone Degradation Score, and Pannus Score. A score for cellular infiltration (inflammation score), cartilage and bone degradation, and pannus formation was assigned to the hematoxylin-eosin

(HE) stained paw specimens according to published methods [29]. Inflammation within the radiocarpal/metatarsal joints was scored as 0 to 3: score 0 ≤ 5% inflammatory cells, score 1 = 6–20%, score 2 = 21–50%, and score 3 = more than 50% inflammatory cells. Cartilage/bone degradation was scored: 0 = normal bone integrity, 1 = mild cartilage destruction, 2 = evidence of both cartilage and bone destruction, 3 = severe cartilage and bone destruction. Pannus formation was scored as 0 = no pannus formation and 1 = pannus formed. Histological scoring was performed by two independent observers that were blinded as to the group allocation.

2.4.2. TRAP Staining. TRAP staining was performed based on a previously published method [34–36]. TRAP positive cells with 3 or more nuclei within the metatarsal and metacarpal joints of the paws were counted using a light microscope with 100x magnification (NIKON D1 digital camera attached to NIKON FXA research microscope, Japan).

2.4.3. XIAP Immunohistochemistry. XIAP expression was detected in paw tissues from all mice using the Vectastain Elite ABC Kit Universal (PK-6200, Vector Labs, Burlingame, CA), according to published methods [37]. The primary antibody was rabbit polyclonal anti-mouse XIAP (Abcam, Ab21278, Sapphire Bioscience Pty. Ltd., NSW, Australia) at 2 μg/mL. Negative controls included omission of primary antibodies or isotype control (rabbit universal isotype IgG, DakoCytomation, Glostrup, Denmark). Sections were stained with 3-amino-9-ethylcarbazole (AEC) dye (Cat. K3469; DakoCytomation, Glostrup, Denmark) and counterstained with hematoxylin and lithium carbonate. Semiquantitative analysis of XIAP immunostaining was performed according to previously published method [38].

2.4.4. Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling (TUNEL). TUNEL was performed on paw tissues for apoptosis assessment using an *in situ* cell death detection kit AP (Roche Diagnostic Australia Pty. Ltd., NSW, Australia), as previously published [16]. Tissue was incubated with label solution only for a negative control or with DNA-ase for a positive control for the presence of fragmented DNA. Colour was developed using fast red (Vector Labs, CA, USA) and counterstained with hematoxylin and lithium carbonate.

2.4.5. Serum CTX-1 ELISA. Serum CTX-1 was measured in duplicate for each sample or standard using Ratlaps CTX-1 ELISA according to the manufacturer instructions (Immunodiagnostic Systems, Nordic) [39]. The optical density was measured at 450 nm using a Power-Wave ELISA plate reader and software KC4 (Biotek Instruments, Winooski, VT, USA). Serum CTX-1 concentration was interpolated from the standard curve generated.

2.4.6. Statistical Analysis. Differences in mean values of each parameter between groups were analysed using the Kruskal-Wallis statistical test and differences between two groups were analysed using Mann-Whitney *U* test. Correlation between two parameters was analysed using Kendall's tau b-test. All statistical analysis was performed using SPSS version 20 (Chicago, IL, USA). A P value of less than 0.05 was considered statistically significant.

3. Results

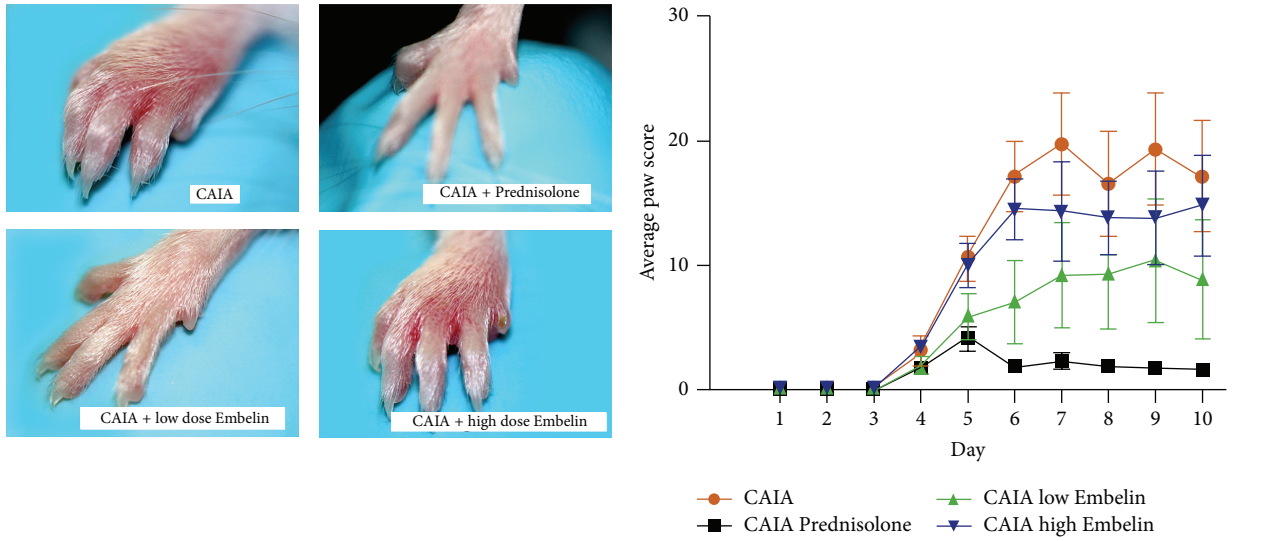
3.1. CAIA Mice Treated with Low Dose Embelin Demonstrated Lower Paw Scores Than Untreated CAIA Mice. The front paws in the CAIA mice exhibited inflammation as assessed by clinical paw score (Figure 1(a)). Prednisolone treated CAIA mice (a positive treatment control) consistently demonstrated lower mean paw scores compared to CAIA untreated mice throughout the experiment ($P < 0.05$). CAIA mice treated with a low dose of Embelin also demonstrated markedly lower paw scores throughout the experiment (Figure 1(b)) with statistically significant differences observed on day 6 ($P < 0.05$). Although the mice treated with high dose Embelin demonstrated lower mean paw scores, these were not significant statistically when compared to the CAIA untreated group.

The mice body weights did not differ between groups over the course of the experiment (data not shown).

3.2. CAIA Mice Treated with Low Dose Embelin Demonstrated Lower Scores for Inflammation, Cartilage and Bone Degradation, and Pannus Formation. Histological evaluation of all four paws of all mice showed that CAIA mice treated with low dose Embelin had lower scores for cellular infiltration ($P = 0.05$), cartilage and bone degradation ($P = 0.071$), and pannus formation ($P = 0.167$) compared to CAIA untreated mice. Representative HE stained images of the front right paw from each group are shown (Figure 2(a)). All histological scores were significantly lower in Prednisolone treated CAIA mice compared to untreated CAIA mice (Figure 3).

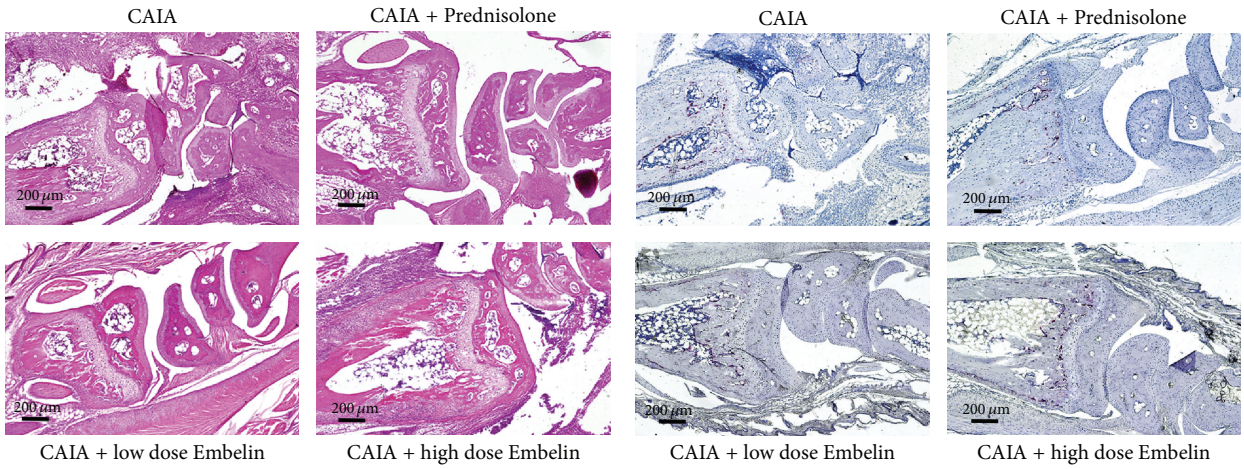
3.3. CAIA Mice Treated with Low Dose Embelin Demonstrated Lower but Not Significantly Different Number of TRAP Positive Osteoclasts. TRAP staining was performed on sections from all four paws of each mouse to identify the number of preosteoclast/osteoclast cells. Representative images of TRAP staining of the front right paw from each group are presented in Figure 2(b). Multiple TRAP positive cells were observed within the joint and pannus region of CAIA mice (mean ± standard error of the mean, 57.27 ± 17.52) and this was reduced with Prednisolone treatment (20.43 ± 7.31) and to a lesser degree in CAIA treated with low dose Embelin (45.33 ± 15.13). Interestingly, CAIA mice treated with high dose Embelin demonstrated similar number of TRAP positive cells to CAIA untreated mice (57.09 ± 21.00) (Figures 2(b) and 3).

3.4. There Was No Difference in XIAP Protein Expression between CAIA Mice Treated with Low or High Dose Embelin and CAIA Untreated Mice. Semiquantitative analysis of XIAP immunostaining revealed that mouse paws from all groups expressed XIAP proteins within cells in pannus, periosteal tissue, articular cartilage, and bone marrow (Figure 4(a)). There was no statistical significant difference in the XIAP expression within the joint space between the groups. Mean



(a) (b)

Figure 1: (a) Representative clinical features of mouse paws in CAIA untreated mice, Prednisolone treated CAIA mice, CAIA mice treated with low dose Embelin, and CAIA mice treated with high dose Embelin. (b) Mean clinical paw scores of each study group throughout the experiment. Error bars represent standard error of the mean (SEM).



(a) (b)

Figure 2: (a) Representative hematoxylin-eosin staining within the radiocarpal joint from each mouse group. (b) Representative TRAP staining (red) in the radiocarpal joint from each mouse group. Hematoxylin counterstaining.

(±SEM) score for XIAP was 0.59 ± 0.3 for CAIA untreated mice, 0.85 ± 0.05 for CAIA treated with Prednisolone, 0.62 ± 0.29 for CAIA treated with low dose Embelin, and 0.81 ± 0.39 for CAIA treated with high dose Embelin.

3.5. CAIA Mice Treated with Low and High Dose Embelin Demonstrated Increased Numbers of Apoptotic Cells Compared with CAIA Untreated Mice. To investigate if Embelin induced apoptosis in CAIA mice, evidence of fragmented DNA was assessed using TUNEL. Fewer TUNEL positive cells were observed within the pannus of the CAIA mice, compared to prednisolone treated CAIA mice and both low and high dose Embelin treated CAIA mice (Figure 4(b)).

3.6. Higher but Not Significant Bone Volume (BV) Was Observed in CAIA Mice Treated with Low and High Dose Embelin Compared to CAIA Untreated Mice. Three-dimensional reconstruction of front right paws by micro-CT demonstrated severe osteolysis within their radiocarpal and PIP (proximal interphalangeal) joints of the untreated CAIA mice in marked contrast to Prednisolone treated CAIA mice (Figure 5(a)). There was a significantly higher mean BV

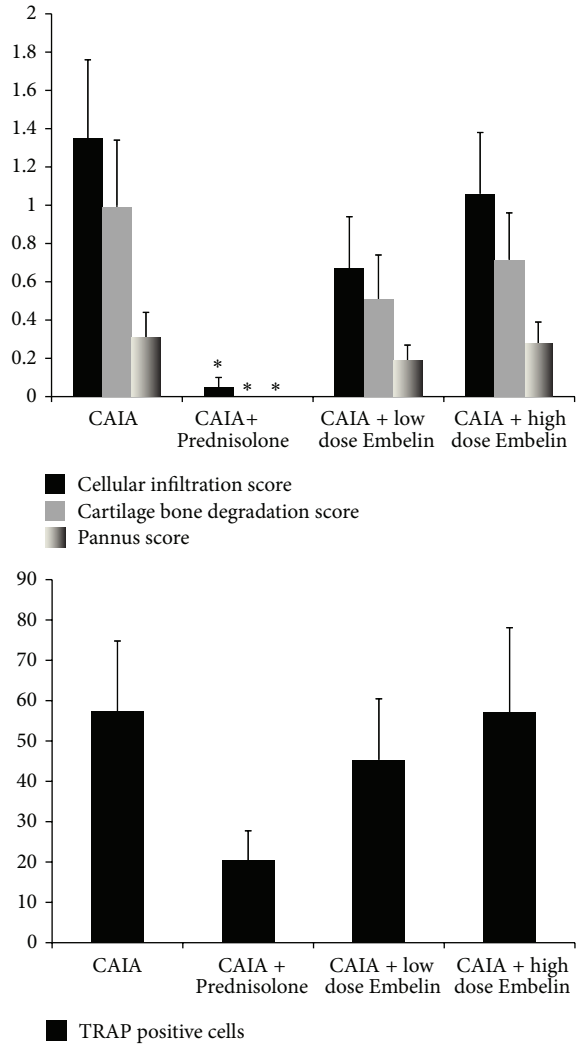


Figure 3: Histological and TRAP scores (obtained using scores as described in Section 2) on paw tissues at day 10. Bars represent mean ± SEM. $^{*}P < 0.05$.

within the radiocarpal region in the Prednisolone treated CAIA mice, compared to CAIA untreated mice (1.73 ± 0.02 versus 1.43 ± 0.06, $P = 0.011$) (Figures 5(b) and 5(c)). Although the mean BV was higher in both low and high dose Embelin treated groups (1.47 ± 0.05 and 1.57 ± 0.06, resp.) compared to the CAIA untreated group, there was no statistically significant difference (Figure 5(c)).

3.7. CAIA Mice Treated with Low Dose Embelin Demonstrated Significantly Lower Serum CTX-1 Levels Than the Untreated CAIA Mice. Serum CTX-1 was measured as a marker of systemic bone resorption in all groups. Levels were significantly lower in the Prednisolone treated CAIA group (25.28 ± 3.35 ng/mL) as compared to CAIA untreated mice (33.27 ± 0.85, $P = 0.017$). There was significantly lower levels of serum CTX-1 (25.12 ± 1.91) in mice treated with low dose Embelin compared to CAIA untreated mice ($P = 0.016$). Although CTX-1 levels were lower in the high dose Embelin treated group, they were not statistically significant (Figure 5(d)).

3.8. Correlation Analysis of Parameters. Correlations between clinical and histological scores were made using data from four paws of each of the mice. Only data from the front right paw was correlated with the micro-CT data. Overall, there was a strong and significant correlation between the clinical paw score and each of the histological scores (Figure 6). There was also a significant strong inverse correlation ($\tau = -0.525$, $P = 0.004$) between BV as assessed by micro-CT and the cartilage and bone scores assessed histologically. The inverse correlation between BV assessed by micro-CT and the clinical paw score was also strong ($\tau = -0.531$) and statistically significant ($P = 0.004$).

4. Discussion

This study demonstrates low dose Embelin suppressed joint inflammation and bone erosion in a CAIA model in mice. The findings support a previous study in a model of antigen induced arthritis mice by Mayer et al. [21] where inhibition of IAP using a Smac mimetic suppressed disease activity. In the study by Mayer et al. an antigen induced arthritis was used to assess the initial immune response, whereas the current results demonstrate effects of an XIAP inhibitor on the effector phase of disease. In the antigen induced arthritis model, disease induction involves stimulation of antibody production by the host, via administration of antigen (bovine serum albumin), while in CAIA model this initiation stage is bypassed by the administration of antibodies to collagen antibody type 2. While the IAP inhibitor A-4.10099.1 (ABT) was given intravenously in the Mayer study [21], the XIAP inhibitor (Embelin) used in our study was given via oral gavage, suggesting a potential oral treatment for RA patients. It is important to note that the IAP inhibitor A-4.10099.1 (ABT) was given prior to the disease onset (prophylactic scheme) while in our study the Embelin was assessed for its therapeutic effects with treatment commencing following disease induction.

Mayer et al. [21] elucidated the molecular mechanism involved in suppression of inflammation using a Smac mimetic, IAP antagonist A-4.10099.1 (ABT) with a particular focus on endothelial signaling. However, the study did not report microscopic features of mouse joint damage or assess bone erosion. In the current study, we demonstrate that the XIAP inhibitor, Embelin, used in low dose, suppressed inflammation clinically and microscopically in CAIA mice and we provided evidence of suppression of bone erosion using histological assessment, TRAP counting, micro-CT, and serum CTX-1 assay.

The mechanism by which Embelin suppresses inflammation has been investigated in various *in vivo* models of inflammation. In an LPS induced mouse model of skin inflammation, Embelin was reported to reduce cutaneous TNF-α expression [24]. In acetic acid induced colitis Embelin reduced the activity of colonic myeloperoxidase (MPO), lipid peroxides, and serum lactate dehydrogenase as well

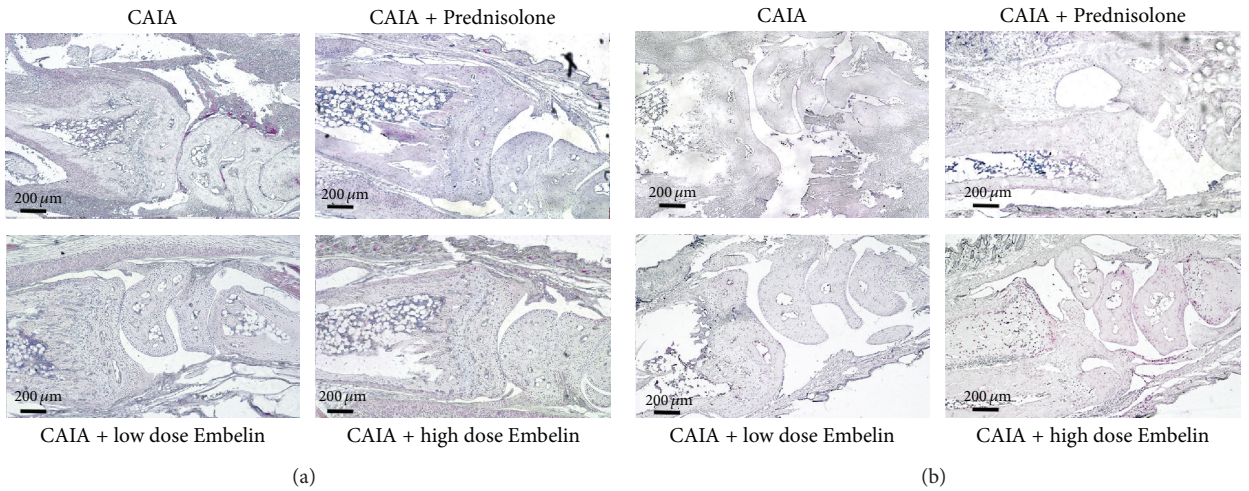


Figure 4: (a) XIAP protein expression (red) in the radiocarpal joint in CAIA mice, in Prednisolone treated mice, in CAIA mice treated with low dose Embelin, and in CAIA mice treated with high dose Embelin. (b) Representative TUNEL staining (indicated in red) in the radiocarpal joint of each mouse group. Hematoxylin counterstaining.

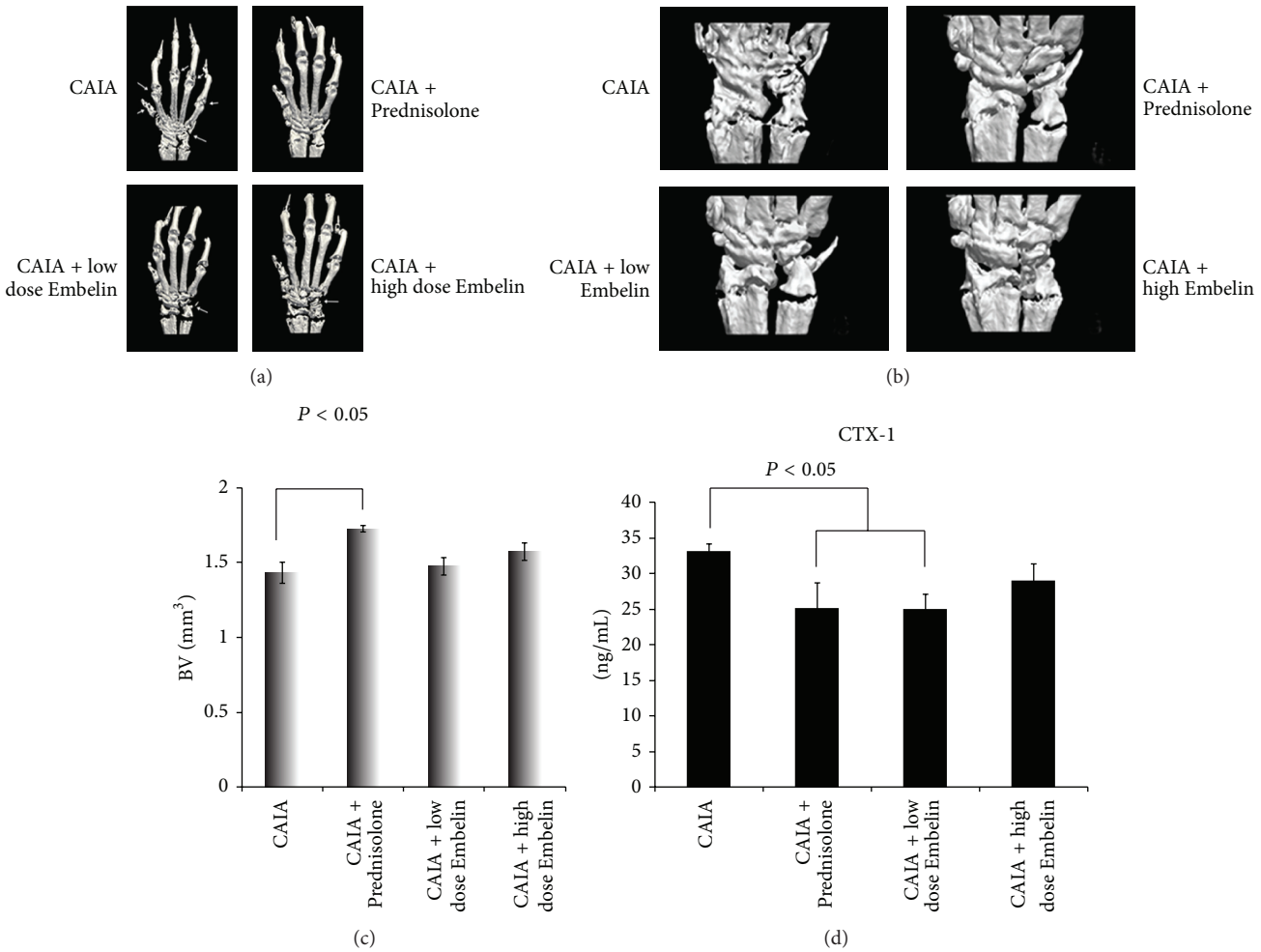


Figure 5: (a) Three-dimensional reconstruction of front right paw in each study group. Arrows point out bone area with erosion. (b) Three-dimensional reconstruction of the radiocarpal region from each mouse group chosen for quantitation of bone volume. (c) Mean bone volume (BV, expressed in mm^3) analysed by micro-CT in each group. (d) Mean serum CTX-1 level in each mouse group. Error bars represent standard error of the mean (SEM).

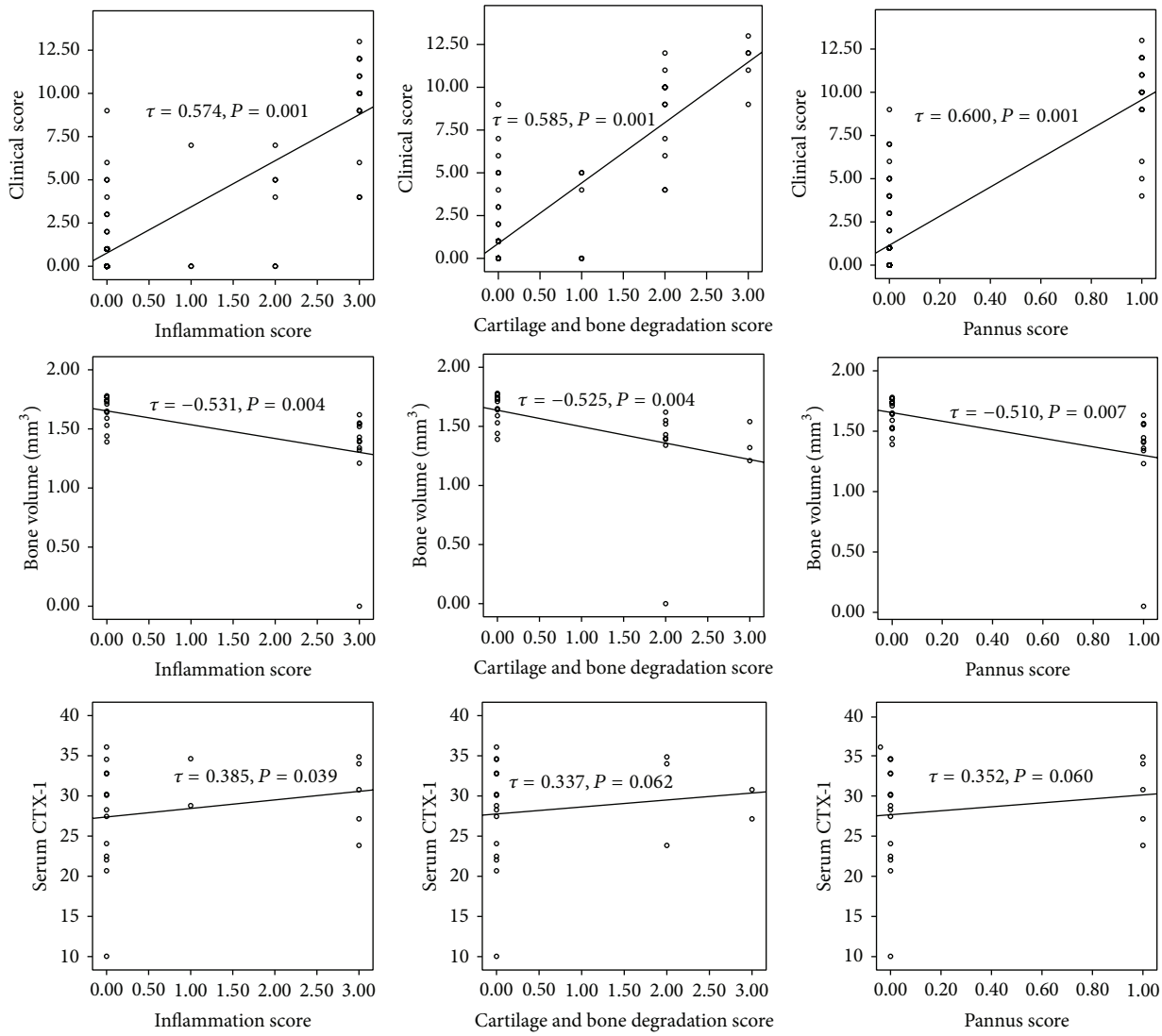


Figure 6: Bivariate correlations between clinical score, bone volume or serum CTX-1 (y-axis), and histological scores (inflammation score or cellular infiltration score, cartilage and bone degradation score, and pannus score).

as significantly increasing the reduced glutathione [40]. In dextran sodium sulphate (DSS) induced colitis in mice, Embelin reduced the mRNA expression of TNF-α, IL-1, IL-6, and colonic MPO [41].

The Mayer et al. study suggested that apoptosis was not the mechanism of action of the IAP antagonist A-4.10099.1 (ABT) in abrogating inflammation in antigen induced arthritis mice. They reported that A-4.10099.1 (ABT) regulated the inflammatory processes by attenuation of leucocyte-endothelial cell interaction and decreased TNF-α induced activation of TGFβ activated kinase-1, p38, and cJun N terminal kinase. Smac mimetics can target at least three members of IAPs (cIAP1, cIAP2, and XIAP) and therefore are a broader IAP inhibitor than Embelin. cIAPs have been reported to interfere with the type 2 TNF receptor signaling [6] in addition to directly binding to cleaved caspases 3, 7, and 9 [42].

We demonstrated positive TUNEL staining in the residual inflammatory cells in mice treated with Embelin suggesting that Embelin induced apoptosis in these cells. However, nonresponder mice in the Embelin treated groups demonstrated a very low positivity of TUNEL staining within the pannus suggesting some resistance of inflammatory cells to Embelin induced apoptosis. This correlates with our immunohistochemical evaluation of XIAP protein expression. We observed Embelin reduced XIAP expression within the pannus of responder mice in the group treated with low dose Embelin ($n = 4$), while in the nonresponder mice ($n = 2$) there were very low XIAP levels, low TUNEL staining, and persistence of pannus despite receiving Embelin treatment (data not shown). This suggests that Embelin may only be beneficial if the pannus has high levels of XIAP expression. An *in vitro* study using leukemia cells observed that Embelin reduced XIAP protein expression as detected by western

blot [43]. However, a study on glioma cells demonstrated no suppression of XIAP protein despite induction of apoptosis, suggesting suppression of other antiapoptotic molecules such as Bcl2 and Bcl-XL in these cells [44].

The mechanism of action of Embelin in suppressing bone resorption has only been investigated *in vitro*. In this study Embelin suppressed RANKL induced activation of osteoclasts via inhibiting NFκB, inhibition of IκBα phosphorylation, and IκBα degradation in RAW 267.4 cells [28]. Consistent with this, our study is the first to show Embelin suppression of bone erosion in an *in vivo* model.

Dose-dependent effects of Embelin (10, 30, and 50 mg/kg) have been reported previously in DSS induced colitis in mice [41]; however, in our study the lower dose was more effective than the higher dose. This is also in agreement with a previous study that reported the ED50 of Embelin in LPS induced mouse model of skin inflammation to be 9.8 mg/kg [24]. A similar trend to what we observed with Embelin was seen in a study which investigated the effect of methotrexate in CIA mice [45]. They observed that methotrexate given at 2.5 mg/kg was the most effective dose as compared to 0.1 and 5 mg/kg. The highest dose (5 mg/kg) in that study resulted in a higher clinical score, similar to what we observed with a higher dose of Embelin, which indicates that an optimal dose to reduce the inflammation in arthritis model is not necessarily the highest dose. It is possible that higher dose Embelin failed to suppress inflammation in this model because it has reached a threshold where upregulation of cIAPs occurred as a compensatory mechanism for higher level of XIAP suppression [46]. cIAPs upregulation subsequently can form a complex with TRADD resulting in activation of downstream inflammatory signalling pathways which may maintain inflammation. Further studies to elucidate the involvement of other IAP members in compensatory inhibition of IAP members in this setting are warranted.

In this study, both front paws of each mouse had higher paw scores with most of the mice having highest paw score in the front right paws and hind paws were rarely involved. Based on this observation, micro-CT evaluation was performed on the front right paws. The reduced involvement of hind paws in our CAIA model could possibly be due to the administration of a lower dose of both the cocktail antibody to collagen type 2 and LPS compared to other studies [47]. However, the use of a lower concentration of antibody and LPS resulted in less harmful effects to these mice and possibly is more relevant to the clinical situation in human RA where involvement of all joints is rarely seen.

Our micro-CT analysis showed that mice treated with low dose Embelin exhibited less bone erosion compared to CAIA untreated mice, although the difference was not statistically significant, possibly because a small number (only front right paws from each mouse) was analysed. The evidence of reduced bone damage was supported by the significant decrease in serum CTX-1 level. Nonetheless, BV assessed by micro-CT demonstrated significant positive correlations with both clinical and histological scores and at the same time demonstrated significant negative correlations with serum CTX-1. Overall, this supports the evidence of increased bone damage in untreated CAIA mice compared to CAIA mice treated with Prednisolone and low dose Embelin. Moreover, this confirms micro-CT as an objective quantitative method for assessing bone erosion in a rodent model of arthritis [48, 49].

A recent study involving 108 RA patients receiving low dose Prednisolone and 117 healthy controls has reported that serum CTX-1 (a marker for bone resorption) measured at 0, 3, and 12 months following treatment was significantly lower in the Prednisolone treated group [50]. The current study indicated differences in CTX-1 levels between being untreated and Prednisolone or low dose Embelin treatment, suggesting that serum CTX-1 is sensitive for measurement of bone damage.

Our study supports the induction of apoptosis using low dose Embelin to reduce inflammation and bone erosion in a murine model of inflammatory arthritis. Given the multiple roles of XIAP, further studies on Embelin's mechanism of action to interfere with various signalling pathways in this model are needed. The present study investigated the effect of Embelin in an inflammatory arthritis model that mimics the effector phase of RA; thus studies using Embelin in animal models mimicking the initial stage of RA, such as collagen induced arthritis (CIA) or antigen induced arthritis, will provide further information on the effect of Embelin in the initiation phase of the disease.

Conflict of Interests

The authors declare that they have no competing interests.

Acknowledgments

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Review Article

The Emerging and Diverse Roles of Src-Like Adaptor Proteins in Health and Disease

Nikolett Marton,[1] Eszter Baricza,[1] Barbara Érsek,[1,2] Edit I. Buzás,[1] and György Nagy[1,3]

[1]*Department of Genetics, Cell- and Immunobiology, Semmelweis University, 4 Nagyvárad Square, Budapest 1089, Hungary*
[2]*MTA-SE "Lendület" Experimental and Translational Immunomics Research Group, 4 Nagyvárad Square, Budapest 1089, Hungary*
[3]*Department of Rheumatology, Faculty of Medicine, Semmelweis University, 4 Frankel Leó Street, Budapest 1023, Hungary*

Correspondence should be addressed to György Nagy; gyorgyngy@gmail.com

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Although Src-like adaptor proteins (SLAP-1 and SLAP-2) were mainly studied in lymphocytes, where they act as negative regulators and provide fine control of receptor signaling, recently, several other functions of these proteins were discovered. In addition to the well-characterized immunoregulatory functions, SLAP proteins appear to have an essential role in the pathogenesis of type I hypersensitivity, osteoporosis, and numerous malignant diseases. Both adaptor proteins are expressed in a wide variety of tissues, where they have mostly inhibitory effects on multiple intracellular signaling pathways. In this review, we summarize the diverse effects of SLAP proteins.

1. Introduction

Signal transducing adaptor proteins are a group of intracellular and transmembrane molecules which are crucial supplementary factors of signaling pathways. They mediate interactions between different molecules and contribute to the formation of signaling complexes. Adaptor proteins lack enzymatic activity and interaction domains enable them to connect with other molecules (e.g., proteins, lipids). Src-like adaptor protein 1 (SLAP-1) was cloned in a yeast two-hybrid screen with the cytoplasmic domain of the receptor tyrosine kinase ephrin type-A receptor 2 as decoy [1, 2]. SLAP proteins are not identical in size; SLAP-1 (or often quoted as SLAP) contains 276 amino acids while SLAP-2 consists of 261 amino acids (Figure 1). SLAP-1 and SLAP-2 contain common SH2-SH3 domains. SH2 domains allow proteins to dock to phosphorylated tyrosine containing epitopes. SH3 domains bind to hydrophobic amino acid and proline rich molecules. SH domains of SLAP-2 form continuous β-sheet that stretches the modular domains [3].

SLAP proteins were named after Src family kinases which have the same SH sequences. Src protein tyrosine kinases (PTKs) are protooncogenes that play a role in cell proliferation, survival, and morphology. Unlike Src PTKs, SLAP molecules do not have tyrosine kinase domains. In addition to the myristoylated amino-terminal and SH domains, SLAP family members contain unique carboxy-terminal sequences as well. SLAP-1 has a longer carboxy-tail than SLAP-2, but the N-terminal is longer in SLAP-2. Myristoylated part of the SLAP promotes the association with membranes, while isoforms without the myristoylated N-terminal are located in the nucleus (Figure 1). Human SLAP is coded by a 64 kb intron of the thyroglobulin gene, on chromosome 8q24.23 in the candidate territory for a recessive demyelinating neuropathy. Sequence analysis could not find any mutations suggesting that this gene is not responsible for the disease [4]. Human SLA2 gene is located on 20q11.23 [1].

Human SLAP-1 and SLAP-2 molecules show sequence homology similarly to mouse SLAP proteins. SH domains are the most similar parts of the two SLAP molecules, in which the sequence homology is 59%, while the N-terminals are the most different parts; they have only 19% identity. SLAP-2 has a shorter alternative splice variant, called SLAP-2v. This isoform contains only 210 amino acids due to the deletion of 50 bp from exon 6, which results an alternative reading frame. The splicing variant molecule does not have

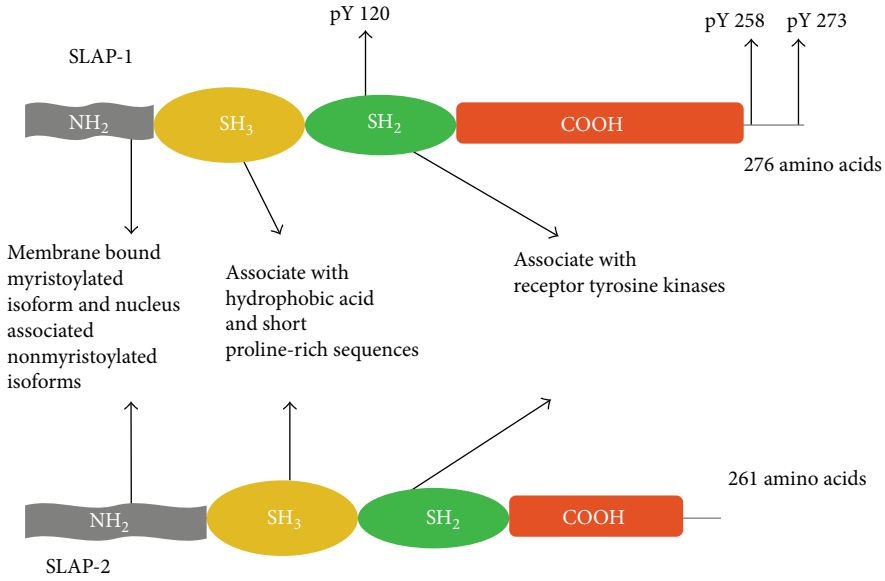

Figure 1: The schematic structure of SLAP molecules. SLAP-1 and SLAP-2 contain unique carboxy-terminal sequences, common SH2-SH3 domains and amino-terminals which exist in myristoylated and nonmyristoylated isoforms. SH2 domains help molecules to bind to phosphorylated tyrosine containing epitopes. The SH3 domains connect the proline and hydrophobic amino acid containing molecules.

Table 1: The expression of SLAP proteins in different tissues and cell lines.

Tissue/organ	Expressed molecule	Reference	Cell line	Expressed molecule	Reference
Blood	SLAP-1, SLAP-2	[1, 2]	BaF3	SLAP-1, SLAP-2	[5]
Bone marrow	SLAP-1, SLAP-2	[9, 10]	BJAB	SLAP-2	[35]
Brain	SLAP-1	[9]	D011.10T	SLAP-2	[5]
Colon	SLAP-2	[10]	EL4	SLAP-1	[9]
Heart	SLAP-1, SLAP-2	[10]	FLI-1 transformed erythroblasts	SLAP-1	[8]
Kidney	SLAP-1	[9]	HeLa	SLAP-1	[7]
Liver	SLAP-1	[9]	HL60	SLAP-1	[12]
Lung	SLAP-1, SLAP-2	[9]	Jurkat	SLAP-1, SLAP-2	[7, 35]
Lymph nodes	SLAP-1	[7]	NB-4	SLAP-1	[12]
Pancreas	SLAP-1	[10]	NIH3T3	SLAP-1	[10]
Placenta	SLAP-1, SLAP-2	[10]	RBL-2H3	SLAP-1	[11]
Prostate	SLAP-2	[10]	U.937	SLAP-1	[12]
Skeletal muscle	SLAP-1, SLAP-2	[9, 10]			
Small intestine	SLAP-1, SLAP-2	[10]			
Skin	SLAP-1, SLAP-2	[10]			
Spleen	SLAP-1, SLAP-2	[9, 10]			
Thymus	SLAP-1, SLAP-2	[29, 30]			

any c-Cbl interacting site. The biological relevance of SLAP-2v is yet unknown [5, 6]. Although expression of SLAP-1 and SLAP-2 mRNAs has been most extensively studied in lymphocytes, they are also expressed by numerous human and murine tissues and cell lines [1, 2, 7–12] (Table 1). Several proteins have been reported to interact with SLAP-1 and SLAP-2 [5, 8, 9, 13–15] (Figure 2). SLAPs are involved in a broad range of cellular processes, for example, lymphocyte development, neuronal excitotoxicity and platelet activation. SLAP molecules may participate in several pathological conditions of the immune system as well. In the present review, we will discuss the role of both SLAP proteins in different cell types and overview our current understanding regarding their relevance in pathological conditions.

2. SLAP Proteins in Immune Cells

2.1. SLAP-1 in T Lymphocytes. SLAP proteins were extensively investigated in lymphocytes where they are strongly expressed. SLAP-1 is involved in the regulation of the TCR signal transduction pathway. TCR consists of a ligand binding $\alpha\beta$ heterodimer and the CD3 complex that includes the $\gamma\varepsilon$

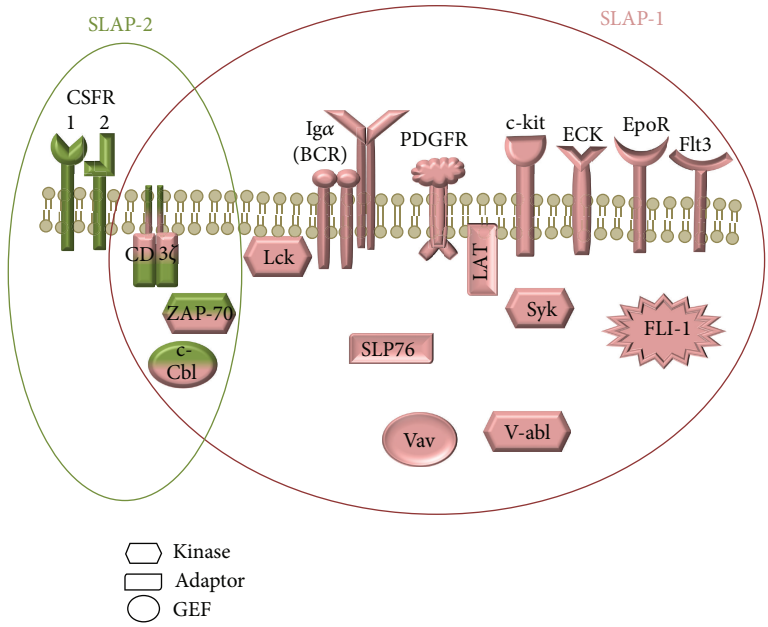

Figure 2: The interaction partners of SLAP-1 (pink) and SLAP-2 (green). Several proteins have been reported to interact with SLAP-1 molecule: c-Cbl, CD3 ζ chain, ECK, EpoR, Igα, LAT, Lck, PDGFR, SLP-76, Syk, v-abl, Vav (protooncogene vav), ZAP70, c-kit, Flt3, and FLI. SLAP-2 interacts with c-Cbl, CD3 ζ chain, CSFR, and ZAP70.

and δε heterodimers and the ζζ homodimer. The expression of the ζ-chains is about the 10% of the other subunits of the TCR complex. SLAP-1 may interact with many molecules involved in the TCR signal transduction (ZAP-70, Syk, LAT, CD3 ζ-chain, Vav, and Lck) through the SH2 domain [9, 16].

SLAP-1 reduces the production of IL-2 and the transcription of NFATc1 and AP-1, thereafter functions as a negative regulator of the TCR signaling; both SH2 and SH3 domains are necessary for this effect. However, upon ionomycin or PMA activation of lymphocytes, these inhibitory effects are absent suggesting that SLAP-1 regulates the proximal part of the TCR pathway [7]. SLAP may associate with the N-terminal of the E3 ubiquitin ligase c-Cbl in a tyrosine phosphorylation independent way [16]. The simultaneous expression of SLAP-1 and c-Cbl promotes the ubiquitination and degradation of the ζ-chain, consequently enhancing the recycling and preventing the accumulation of the receptor complexes [17]. By contrast, similarly to SLAP −/− lymphocytes, c-Cbl −/− lymphocytes overexpress the ζ-chain due to its abolished degradation [18]. For successful operation, SLAP-1 requires the phosphorylation of the cytoplasmic domains of the TCR ζ-chains and the activation of Lck, but not of ZAP70 [19]. It has been shown that the phosphorylated proportion of the ζ-chains is slightly traceable in Lck −/− lymphocytes [20, 21]. SLAP colocalizes with early endosomes according to confocal microscopy images [7] (Figure 3). It is noteworthy that downregulation of ζ-chain of T-cells has been observed in many pathological conditions including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), human immunodeficiency virus (HIV) infection, and various cancers [22–28].

Thymic selection is essential in shaping the peripheral T cell repertoire. The central role of SLAP in lymphocyte development has been also described. The fate of the early lymphocytes depends on the TCR-mediated signals: both too strong and too week signals through the TCR lead to cell death (during positive and negative selections, resp.). The expression of SLAP is strictly regulated during the thymic development of lymphocytes. It is expressed at low levels in CD4− CD8− cells and at high levels in CD4+ CD8+ thymocytes. SLAP as a regulator of the TCR expression plays a pivotal role in the downregulation of TCR complexes of the developing lymphocytes. Enhanced positive selection was observed in SLAP deficient mice. Moreover, the apoptosis of ZAP70 −/− cells was inhibited in the absence of SLAP [29]. It has been reported that the proline rich-sequence (PRS) of CD3ε act together with SLAP in the regulation of TCR expression in CD4+ CD8+ thymocytes. CD3ε PRS deficient cells were unable to degrade the ζ-chain [30]. In double positive thymocytes, the chains of the TCR complex are constitutively ubiquitinated, but the ubiquitination is absent in mature cells. It has been shown that CD3ε PRS, Lck, c-Cbl, and SLAP are required for the ubiquitination and degradation of the ζ-chains. In the absence of ubiquitination, both the lysosomal sequestration and degradation are failed, and TCR chains are upregulated in CD4+ CD8+ lymphocytes. In addition, modified TCR complex ubiquitination influences the formation of the immunological synapse and alters the selection of the immature cells [31]. The lack of SLAP increases the avidity of the TCR which leads to the negative selection of antigen specific CD8+ cells. All these data suggest that SLAP plays a major role in the TCR repertoire configuration [32].

2.2. SLAP-1 in B Lymphocytes. SLAP is associated with c-Cbl in B lymphocytes, leading to BCR recycling [33] (Figure 4).

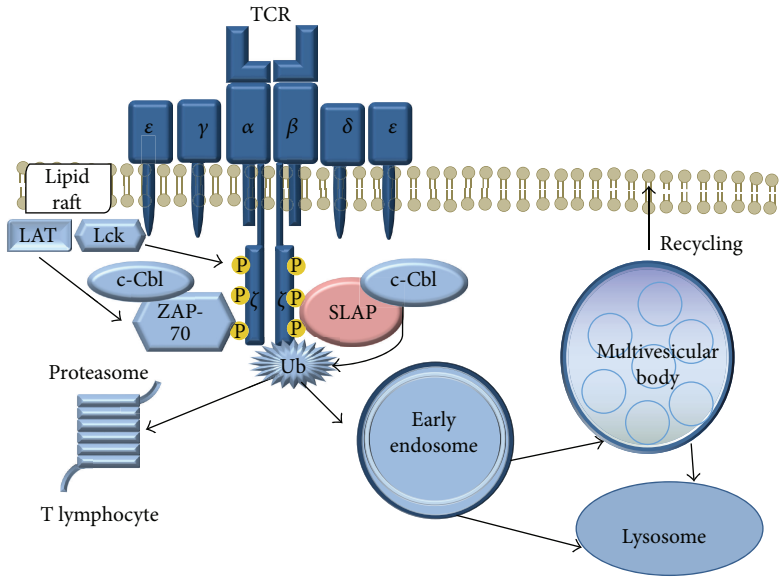

Figure 3: The role of SLAP in T lymphocytes.

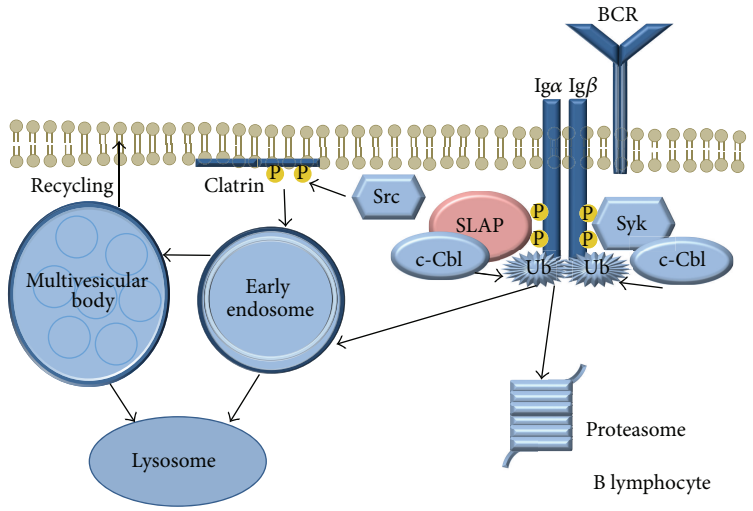

Figure 4: The role of SLAP in B lymphocytes.

SLAP deficiency increases the BCR levels of immature B cells in HEL-specific MD4- transgenic mice, which is a frequently used model to study naïve B lymphocytes. In this model the upregulated receptor complex levels lead to increased signal transduction. SLAP deficient mice have an increased number of splenic B cells, but the surface expression of BCR and IgM of mature lymphocytes is decreased. Furthermore, the activation induced calcium flux is diminished in SLAP KO B cells. In virtue of the previous data SLAP regulates the level of BCR s which is essential for the adequate development and function of B cells [34].

2.3. SLAP-2 in T and B Lymphocytes. Similarly to SLAP-1, SLAP-2 also has an effect on lymphocyte receptor signalization. SLAP-2 has a negative regulatory role on the antigen receptor signaling of T and B lymphocytes. The overexpression of SLAP-2 reduces the surface levels of CD3 [5]. The overexpression of SLAP-1 and SLAP-2 inhibits the upregulation of CD69 after antigen receptor cross-linking. CD69 is an inducible cell surface glycoprotein, upregulated during lymphocyte activation. SLAP-2 suppresses the antigen binding induced calcium influx in T (Jurkat) and B (BJAB) cell lines. SLAP-2 reduces the CD69 expression in Jurkat cells (61%) more significantly than in BJAB cells (28%). Similarly to SLAP-1, SLAP-2 does not impair the ionomycin and PMA induced signalization [35]. SLAP-2 is associated with ubiquitin ligase c-Cbl similarly to SLAP-1, deletion of the carboxy-terminal of SLAP-2 was reported to inhibit this connection [35]. In activated Jurkat cells SLAP-2 binds to c-Cbl, ZAP-70, and CD3 ζ in a phosphorylation independent manner [1]. The coexpression of SLAP-2 and ZAP-70 or Syk in T-cell lines lead to the degradation of both kinases. Thus,

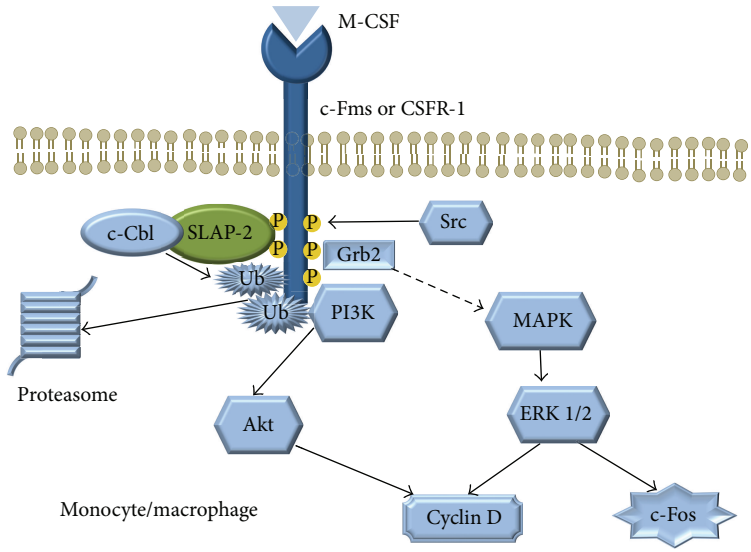

Figure 5: The role of SLAP-2 in monocytes/macrophages.

SLAP-2 induces the c-Cbl dependent degradation of tyrosine kinases and downregulates CD3 expression.

2.4. SLAP Molecules in Monocytes and Dendritic Cells. SLAP-2 is expressed in human monocytes and bone marrow cells [1, 35], but not in CSF1 independent monocyte cell lines such as RAW264.7 [5]. It was shown that SLAP-2 may bind to both c-Fms and c-Cbl, and several domains of SLAP-2 are involved in this interaction (Figure 5). The overexpression of SLAP-2 in murine bone marrow reduced the M-CSF-induced tyrosine phosphorylation [36]. SLAP-2 downregulates the c-Fms signaling through a c-Cbl dependent internalization and degradation of the CSF-1R, providing a negative feedback of the M-CSF pathway [37]. In addition, c-Fms stimulation induces the phosphorylation of the serine rich N-terminal domain of the SLAP-2 by a JNK dependent pathway [36].

The activation-induced GM-CSFR downregulation is attenuated in SLAP-1 and SLAP-2 deficient bone marrow dendritic cells (BM-DC), which is associated with enhanced MAP/Erk and Akt pathways upon GM-CSF stimulation. The inhibited activation through GM-CSFR impairs the bone marrow derived dendritic cell maturation. SLAP-1 and SLAP-2 deficient BM-DC cells produce less IL-12 and TNF-α upon lipopolysaccharide (LPS) stimulation and induce significantly less IFN-γ secretion of T-lymphocytes than the wild type cells [38]. These data suggest that SLAP proteins are necessary for monocyte and dendritic cell maturation and activation.

2.5. SLAP Proteins in Mast Cells. SLAP has a prominent role in the regulation of intracellular signal transduction in mast cells. SLAP-specific small interfering RNAs inhibit the effect of dexamethasone on the phosphorylation of PLC γ, LAT, Syk, and ERK [39]. Actinomycin D inhibits the transcription of SLAP following dexamethason treatment, suggesting that the upregulation for SLAP-1 upon glucocorticoid treatment occurs at the transcriptional level. Glucocorticoid receptor activation is required for the elevation of the SLAP transcription [40]. SLAP-1 plays a role in the negative regulation of antigen-stimulated mast cells as well. Upon antigen stimulation of the RBL-2H3 mast cell line, elevated transcription of the SLA gene and upregulation of the SLAP-1 (but not SLAP-2) protein were reported. An increased amount of SLAP-1 was detected after 60 minutes stimulation and it reached the maximum after 2-3 hours. By contrast, after silencing of SLAP, increased IL-3 and MCP-1 production was detected. Knock-down of SLAP by using siRNA increases the expression of FcεRI [39]. Thus, SLAP appears to be a crucial regulator of mast cell function.

3. The Function of SLAP Molecules in Other Cell Types

3.1. SLAP-1 in Osteoclasts. Osteoclasts play a fundamental role in the pathogenesis of osteoporosis. Inhibiting the development and activation of osteoclasts is currently the gold standard therapeutic strategy in this disease. Although today several antiosteoporotic drugs are widely used in the clinical practice, the effect of these medications on increasing bone density and strength is moderate. There is an inverse correlation between both SLAP-1 and SLAP-2 and the tartrate-resistant acid phosphatase (osteoclastogenesis marker) mRNA expression. The level of osteoclast-specific protein mRNAs (e.g., catepsin K and MMP-9) are elevated in the SLAP-1 −/− preosteoclasts. SLAP-1 has an inhibitory effect on the MAP kinase pathway which is initiated with the binding of M-CSF to its receptor c-Fms. This receptor is a tyrosine kinase transmembrane protein which accumulates in lipid rafts where it associates with SLAP-1. The lack of SLAP enhances osteoclastogenesis without changing the resorptive function of the individual cells [41]. In addition, SLAP-1 deficiency increases the apoptosis of the mature

polykariotic osteoclasts without altering the viability of the precursors. According to our current understanding, SLAP-1 has a regulatory effect on osteoclastogenesis and mature cell survival through the M-CSF pathway [41].

3.2. SLAP Molecules in Platelets and Fibroblasts. SLAP-2 is expressed in human platelets and may associate with Syk, c-Cbl, and LAT. After its activation, SLAP-2 promotes Syk and c-Cbl to approach their substrates. SLAP-2 inhibits glycoprotein VI (GPVI) initiated signal transduction of platelets through the connection with c-Cbl [42]. This inhibitory effect on thrombocyte activation is similar to those described previously in lymphocytes [1, 5].

SLAP may also associate with platelet derived growth factor receptor (PDGFR) in NIH3T3 mouse embryonic fibroblast cell line. Overexpression of SLAP in NIH3T3 cells inhibits PDGF-induced mitogenesis [43] suggesting that SLAP is a negative regulator of growth factor initiated signaling.

3.3. SLAP-1 in Neurons. Although in embryonic rat telencephalon sections SLAP-1 mRNA is absent in migrating neurons, it is highly and selectively expressed in neurons which have reached their final location. SLAP-1 has a characteristic expression pattern during the development of the cortex. It is expressed mostly by deeper stratum cells especially by pyramid cells. Pyramid cells are typically located in the deeper layers of the cortex, and they have connection with the subcortical areas. It is not yet clear whether SLAP-1 plays a role in the axon guidance during development but apparently has a remarkable expression. There is also a possible interaction between SLAP-1 and EphA receptors [44].

Western blot analysis and immunolabeling of rat brain extract showed that SLAP-1 is located in the postsynaptic membrane. SLAP-1 has an association with EphAs and NMDARs have a connection with EphBs [44, 45]. EphB may excite the enlistment of EphAs and promote the recruitment of SLAP-1 and NMDARs. Despite the analogous localization, SLAP-1 does not affect the baseline activity of NMDARs through a Src-dependent stimulation of the receptors. SLAP-1 may have a role in the negative feedback which regulates the number of the NMDARs and prevent excitotoxicity. This effect of SLAP-1 is based on the proteasomal degradation of redundant NMDARs in neurons [46]. The described role of SLAP in neurons seems to be essential for the adequate neuronal functions.

4. SLAP in Pathological Conditions

4.1. The Role of SLAP in Rheumatoid Arthritis. Rheumatoid arthritis (RA) is a common autoimmune disease that is associated with progressive disability and systemic complications. Symmetric synovial inflammation of multiple joints, especially the small joints of the hands and the feet, is characteristic for the disease. The ongoing inflammation leads to cartilage destruction, bone erosions, and subsequent joint deformities. Although the current treatment strategy, especially the use of biologicals, improved largely the outcome of the disease, still only a small portion of the patients are in sustained remission and systemic complications (including cardiovascular risk) still represent a significant challenge. TNF-α plays an essential role in the pathogenesis of RA through promotion of angiogenesis, suppression of regulatory T-cell activation, and cytokine and chemokine expression.

According to the recently published data of our research group, TNF-α treatment downregulates the expression of ζ-chain of CD4 T-lymphocytes reversibly and selectively in a dose dependent way. Decreased ζ-chain expression leads to the hyporesponsiveness of T-cells. TNF-α induces the expression of SLAP which promotes the proteasomal but not the lysosomal degradation of the ζ-chain. Silencing SLAP with short interfering RNAs inhibits the TNF-α induced ζ-chain degradation. TNF-α treatment does not alter the SLAP mRNA level, suggesting that TNF-α controls SLAP activity through miRNA mediated posttranscriptional silencing. CD4+ T-lymphocytes isolated from RA patients expressed more than 2-fold higher SLAP levels than the T-cells of healthy donors. TNF-α treatment enhances the expression of SLAP in the CD4+ T-lymphocytes of healthy donors and DMARD treated RA patients but does not alter the expression of CD4+ T-cells isolated from biological DMARD (etanercept, certolizumab pegol) treated patients [47].

A spontaneous mutation in ZAP70 protein uncoupled proximal TCR signal transduction and led to severe symmetrical arthritis in SKG mice upon exposure to zymosan. According to recently published data, SLAP deficiency dramatically reduced both the incidence and severity of zymosan-induced chronic autoimmune arthritis in SKG mice [48]. The protective role of SLAP deficiency was associated with the increased number of regulatory T-cells and decreased amount of Th17 cells [48].

4.2. The Role of SLAP in Malignant Diseases and in Erythropoiesis. SLAP expression is increased in several cancers including chronic myeloid leukemia (CML), chronic lymphocytic leukemia (CLL), and prostate cancer. By contrast, its expression is decreased in acute myeloid leukemia (AML), myeloma, and colon cancer [13]. Furthermore, SLAP is associated with several oncogenic signaling pathways.

It was recently reported that the transmembrane protooncogene c-kit is degraded through a SLAP dependent pathway. SLAP binds to the WT c-kit and initiates its ubiquitylation and degradation in the proteasome. By contrast, SLAP does not have a similar regulatory effect on the oncogenic c-kit variant (c-kit D816V). Oncogenic c-kit associates with SLAP and phosphorylates it on three different tyrosines (Y120, Y258, and Y273); the phosphorylated forms of SLAP do not alter the downstream signaling of the mutant c-Kit. These data suggest that SLAP regulates WT c-kit signaling, but the oncogenic variant escapes from this negative feedback regulation [49].

The Fms like tyrosine kinase (Flt3) is a receptor tyrosine kinase, which is predominantly expressed in hematopoietic progenitor cells. SLAP associates with both the WT and

mutated, oncogenic Flt3 (Flt3-ITD). Silencing of SLAP with short interfering RNAs leads to an attenuated MAPK signal pathway. After ligand stimulation, SLAP colocalizes with the Flt3-ITD and targets it for c-Cbl dependent ubiquitylation and proteasomal degradation. About 30% of patients with AML have mutation in the Flt3 gene and gain of function mutations contribute to the initiation of AML. The expression of SLAP is increased in patients with acute promyelocytic leukemia (APL) carrying Flt3-ITD mutation as compared to the Flt3-WT [13].

FLI-1 is a transcription factor, a member of the E26 transformation-specific (ETS) protein family. Furthermore, the FLI-1 locus is an integration site for the Friend murine leukemia virus, which induces erythroleukaemia in responsive mice. SLAP protein and mRNA levels are overexpressed in FLI-1 transformed erythroblasts [8]. SLAP binds to both the phosphorylated and unphosphorylated forms of the erythropoietin receptor (EpoR). SLAP expression prevents the EPO-induced differentiation, whose effect is associated with the inhibition of STAT5 activation and BCL-X upregulation. Both STAT5 and BCL-X are critical in EPO-induced signaling [8]. SLAP may mediate the erythropoiesis in this manner.

According to recently published data, SLAP is expressed in colon epithelium, but it is significantly downregulated in colorectal tumors [15]. Interestingly, silencing of SLAP promotes tumor progression, while overexpression inhibits tumor growth and invasiveness. SLAP promotes the destabilization of the Src substrate ephrin type-A receptor 2 (EphA2) in the intestinal cells. EphA2 plays a critical role in the regulation of several intracellular signal pathways which mediate cell migration, invasion, and angiogenesis. The inhibitory effect of SLAP appears to be independent from c-Cbl but has an association with the ubiquitination factor UBE4A and with the pTyr594-EphA2. These results suggest a tumor-suppressive effect of SLAP in colorectal cancers [15].

5. Conclusions and Future Perspectives

SLAP proteins are expressed in a variety of cell types which indicates a conserved function of these proteins. Both SLAP-1 and SLAP-2 have a prominent role in the negative regulation of several membrane bound receptors and receptor tyrosine kinases, thereafter SLAPs play a central role in the regulation of intracellular signal transduction and cell reactivity. The proper function of SLAPs is necessary for immunoreceptor repertoire configuration and helps to avoid uncontrolled cell activation, proliferation, and migration. SLAP molecules are involved in the ubiquitination of proteins, which may lead to proteasomal degradation. Selective regulation of SLAP molecules in different cell types may allow the fine control of cell activation and differentiation. Exploring the precise role of SLAP proteins will contribute to the understanding of many yet unknown physiological regulatory processes. In addition, tissue specific targeting of SLAP may provide valuable therapeutic approaches in diverse diseases including RA, osteoporosis, immunodeficiency, and different cancers.

Abbreviations

AP-1:	Activator protein 1
AML:	Acute myeloid leukaemia
BCLXL:	B-cell lymphoma extra-large transmembrane molecule
BCR:	B cell receptor
BJAB:	EBV-negative, Burkitt-like lymphoma
c-Cbl:	E3 Ubiquitin-protein ligase casitas B-linage lymphoma protooncogene
CD:	Cluster of differentiation
CD3 ζ-chain:	T-cell receptor complex zeta chain
c-Fms:	Colony stimulating factor 1 receptor
c-kit:	Mast/stem cell growth factor receptor
CSF1:	Colony stimulating factor 1
DMARDs:	Disease modifying antirheumatic drugs
ECK:	Ephrin type-A receptor 2
Eph:	Ephrin type receptor
EphA2:	Ephrin type-A receptor 2
EphB:	Ephrin type-B receptor
Epo:	Erythropoietin
EpoR:	Erythropoietin receptor
ERK:	Extracellular signal-related kinases
FcεRI:	Fc ε receptor I
FLI1:	Friend leukemia integration 1 transcription factor
Flt3:	Fms-like tyrosine kinase 3
GM-CSFR:	Granulocyte macrophage colony stimulating factor receptor
GPVI:	Glycoprotein VI
HIV:	Human immunodeficiency virus
Igα:	B-cell receptor antigen complex-associated protein alpha
IFN γ:	Interferon γ
IL:	Interleukin
ITD:	Internal tandem duplications
JNK:	c-Jun N-terminal kinase
kb:	Kilobase
LAT:	Linker for activation of T-cells family member
Lck:	Protooncogene tyrosine-protein kinase
MAP:	Microtubule associated protein
MAPK:	Microtubule associated protein kinase
MCP-1:	Monocyte chemotactic protein 1
M-CSF:	Macrophage colony stimulating factor
MMP9:	Matrix metallopeptidase 9
mRNA:	Messenger ribonucleic acid
miRNA:	Microribonucleic acids
NFATc:	Nuclear factor of activated T-cells
NMDAR:	N-methyl-D-aspartate receptor
PDGF:	Platelet derived growth factor
PDGFR:	Platelet derived growth factor receptor
PLC γ:	Phospholipase C γ
PMA:	Phorbol myristate acetate
PTK:	Protein tyrosine kinase
RA:	Rheumatoid arthritis
RNA:	Ribonucleic acid
SH:	Src homology domain
SLA:	Src-like adaptor

SLAP-1: Src-like adaptor protein 1
SLAP-2: Src-like adaptor protein 2
SLP-76: SH2 domain-containing protein of 76 kDa
Src: Sarcoma
STAT5: Signal transducer and activator of transcription 5
Syk: Spleen tyrosine kinase
Th17: T helper 17
TNF-α: Tumor necrosis factor α
TCR: T-cell receptor
UBE4A: Ubiquitin conjugation factor E4A
V-abl: Tyrosine-protein kinase transforming protein Abl
Vav: Protooncogene vav
WT: Wild- type
ZAP70: Zeta-associated protein of 70 kDa.

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

Inflammatory Bowel Disease: An Overview of Immune Mechanisms and Biological Treatments

Bruno Rafael Ramos de Mattos,[1] Maellin Pereira Gracindo Garcia,[1,2] Julia Bier Nogueira,[1] Lisiery Negrini Paiatto,[1] Cassia Galdino Albuquerque,[1] Caique Lopes Souza,[1] Luís Gustavo Romani Fernandes,[2,3] Wirla Maria da Silva Cunha Tamashiro,[1] and Patricia Ucelli Simioni[1,2,4]

[1]*Department of Genetics, Evolution and Bioagents, Institute of Biology, University of Campinas (UNICAMP), 13083-970 Campinas, SP, Brazil*
[2]*Department of Biomedical Science, Faculdade de Americana, Avenida Joaquim Boer 733, 13477-360 Americana, SP, Brazil*
[3]*Medical School, UNICAMP, 13083-887 Campinas, SP, Brazil*
[4]*Department of Biochemistry and Microbiology, Institute of Biosciences, Universidade Estadual Paulista (UNESP), Avenida 24 A 1515, 13506-900 Rio Claro, SP, Brazil*

Correspondence should be addressed to Patricia Ucelli Simioni; psimioni@gmail.com

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Inflammatory bowel diseases (IBD) are characterized by chronic inflammation of the intestinal tract associated with an imbalance of the intestinal microbiota. Crohn's disease (CD) and ulcerative colitis (UC) are the most widely known types of IBD and have been the focus of attention due to their increasing incidence. Recent studies have pointed out genes associated with IBD susceptibility that, together with environment factors, may contribute to the outcome of the disease. In ulcerative colitis, there are several therapies available, depending on the stage of the disease. Aminosalicylates, corticosteroids, and cyclosporine are used to treat mild, moderate, and severe disease, respectively. In Crohn's disease, drug choices are dependent on both location and behavior of the disease. Nowadays, advances in treatments for IBD have included biological therapies, based mainly on monoclonal antibodies or fusion proteins, such as anti-TNF drugs. Notwithstanding the high cost involved, these biological therapies show a high index of remission, enabling a significant reduction in cases of surgery and hospitalization. Furthermore, migration inhibitors and new cytokine blockers are also a promising alternative for treating patients with IBD. In this review, an analysis of literature data on biological treatments for IBD is approached, with the main focus on therapies based on emerging recombinant biomolecules.

1. Introduction

The role of intestinal milieu in immune homeostasis appears to be of greater significance than it was previously thought. This complex interplay of genetic, microbial, and environmental factors culminates in a sustained activation of the mucosal immune and nonimmune responses. Under normal situations, the intestinal mucosa is in a state of "controlled" inflammation regulated by a delicate balance of Th1, Th17, Th2, Th3, Th9, and Treg cells [1–6].

Inflammatory bowel diseases (IBD) are related to an immunological imbalance of the intestinal mucosa, mainly associated with cells of the adaptive immune system, which respond against self-antigens producing chronic inflammatory conditions in these patients. Ulcerative colitis (UC) and Crohn's disease (CD) are the most studied types of inflammatory bowel diseases, having the highest prevalence in the world population. The pathophysiological mechanisms of IBD are not fully understood, although these diseases have been discovered several decades ago [7–10]. In the present work, we aim to review the current approaches for treating IBD, focusing on the new therapies based on biological molecules.

2. Inflammatory Bowel Disease

It is widely known that the number of bacteria in the gastrointestinal tract is about 10 times higher when compared to eukaryotic cells in the body. Also, the normal enteric bacterial flora is a complex ecosystem of approximately 300–500 bacterial species [11, 12]. Moreover, the balance of the innate and adaptive immunity is critical for this microenvironment homeostasis. In this sense, the immune system has the important role of promoting immune tolerance, thereby avoiding the specific immune response against the large mass of commensal bacteria. The local immunity in intestinal mucosa is basically ensured by gut associated lymphoid tissue (GALT), constituted by Peyer's patches, lymphoid follicles, and mesenteric lymph nodes [13]. Along with cellular, environmental, and genetic factors, deregulation of immune responses in the intestinal mucosa has been associated with the etiology of IBD. Alterations in the autophagy—a cellular process related to the degradation of intracellular pathogens, antigen processing, regulation of cell signaling and T cell homeostasis—usually results in reduced clearance of pathogens, thus contributing to the onset of inflammatory disorders in susceptible subjects [14, 15]. In this sense, mutations on ATG16L1 gene, a member of a family of genes involved in autophagy, were detected in patients with CD [16].

The breakage of self-antigens tolerance in the intestinal mucosa, by injury or genetic predisposition, may lead to CD or UC [17, 18]. Cells of the innate immunity, such as macrophages and dendritic cells, are specialized in identifying microorganism's molecular patterns by using the pattern recognition receptors (PRR), such as toll-like receptors (TLR) and nucleotide-binding oligomerization domains (NOD). In this regard, mutations in the caspase recruitment domain-containing protein 15 (CARD-15) gene encoding the NOD-2 protein were associated with the occurrence of IBD, especially CD. NOD2 is an intracellular microbial sensor that acts as a potent activator and regulator of inflammation. Therefore, deficiency in this protein promotes important changes on the immune response in the lamina propria, producing a chronic inflammation in the tissue. Clinically, it is of interest to determine the relationship between NOD2 gene status and the efficacy of antibiotic treatment in CD [19–22].

Likewise, the imbalance between Th1 and Th2 cytokines released by the intestinal mucosa determines the intensity and duration of the inflammatory response in experimental colitis [23]. The secretion of certain cytokines such as tumor necrosis factor-alpha (TNF-α) [24, 25], transforming growth factor-beta (TGF-β) [23, 26, 27], and interferon-gamma (IFN-γ) [28, 29] as well as the response to self-antigens [30–32] are factors that seem to be related to the onset and establishment of IBD. Although UC is often described as Th2-mediated diseases while CD is known as a Th1 condition, the classic paradigm has recently been changed, since cytokines can have diverse and opposing actions [33].

Recent data showed that Th17 cells and other cells producing interleukin- (IL-) 17 play a crucial role in the intestinal inflammatory manifestations. IL-17 and IL-22 appear to be related to the induction of colitis, since these cytokines initiate and amplify the local inflammatory signs and promote the activation of counter-regulatory mechanisms targeting intestinal epithelium cells [34]. Also IL-23, released by macrophages and dendritic cells located in the intestinal mucosa, activates signal transducer and activator of transcription- (STAT-) 4 in memory T lymphocytes, stimulating the production of IFN-γ. In turn, IFN-γ is responsible for triggering the production of inflammatory cytokines in cells of the innate immune system, contributing to the increase of the inflammation present in colitis [35]. Latest results from Neurath group [3] identified a pathogenic role of IL-9 in experimental and human ulcerative colitis by regulating intestinal epithelial cells.

It is also important to report that environmental factors can play a significant role in the development of IBD, although this relationship is poorly understood. Particularly, there are several evidences that tobacco could have an important role in triggering this type of intestinal inflammation [36, 37].

2.1. Crohn's Disease. Crohn's disease, one of the most frequent forms of inflammatory disease worldwide, is characterized by the formation of strictures, fistulas, ulcers, and granulomas in the mucosa. Although the CD's gastrointestinal manifestation can primarily affect the terminal ileum region, it can also compromise any region from the mouth to the rectum of affected patient. The clinical manifestations of CD can include diarrhea or bloody diarrhea, malnutrition, abdominal pain, and weight loss. Extraintestinal findings, for instance, arthropathy or skin disorders, rarely occur. However, manifestations on skin, muscle, or bone of metastatic Crohn's disease can actually lead to recognition of occult intestinal disease [80–82]. In general, CD has a genetic background and the first-degree relatives of affected individuals have a fivefold greater risk of developing the disease [83, 84].

The localized release of certain cytokines, such as IL-12, IL-17, TNF-α, and IFN-γ, has been implicated in the chronic intestinal inflammation observed in CD patients [51, 85]. The production of IL-12 and IL-18 by antigen-presenting cells (APC) and macrophages generates a polarized differentiation towards Th1 lymphocyte, leading to an increased release of proinflammatory cytokines, including TNF-α and IFN-γ. Additionally, Th1 cytokines stimulate the antigen-presenting cells to secrete a wider spectrum of inflammatory cytokines such as IL-1, IL-6, IL-8, IL-12, and IL-18, resulting in a self-sustained cycle [86].

2.2. Ulcerative Colitis. Ulcerative colitis is another form of IBD characterized by superficial ulcerations, granularity, and a vascular pattern. In contrast with the inflammation found in CD—transmural and being able to occur throughout the entire gastrointestinal tract—inflammation in UC is limited to the mucosal layer of the colon [87, 88]. Although Montreal classification—a system to classify IBD phenotypes including UC—is widely used, data on its reliability are very limited due to the great variety of clinical presentations of UC [88]. In general, the clinical manifestation of UC can include release of blood and mucus, petechial hemorrhage, and granulation

tissue, among others. However, in periods of remission, the mucosa may have normal appearance. In most severe forms of the disease, the intestine can get distended, presenting deep ulceration and possibly intestinal perforation [87, 89].

In UC, there is a substantial increase in the secretion of IL-13, the main interleukin responsible for the inflammation and chronicity of this condition [87]. Despite the Th1 involvement, UC patients also present a Th2 response with increased secretion of IL-4, IL-5, and IL-9 [3, 5]. It has been suggested that the expression of the PU.1 transcription factor, a regulator of cellular communication, and the production of IL-9 by effectors' Th9 cells block the proliferation of intestinal epithelial cells and regulate the expression of several tight-junction proteins. Together, these aspects favor the translocation of specific bacterial species with subsequent activation of immune cells and mucosal inflammation in experimental and human UC [3]. As in CD, Th17-related cytokines are also increased in UC [34, 90].

3. Treatments for IBD

In order to better illustrate the relevance of each of the different IBD treatments, Table 1 compares different forms of treatment, mechanisms of action, patterns, and adverse effects of each form of therapy.

3.1. Classical Treatments for IBD. In ulcerative colitis, treatment decision is dependent on the stage of the disease: patients with mild manifestations are usually treated with aminosalicylates, whereas corticosteroids are prescribed for those with moderate disease and cyclosporine is given to patients with severe disease. In Crohn's disease, decisions about drug therapy are dependent on both location and behavior of the disease. Despite that, the medication in CD usually includes aminosalicylates and antibiotics to treat mild mucosal disease, corticosteroids to moderate disease, and biological molecules to treat fistulizing disease. Also, aminosalicylates, azathioprine, mercaptopurine, methotrexate, metronidazole, and associations can be used as maintenance therapies [38, 39, 42, 80, 91–94]. Notwithstanding their reduced cost, these drugs can generate several side effects. Moreover, these therapies do not achieve clinical remission and they can lead to the onset of other conditions such as renal impairment [39, 49, 50].

At the same time the classical treatments are widely used, new therapies are under development in the attempt of improving the patient's life quality. The new therapies aim to reduce the side effects and to treat patients who do not respond satisfactorily to conventional therapies [51, 52, 74, 95, 96].

Other therapeutic strategies, not covered in this review, are in very early evaluation. These involve the manipulation of the microbiome using antibiotics, probiotics, prebiotics, diet, and fecal microbiota transplantation [93, 97, 98].

3.2. Biological Therapies. The application of biological therapy for the treatment of inflammatory diseases can be associated with some studies that identified the proinflammatory cytokines present in the gut lamina propria of the IBD patients. These cytokines, in particular TNF-α, play a crucial role in the maintenance of chronic inflammation of the intestinal mucosa [53, 54]. Among the biological molecules, the use of monoclonal antibodies specific against TNF-α, cytokine related to the establishment of IBD, seems to be a relevant alternative. These antibodies may activate various mechanisms involved in the immune response, such as induction of apoptosis as well as the blockage of growth factors for the Th cells, antibody production, and complement activation [99].

Although the treatment of IBD with biological molecules, specifically with monoclonal antibodies, presents high specificity and directed mechanism of action, the high cost of this therapy still represents a barrier to be overcome. For this reason, together with this being a therapy in early stages of development, these drugs are generally used as an alternative for patients that are refractory to corticosteroid and aminosalicylates treatments [55, 100].

Also, long-term therapy with biological molecules can cause immunogenicity by generating anti-drug antibodies. These antibodies can promote acute and delayed infusion reactions and can reduce the duration of the patient response to each infusion or injection [101, 102]. In this sense, there is a potential contribution of the complement system as well as of the formation of immune complexes in the augmentation of immunogenicity [103, 104]. In some patients, immunogenicity is restricted to transient low level of antibodies, presenting no clinical effects. However, patients with high levels of anti-drug antibodies are more likely to present a loss of response by the reduction of drug levels, compromising the long-term therapy [101, 102]. As an alternative, concomitant immunosuppression appears to reduce immunogenicity and improve therapeutic control, even though it can present an increased risk for infection and malignancy [105].

Immunogenicity will depend on structure and origin of biologic agents. Biologicals can be fusion protein or a chimeric, and a humanized, or fully human antibody [106]. Also, the administration route, dosing schedule, and individual characteristics can have a great impact on immunogenicity [104, 106]. It is necessary to determine the optimal treatment regimen in order to minimize the likelihood of anti-drug antibody formation.

3.2.1. Anticytokine Agents. Currently, some anticytokine agents have been showing relevant results for the treatment of IBD. It is already known that antibodies specific for TNF-α play an important role in maintaining the remission of CD, in both severe and moderate forms of the disease. These molecules were effective in inducing mucosal healing and clinical remission, reducing the cases of hospitalization and surgical procedures in affected individuals [52, 56, 57].

The first commercially available anti-TNF molecule was infliximab (IFX), a chimeric monoclonal IgG1 antibody [52, 56–58] formed by a segment of the native mouse protein containing the binding site for the TNF-α and a portion of human immunoglobulin responsible for the effector function of the antibody molecule [25, 54, 59]. With the introduction

Table 1: IBD treatments: drugs in use, mechanisms of action, and side effects.

Treatment type	Related drugs	Mechanism of action	Features	Potentials adverse effects	References
Aminosalicylates	Mesalamine Olsalazine Balsalazide Sulfasalazine	Inhibition of IL-1, TNF-α, and platelet activating factor (PAF), decreased antibody secretion.	Locally immunosuppressive, nonspecific inhibition of cytokines; medium cost.	Headache, dizziness, dyspepsia, epigastric pain, abdominal pain, nausea, vomiting, and diarrhea.	[38–41]
Immunomodulators	Azathioprin 6-mercaptopurin Methotrexate	Blockage of *de novo* pathway of purine synthesis.	Antiproliferative effects, reduction of inflammation.	Black, tarry stools, bleeding gums, chest pain, fever, chills, swollen glands, pain, cough, and weakness.	[42–48]
Corticosteroids	Prednisone Methylprednisolone Hydrocortisone Budesonide	Blockage of phospholipase A2 in the arachidonic acid cascade altering the balance between prostaglandins and leukotrienes; stimulation of apoptosis of lamina propria lymphocytes; suppression of the transcription of cytokines.	High immunosuppression, risk of potential infections, adverse effects with long periods of use, low cost.	Full moon face, difficulty of healing, acne, sleep and mood disturbances, glucose intolerance, osteoporosis, osteonecrosis, subcapsular cataracts, myopathy, infections, acute adrenal insufficiency, myalgia, malaise, arthralgia or intracranial hypertension, and pseudorreumatism syndrome.	[49, 50]
Biologicals: anti-cytokine drugs	Infliximab Adalimumab Certolizumab-pegol Golimumab Ustekinumab (phase 3 trial)	Induction of apoptosis in proinflammatory cells; binding specifically to TNF-α, blockage of the interaction the receptor.	Specific inhibition of cytokine, immunosuppression, high cost, advanced technology required.	Abdominal or stomach pain, chest pain, chills, cough, dizziness, fainting, headache, itching, muscle pain, nasal congestion, nausea, sneezing, weakness, vomiting, bloody urine, cracks in the skin, diarrhea, pain, fever, abscess, back or side pain, bone or joint pain, constipation, falls, facial edema, general feeling of illness, hernia, irregular heartbeat, unusual bleeding, weight loss, increased risk of reactivation of latent tuberculosis, and increased risk for developing infections and lymphoma.	[24, 51–73]
Biologicals: anti-cell adhesion molecule	Vedolizumab Natalizumab	Inhibition of migration.	Specific inhibition of cell adhesion molecules high cost, advanced technology required.	Nasopharyngitis, headache and abdominal pain, increased risk of infections, serious infections, and progressive multifocal leukoencephalopathy (natalizumab).	[63, 74–79]

of IFX in market there was a considerable increase of clinical remission in CD and UC patients. In fact, IFX is currently the approved biologic agent for the treatment of inflammatory and fistulizing Crohn's disease and UC in several countries [52, 56, 57].

In order to treat nonresponsive patients to IFX, adalimumab (ADA), a fully human IgG1 monoclonal antibody, has emerged as an alternative molecule [58]. ADA is the first biotechnological product that is produced by using phage display technology approved by US Food and Drug Administration (FDA). Administration of ADA is indicated for individuals with CD in the moderate and severe forms, also showing positive results for the treatment of UC [58, 60]. The ADA induces apoptosis of proinflammatory cells

by specifically binding to TNF-α molecule, leading to the blockage of the interaction of this cytokine with their surface receptors p55 and p75 [58].

Biological therapy with monoclonal antibodies IFX and ADA has been increasingly employed in the treatment of IBD, showing a significant improvement of the patient's clinical condition. In general, 45–70% of IBD cases presented clinical remission after being treated with IFX [43, 61, 107], while patients treated with ADA presented clinical remission near 30–60% [55, 61, 108].

However, the treatment with these molecules involves several risks and generates side effects. Renal complications, infusion reaction, delayed hypersensitivity-like reaction, new onset of autoimmunity (with rare cases of drug-induced lupus and new-onset demyelination) and opportunistic infections are some examples of complications resulting from the immunosuppression induced by biological therapy [25, 53, 54, 59, 62]. The fact that these drugs have been recently approved for clinical practice explains why their side effects are not thoroughly known. Because of that, more studies should be conducted in order to fully understand the mechanisms and the consequences of the use of these drugs. Furthermore, these anti-TNF agents did not present relevant effects in all treated patients, indicating another aspect worth investigating [55, 61, 109–111].

In this context, other anti-TNF biologic agents have emerged, including CDP 571, etanercept, and onercept. These humanized or fully human anti-TNF biotechnological agents are theoretically less immunogenic than the chimeric IFX. However, biopharmaceuticals such as etanercept (TNF inhibitor), onercept (recombinant TNF p55 receptor monomer), and CDP571 (recombinant humanized MAb against TNF-α) were not effective for active Crohn's disease [63, 112, 113].

Certolizumab pegol (CDP 870), a pegylated and fully humanized monoclonal antibody fragment, is an anti-TNF agent that has recently been approved by FDA for CD treatment with sustained remission [114–116]. Studies show that this drug seems to be more effective and less immunogenic than IFX and ADA [63, 64, 117]. Additionally, golimumab, a human IgG1 TNF-α antagonist monoclonal antibody, also showed significant results in inducing and maintaining remission in CD and UC patients, with a rate of adverse events similar to the placebo [63, 65, 118].

3.2.2. Leukocyte Migration and Signaling Inhibitors. Since the application of biological drugs for treating IBD is a novel approach, there are several new biologic agents that have been recently approved or included in clinical trials or are under evaluation for determining their clinical efficacy and safety profile.

Currently, therapies that manipulate leukocyte adhesion, costimulatory signaling and cytokine receptors are being evaluated as potential treatments for IBD. These alternative treatments emerged when it was observed that some of the patients under current biologic therapies with anti-TNF-α agents were primarily nonresponders or experience a loss of response, intolerance, or even presented side effects [63, 75, 119–122].

Lymphocyte-endothelial interactions, mediated by adhesion molecules, are important in leukocyte migration and recruitment to sites of inflammation. The selective blockage of these adhesion molecules is a new and promising approach to treat CD. Recently approved by FDA, anti-α4 integrin monoclonal antibodies, specifically natalizumab and vedolizumab, were effective in the treatment of moderately to severely active CD (natalizumab and vedolizumab) and for UC (vedolizumab) patients [10, 63, 123]. The blockage of the T cell migration into the intestine by using anti-$\alpha 4\beta 7$ antibody vedolizumab, approved to treat adult patients, resulted in a selective barrier for the trafficking of CD4+CD45RO+ T cells. It also reduced the UC clinical score, presenting a successful remission in 33% to 50% of cases [74, 76, 124].

Meanwhile, clinical efficacy of some therapeutic agents, such as inhibitors of leukocyte trafficking, including alicaforsen, an oligodeoxynucleotide that inhibits intercellular adhesion molecule 1 (ICAM-1) expression, are still under evaluation [123, 125–127]. Among the new drugs being tested, ustekinumab, a monoclonal antibody against the p40 subunit of interleukin-12/23, approved to use in patients with moderate or severe psoriasis and psoriatic arthritis, was able to induce clinical response in patients with moderate-to-severe CD, especially in those previously treated with IFX [51, 66]. Etrolizumab, a humanized monoclonal antibody that selectively binds to the $\beta 7$ subunit of the heterodimeric integrins $\alpha 4\beta 7$ and $\alpha E\beta 7$, was well tolerated in moderate to severe UC on phase II studies [128, 129]. Additionally, tofacitinib, a small molecule targeting Janus-activated kinase (JAK), was shown to particularly inhibit JAK1 and JAK3, also interfering with several cytokine receptors. However, there are no relevant clinical data related to this molecule [130].

Since one of the most important mechanisms in establishing gastrointestinal inflammatory conditions is the activation of different populations of T cells, the balance among effectors and regulatory populations is crucial for driving the immune response in GALT [131]. In this way, costimulatory signaling of T cell activation has been investigated as a potential target to block unwanted and deleterious inflammatory response. One of these targets is the CTLA-4 molecule, expressed on the surface of T cells, which selectively competes with CD28 molecule for binding to CD80 and CD86 molecules present on APCs. Besides that, it was recently demonstrated that both T regulatory and T conventional cells exert a suppressive function on the externalization of CTLA-4 protein [132]. Thus, new clinical approaches have used the biological molecule abatacept, a fusion protein composed of the Fc portion of IgG together with the CTLA4 molecule (CTLA4-Ig), to treat different inflammatory disorders such as psoriatic arthritis, type 1 diabetes, multiple sclerosis, and systemic lupus erythematosus. However, a phase III trial in moderate-to-severe CD and UC showed no therapeutic benefits with the use of the abatacept, indicating that blocking the T cell activation possibly compromises the activation of important regulatory T cells subsets in IBD patients [133].

3.2.3. Biological Drug Dosage. Nowadays, five biologic agents are approved by FDA for the treatment of IBD: adalimumab, infliximab, golimumab, certolizumab pegol, and vedolizumab. In order to reach an effective disease remission of IBD patients, IFX standard dosage for UC and CD is usually 5 mg/kg by intravenous infusion at weeks 0, 2, and 6, followed by a maintenance regimen every 8 weeks. However, some data shows that the dosage of 10 mg/kg seems to maintain the remission for a longer period [134–137]. On the other hand, ADA has shown to be effective to UC and CD by the subcutaneous administration with an initial dose of 160 milligrams, a second dose two weeks later of 80 mg, and a maintenance dose of 40 mg every other week, although it has also been shown that there is a dose-dependent effectiveness related to this drug [138–140].

In patients with moderate to severe CD, subcutaneous administration of certolizumab pegol on subcutaneous doses of 400 mg once every 4 weeks was effective as induction and maintenance therapy. In case of lack of response, it should be given every 2 weeks [141]. The recommended golimumab initial regimen for the treatment of UC is a 200 mg subcutaneous dose at week 0 followed by 100 mg at week 2. The maintenance therapy is 100 mg every 4 weeks [67]. Vedolizumab was recently approved by FDA for the treatment of adults with moderately to severely active UC and CD. Dose regimen is 300 mg infused intravenously at 0, 2, and 6 weeks and the maintenance therapy at every 8 weeks thereafter [74].

Despite the fact that there are no suficient comparative trial data available between infliximab, adalimumab, and certolizumab pegol, they are considered as having comparable efficacy, especially when the maintenance of remission is taking into consideration [68, 137]. An advantage of ADA, golimumab, and certolizumab in comparison with IFX and vedolizumab is that they can be administered by a subcutaneous injection. It is important to mention that patient's history, drug regimen, and drug efficacy create a singular scenario that should be taken into account before choosing the appropriate therapy.

3.2.4. Biosimilars. Additionally, it is important to mention that infliximab's patent has already expired in many countries (e.g., Brazil, Argentina, Canada, South Korea, and some Eastern Europe countries) and is about to in USA and in Western Europe countries, opening the opportunities for biosimilar drugs to reach the market. Nowadays, IFX-biosimilars are already used in some countries for the treatment of IBD and present a significant reduction in costs.

Biosimilars present the same amino acid sequence and a highly similar glycosylation pattern when compared with the original product. Accordingly, biosimilars represent a future tendency and a promising new option for IBD patients with the main advantage of being less expensive, which may affect the availability of the biological treatment for patients around the world. However, concerns about the efficacy, safety, and immunogenicity of biosimilars still exists [142, 143].

4. Final Considerations

Gastrointestinal immune disorders are most likely facilitated by defects in the intestinal epithelial barrier and in the mucosal immune system, resulting in an active inflammation and tissue destruction. The mucosal immune system is essential for the establishment and controlling of the intestinal inflammation and injury, with cytokines playing a central role in modulating IBD. Therefore, using specific inhibitors or blockers targeting cytokines and chemokines may be a strategic move for treating IBD. Essentially, approved biological molecules for commercial use act as specific inhibitors of inflammatory cytokines related to autoimmune diseases. Among approved TNF blockers, IFX and ADA are the most commonly used biological drugs for the treatment of IBD. Despite the fact that half of the patients treated with these biological molecules have shown clinical remission and its clinical benefits seemed to outweigh the risks involved, there is a growing concern regarding the development of immunogenicity against the biologics, since some patients may develop anti-drug antibodies.

Albeit many studies are still ongoing with the goal of using biological therapy, the effective cost of its production is very elevated in comparison with other drugs, which might make this a hard to implement treatment. Furthermore, treatment with biologicals must be defined carefully, since several drugs need further preclinical and clinical studies prior for their use to be considered as a first option treatment. In this sense, more controlled clinical trials are currently being conducted, exploring the safety and efficacy of old and new biologic agents. Regardless, the most recently engineered biological drugs will certainly open a fresh and exciting perspective on the development and improvement of therapies for IBD.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Systemic Autoimmune, Rheumatic Diseases and Coinciding Psoriasis: Data from a Large Single-Centre Registry and Review of the Literature

Anna Bazsó,[1] Péter Szodoray,[2] Ágnes Szappanos,[1] Judit Korda,[1] Patrícia Pálfi,[1] Emese Kiss,[1] and Gyula Poór[1]

[1]*National Institute of Rheumatology and Physiotherapy, Frankel Leó Utca 38-40, Budapest 1023, Hungary*
[2]*Institute of Immunology, Rikshospitalet, Oslo University Hospital, 0027 Oslo, Norway*

Correspondence should be addressed to Anna Bazsó; bazsoanna@yahoo.com

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Psoriasis is a systemic immune-inflammatory disease characterized by chronic or recurrent skin symptoms, psoriatic arthritis, enthesopathy, and uveitis. Psoriasis has recently been published to appear with various autoimmune disorders, but the coexistence has been systematically reviewed by only few studies until now. In the present study, charts and electronic database of 4344 patients with various systemic autoimmune disorders, under regular medical control at our department, were reviewed retrospectively searching for association with psoriasis. Hereby, we demonstrate 25 psoriatic patients coinciding with various systemic autoimmune diseases. The coexistence of psoriasis and autoimmune diseases resulted in the worsening of the clinical outcome of the autoimmune diseases as indicated by higher frequency and dosages of glucocorticoid use, need for biologicals, and other comorbidities. These results suggest common environmental and genetic background as well as therapeutic possibilities in the future.

1. Introduction

Autoimmunity is characterized by the breakdown of self-tolerance leading to a state of abnormal humoral and cell-mediated responses against self-components. Psoriasis is an immune-inflammatory skin disease affecting 2-3% of the general population which can be associated with psoriatic arthritis (PsA), enthesopathy, uveitis, and an increased prevalence of cardiovascular morbidity [1]. The association between psoriasis and systemic autoimmune, rheumatic diseases is rare and little is known about its exact incidence. The pathogenesis of both disease entities involves genetic background and environmental triggers. A potential role of molecular mimicry has previously been described in the pathogenesis not only of autoimmune disease but also of psoriasis [2]. Several autoantigens have been implicated in psoriasis, amongst which are keratin 13 (K13), heterogeneous nuclear ribonucleoprotein-A1 (hnRNP-A1), and Rab coupling protein isoform 3 (FLJ00294) (RAB11FIP1), although the epidermal autoantigens have not been conclusively identified [3]. Underlying the importance of genetic associations, previously a clear correlation has been shown between psoriasis and risk of the development of diseases with autoimmune background, such as rheumatoid arthritis (RA), type 1 diabetes, celiac disease, or Crohn's disease, based on the single nucleotide polymorphism (SNP) analysis of the TNFAIP3 gene [4].

In this work, we demonstrate 25 patients with psoriasis and various systemic autoimmune diseases. Among the patients with autoimmune diseases included in our database we selected those who were associated with psoriasis. Our survey aimed to determine the prevalence of coinciding psoriasis in autoimmune conditions and whether psoriasis has an impact on the outcome of associated autoimmune diseases.

2. Materials and Methods

In this retrospective study medical charts and electronic database of patients, regularly followed at the National

Institute of Rheumatology and Physiotherapy, were systematically reviewed searching for psoriasis as comorbidity. As psoriasis associated with the highest frequency to RA and SLE the same number of patients with and without psoriasis was selected and matched according to gender and age at onset, and as such case-control study could be performed. Patients in these subgroups were compared regarding the onset of the autoimmune diseases, clinical symptoms, and disease duration, as well as dose of corticosteroid and response to conventional and biological immunosuppressive therapies. In case of other autoimmune diseases only few patients belonged to subgroups with psoriasis; therefore a case-control study would not have been informative by statistical respect. Patients with psoriatic arthritis fulfilled the diagnostic criteria by laboratory markers, symptoms, and radiographic images and were distinguished from the joint manifestations of the coexisting autoimmune diseases.

2.1. Study Population. Out of the 4344 investigated patients (1450 with RA, 835 with Sjögren's syndrome, 807 with SLE, 486 with Raynaud's syndrome, 113 with undifferentiated connective diseases (UCTD), 313 with primary antiphospholipid syndrome (PAPS), 144 with polymyositis (PM), 127 with primary systemic vasculitis, 85 with systemic sclerosis, and 69 with mixed connective tissue diseases (MCTD)), 25 had coinciding psoriasis. Psoriatic arthritis was present in 14 cases. All patients fulfilled the corresponding classification criteria of the above-mentioned autoimmune diseases [1, 5–16]. Psoriasis coexisted with SLE ($n = 8$), rheumatoid arthritis ($n = 5$), primary Sjögren's syndrome ($n = 5$), primary Raynaud's syndrome ($n = 4$), primary systemic vasculitis ($n = 3$), APS ($n = 2$), systemic sclerosis ($n = 2$), UCTD ($n = 1$), polymyositis ($n = 1$), and MCTD ($n = 1$). Various other comorbidities also associate with different autoimmune diseases, such as hypertension, crystal arthritis, interstitial lung disease, ischemic heart disease, cataract, and glaucoma.

2.2. Data Collection. The clinical and laboratory data were collected from the institute's electronic patient databases from inpatient and outpatient visits. The following diseases were investigated: SLE, primary systemic vasculitis, PAPS, UCTD, primary Raynaud's syndrome, PM, systemic sclerosis, MCTD, primary Sjögren's disease, and RA. Each specific disease was treated as an outcome variable. All diagnoses for these conditions were recorded from September 2007 to November 2013. In our database the following data were detected: age at the onset of the autoimmune diseases, clinical symptoms, immune serology, associated diseases, disease duration, coexistence of psoriatic arthritis, actual clinical state, and average dose of corticosteroid, immune suppressive therapy, and response to the therapy.

2.3. Statistical Analysis. All statistical analyses were performed using IBM SPSS 20 software. Fisher's exact test was utilized to assess the average age of appearance of psoriasis and psoriatic arthritis and Mann-Whitney U test was performed to measure the average of corticosteroid usage.

3. Results

We determined the frequency of psoriasis in various autoimmune diseases and also assessed the rate of the psoriatic arthritis. We also aimed to compare demographic and disease-specific characteristics of RA and SLE with and without associating psoriasis.

There were 25 eligible individuals who fulfilled the criteria for psoriasis in the study population. The frequency of coinciding psoriasis was 0.99% in RA, 0.34% in SLE, 0.59% in Sjögren's syndrome, 0.82% in Raynaud's syndrome, 3.29% in systemic vasculitis, 6.3% in PAPS, 0.69% in PM, 2.35% in systemic sclerosis, 1.17% in UCTD, and 1.44% in MCTD. Out of the psoriatic cases 62.5% ($n = 15$) had psoriatic arthritis. Compared to the estimated vary of the population from 0.3% to 1% [15]. Psoriatic arthritis was diagnosed and distinguished from the musculoskeletal manifestations of the autoimmune diseases by the CASPAR criteria [16]. The median (min-max) age at autoimmune disease onset was 48 (24–68) years. Of those with psoriasis 12% were male and 88% were female. In 18 patients psoriasis developed first. In psoriatic patients who also suffered from different kinds of autoimmune diseases an increased rate of comorbidities was observed.

The second goal was to analyze demographic characteristics and the outcome of clinical symptoms in RA and SLE. In the case-control study the same number of patients with and without psoriasis was selected and matched out of our entire RA and SLE population. Demographic and disease-specific and treatment-associated data were compared in psoriatic and nonpsoriatic SLE and RA groups. The average age of appearance of psoriasis was 48 (24–68) years. The female to male ratio was 3 : 22 (12% and 88%). The appearance of psoriasis before and after the age of 40 years was similar (13/11 or 54% and 46%); however the frequency of psoriatic arthritis was significantly higher after 40 years of age (1/14 or 7% and 93%). Significantly higher doses of glucocorticoid (GC) were required in the SLE with psoriasis group (16.88 (10–30)) compared to SLE without psoriasis (11.4 (7.5–20)) (Tables 3 and 4). On the contrary in RA patients with psoriasis both the number of patients on GC and both the used GC doses were lower as compared to those with RA patients without associating psoriasis (Tables 1 and 2). The fact can be explained by differences in the usage of biological therapies, as all patients from the RA + psoriasis group were on TNF-alpha inhibitors, while in the control group only 1 patient received biological therapy. Both the SLE- and RA-associated psoriasis groups required intensified immune suppressive therapy. The association of psoriasis in both RA and SLE groups was characterized by worse laboratory markers, diseases outcome, and response to therapy. In the RA + psoriasis group 4 patients (80%) had other coinciding diseases, such as hypertension, neurofibromatosis, Sjögren's syndrome, and systemic sclerosis, as compared to the RA group where 2 patients (40%) had hypertension and Sjögren's syndrome. There were no significant differences of the immunological serology markers between the 2 groups. The responses to disease-modifying antirheumatic drugs (DMARDs) therapy were significantly worse in the RA + psoriasis group, since none of the patients responded or had side effects of the

Table 1: Patient characteristics with rheumatoid arthritis associated with psoriasis.

The age of RA onset	Comorbidity	Immunoserology	Actual clinical status	DMARD therapy	Response to DMARD	Corticosteroids (mg PED)	Biological therapy	Response to biology therapy	Psoriatic arthritis
63 yrs	Hypertension	ANA, ACPA	Remission	Sulfasalazine-leukopenia, oral methotrexate-gastrointestinal side effect, cyclosporine-with golimumab	Nonresponder	—	Golimumab	Remission	Axial
46 yrs	—	RF, ACPA, ANA	Remission	Oral methotrexate-ineffective, sulfasalazine-gastrointestinal side effect	Nonresponder	—	Etanercept	Remission	Peripheral
59 yrs	Systemic sclerosis	RF, ACPA, ANA	Remission	Chloroquine-gastrointestinal side effect sulfasalazine	Nonresponder	—	Adalimumab-ineffective, rituximab	Remission	Peripheral
32 yrs	Hypertension	RF, ACPA, ANA	Active polyarthritis	Oral methotrexate-bone marrow toxicity azathioprine-ineffective cyclosporine-ineffective combination of chloroquine, sulfasalazine, oral methotrexate-GI side effect subcutaneous methotrexate	Ineffective	>7.5 continuously	Adalimumab-ineffective etanercept-ineffective golimumab-ineffective	Nonresponder	Peripheral and axial
40 yrs	Sjögren's syndrome, neurofibromatosis	RF, ACPA, ANA, aCL IgM	Severe glandular symptoms, active polyarthritis	Leflunomide-ineffective, oral methotrexate-hepatotoxicity	Nonresponder	—	Etanercept	Just started	Peripheral

RA: rheumatoid arthritis, ANA: anti-nuclear antibody, ACPA: anti-citrullinated peptide antibody, aCL IgM: anti-cardiolipin antibody immunoglobulin M, DMARD: disease-modifying antirheumatic drugs, and PED: prednisolone equivalent dose.

Table 2: Patient characteristics with rheumatoid arthritis only.

At the age of RA onset	Comorbidity	Immunoserology	DMARD therapy	Response to DMARD	Corticosteroids (mg PED)	Biological therapy	Response to biology therapy
63 yrs	Hypertension	RF, ACPA	Sulfasalazine-ineffective, leflunomide-allergic side effect, oral methotrexate	Partial response	<7.5	Tocilizumab	Remission
46 yrs	—	RF, ACPA, ANA	Combination of oral methotrexate and chloroquine	Remission	10	—	—
59 yrs	Sjögren's syndrome	RF, ACPA, ANA	Combination of oral methotrexate and chloroquine	Remission	10	—	—
32 yrs	—	RF, ACPA, ANA	Combination of subcutaneous methotrexate and chloroquine	Remission	—	—	—
40 yrs	—	RF, ACPA	Subcutaneous methotrexate	Remission	—	—	—

RA: rheumatoid arthritis, ANA: anti-nuclear antibody, ACPA: anti-citrullinated peptide antibody, DMARD: disease-modifying antirheumatic drugs, and PED: prednisolone equivalent dose.

DMARDs. All patients of the RA + psoriasis group were treated with biologicals and 2 patients (40%) needed to switch to another biological therapy, while in the RA group only 1 patient (20%) received biological therapy and all (100%) patients were in remission (defined as Das28 <2.1) (Tables 1 and 2). In the SLE + psoriasis group 7 patients had comorbidities (87.5%) as compared to the 6 patients (75%) in the SLE-only group. Similar to the RA groups there were no significant differences in immunological markers between the 2 groups. Otherwise, in the SLE + psoriasis group 5 patients (62.5%) had relapse, 2 patients (25%) had worsening outcome, and only 1 patient (12.5%) was in remission when compared to the SLE group, where all patients were in remission. In the SLE + psoriasis group all patients received more than 1 immunosuppressive agent and the systemic lupus international collaborating clinics (SLICC) index was elevated in 7 patients (87.5%) compared to the SLE group (37.5%). The average dose of corticosteroids (PED) was 17.5 mg in the SLE + psoriasis group and 11.4 mg in the SLE-only group (Tables 3 and 4).

4. Discussion

The overlap between psoriasis and autoimmune diseases is unusual. Differential diagnostic problems may also occur. The prevalence of psoriasis in African Americans is 1.3% compared to the 2.5% in Caucasians [17]. In our database we found 25 patients (0.545%) with psoriasis from the 4344 autoimmune disorders. In the general population the prevalence of skin psoriasis is around 2-3%. The lower prevalence, found in our data base, can probably be attributed to concurrent existence of national tertiary dermatology centre taking care of psoriatic patients. Therefore, our centre followed those patients who suffer from psoriatic arthritis or other autoimmune disorders. We have confirmed that the overlapping psoriasis and autoimmune diseases result in the worsening of the autoimmune diseases, reflected by the increased corticosteroid usage, worse response to the therapy, and the appearance of other comorbidities. We have not observed a significantly higher tendency to develop more autoimmune diseases in patients with psoriatic arthritis. Particularly, in case of patients with RA and psoriasis, physicians should be aware of the possible development of psoriatic arthritis. Despite the unusual association there are several similarities in the innate and adaptive pathways and genetic and environmental—including the infections—background between psoriasis and autoimmune diseases.

Th1, Th17, and IL-22-producing CD4 T cells in psoriasis and autoimmunity have recently been supposed. The concept of molecular mimicry is based on a structural similarity between a pathogen and a metabolite and self-structures. Epidemiological findings show a strong correlation between EBV infection and the risk of developing sclerosis multiplex, SLE, RA, and primer SS [18]. There are some gene variants evaluated to be involved and are common in the pathogenesis of the four diseases of cytokine pathways (e.g., IRF5, STAT4, and TNFSF4) leading to the development of autoimmunity [19]. Regarding the genetic association between psoriasis and autoimmunity, TNF-alpha-induced protein 3 (TNFAIP3) has been shown to be a major candidate. Multiple variants of TNFAIP3 could modulate development of autoimmunity in different diseases. The TNFAIP3 gene region has been implicated in the susceptibility to multiple autoimmune diseases in Europe. SNPs ~ 185 kb upstream from the TNFAIP3 gene have a strong association with risk of RA, type 1 diabetes (T1D), celiac disease (CeD), and Crohn's disease [20]. Two independent studies regard the association between TNFAIP3 and SLE in a European cohort [21, 22]. Wu et al. have confirmed in a well-powered, genetic case-control study

Table 3: Patient characteristics with SLE associated with psoriasis.

The age of SLE onset	Comorbidity	Immunoserology	Actual clinical status	Therapy	Arthritis psoriatica	Corticosteroids (mg PED)	SLICC
27 yrs	Lupus nephritis, autoimmune thyroiditis	ANA, aSSA, aSSB, aTPO	Relapsing, remission by MMF	Cyclophosphamide, mycophenolate mofetil, methylprednisolone	Peripheral	25	1
54 yrs	Hypertension, gout, ischaemic heart disease, COPD, cataract	ANA, a-dsDNA	Relapsing	Chloroquine, azathioprine, methylprednisolone, etanercept	Axial	12.5	4
45 yrs	—	ANA, a-SM, a-SSA	Remission	Oral methotrexate, leflunomide, chloroquine	—	—	0
24 yrs	Raynaud's syndrome, cutaneous vasculitis	ANA, a-dsDNA, a-SSA, aSSB, β2GPI IgG, hypocomplementaemia	Relapsing	Methylprednisolone, azathioprine, intravenous cyclophosphamide, IVIG, plasmapheresis,	—	30	1
33 yrs	Autoimmune thyroiditis, nephrotic syndrome	ANA, a-TPO, lupus anticoagulant, aCL IgM	Relapsing	Methylprednisolone, chloroquine, oral methotrexate,	—	10	1
57 yrs	Sjögren's syndrome	ANA, a-SSA, a-SSB, a-dsDNA, hypocomplementaemia	Relapsing	Oral methotrexate, methylprednisolone, sulfasalazine, intravenous cyclophosphamide	—	20	1
58 yrs	Sjögren's syndrome, urticaria vasculitis, ILD	ANA, a-dsDNA, a-chromatin, hypocomplementaemia	Worsening of ILD, psoriasis, immunoserology	Oral methotrexate, sulfasalazine, intravenous cyclophosphamide	Axial	30	1
29 yrs	Sjögren's syndrome	ANA, a-dsDNA, a-SSA, a-SSB, anti-RNP, a-U1RNP, a-TG	Worsening of psychosis, thrombocytopenia	Methylprednisolone	—	12.5	1

SLE: systemic lupus erythematosus, COPD: chronic obstructive pulmonary disease, ILD: interstitial lung disease, ANA: anti-nuclear antibody, ACPA: anti-citrullinated peptide antibody, a-SSA: anti-Sjögren's syndrome A antibody, a-SSB: anti-Sjögren's syndrome B antibody, anti-RNP: anti-ribonucleoprotein antibody, a-dsDNA: anti-double stranded deoxyribonucleic acid antibody, β2GPI IgG: beta 2 glycoprotein I IgG, aCL IgM: anti-cardiolipin antibody immunoglobulin M, aU1RNP: anti-U1 ribonucleoprotein antibody, a-TG: anti-thyroglobulin antibody, a-TPO: anti-thyreoid peroxidase antibody, MMF: mycophenolate mofetil, PED: prednisolone equivalent dose, and SLICC: systemic lupus international collaborating clinics.

that both psoriasis and autoimmune diseases have complex genetic basis; multiple genes contribute to disease risk [23]. Overlapping of some gene locations of different autoimmune diseases has been known and supports common pathogenic gene variants (PTPN 22, Csk, PAG, PSTPIP1, PDCD1, SLC9A3R1, CARD15, and SUMO4) transcript within these diseases [24]. TNF-alpha polymorphism or TNF-alpha can increase the development of psoriasis or psoriatic arthritis. TNF inhibitors are effective in the treatment of psoriasis as well as in RA; however they can induce antinuclear antibodies and even lupus [25].

Interleukin-17 is a Th17 cytokine associated with inflammation, autoimmunity, and defence against some bacteria; it has been implicated in many chronic autoimmune diseases including psoriasis, Crohn's disease, autoimmune uveitis, SLE, ankylosing spondylitis, asthma, multiple sclerosis, and systemic sclerosis [26]. The pathophysiologic relevance of the IL-23-IL-17 axis in autoinflammatory diseases is highlighted by the clinical efficacy of antibodies targeting IL-23/IL-12 p40 and IL-17 in treating psoriasis, as well as the other systemic autoimmune diseases [27]. The level of IL-17 was significantly higher in serum of SLE patients than in normal controls, indicating that IL-17 may trigger the inflammatory process, although no correlation was found between serum IL-17 levels and disease manifestation or SLEDAI [28]. In general, the common IL-23-IL-17 axis may also initiate and maintain the coexistence of psoriasis and other systemic autoimmune diseases [27, 28].

The other prominent cytokine highlighting the common pathway is interferon- (IFN-) alpha, mainly produced by

Table 4: Patient characteristics with SLE only.

The age of SLE onset	Comorbidity	Immunoserology	Actual clinical status	Therapy	Corticosteroids (mg PED)	SLICC
27 yrs	—	ANA, a-SSA, a-SSB, a-U1RNP, hypocomplementaemia	Remission	Azathioprine, low-dose corticosteroid	13.75	0
54 yrs	Gout, diabetes mellitus, COPD, ischemic heart disease	ANA, a-dsDNA, a-chromatin	Remission	Low-dose corticosteroid	12.5	1
45 yrs	—	ANA, a -Sm, a-SSA, a-U1RNP, aCL IgM	Remission	—	20	2
24 yrs	Raynaud's syndrome, Sjögren's syndrome, Hashimoto-thyroiditis, seronegative rheumatoid arthritis (erosive polyarthritis), idiopathic thrombocytopenic purpura	ANA, a-ENA, a-SSA, a-dsDNA, a-Sm, a-TPO	Remission	—	10	0
33 yrs	Sjögren's syndrome	ANA, a-dsDNA, a-SSA, a-SSB, aCL IgM	Remission	—	8.7	0
58 yrs	Sjögren's syndrome, type 2 diabetes mellitus	ANA, a-dsDNA, a-SSA, a-SSB,	Remission	—	8.75	0
57 yrs	Rheumatoid arthritis, sacroiliitis	ANA, a-ACPA, a-dsDNA, a-Sm, a-U1RNP	Remission	—	7.5	0
29 yrs	Rheumatoid arthritis, asthma bronchial, aseptic femur-head necrosis, cataract, glaucoma	ANA, a-ACPA, a-dsDNA	Remission	—	10	2

SLE: systemic lupus erythematosus, COPD: chronic obstructive pulmonary disease, ILD: interstitial lung disease, ANA: anti-nuclear antibody, a-SSA: anti-Sjögren's syndrome A antibody, a-SSB: anti-Sjögren's syndrome B antibody, a-chromatin: anti-chromatin antibody, anti-RNP: anti-ribonucleoprotein antibody, a-dsDNA: anti-double stranded deoxyribonucleic acid antibody, anti-Sm: anti-Smith antibody, aCL IgM: anti-cardiolipin antibody immunoglobulin M, a-U1RNP: anti-U1 ribonucleoprotein antibody, a-TPO: anti-thyreoid peroxidase antibody, MMF: mycophenolate mofetil, PED: prednisolone equivalent dose, and SLICC: systemic lupus international collaborating clinics.

plasmacytoid dendritic cells (PDCs) [29]. IFN-alpha plays a pivotal role in the development of SLE, insulin-dependent diabetes mellitus (IDDM), or RA. In psoriatic lesions plasmacytoid dendritic cell infiltrations have been shown, indicating that IFN-alpha may contribute to the pathogenesis of these diseases [29–32].

The humoral and cellular immunity have been shown to act against endothelial antigens and moreover it has a greater risk of atherosclerosis in both diseases as RA and SLE. So, these processes highlighted the significance of autoimmunity in atherosclerotic processes. Both angiogenic and oxidative pathways have a common role in the pathophysiology of psoriasis and atherosclerosis. Psoriasis and autoimmune diseases have a strong relationship with lipid metabolism and oxidative stress. Heat shock protein (Hsp) and human Hsp are known as possible pathogenic links between infection and atherosclerosis, as well as infection and autoimmunity [33, 34]. However, the immune-mediated inflammatory disease (IMID) is a group of diseases without exact etiology, but involving common inflammatory pathways resulting in many diseases as psoriasis, psoriatic arthritis and atherosclerosis, also. Psoriasis and autoimmune diseases also associate with an increased risk of atherosclerosis. Activated inflammatory cells and proinflammatory cytokines contribute to the psoriatic lesions and the rupture of atherosclerotic plaque. Macrophages also interact with T cells and other cells via activation of the CD40-CD40 ligand pathway, which contributes to the atheromatous plaque rupture [34]. Anti-CD40 therapy has been shown to be efficacious in some autoimmune diseases, such as SLE, vasculitis, and pSS [35]. Several studies have shown the endothelial cell dysfunction, the deficiency of nitric oxide (NO)2, elevated endothelin 1 (ET-1), angiotensin II (Ang II), plasminogen activator inhibitor 1 (PAI-1), and cellular adhesion molecules. Furthermore, other common pathognomonic factors such as the Toll-like receptors (TLR2 and TLR4) play key roles in atherosclerosis. TLR2 and TLR4 bind to components of gram positive and gram negative bacteria which could be a pathognomonic factor in autoimmunity, as mentioned above [34].

Serum leptin, resistin, and lipocalin are increased in psoriasis patients and have a potential important role for developing insulin resistance and cardiovascular disease in psoriasis. An adipose tissue secreted cytokine, called adiponectin, is able to improve insulin resistance. Its serum level is decreased in psoriatic patients. The decreased level of different adipokines and Th17 cytokine has also associated in patients with psoriasis and autoimmune diseases, as well. However, leptin, adiponectin, resistin, and visfatin play a significant role in physiopathology of several inflammatory diseases. Moreover, all are involved strongly in other relevant inflammatory conditions and autoimmune disorders [36].

Nutritional compounds and drugs also trigger autoimmune disease. There are some data about the association of the early exposure to dietary cow's milk proteins and a decreased risk of T1D; tienilic acid, dihydralazine, and halothane have been reported to induce autoimmune hepatitis. Stress and smoking are also associated susceptibilities to many autoimmune diseases [37].

Moreover, there is also strong association between autoimmunity and psoriasis when analyzing new bone formations and bone erosion as cellular biomarkers such as osteoclast precursors; osteoprotegerin (OPG), matrix metalloproteinase 3, serum IL-6, and IL-2R alpha were found elevated. OPG is produced not only in bone but also in several other tissues, including the cardiovascular system, lungs, kidneys, immune tissues, and blood vessels. In vascular system, increased OPG levels may be related to endothelial lesion, intimal hyperplasia, smooth cell hypertrophy, or advanced plaque calcification [38].

5. Summary

We believe that further prospective, cohort studies are required to determine real frequency of psoriasis in various autoimmune diseases as well as the incidence of autoimmune diseases within psoriatic patients. Positive and negative prognostic factors are still to be identified. The characteristics and outcome of autoimmune diseases and psoriasis also have to be followed.

Our present findings suggest that psoriasis exists as a negative predictive factor for the clinical outcome of autoimmune diseases. Despite there being several similarities between the pathogenesis of psoriasis and autoimmune diseases it was surprising to find the low frequencies of coexistence.

Patients with RA and psoriasis are more likely to receive biological therapy, while patients with SLE and psoriasis need significant higher doses of glucocorticoids. TNF-α inhibitors are effective in the treatment of psoriasis; however they can induce antinuclear antibodies and even lupus. Therapeutic considerations have to be done in overlapping cases. Biologicals with different ways of action, for example, targeting of IL-17 and IL-23, dendritic cell suppression, might reduce activity of both diseases. Röhn et al. and de Carvalho et al. explored the fact that neutralization of IL-17 by passive and active vaccination may be a novel therapeutic approach for the treatment of SLE and atherosclerosis [39, 40]. Targeting IFN-alpha, RANK-RANKL, and CD40-CD40L system could also be beneficial for the prevention and early therapeutic intervention in psoriasis and other related autoimmune diseases [30, 34, 35, 38].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Role of Poly(ADP-ribose) Polymerase-1 in Rheumatoid Arthritis

Samuel García[1] and Carmen Conde[2]

[1]*Laboratory of Translational Immunology and Department of Rheumatology and Clinical Immunology, University Medical Center, Lundlaan 6, 3508 GA Utrecht, Netherlands*
[2]*Laboratorio de Investigación 8, Instituto de Investigación Sanitaria (IDIS), Hospital Clínico Universitario de Santiago de Compostela (CHUS), SERGAS, Travesia da Choupana s/n, 15706 Santiago de Compostela, Spain*

Correspondence should be addressed to Carmen Conde; carmen.conde.muro@sergas.es

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Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear enzyme with a crucial role in the maintenance of genomic stability. In addition to the role of PARP-1 in DNA repair, multiple studies have also demonstrated its involvement in several inflammatory diseases, such as septic shock, asthma, atherosclerosis, and stroke, as well as in cancer. In these diseases, the pharmacological inhibition of PARP-1 has shown a beneficial effect, suggesting that PARP-1 regulates their inflammatory processes. In recent years, we have studied the role of PARP-1 in rheumatoid arthritis, as have other researchers, and the results have shown that PARP-1 has an important function in the development of this disease. This review summarizes current knowledge on the effects of PARP-1 in rheumatoid arthritis.

1. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an autoimmune disease that is characterized by chronic inflammation that affects the peripheral joints and leads to the progressive destruction of the cartilage and bone. RA has a prevalence of 0.5–1% in the population worldwide, and genetic and environmental factors have been implicated in its aetiology. The age of onset is between 35 and 50 years and it is more common in women than in men (a 3 : 1 ratio), suggesting that hormonal factors are related to the development of the disease. Approximately 30% of RA patients have extra-articular manifestations, which contribute to the morbidity and mortality of the disease. Furthermore, this disease leads to a reduction in life expectancy between 3 and 10 years [1–4].

In RA, the initiation of an immune response against unknown antigens leads to the infiltration of the immune cells, primarily the monocytes/macrophages and B and T cells in the affected joints, and also to the activation and proliferation of the stromal cells of the joints, the fibroblast-like synoviocytes (FLS). The activated immune cells and FLS release inflammatory mediators, such as cytokines, chemokines, growth factors, and prostanoids, that perpetuate the inflammatory process and promote the hyperplasia of the synovial membrane; they also release matrix metalloproteinases (MMPs) and aggrecanases that digest the extracellular matrix and articular structures. These mediators also contribute to the formation of new blood vessels from the existing vasculature (angiogenesis), which provide nutrients to the inflamed joint and allow the infiltration of the immune cells into the synovium, thereby perpetuating the inflammatory process [4–7].

The final consequences of these processes are the destruction of the cartilage and the erosion of bone, leading to joint deformity and disability.

Among the plethora of inflammatory mediators playing a role in RA, interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α) have been shown to be the most relevant cytokines in the pathology. These cytokines are related to different processes in RA, such as the induction of inflammatory mediators and MMP expression by the FLS, monocytes/macrophages, and T cells; the activation, proliferation, and differentiation of the T cells; the induction of the B cells' proliferation and antibody production and

the osteoclast activation. Blocking these cytokines has shown therapeutic efficacy, and three of the current biological therapies used for the treatment of RA target these cytokines [8, 9].

1.1. Molecular Pathways in Rheumatoid Arthritis. Although there are multiple signal transduction pathways involved in RA, the mitogen activated protein kinase (MAPK) pathways play a primary role in the inflammatory processes of the disease [10, 11]. ERK, JNK, and p38 are expressed and activated in the synovial tissue of RA patients, and IL-1β and TNF-α induce ERK, p38, and JNK activation in RA FLS [12]. The key role of the MAPKs in RA pathogenesis has been demonstrated in different studies, in which the absence or inhibition of MAPKs has been shown to reduce the severity of several models of arthritis [13–16].

Numerous reports have also shown the activation of different transcription factors in the synovium of RA patients, such as NF-κB, AP-1, IRFs, and FoxO, as well as STAT family members [17].

NF-κB is activated in the synovial tissue from RA patients; both subunits p50 and p65 are located in the nuclei of the synovial macrophages, and the RA FLS and TNF-α and IL-1β induce a rapid NF-κB translocation in the RA FLS. Moreover, pharmacological NF-κB inhibition and genetic NF-κB deletion or modification decrease the severity and bone erosion in different arthritis models in animals [18, 19].

AP-1 is another transcription factor that is involved primarily in the process of joint destruction by inducing the expression of MMPs, but it also elicits other roles in RA, such as the production of inflammatory mediators and the induction of T cell differentiation and osteoclast formation. The key role of AP-1 in RA has been shown in animal models, in which the deletion or pharmacological inhibition of AP-1 reduces both the severity of arthritis and the production of inflammatory cytokines and MMPs [17, 20].

1.2. Experimental Arthritis Models. Several experimental models have been used to characterize the mechanisms involved in the pathogenesis of arthritis and to test new therapeutic strategies. Such models include collagen-induced arthritis (CIA), collagen-antibody-induced arthritis (CAIA), adjuvant-induced arthritis (AIA), and spontaneous arthritis models such as TNF-transgenic mice and the K/BxN mice.

The CIA model shares many clinical and histological features with human RA [21]. Similar to RA, mice develop symmetric peripheral joint inflammation, synovitis, and cartilage and bone damage. Mice also develop hyper γ-globulinemia, antibodies to type-II collagen, and rheumatoid factor. In this model, DBA/1 mice with the MHC class-II I-A^q haplotype develop arthritis following the injection of type-II collagen in complete Freund's adjuvant (CFA). The initial injection is usually followed by an intraperitoneal collagen booster 21 days later. Following immunisation, mice develop polyarthritis that reaches its severity peak at about day 35 [21–23]. One of the advantages of this model is that it allows the study of two phases in the development of arthritis: the initial autoimmune response, in which collagen-specific T and B cells are produced, and the effector phase, consisting of joint inflammation, cartilage damage, and bone erosion. On the other side, a notable disadvantage of this model is the variable incidence and progression of diseases between different laboratories. The CIA model has been a valuable tool to identify the involvement of inflammatory cytokines, autoantibody responses, and T cells in arthritis and for the preclinical testing of new treatments for RA [23].

CAIA is a variant of CIA, in which arthritis is induced by intravenous injection of an arthritogenic cocktail of monoclonal anti-type-II collagen antibodies [24]. This model reflects only the effector phase of arthritis, which develops within 48 hours of antibody injection. Clinical and histopathological features of CAIA are similar to CIA, although the infiltrate is composed mainly of macrophages and neutrophils. In addition, the incidence of arthritis reaches 100% and is independent of the MHC class-II haplotype. These two characteristics make this model particularly useful for studying arthritis in genetically modified mice [24, 25].

The adjuvant-induced arthritis (AIA) model is elicited by intradermal injection of CFA at the base of the tail of rats [26]. AIA is characterized by the rapid onset and progression of joint inflammation with marked cartilage and bone resorption. It shares features of human RA including joint inflammation, cartilage damage, and bone erosion. However, AIA also affects the spine, the skin, the eyes, and the gastrointestinal and genitourinary tracts, which are not involved in human RA [22]. The main advantage of this model is its reproducibility, as 100% of the animals develop arthritis by 14 days after inoculation of CFA, and its main disadvantages are the absence of humoral component and the dissimilarities with human RA regarding tissue damage.

On the side of the spontaneous arthritis models, the first developed was the TNF transgenic mouse, which was reported by Keffer et al. in 1991 [27]. The mice overexpress human TNF (Tg197) and spontaneously develop an erosive chronic polyarthritis that closely mimics human RA, with synovial hyperplasia, pannus formation, cartilage destruction, and bone erosion. This model has established that TNF plays a fundamental role in the pathogenesis of RA and it has been very useful in the assessment of the anti-TNF treatment. The main limitation of this model is the absence of the early autoimmune phase of arthritis.

Another widely used spontaneous model is the K/BxN arthritis model, described by Kouskoff et al. [28]. In this model, a T cell receptor (TCR) transgene recognizes endogenous glucose-6-phosphate isomerase presented on the MHC class-II molecule I-A^{g7}. This autoimmune reaction induces an early and rapidly progressive arthritis that is T and B cell dependent and similar to human RA. It is a very reproducible model in which robust arthritis is developed in 100% of the transgenic mice. This model allows also transferring the disease to a large variety of strains using serum from K/BxN mice, which contains anti-glucose-6-phosphate isomerase pathogenic antibodies. The advantages of this model are its reproducibility and the ability to separate the immunization and effector phases of arthritis [29]. Its main limitation is that the pathogenic autoantibodies of this

model are absent in human RA. The K/BxN transfer model has led to reappreciating the role of the humoral response in RA pathogenesis.

2. Poly(ADP-ribose) Polymerase-1

Several studies using experimental arthritis models [30–37] or pharmacological and genetic inhibition of PARP-1 in FLS from RA patients [38] have revealed the involvement of this protein in the pathogenesis of arthritis and the potential therapeutic effects of PARP-1 inhibition.

PARP-1 (EC2.4.2.30) is a nuclear enzyme with a key role in the maintenance of genomic integrity. PARP-1 is a highly conserved protein of 113 KDa with a ubiquitous expression encoded by the *PARP-1* gene, located in the human 1q41-42 chromosome. PARP-1 has three primary domains: an amino- (N-) terminal DNA binding domain (DBD), an automodification domain, and a carboxy- (C-) terminal catalytic domain. PARP-1 is the foundation and the most abundant member of the PARP family, which includes 18 members. All PARP members have a characteristic conserved catalytic domain located in the C-terminal region. According to their functional domains and functions, the members of the PARP family can be divided into five groups: DNA-dependent PARPs, tankyrases, CCCH-type zinc-finger PARPs, macroPARPs, and other PARPs [39–41].

PARP-1 is the most important member, exhibiting poly (ADP-rybosil)ation activity; in fact, 80–85% of this activity is mediated by PARP-1. The remaining poly(ADP-rybosil)ation activity is mediated by other members of the family, such as PARP-2, PARP-3, PARP-4, and tankyrases 1 and 2. Poly(ADP-rybosil)ation is a protein postransductional modification essential to cellular processes, such as the regulation of DNA reparation, the maintenance of chromatin function and genomic stability, the regulation of transcription, cell cycle progression, and cell death [39, 41].

In the poly(ADP-rybosil)ation process, PARP cleaves the NAD^+ in the nicotinamide and ADP-ribose to form long and branched (ADP-ribose) polymers (PAR). The PAR binds to the acceptor proteins (including PARP-1 itself) through ester bonds to the residues of carboxyl-γ of glutamic acid and regulates their enzymatic activity or macromolecular interactions with other proteins or DNA or RNA molecules [40, 42].

PARP-1 has a key role in the maintenance of genomic stability, and the absence or deficiency of PARP-1 leads to defects in the repair of DNA breaks, an increase in homologous recombination, sister chromatic exchange, and micronuclei formation [43]. PARP-1 is also involved in the regulation of diverse DNA reparation pathways, such as the BER (Base Excision Repair), SSBR (Single Strand Break Repair), and DSBR (Double Strand Break Repair) pathways [41, 44, 45]. Moreover, PARP-1 has been related to DNA damage-induced cell death and apoptosis, both caspase dependent and caspase independent, and the results suggest that the role of PARP-1 in cell death depends on the cell type and the type of stimulus [45–50].

2.1. PARP-1 in Inflammation. In recent years, new roles of PARP-1 have been discovered, and one of the most important is the role of PARP-1 in inflammatory processes. Different works have reported that both the pharmacological inhibition of PARP and a deficiency of PARP-1 have a protective role in inflammatory diseases, such as LPS-induced septic shock, uveitis, colitis, streptozotocin-induced diabetes, asthma-related lung inflammation, and atherosclerosis [33, 51–55]. In these models, PARP inhibition showed an anti-inflammatory effect, due primarily to a reduction in the expression of inflammatory mediators, the impaired recruitment of inflammatory cells, the inflammation sites, and the reduction of necrotic cells in the inflamed areas.

There are two possible molecular mechanisms that have been proposed to explain this resistance. The first mechanism is related to the overactivation of the PARP-1 and the depletion of NAD^+ and ATP. In the inflammatory processes, the oxygen and nitrogen free radicals that are released result in DNA damage that leads to the constant activation of PARP-1 and consequently to the depletion of the cellular levels of NAD^+ and ATP. The depletion of the cellular NAD^+ and ATP pools produces an irreversible cellular energetic failure and cell death by necrosis. The necrotic cells release their cellular content into the extracellular space, perpetuating the inflammatory processes [40, 45, 56].

The second mechanism is related to the role of PARP-1 as a transcriptional regulator and to the ability of PARP-1 to modulate the expression of proinflammatory genes, primarily the cytokines and chemokines. PARP-1 regulates genomic transcription through 2 independent processes: the regulation of the chromatin structure and the regulation of the activity of the transcription factors. In the first mechanism, PARP-1 mediates the poly(ADP-rybosil-)ation of the chromatin-associated protein-like histones, leading to the dissociation of the nucleosomes dissociation and the relaxation of the chromatin structure. The relaxation of the chromatin allows the access of proteins implicated in the transcription into the DNA and the subsequent genomic expression. PARP-1 also regulates, through PARP-1 enzymatic activity, the activation of different transcription factors, such as NF-κB, AP-1, AP-2, YY-1, Oct-1, Stat-1, B-MYB, HIF-α, and SP-1. Moreover, PARP-1 also modulates the activation of other transcription factors, including Oct-1, YY-1, B-MYB, and AP-2, through direct protein-protein interactions [40, 45, 56].

Of special interest is the role of PARP-1 in the transcriptional activation of NF-κB and AP-1, as both transcription factors are related to different pathologies. PARP-1 regulates the activation of both transcription factors, and PARP inhibition or PARP-1 deficiency reduces the transcriptional activity of NF-κB and/or AP-1 in models of septic shock, inflammation, and ischemic-reperfusion, as well as in cancer and RA [51, 52, 57–59].

Beyond the modulation of the transcription factors' activity, PARP-1 has also been related to the regulation of the MAPK pathways, and it has been shown that PARP inhibition reduces the activation of ERK, JNK, and p38 in inflammatory as well as in ischemic and oxidative stress processes [60–63].

Therefore, the protective effect of PARP-1 inhibition in inflammatory models is due primarily to the reduction of

Table 1: Effect of PARP inhibition/deletion in experimental arthritis.

Treatment/genetic approach	Arthritis model	Major findings	Reference
Nicotinamide	Potassium peroxochromate-induced arthritis	Reduced arthritis severity. Reduction of phagocytic generation of reactive oxygen species.	[30]
Nicotinic acid amide	Collagen-induced arthritis	Reduced arthritis severity. Synergistic effect with thalidomide.	[31]
INH_2BP	Collagen-induced arthritis	Reduced arthritis severity and incidence. Reduced neutrophil infiltration. Reduced O_2 and N_2 derived free radical production.	[33]
PJ34	Collagen-induced arthritis	Reduced arthritis severity and incidence. Reduced neutrophil infiltration. Reduced O_2 and N_2 derived free radical production.	[34]
AIQ	Collagen-induced arthritis	Reduced arthritis severity and incidence. Reduced inflammatory response. Reduced Th1-driven autoimmune response.	[35]
PARP-1 deficient mice	Arthritis induced by anti-collagen antibodies	Reduced arthritis severity. Reduced synovial inflammation and cartilage damage. Reduced IL-1β and MCP-1 expression.	[36]
GPI6150	Adjuvant-induced arthritis	Reduced paw edema. Reduced neutrophil infiltration.	[37]

the transcription factors and the activation of the signalling pathways, leading to the downregulation of the expression of the proinflammatory mediators.

3. PARP-1 in Rheumatoid Arthritis

The studies of the role of PARP-1 in experimental arthritis models (Table 1) started in the 1990s, using unspecific inhibitors such as nicotinamide (NA) and nicotinic acid amide (NAA). These studies showed that PARP inhibition, alone or in combination with the TNF-α inhibitor thalidomide, reduced the severity of two different models of arthritis, potassium peroxochromate-induced arthritis and the collagen-induced arthritis (CIA) [30, 31]. However, due to the high unspecificity of the inhibitors used, the protective role of PARP inhibition in arthritis could not be concluded in these studies.

In the late 1990s, the second generation of PARP inhibitors was developed (Table 1). These inhibitors, such as 6-iodo-5-amino-1, 2-benzopyrone (INH_2BP), N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-(N,N-dimethylamino)acetamide hydrochloride (PJ34), 3,4-dihydro-5-[4-1-(1-piperidinyl) butoxy]-1-(2H)-isoquinolinone (DPQ), and 4-amino-1, 8-naphthalimida (ANI), are based on the pharmacophore benzamide (the analogue of NA), and they are very specific and strong PARP inhibitors [32, 45].

Independent studies have shown that the PARP inhibitors INH_2BP and PJ34 reduce the incidence and severity of the CIA model. Interestingly, the therapeutic effects of both PARP inhibitors were associated with a reduction in the oxygen- and nitrogen-derived free radicals and a decrease in the neutrophil infiltration into the joints [33, 34]. Gonzalez-Rey et al. [35] also investigated the impact of the PARP-1 inhibitor AIQ in experimental arthritis. AIQ reduced the incidence and severity of established collagen-induced arthritis, completely abrogating the joint swelling and destruction of the cartilage and bone. The therapeutic effect of AIQ was due to the reduction of the inflammatory response and to the reduced Th1-driven autoimmune response, demonstrating the role of PARP-1 in the two crucial processes of RA, the initiation of the immune response and the initiation and perpetuation of inflammation [35]. Interestingly, the PARP-1 inhibitors PJ34 and AIQ also showed a prophylactic effect, as a protective effect was observed when the inhibitors were administered after the onset of arthritis.

Our group investigated the impact of the selective suppression of PARP-1, using PARP-1 deficient mice, in a model of arthritis induced by anti-collagen antibodies (CAIA). We observed that the absence of PARP-1 reduced the severity of the disease, although the incidence was not affected. This reduction was due to the reduced joint expression of the cytokine IL-1β and the chemokine MCP-1 (Table 1). Indeed, we showed that it is likely that PARP-1 is the member of the PARP family that is involved in arthritic inflammation, as the reduction in the severity of arthritis was similar in the arthritic PARP-1 deficient mice regardless of whether they were treated with the PARP inhibitor DPQ [36].

The role of PARP in arthritis has also been analysed in other animal models, and comparable results have been found. Mazzon et al. [37] showed that PARP inhibition by GPI 6150 treatment significantly reduced paw edema in the acute and delayed phases of inflammation in the rat adjuvant-induced arthritis model.

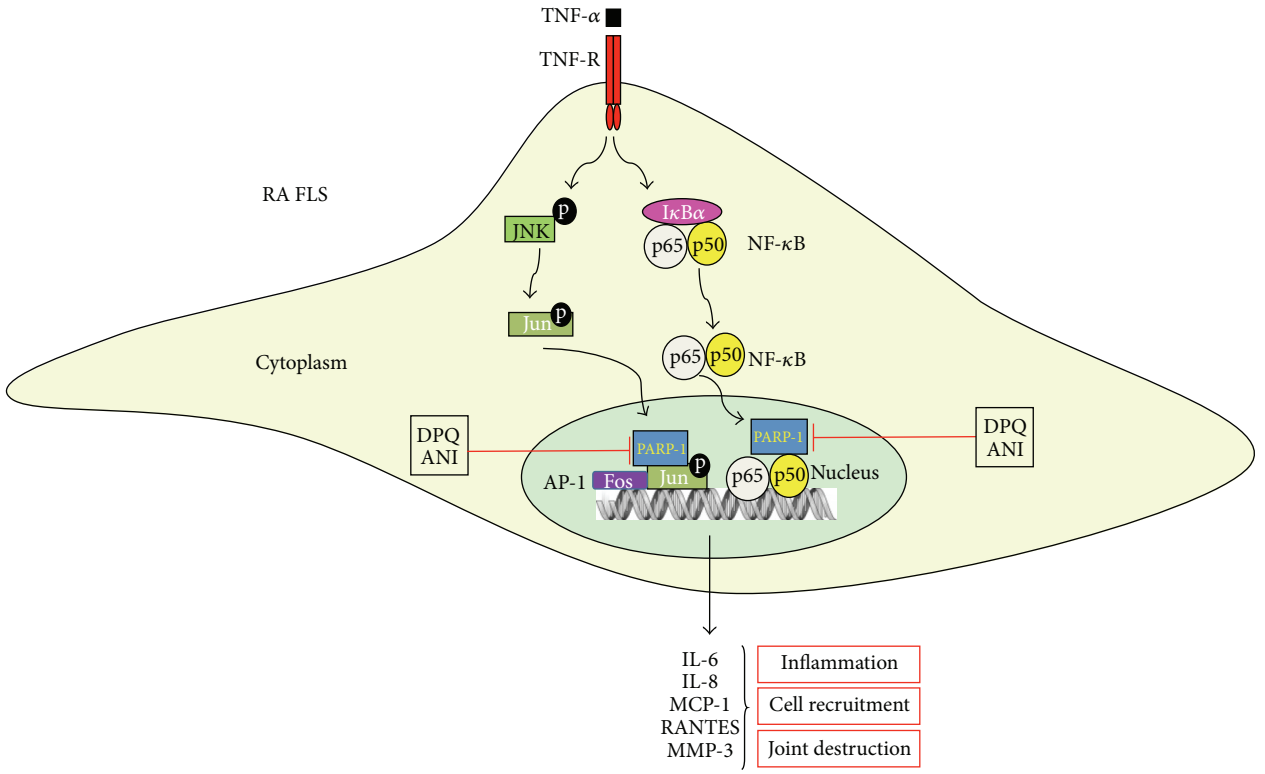

Figure 1: *Effect of PARP-1 on the TNF-α induced production of inflammatory mediators in RA FLS.* TNF-α stimulation induces the activation and the translocation to the nucleus of the transcription factors NF-κB and AP-1. In the nucleus, PARP-1 enzymatic activity and/or direct protein interaction enhances the transcriptional activity of both NF-κB and AP-1, leading to the production of inflammatory mediators.

To extend the knowledge about the role of PARP-1 in rheumatoid arthritis, our group analysed the effect of PARP inhibition on the cell proliferation, production of inflammatory mediators, and activation of molecular pathways in the RA FLS. The results showed that PARP-1 inhibition reduced the FLS proliferation and the TNF-induced IL-6, IL-8, MCP-1, RANTES, and MMP-3 production. PARP-1 suppression by siRNA confirmed the reduction of the production of the TNF-induced inflammatory mediators, suggesting that the reduced inflammatory response we observed was due to the inhibition of poly(ADP-ribosil)ation and not to effects other than the inhibition of the PARP function. Moreover, the results also suggest that PARP-1 is the PARP family member responsible for this reduction. Indeed, in this work we have suggested that the reductions in the production of inflammatory mediators and proliferation are most likely due to the reduced activation of NF-κB and AP-1, as we observed a partially impaired NF-κB and AP-1 binding activity after the treatment with the PARP inhibitors or after the siRNA PARP-1 transfection (Figure 1) [38].

Therefore, this work supports the previous results in animal models showing that PARP inhibition reduced the production of the inflammatory mediators involved in RA.

Taken as a whole, these studies suggest that PARP is involved in the progression of the inflammatory process of RA and that the pharmacological inhibition of PARP has an anti-inflammatory potential in arthritis diseases.

4. PARP-1 Inhibitors in Clinical Trials

Due to the crucial role of PARP-1 in the pathophysiology of different diseases, multiple clinical trials using PARP inhibitors have been performed, primarily for the treatment of cancer. One of the key findings was the discovery of a potent and specific beneficial effect of PARP inhibition in BRCA2-deficient tumours, due to the defect in homologous recombination repair (HRR) in the BRCA2-deficient cells [64, 65]. In fact, most of the clinical trials using PARP inhibitors for the treatment of different tumours that have been performed in the past or are currently being performed are based on the role of PARP-1 in the maintenance of genomic stability and integrity. Interestingly, preclinical and clinical trials have been successful not only in tumours with deficiencies in the double-stranded DNA repair, such as mutations in BRCA1 and BRCA2, but also in tumours that have no defects in the HRR [66–68].

Importantly, a protective effect of PARP inhibition has also been found in breast cancer through the attenuation of the NF-κB-mediated signalling, independently of any defect in the homologous recombination DNA repair. PARP-1 inhibition may have a clear benefit in tumour treatment by limiting the rate of cell proliferation and activation of NF-κB, leading to the suppression of both the inflammation and the expression of genes related to tumour progression [69]. These results suggest that PARP inhibitors might also

be used in other inflammatory diseases in which NF-κB has a critical role. Moreover, there is preclinical evidence showing that PARP-1 inhibitors would be beneficial in acute diseases, such as stroke, traumatic brain injury, circulatory shock, and acute myocardial infarction, as well as in chronic diseases such as chronic heart failure and asthma (reviewed by Curtin) [67].

Two important aspects of the PARP-1 inhibitors for their use as therapeutic target are the specificity and the toxicity and tolerability of the inhibitors. Regarding the specificity, most of the PARP-1 inhibitors that are currently in clinical trials also inhibit PARP-2 activity, as both PARP-1 and PARP-2 share significant sequence homology in their catalytic domains and these PARP inhibitors inhibit the PARP activity [40, 70]. The inhibition of both PARP-1 and PARP-2, rather than being a problem, may be beneficial in tumors associated with defective DNA repair, since PARP-2 is also related to the maintenance of the genomic stability. Regarding the toxicity and tolerability of the PARP-1 inhibitors, all the PARP-1 inhibitors which are or have been tested in clinical trial have not shown unacceptable side effect at the doses used. Alone or in combination with other drugs, PARP-1 inhibitors showed mild effects like nausea, vomiting, diarrhea, and fatigue [70–72].

Therefore, as PARP-1 inhibitors inhibit specifically the PARP enzymatic activity and are well tolerated for the patients, clinical trials are really promising and we can expect the use of PARP inhibitors for the treatment of different pathologies in the near future.

5. Conclusion

Intensive research on the pathology of RA has stimulated the introduction of novel approaches aimed at blocking the inflammatory cytokine pathways in the joint. The best example is the TNF blockade, which allows at least 20% improvement in approximately 70% of the patients. However, as many patients do not respond and remission is rarely achieved, there is a clear need for identifying novel therapeutic strategies. PARP-1, which has been shown to be an important regulator of inflammation in RA, is an interesting therapeutic target. The use of pharmacological PARP inhibitors, PARP-1-deficient mice, and siRNA technology have led to a better understanding of the role of PARP in arthritis and have indicated the potential therapeutic effects of PARP inhibition. Specifically, these studies have shown that PARP-1 is involved in the pathogenesis of RA and that PARP-1 inhibition may be used therapeutically in RA patients.

However, an important aspect of PARP biology that must be considered in the treatment of chronic diseases such as RA is the involvement of PARP in the maintenance of genomic stability. In RA, the long-term inhibition of PARP might increase the DNA mutation rate, thereby increasing the probability of tumour development. However, bearing in mind that the protective effect against arthritis of AIQ in the CIA model was mediated by a reduction of the autoimmune response [35], it is also possible to speculate that, in the future, PARP inhibitors will be developed that induce a strong diminution of the immune response, leading to the remission of RA and avoiding the need for chronic treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Serum IL-6 and IL-23 Levels and Their Correlation with Angiogenic Cytokines and Disease Activity in Ankylosing Spondylitis, Psoriatic Arthritis, and SAPHO Syndrome

Hanna Przepiera-Będzak,[1] Katarzyna Fischer,[2] and Marek Brzosko[1]

[1] *Department of Rheumatology and Internal Diseases, Pomeranian Medical University in Szczecin, Unii Lubelskiej 1, 71-252 Szczecin, Poland*
[2] *Independent Laboratory of Rheumatic Diagnostics, Pomeranian Medical University in Szczecin, Unii Lubelskiej 1, 71-252 Szczecin, Poland*

Correspondence should be addressed to Hanna Przepiera-Będzak; hannapb@wp.pl

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Objectives. To assess serum interleukin-6 (IL-6) and interleukin-23 (IL-23) and their correlation with angiogenic cytokines and disease activity in ankylosing spondylitis (AS), psoriatic arthritis (PsA), and SAPHO syndrome. *Patients and Methods.* We studied 152 spondyloarthritis (SpA) patients: 69 PsA, 61 AS, 22 SAPHO, and 29 controls. We recorded age, sex, disease duration, and treatment. We assessed BASDAI, VAS, and PASI scores. Serum IL-6, IL-23, VEGF, EGF, FGFb, and FGFa levels were determined using ELISA. We estimated ESR and CRP. *Results.* Serum IL-6 and IL-23 levels were higher in SpA than in control ($P < 0.00001$ and $P = 0.0004$, resp.). There was a positive correlation between serum IL-6 and CRP in AS ($P = 0.000001$), PsA ($P = 0.000001$), and SAPHO ($P = 0.0003$) patients. There was a positive correlation between serum IL-6 and ESR in AS ($P = 0.000001$), PsA ($P = 0.002$), and SAPHO ($P = 0.02$) patients. There was no correlation of serum IL-6 and IL-23 with VAS, BASDAI, and angiogenic cytokines in SpA. *Conclusions.* Serum IL-6 but not serum IL-23 correlated with ESR and CRP in SpA. No correlation was found of serum IL-6 and IL-23 with VAS, BASDAI, and angiogenic cytokines.

1. Introduction

Spondyloarthropathies (SpA) are a group of chronic, inflammatory, immune-mediated disorders of the axial and peripheral joints. This group encompasses ankylosing spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis, enteropathic arthritis, and undifferentiated seronegative arthritis. SAPHO (synovitis, acne, pustulosis, hyperostosis, and osteitis) syndrome is always considered as a SpA. The aetiology of SpA is unknown, but some cytokines such as interleukin-6 (IL-6) and interleukin-23 (IL-23) are considered to be associated with the pathogenesis of these disorders [1–7].

IL-6 is a pleiotropic cytokine that plays role in arthritis but its role in the pathogenesis of AS remains controversial [1, 3]. IL-23 is produced by dendritic cells, macrophages, keratinocytes, and other antigen-presenting cells. There are some data showing that IL-23 plays an important role in the pathogenesis of spondyloarthritis [4]. In the available literature, we found that few reports assessed serum levels of IL-23 in small groups of AS and PsA patients [8, 9]. There were no data in the available literature concerning serum levels of IL-6 and IL-23 in SAPHO syndrome.

Angiogenesis also plays an important role in the pathogenesis of SpA. The group of cytokines involved in angiogenesis includes vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and basic and acidic fibroblast growth factors (FGFb and FGFa, resp.) [10–13].

There were no data available in the literature comparing serum levels of IL-6 and IL-23 and angiogenic cytokines in AS, PsA, and SAPHO.

2. Objectives

The aim of this study was to assess serum levels of IL-6 and IL-23 and their association with disease activity in AS, PsA, and SAPHO patients.

3. Materials and Methods

This study was approved by the Local Ethics Committee of Pomeranian Medical University in Szczecin. Informed consent was obtained from all patients.

All patients were Caucasian. We studied 152 patients: 69 had PsA, 61 had AS, and 22 had SAPHO. The controls were 29 healthy volunteers.

The diagnosis of AS was made according to modified New York criteria [14]. The diagnosis of PsA was made according to the Caspar classification criteria [15]. The diagnosis of SAPHO syndrome was made according to the Kahn criteria [16].

The following data were recorded: age, sex, disease duration, presence of peripheral or axial joint involvement, type of skin psoriasis, nail involvement, and treatment. In the PsA group, skin changes were assessed according to the Psoriasis Area and Severity Index (PASI) [17].

We assessed the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI). This index has a possible score of 0–10, with a higher score indicating greater disease activity. We regarded patients as active if the BASDAI score was >4 [18]. The patient's pain due to the disease at the time of examination was assessed by a visual analogue scale (VAS).

Blood was taken for the assessment of ESR and C-reactive protein (CRP) (turbidimetric nephelometry, rate of reaction). Serum was stored at −70°C until analysis for IL-6, IL-23, VEGF, EGF, FGFb, and FGFa using a sensitive sandwich ELISA method using the Human IL-6 Immunoassay Quantikine ELISA kit (the minimum detectable dose less than 0.7 pg/mL), Human IL-23 Immunoassay Quantikine ELISA kit (the minimum detectable dose less than 6.8 pg/mL), Human VEGF Immunoassay Quantikine ELISA kit (the minimum detectable dose less than 5.0 pg/mL), Human EGF Immunoassay Quantikine ELISA kit (the minimum detectable dose less than 0.7 pg/mL), Human FGF Basic Immunoassay Quantikine ELISA kit (the minimum detectable dose less than 3 pg/mL), and Human FGF Acidic Immunoassay Quantikine ELISA kit (the minimum detectable dose less than 5.68 pg/mL). All kits were from R&D System, Minneapolis, USA. The system uses microplates with the walls coated with a monoclonal antibody and an enzyme-linked polyclonal antibody specific for IL-6, IL-23, VEGF, EGF, FGFb, or FGFa. All analyses and calibrations were performed in duplicate and were read using BioTek PowerWave XS, BioTek Instruments, Winooski, USA.

Data distributions were assessed using Shapiro-Wilk test. We used the rank Spearman test to calculate correlations. R values of correlations were determined and corresponding P values < 0.05 were considered significant. The groups were compared using Mann-Whitney U test and Kruskal-Wallis test. To assess parameters associated with serum levels of IL-6 and IL-23 a Pearson chi-squared test (χ^2), logistic regression analysis, and stepwise analysis were performed.

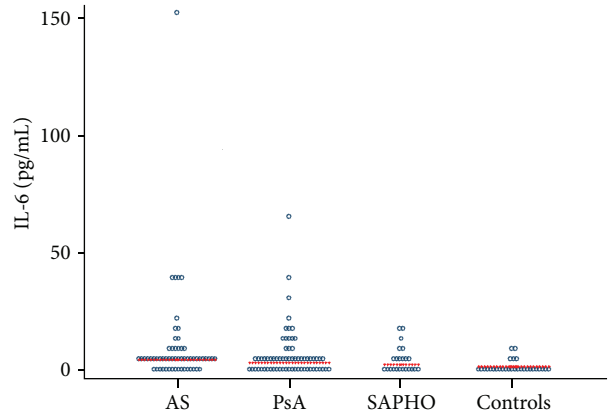

Figure 1: Serum levels of interleukin-6 (IL-6) in patients with psoriatic arthritis (PsA), ankylosing spondylitis (AS), and SAPHO syndrome (SAPHO) and controls.

The level of significance was set at $P < 0.05$. Statistical analysis was performed using STATISTICA version 6.0.

4. Results

The clinical and laboratory characteristics of the patients and healthy controls are presented in Table 1.

In the AS group, 24 patients received nonsteroidal anti-inflammatory drugs (NSAIDs), 26 received sulfasalazine 2 g/day, and 11 received methotrexate 15 mg/week. In the PsA group, 17 patients received methotrexate 15 mg/day, 2 received methotrexate 15 mg/week in combination with cyclosporine A 3 mg/kg, 35 received sulfasalazine 2 g/day, and 15 received NSAIDs. In the SAPHO group, 10 received methotrexate 15 mg/week, 8 received sulfasalazine 1 g/day, and 4 received NSAIDs. No patients received biological therapy.

Serum IL-6 levels were significantly higher in SpA patients than in the control group ($P < 0.00001$). Serum IL-6 levels were significantly higher in AS than in SAPHO patients ($P = 0.04$). No differences were found between AS and PsA patients ($P = 0.21$) or between PsA and SAPHO patients ($P = 0.27$) in terms of IL-6 levels (Figure 1).

Serum IL-23 levels were significantly higher in SpA patients than in the control group ($P = 0.0004$). Serum IL-23 levels were significantly higher in AS than in SAPHO patients ($P = 0.03$). No differences were found between AS and PsA patients ($P = 0.17$) or between PsA and SAPHO patients ($P = 0.21$) in terms of IL-6 levels (Figure 2).

Serum VEGF, EGF, FGFb, and FGFa levels were similar in AS, PsA, and SAPHO patients and controls (Kruskal-Wallis test: $P > 0.05$).

All PsA patients had plaque-type psoriasis. IL-23 and IL-6 levels were not correlated with PASI score (Tables 2 and 3). Among PsA patients, 57 (82.6%) had peripheral arthritis (31 had polyarthritis, 20 had oligoarthritis, and 6 had distal arthritis) and 12 (17.4%) had axial disease. No differences were found between patients with different forms of PsA presentation in terms of IL-6 levels (Kruskal-Wallis test: $P > 0.05$) and IL-23 levels (Kruskal-Wallis test: $P > 0.05$).

Table 1: Clinical and laboratory characteristics of ankylosing spondylitis, psoriatic arthritis, and SAPHO syndrome patients and healthy controls.

Assessed parameter	Ankylosing spondylitis patients (n = 61) Mean ± SD Median (Q1, Q3)	Psoriatic arthritis patients (n = 69) Mean ± SD Median (Q1, Q3)	SAPHO syndrome patients (n = 22) Mean ± SD Median (Q1, Q3)	Healthy controls (n = 29) Mean ± SD Median (Q1, Q3)
Age (years)	43.3 ± 13.2	52.0 ± 12.0	54.8 ± 12.3	48.2 ± 13.5
Sex	12 F. 49 M	39 F. 30 M	19 F. 3 M	19 F. 10 M
Disease duration (years)	10.0 (5.0, 18.0)	5.0 (2.0, 8.0)	2.0 (1.0, 4.0)	0
PASI	0.0	1.2 (0.0, 4.8)	—	0.0
BASDAI	5.72 (4.0, 7.8)	3.4 (2.3, 5.3)	3.6 (2.5, 5.0)	0
VAS pain (mm)	60.0 (40.0, 80.0)	40.0 (30.0, 60.0)	50.0 (40.0, 60.0)	0.0
CRP (mg/L)	9.93 (3.89, 17.1)	4.15 (1.85, 10.9)	4.2 (1.0, 10.6)	0.0
ESR (mm/h)	15.0 (7.0, 28.0)	13.0 (6.0, 22.0)	16.0 (6.0, 30.0)	9.4 (1.0, 26.0)
IL-23 (pg/mL)	0.3 (0.0, 2.8)	0.0 (0.0, 1.5)	0.0 (0.0, 0.3)	0.0 (0.0, 0.0)
IL-6 (pg/mL)	4.09 (1.8, 7.9)	2.83 (1.5, 6.2)	2.16 (1.0, 5.7)	1.2 (0.7, 1.5)
VEGF (pg/mL)	395.2 (220.0, 680.0)	291.4 (205.7, 652.7)	33.1 (222.5, 372.5)	300.1 (217.0, 437.6)
EGF (pg/mL)	120.0 (174.0, 186.0)	120.0 (60.0, 186.0)	126.0 (70.0, 208.0)	93.0 (45.0, 192.5)
FGFb (pg/mL)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)
FGFa (pg/mL)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 24.3)	0.0 (0.0, 0.0)

Data are presented as number or mean ± standard deviation (Q1, Q3).
CRP: C-reactive protein.
EGF: epidermal growth factor.
ESR: erythrocyte sedimentation rate.
FGFa: acidic fibroblast growth factor.
FGFb: basic fibroblast growth factor.
IL-6: interleukin-6.
IL-23: interleukin-23.
n: number of patients.
PASI: Psoriasis Area and Severity Index.
SD: standard deviation.
VAS pain: visual analogue scale of patient's pain.
VEGF: vascular endothelial growth factor.

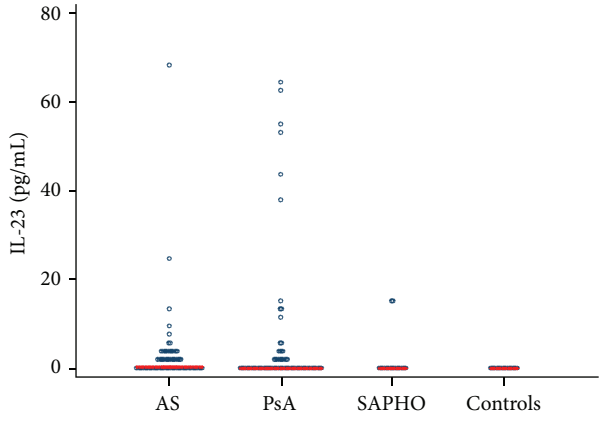

Figure 2: Serum levels of interleukin-23 (IL-23) in patients with psoriatic arthritis (PsA), ankylosing spondylitis (AS), and SAPHO syndrome (SAPHO) and controls.

Among AS patients, peripheral arthritis was present in 23 (37.7%). No differences were found between patients with different forms of AS presentation in terms of IL-6 levels ($P > 0.05$) and IL-23 levels ($P > 0.05$).

There was a positive correlation between serum IL-6 and CRP in AS ($P = 0.000001$), PsA ($P = 0.000001$), and SAPHO ($P = 0.0003$) patients (data not shown). There was a positive correlation between serum IL-6 and ESR in AS ($P = 0.000001$), PsA ($P = 0.002$), and SAPHO ($P = 0.02$) patients (data not shown).

There was no correlation between IL-6, VAS, BASDAI, and angiogenic cytokines (VEGF, EGF, FGFb, and FGFa) ($P > 0.05$) in SpA patients (data not shown). There was no correlation between serum IL-23, CRP, ESR, VAS, BASDAI, and angiogenic cytokines ($P > 0.05$) in SpA patients (data not shown).

No differences were found between SpA groups of various treatment regimens in terms of IL-6 (Kruskal-Wallis test: $P > 0.05$) and IL-23 levels (Kruskal-Wallis test: $P > 0.05$).

The results of univariable and multivariable logistic regression analysis and stepwise analysis of serum IL-6 levels in SpA patients with adjustment to CRP, BASDAI, and DMARDs showed no association with BASDAI and treatment with DMARDs. The adjusted OR for serum IL-6 ≥ 1.53 pg/mL in SpA patients with increased CRP level (≥5 mg/L) was 7.68 (95% CI 2.93–20.16), $P < 0.0001$ (Table 2). In SpA patients with increased CRP level (≥5 mg/L) 63.06%

Table 2: A logistic regression model of the OR of the increased serum level of interleukin-6.

Covariates	Serum IL-6 ≥ 1.53 pg/mL		Serum IL-6 ≥ 6.64 pg/mL	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Ankylosing spondylitis	2.04 (0.87–4.78)	0.10	1.30 (0.6–2.77)	0.51
Psoriatic arthritis	0.70 (0.32–1.51)	0.36	0.86 (0.40–1.84)	0.69
SAPHO	0.61 (0.23–1.65)	0.33	0.82 (0.28–2.41)	0.72
CRP ≥ 5 mg/L	7.68 (2.93–20.16)	<0.0001	15.76 (4.54–54.66)	<0.0001
BASDAI ≥ 4	2.90 (1.16–7.25)	0.02	1.11 (0.50–2.45)	0.79
NSAIDs	1.15 (0.42–3.11)	0.78	0.58 (0.20–1.65)	0.31
Sulfasalazine	0.64 (0.29–1.39)	0.26	1.07 (0.50–2.28)	0.85
Methotrexate	1.71 (0.71–4.15)	0.23	1.42 (0.65–3.11)	0.38

IL-6: interleukin-6.
OR: odds ratio.
95% CI: 95% confidence interval.
NSAIDs: nonsteroidal anti-inflammatory drugs.

Table 3: A logistic regression model of the OR of the increased serum level of interleukin-23 in spondyloarthritis patients.

Covariates	Serum IL-23 > 0.0 pg/mL		Serum IL-23 ≥ 2.5 pg/mL	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Ankylosing spondylitis	1.83 (1.02–3.29)	0.04	2.07 (1.06–4.05)	0.03
Psoriatic arthritis	0.73 (0.41–1.31)	0.29	0.71 (0.36–1.40)	0.33
SAPHO	0.47 (0.18–1.26)	0.135	0.27 (0.06–1.21)	0.08
CRP ≥ 5 mg/L	1.02 (0.57–1.83)	0.94	0.93 (0.48–1.81)	0.82
BASDAI ≥ 4	1.52 (0.80–2.86)	0.19	1.47 (0.70–3.08)	0.30
NSAIDs	1.40 (0.70–2.80)	0.35	2.42 (1.15–5.07)	0.02
Sulfasalazine	1.08 (0.60–1.93)	0.79	0.58 (0.29–1.15)	0.12
Methotrexate	0.70 (0.37–1.30)	0.25	0.92 (0.45–1.86)	0.81

IL-23: interleukin-23.
OR: odds ratio.
95% CI: 95% confidence interval.
NSAIDs: nonsteroidal anti-inflammatory drugs.

had serum IL-6 ≥ 1.53 pg/mL whereas 18.18% had serum IL-6 < 1.53 pg/mL ($P < 0.00001$).

The results of univariable and multivariable logistic regression analysis and stepwise analysis of serum IL-23 levels in SpA patients with adjustment to CRP, BASDAI, and DMARDs showed no association. The adjusted OR for serum IL-23 ≥ 2.5 pg/mL in SpA patients treated with NSAIDs was 2.42 (95% CI 1.15–5.07) compared to patients not treated with NSAIDs ($P = 0.02$) (Table 3). The adjusted OR for serum IL-23 ≥ 2.5 pg/mL in AS patients was 2.07 (95% CI 1.06–4.057) compared to other SpA patients ($P = 0.03$) (Table 3).

5. Discussion

In this study, we evaluated the relationship between serum levels of IL-6 and IL-23 and activity of AS, PsA, and SAPHO syndrome. The role of IL-6 in SpA is so far not clearly demonstrated. Elevated levels of IL-6 have been described in patients with PsA compared with patients with psoriasis [19]. IL-6 was found to be increased in the serum and in the sacroiliac joints of patients with AS [1]. A correlation was found between serum levels of IL-6 and spinal inflammation as detected by magnetic resonance imaging (MRI) in AS [20]. A positive correlation has been reported between serum IL-6 and severity indexes such as those for vertebral mobility in AS [1]. A correlation was found between IL-6 level and BASDAI in AS [20]. Serum IL-6 levels were shown to be elevated in AS patients, along with factors associated with poor prognosis such as positive HLA-B27, inflammatory lower back pain, and arthritis [21]. A correlation between serum IL-6, CRP, and ESR in AS was shown [2]. Isolated cases of good treatment effect with tocilizumab in patients with AS and PsA have been reported [22–25].

On the other hand, data showing that IL-6 inhibition is not effective in SpA were reported [26, 27]. Treatment with tocilizumab was not effective in axial symptoms in AS patients, although it had the effect of reducing CRP [26]. A similar situation was described in PsA patients treated with tocilizumab; despite a complete normalisation of serum CRP, there was no improvement in either the joint or skin disease [27]. The role of IL-6 was not considered crucial in an animal model of tumour necrosis factor- (TNF-) mediated bilateral sacroiliitis [3].

In our study, we have shown increased serum IL-6 compared with control and found a positive correlation between serum IL-6 and disease activity measured by serum CRP and ESR in patients with AS, PsA, and SAPHO. This is in agreement with other publications [1, 2, 19]. In our opinion,

this confirms the previously suggested pathogenic role of this cytokine in SpA. It will be necessary to conduct further studies on the possibility of using antibodies against IL-6 in selected patients with SpA.

IL-23 is a heterodimeric cytokine composed of two subunits, p40 (common with interleukin-12 (IL-12)) and p19. The relationship between IL-23 receptor polymorphism and the occurrence of AS and PsA has been described [28, 29]. A good clinical response to the use of antibodies against IL-23 in patients with PsA was also reported [30, 31]. All of this could have to confirm the role of IL-23 in SpA.

In previous small case studies, authors did not find a correlation between serum IL-23 and disease activity in AS and PsA patients [8, 9]. Wendling et al. [8] found no correlation between serum IL-12/IL-23 p40 and ESR, CRP, and BASDAI in 27 nonselected SpA patients. Melis et al. [9] showed a positive correlation between serum IL-23 levels and disease activity measured by CRP, ESR, and the number of swollen joints in patients with rheumatoid arthritis (RA), but they did not find such a correlation in 52 SpA patients (the group consisted of PsA and non-PsA spondyloarthritis patients). Histological changes of the synovial membrane correlated with the concentration of IL-23 in RA patients but did not in SpA patients. Furthermore, in RA patients they observed a reduction of serum IL-23 levels after treatment with TNF-alpha blockers, but this was not observed in patients with SpA [9]. The exacerbation of psoriasis induced by anti-TNF therapy has also been described in a patient with AS after switching to treatment with ustekinumab [32].

The results of our study, carried out on a larger group of SpA patients (152 patients), are in line with those described by other authors [8, 9]. We also did not observe a correlation between disease activity measured by ESR, CRP, and BASDAI and the serum concentration of IL-23 in SpA patients. There was also no difference between the serum concentration of IL-23 in the peripheral and axial forms of PsA and AS. However, SpA patients had significantly higher levels of IL-23 in comparison with the control group. Additionally AS patients had increased risk of increased serum IL-23 levels compared to other SpA patients. This could confirm suggestions about the role of this cytokine in the pathogenesis of SpA.

In situ analysis of IL-23-positive cells in the spine of patients with AS showed that IL-23 was expressed in the subchondral bone marrow and in fibrous tissue replacing bone marrow in facet joints of patients with AS [33]. The lack of relationship between serum IL-23 and disease activity in SpA can be explained by the fact that it acts locally in the joints and bones with no systemic effects [33]. It could confirm the previously suggested independent processes of ossification and inflammation in patients with AS. IL-23 might have a role in chronic changes in AS joints with no influence on laboratory disease activity. It will be necessary to conduct further studies on the possibility of using antibodies against IL-23 in AS patients. Perhaps the local rather than systemic application of IL-23 blockers had to make sense in the treatment of AS.

Despite the fact that, in our study, no correlation was found between serum IL-23 and disease activity in AS patients, this group of patients had an increased risk of elevated levels of IL-23.

Angiogenesis also plays a role in arthritis. Serum VEGF levels may be a marker of inflammatory activity in arthritis. In our previously published studies, we showed that serum VEGF levels correlated positively with disease activity assessed by CRP in PsA, but no significant correlations were found between levels of angiogenic cytokines and clinical presentation in SAPHO patients [34]. In our current study, we failed to show a correlation between serum IL-6 and IL-23 and the concentration of angiogenic cytokines in AS, PsA, and SAPHO.

The value of our work is the much larger number of SpA patients than that of previously reported studies. Moreover, no study has evaluated the relationship between serum IL-6 and IL-23 and angiogenic cytokines. The novelty of our work is that we assessed the association between IL-6, IL-23, and disease activity in patients with SAPHO syndrome. Little is known about the role of various cytokines in SAPHO. In the available literature, we did not find data concerning the role of IL-6 and IL-23 in SAPHO syndrome.

In summary, we emphasise that we have shown the relationship between serum IL-6 concentration and disease activity measured by ESR and CRP in AS, PsA, and SAPHO patients, while we have not found such a relationship in the case of serum IL-23. There was no correlation of serum IL-6 and IL-23 with clinical disease activity indexes in SpA patients.

The mechanism of action of IL-6 and IL-23 in patients with SpA requires further study to clarify their role in the pathogenesis of these diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Hanna Przepiera-Będzak and Marek Brzosko conceived the idea for the study, contributed to the design of the research, were involved in data collection, and analyzed the data. Hanna Przepiera-Będzak coordinated funding for the project. Katarzyna Fischer was involved in laboratory analysis. All authors edited and approved the final version of the paper.

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Clinical Study

Response to Infliximab in Crohn's Disease: Genetic Analysis Supporting Expression Profile

Luz María Medrano,[1] Carlos Taxonera,[2] Cristina González-Artacho,[3] Virginia Pascual,[1] María Gómez-García,[3] Manuel Barreiro-de Acosta,[4] José L. Pérez-Calle,[5] Fernando Bermejo,[6] Antonio López-Sanromán,[7] Dolores Martín Arranz,[8] Javier P. Gisbert,[9,10] Juan Luis Mendoza,[2] Javier Martín,[11] Concepción Núñez,[1] and Elena Urcelay[1]

[1] *Immunology Department, Hospital Clínico San Carlos, Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC), 28040 Madrid, Spain*
[2] *Gastroenterology Department, Hospital Clínico San Carlos, Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC), 28040 Madrid, Spain*
[3] *Gastroenterology Department, Virgen de las Nieves Hospital, 18014 Granada, Spain*
[4] *Gastroenterology Department, Hospital Clínico Universitario de Santiago de Compostela, 15706 Santiago de Compostela, Spain*
[5] *Gastroenterology Department, Alcorcón Hospital, 28922 Madrid, Spain*
[6] *Gastroenterology Department, Fuenlabrada Hospital, 28942 Madrid, Spain*
[7] *Gastroenterology Department, Ramón y Cajal Hospital, 28034 Madrid, Spain*
[8] *Gastroenterology Department, La Paz Hospital, 28046 Madrid, Spain*
[9] *Gastroenterology Department, Hospital Universitario La Princesa, Instituto de Investigación Sanitaria Princesa (IP), 28006 Madrid, Spain*
[10] *Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), 28002 Madrid, Spain*
[11] *Instituto de Parasitología y Biomedicina López-Neyra, IPBLN-CSIC, Armilla, 18100 Granada, Spain*

Correspondence should be addressed to Elena Urcelay; elena.urcelay@salud.madrid.org

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Substantial proportion of Crohn's disease (CD) patients shows no response or a limited response to treatment with infliximab (IFX) and to identify biomarkers of response would be of great clinical and economic benefit. The expression profile of five genes (*S100A8-S100A9, G0S2, TNFAIP6*, and *IL11*) reportedly predicted response to IFX and we aimed at investigating their etiologic role through genetic association analysis. Patients with active CD (350) who received at least three induction doses of IFX were included and classified according to IFX response. A tagging strategy was used to select genetic polymorphisms that cover the variability present in the chromosomal regions encoding the identified genes with altered expression. Following genotyping, differences between responders and nonresponders to IFX were observed in haplotypes of the studied regions: *S100A8-S100A9* (rs11205276*G/rs3014866*C/rs724781*C/rs3006488*A; $P = 0.05$); *G0S2* (rs4844486*A/rs1473683*T; $P = 0.15$); *TNFAIP6* (rs11677200*C/rs2342910*A/rs3755480*G/rs10432475*A; $P = 0.10$); and *IL11* (rs1126760*C/rs1042506*G; $P = 0.07$). These differences were amplified in patients with colonic and ileocolonic location for all but the *TNFAIP6* haplotype, which evidenced significant difference in ileal CD patients. Our results support the role of the reported expression signature as predictive of anti-TNF outcome in CD patients and suggest an etiological role of those top-five genes in the IFX response pathway.

1. Introduction

Crohn's disease (CD) is one of the clinical forms of inflammatory bowel disease (IBD) resulting from a defective regulation of mucosal immune responses to commensal microbiota in genetically susceptible individuals [1]. The last years have contemplated substantial progress in the identification of the genes involved in CD predisposition, boosted by the HapMap project and genome-wide association studies [2]. A better understanding of the biological pathways underlying CD pathogenesis will lead to the development of new therapeutic approaches that specifically target those pathways and will eventually allow personalized treatments. Therefore, an increasing need exists to predict the therapy most fitted to each patient.

Since 1998, when the US Food and Drug Administration approved infliximab (IFX) for treatment of moderate or severe CD that does not respond to a conservative treatment, monoclonal antibodies to tumor necrosis factor alpha (TNF-α) have become the hallmark treatment for refractory CD. Infliximab has proven to be effective for the treatment of both luminal [3] and fistulizing CD [4]. However, a lack of response or a partial response to IFX has been consistently observed and a growing need exists to identify biomarkers of response in order to achieve a more efficacious use of this expensive and potentially toxic therapy. Moreover, data from clinical trials of IFX suggest that high-risk patients and patients with active inflammation may benefit from earlier use of this drug [5]. Clinical parameters such as concurrent therapies, smoking habits, or previous surgery seem to account for only a small amount of the variance in response to anti-TNF therapies [6].

In a recent study by microarray analysis, pretreatment mucosal gene expression profiles predicted response to first IFX treatment in CD patients [7]. The study identified a 100% accurate predictive gene expression signature for response to IFX in Crohn's colitis; class prediction analysis allowed complete separation between responders and nonresponders through a panel of 5 top significant genes: *S100A8* and *S100A9, G0S2, TNFAIP6*, and *IL11*. This biological therapy has been also used for the treatment of other chronic inflammatory diseases such as rheumatoid arthritis (RA). RA and CD probably share a pathogenetic background, as they have been associated with overlapping susceptibility genes. Therefore, it is reasonable to expect that common genes would anticipate the response to this therapy in both conditions. Up to date, several studies used genome-wide expression analysis to identify expression signatures predicting response to anti-TNF treatment in RA patients, but results showed little overlap [8]. The identified expression profiles were often not consistent with each other, and different gene sets were reported to distinguish between responders and nonresponders. Therefore, despite the original promising results in CD, the expression signature could be considered a first step towards a predictive test. Provided corroboration from a genetic standpoint is achieved, the cluster of five genes with different expression in CD responders compared to nonresponders to IFX therapy would be a useful tool to classify patients. With this hypothesis, we aimed at investigating the reported expression profile by exploring the association of tagging variants in those top-five genes with the response to IFX in an independent Spanish cohort of CD patients. Once an association between polymorphisms within those genes and the response to IFX is found, a causal mechanism of IFX response would be envisaged and a simpler way to classify patients could be established, as it is much easier to perform genotyping of the involved genes than to check their levels of expression.

2. Material and Methods

2.1. Study Design and Patients. Overall, 350 unrelated white Spanish patients with active CD were consecutively recruited from 8 centers. Eligible patients were at least 18 years old, had an established diagnosis of CD, and had received at least the 3 induction doses of IFX (5 mg per kilogram) at weeks 0, 2, and 6. Diagnosis of CD was based on standard clinical, radiologic, endoscopic, and histological criteria [9]. IFX was administered to treat either moderate to severe active luminal CD or active fistulizing perianal CD.

Disease phenotype was determined following the Montreal Classification: age at diagnosis (A1: ≤16 years; A2: 17–40 years; A3: >40 years), anatomic location (L1: terminal ileum; L2: colon; L3; ileocolon; L4: upper gastrointestinal tract; and +L4: upper gastrointestinal modifier), and disease behavior (B1: inflammatory; B2: stricturing; B3: penetrating; and p: perianal modifier) [10].

Patients were classified as responders (remission or partial response) or nonresponders to IFX. The response to IFX was determined by a chronological review of the medical records and data were centrally monitored. The response to IFX in patients with luminal disease was evaluated by the Harvey-Bradshaw index (HBI) [11] at the beginning and 10 weeks after the first IFX dose. Partial response was defined as a decrease in the HBI of more than 3 points and absence of concomitant corticosteroids [12]. Remission was defined as a final HBI ≤4 and absence of concomitant corticosteroids [12]. In patients with perianal disease, response was evaluated at week 10 after the first IFX dose. Remission was defined as the complete closure of all fistulas and partial response as a reduction (≥50%) in the number of draining fistulas. Patients either receiving IFX for both luminal and fistulizing disease or achieving remission of any type that justified the maintenance of IFX treatment were considered responders. All patients who did not achieve partial response or remission after the three IFX induction doses were considered nonresponders.

2.2. Genotyping. In order to cover the highest variability within each gene showing altered expression, we chose single nucleotide polymorphisms (SNPs) by aggressive tagging from the HapMap B36 CEU population, which captured markers with $r^2 > 0.8$ (mean $r^2 = 0.93$) and a minor-allele frequency (MAF) >0.1. Genotyping of the Spanish samples was carried out with predesigned TaqMan Assays from Applied Biosystems (Applied Biosystems Inc., Foster City, CA, USA), in a 7900HT Fast Real-Time PCR system, under conditions recommended by the manufacturer. Genotyping call-rate success was over 95% for the SNPs in all groups of patients.

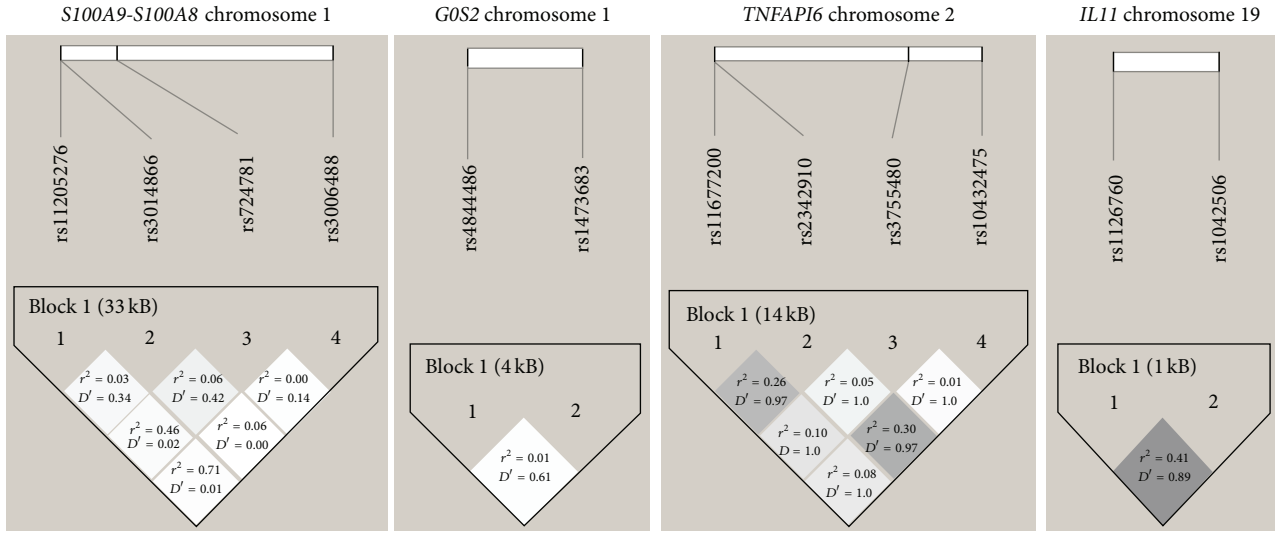

FIGURE 1: Linkage disequilibrium (D' and r^2) between the studied SNPs located in the same genetic region: *S100A9-S100A8, G0S2, TNFAIP6*, and *IL11*.

2.3. Data Analysis. Demographic and baseline characteristics were compared between responders and nonresponders by using the Mann-Whitney U test or the chi-square test, whether continuous or categorical variables were considered. The statistical analysis to compare allelic and genotypic distributions was performed using chi-square test or Fisher's exact test (when expected values were below 5). Odds ratios (ORs) were calculated and their 95% confidence intervals were estimated using the Cornfield method. Haplotypic frequencies were inferred with the expectation-maximization algorithm implemented in the Haploview 4.1 software. Linkage disequilibrium was measured by calculating two parameters: r^2 and D' (Figure 1). Demographic characteristics were analyzed as potential confounding factors of the IFX response using logistic regression.

3. Results

Baseline characteristics of the Spanish patients, classified as responders or nonresponders to IFX therapy, are summarized in Table 1. A total of 285 (82%) patients were classified as responders and 62 (18%) as nonresponders. A statistically significant difference was observed between the years of evolution of disease in both groups.

Tagging polymorphisms in the five previously identified genes were genotyped in the two groups of CD patients, responders, and nonresponders to IFX therapy, and frequencies are shown in Table 2. The studied polymorphisms conformed to Hardy-Weinberg expectations. Although independently only rs11677200 in the *TNFAIP6* gene showed a statistically significant result, the aggressive tagging approach allowed a more thorough scrutiny of these genomic regions by analyzing the haplotypes conformed by those polymorphisms (haplotypic frequencies in responders and nonresponders are summarized in Table 3). In the region where the *S100A9, S100A12*, and *S100A8* genes map, the most frequent haplotype evidenced a significantly higher frequency in CD nonresponders than in responders to IFX ($P = 0.051$; OR (95% CI) = 1.54 (0.97–2.43)). Additionally, one haplotype in the *IL11* region showed a marginal significant association with the response to the anti-TNF treatment ($P = 0.068$; OR (95% CI) = 1.72 (0.91–3.21)), and trends for association could be observed for haplotypes within the other two chromosomal regions explored (*G0S2*: $P = 0.15$; OR (95% CI) = 2.92 (0.45–15.23) and *TNFAIP6*: $P = 0.10$; OR (95% CI) = 0.71 (0.46–1.09)).

The identification of an expression profile to predict response to IFX by Arijs and collaborators [7] was focused in colonic CD patients and therefore we evaluated the specific subgroups of Crohn's patients. Tables 4 and 5 summarize the results in ileal patients and colonic and ileocolonic patients, respectively. As shown, the haplotypes associated in the overall patients were now found significantly associated in the colonic subgroup in the *S100A9-S100A8* (Table 5(a), GCCA haplotype: $P = 0.025$; OR (95% CI) = 1.91 (1.05–3.48)), in the *G0S2* (Table 5(b), AT haplotype: $P = 0.025$; OR (95% CI) = 6.31 (0.82–47.89)) and in the *IL11* (Table 5(d), CT haplotype: $P = 0.025$; OR (95% CI) = 2.76 (1.39–5.44)) regions. In contrast, in the *TNFAIP6* gene, the most frequent CAGA haplotype only showed a trend for association in ileal patients (Table 4(c), CAGA haplotype: $P = 0.1$; OR (95% CI) = 0.57 (0.27–1.17)), while no difference was detected in colonic patients (Table 5(c)).

4. Discussion

Pharmacogenetics has emerged as a promising discipline which opens the possibility of a personalized medicine. However, research has been hampered mainly due to limitations that this kind of studies still shows. Recruitment of a high number of patients with a similar treatment is not an easy task, but difficulty increases because response criteria should be centrally monitored as they were in the present study. Moreover, the high success rate of some pharmacological

Table 1: Characteristics of Crohn's disease patients studied: both responders ($n = 288$) and nonresponders ($n = 62$) to infliximab.

	Responders		Nonresponders		P value
	N	%	N	%	
Age	39.6 ± 0.7		41.9 ± 1.5		0.10
Sex					
Male	135	47.4	26	41.9	0.44
Female	150	52.6	36	58.1	
Years of disease	10.2 ± 0.4		13.2 ± 1.1		0.0067
Age at diagnosis (A)					
A1	36	12.9	8	13.1	0.61
A2	203	72.5	47	77.1	
A3	41	14.6	6	9.8	
Location (L)					
L1	74	26.5	28	46.6	0.014[a]
L2	57	20.4	8	13.4	
L3	137	49.1	24	40.0	
L4	2	0.7	0	0	
L1 + L4	3	1.1	0	0	
L2 + L4	1	0.4	0	0	
L3 + L4	5	1.8	0	0	
Behavior (B)					
B1	79	28.2	19	31.7	0.56
B2	27	9.6	7	11.7	
B3	46	16.5	11	18.3	
B1p	70	25.0	8	13.3	
B2p	9	3.2	2	3.3	
B3p	49	17.5	13	21.7	

Data correspond at first IFX dose.

A1: ≤16 years; A2: 17–40 years; A3: >40 years. L1: terminal ileum; L2: colon; L3: ileocolon; L4: upper GI; L1 + L4: terminal ileum + upper GI; L2 + L4: colon + upper GI; L3 + L4: ileocolon + upper GI. B1: nonstricturing, nonpenetrating; B2: structuring; B3: penetrating; B1p: nonstricturing, nonpenetrating + perianal; B2p: structuring + perianal; B3p: penetrating + perianal.

[a]Excluding categories with L4.

therapies as IFX originates a low number of nonresponders, with the consequent decrease in the statistical power to detect differences. In our patients, induction therapy with IFX achieved a very good outcome with higher rates of response than those reported in controlled trials [3, 4], in agreement with a high response rate to the three induction doses of IFX previously reported in a multicenter study [13]. Moreover, a better response to IFX was observed in patients with an earlier treatment (Table 1), as already reported [14].

The hierarchical cluster analysis performed by Arijs and collaborators [7] identified a profile with five differentially expressed genes which was claimed to predict response to IFX in colonic CD patients with an overall accuracy of 100%. In that work, the authors validated a previously published gene expression signature regarding response to IFX in ulcerative colitis patients [15]. *IL11* was the only overlapping gene between the two predictive top-five gene sets in both clinical forms of IBD. We aimed to investigate the causal implication of the described genes in the mechanism of IFX response through the association study of those genes in a cohort of Spanish CD patients. Moreover, this procedure might provide genetic markers of IFX response that would be easier to test.

In our independent cohort of CD patients, the tagging approach allowed us to explore a higher genetic variability within the chromosomal regions where the five genes map. Two of these five top genes are *S100A8* and *S100A9*; both encode members which belong to the S100 family of calcium-binding proteins and are located in a cluster on chromosome 1q21. Their expression is induced by proinflammatory cytokines such as IL-6 or TNF-α [16]. Calprotectin, the heterodimeric complex of S100A8 and S100A9, shows increased expression at an early step in the neoplastic transformation during colorectal carcinogenesis [17] and it is associated with disease activity in patients with IBD [18] and other inflammatory conditions as rheumatoid arthritis [19] or systemic lupus erythematosus [20]. Moreover, fecal calprotectin concentration is considered a useful surrogate marker for mucosal healing during TNF-α blocking therapy for IBD [21, 22]. In this genetic region, as mentioned, the most frequent haplotype was found significantly associated with response to IFX, mainly in colonic patients (22.7% responders versus 35.9% nonresponders, $P = 0.025$).

Another gene with reported downregulated expression in IFX responders is *IL11*. This interleukin is a member of

Table 2: Genotype frequencies of the polymorphisms located in the genes studied in Crohn's disease patients: both responders and nonresponders to infliximab.

	Responders		Nonresponders	
	N	%	*N*	%
Gene *S100A9*				
rs11205276				
GG	189	67	39	67
GC	88	31	18	31
CC	4	2	1	2
rs3014866				
CC	94	33	22	37
CT	129	46	30	51
TT	58	21	7	12
Gene *S100A12*				
rs724781				
CC	128	46	27	47
CG	115	41	27	47
GG	37	13	4	6
Gene *S100A8*				
rs3006488				
AA	235	83	47	78
AG	44	15	12	20
GG	5	2	1	2
Gene *G0S2*				
rs4844486				
CC	145	52	33	54
CA	115	42	26	43
AA	18	6	2	3
rs1473683				
GG	269	97	54	95
GT	7	3	3	5
TT	0	0	0	0
GENE *TNFAIP6*				
rs11677200*				
TT	79	29	26	45
TC	153	56	24	41
CC	42	15	8	14
rs2342910				
AA	138	52	30	57
AT	113	42	18	34
TT	16	6	5	9
rs3755480				
GG	218	78	44	72
GA	57	20	15	25
AA	6	2	2	3
rs10432475				
AA	224	80	44	77
AG	54	19	12	21
GG	2	1	1	2
Gene *IL11*				
rs1126760				
TT	165	60	28	52
TC	94	34	24	44
CC	16	6	2	4
rs1042506				
TT	195	76	37	77
TG	52	20	11	23
GG	9	4	0	0

*CC genotype, $P = 0.017$; OR (95% CI) = 0.5 (0.27–0.93).

the gp130 family of cytokines that stimulates T-cell dependent development of immunoglobulin-producing B cells, and that was tested as a therapy in CD [23]. In this case, association with borderline significance has been found involving an *IL11* haplotype with decreased frequency in CD responders to IFX. Moreover, a significant difference was evidenced when colonic CD patients were examined (11.5% responders versus 27.2% nonresponders, $P = 0.0012$).

One gene included in the predictive expression panel for IFX responsiveness is *TNFAIP6* (tumor necrosis factor, alpha-induced protein 6), which encodes a multifunctional protein with important roles in inflammation and tissue remodeling. It is upregulated in many inflammatory conditions as rheumatoid arthritis [24], a disease that also benefits from IFX therapy, and in colorectal cancer [25]. Another gene previously showing a differential expression profile predictive of response to IFX is *G0S2* (G0/G1 switch 2), involved in lymphocyte cell cycle regulation and found upregulated in rheumatoid arthritis and psoriasis [26, 27]. The haplotypes studied in these two genes evidenced trends for association with the response to IFX in the overall Spanish CD patients. Furthermore, only the trend for association observed in the *G0S2* gene could be significantly replicated in the colonic subgroup of patients (0.8% responders versus 5.2% nonresponders, $P = 0.036$).

The strategy followed in our study lends support to the reported gene expression profile predictive of anti-TNF therapy in an independent cohort of Spanish CD patients. Genetic studies stand out as approaches to define pathogenic pathways and ultimately the integration of genetic together with functional data promotes a clearer understanding of the mechanisms underlying therapeutic pathways.

Ethical Approval

The Ethics Committees of the participant hospitals (CEIC of the following hospitals from Madrid: Clínico San Carlos, Fuenlabrada, Alcorcón, La Princesa, La Paz, and Ramón y Cajal, from the Hospital Virgen de las Nieves (Granada) and CEIC of Galicia) approved the study.

Table 3: Haplotype frequencies and comparison between Crohn's patients, responders and nonresponders to infliximab therapy, in the chromosomal regions including (a) *S100A9* and *S100A8* genes (rs11205276, rs3014866, rs724781, and rs3006488); (b) *G0S2* gene (rs4844486, rs1473683); (c) *TNFAIP6* gene (rs11677200, rs2342910, rs3755480, and rs10432475); and (d) *IL11* gene (rs1126760, rs1042506).

(a)

Haplotype	Responders (%)	Nonresponders (%)	*P* value
GCCA	22.8	31.4	0.05
GTCA	24.6	18.4	0.16
GCGA	21.5	19.7	0.63
CTCA	9.3	10.9	0.57
GTGA	4.9	1.9	0.11
CCCA	4.1	3.4	0.73
GCCG	3.8	4.4	0.77
GTGG	2.4	4.4	0.28
CCGA	2.1	1.8	0.77
GCGG	1.5	1.7	0.92
GTCG	1.3	1.0	0.72
CTGA	1.1	0.7	0.83

(b)

Haplotype	Responders (%)	Nonresponders (%)	*P* value
CG	72.4	74.3	0.61
AG	26.3	23.0	0.43
AT	0.8	2.4	0.12

(c)

Haplotype	Responders (%)	Nonresponders (%)	*P* value
CAGA	43.0	34.8	0.10
TAGA	17.4	21.6	0.24
TTGA	16.8	15.3	0.68
TAAA	12.3	16.1	0.24
TTGG	10.2	12.1	0.60

(d)

Haplotype	Responders (%)	Nonresponders (%)	*P* value
TT	76.5	72.2	0.38
CG	12.8	10.0	0.50
CT	10.0	15.7	0.07
TG	0.7	2.0	0.25

Table 4: Haplotype frequencies and comparison between Crohn's patients with L1 ileum location, responders and nonresponders to infliximab therapy, in the chromosomal regions including (a) *S100A9* and *S100A8* genes (rs11205276, rs3014866, rs724781, and rs3006488); (b) *G0S2* gene (rs4844486, rs1473683); (c) *TNFAIP6* gene (rs11677200, rs2342910, rs3755480, and rs10432475); and (d) *IL11* gene (rs1126760, rs1042506).

(a)

Haplotype	Responders (%)	Nonresponders (%)	*P* value
GTCA	36	23.7	0.16
GCCA	22.8	28.4	0.48
GCGA	12.2	19.4	0.16
CTCA	10.3	12.6	0.57
GTGG	2.6	5.5	0.39
GTGA	3.7	2.5	0.68
CCCA	3.3	3.3	1.00
CCGA	3.5	2.6	1.00
GCGG	3	1.4	1.00
GCCG	1.6	0.4	1.00

(b)

Haplotype	Responders (%)	Nonresponders (%)	*P* value
CG	71.5	75.8	0.54
AG	25.7	24	0.82
CT	2.1	0.1	0.57

(c)

Haplotype	Responders (%)	Nonresponders (%)	*P* value
CAGA	39.4	27.1	0.14
TAGA	20.2	24.4	0.56
TAAA	14.9	24.1	0.13
TTGA	16.1	10.7	0.37
TTGG	9.5	13.7	0.47

(d)

Haplotype	Responders (%)	Nonresponders (%)	*P* value
TT	77.6	83	0.40
CG	16	13.5	0.74
CT	5.6	3.4	1.00

Consent

All patients provided written informed consent. The informed consent was approved by the Ethics Committee of the leading center (CEIC of Hospital Clínico San Carlos, Madrid).

Disclosure

Elena Urcelay works for the Fundación para la Investigación Biomédica-Hospital Clínico San Carlos (IdISSC).

Conflict of Interests

Carlos Taxonera, Manuel Barreiro-de Acosta, Antonio López-Sanromán, Dolores Martín Arranz, Javier P. Gisbert, and Juan Luis Mendoza have served as speakers, consultants, and advisory members for MSD and Abbott.

Authors' Contribution

Luz María Medrano participated in the design of the study, performed the genotyping, analyzed the data, and drafted the paper. Carlos Taxonera participated in the design of the study and drafted the paper. Virginia Pascual participated

Table 5: Haplotype frequencies and comparison between colonic Crohn's patients with L2 and L3 location, responders and nonresponders to infliximab therapy, in the chromosomal regions including: (a) *S100A9* and *S100A8* genes (rs11205276, rs3014866, rs724781, and rs3006488); (b) *G0S2* gene (rs4844486, rs1473683); (c) *TNFAIP6* gene (rs11677200, rs2342910, rs3755480, and rs10432475); and (d) *IL11* gene (rs1126760, rs1042506).

(a)

Haplotype	Responders (%)	Nonresponders (%)	*P* value
GCGA	25.1	21.5	0.56
GCCA	22.7	35.9	0.025
GTCA	20.7	11.6	0.10
CTCA	8.8	10.8	0.52
GTGA	5.2	1.8	0.33
GCCG	4.1	7.2	0.33
CCCA	4.2	3.5	1.00
GTGG	2.4	1.2	1.00
GTCG	2.1	2.3	1.00
CCGA	1.6	1	1.00
GCGG	1.2	1.6	0.58
CTGA	1.1	0.6	0.52
CCCG	0.8	1.1	0.44

(b)

Haplotype	Responders (%)	Nonresponders (%)	*P* value
CG	72.1	71.7	0.94
AG	27.1	23.2	0.54
AT	0.8	5.2	0.036

(c)

Haplotype	Responders (%)	Nonresponders (%)	*P* value
CAGA	44.5	44.1	0.87
TAGA	17.2	19.4	0.61
TTGA	15.7	16.6	0.86
TAAA	11.9	8.3	0.43
TTGG	10.3	11.6	0.74

(d)

Haplotype	Responders (%)	Nonresponders (%)	*P* value
TT	76	62.8	0.040
CT	11.5	27.2	0.0012
CG	11.7	6.1	0.17
TG	0.8	3.9	0.12

in the genotyping and interpretation of data. Elena Urcelay and Concepción Núñez made substantial contribution to the conception and design of the study, acquisition of data, and coordination and drafting of the paper. Cristina González-Artacho, María Gómez-García, Manuel Barreiro-de Acosta, José L. Pérez-Calle, Fernando Bermejo, Antonio López-Sanromán, Dolores Martín Arranz, Javier P. Gisbert, Juan Luis Mendoza, and Javier Martín participated in the acquisition and interpretation of data. All the authors revised critically the paper, gave necessary attention to ensure the integrity of the work presented, and approved the final version. Luz María Medrano and Carlos Taxonera contributed equally to this work.

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Research Article

Elevated Serum Levels of Soluble TNF Receptors and Adhesion Molecules Are Associated with Diabetic Retinopathy in Patients with Type-1 Diabetes

Shruti Sharma,[1] Sharad Purohit,[1] Ashok Sharma,[1] Diane Hopkins,[1] Leigh Steed,[1] Bruce Bode,[2] Stephen W. Anderson,[2] Ruth Caldwell,[3] and Jin-Xiong She[1]

[1]*Center for Biotechnology and Genomic Medicine, Georgia Regents University, 1120 15th Street, Augusta, GA 30912, USA*
[2]*Pediatric Endocrine Associates, Atlanta, GA 30342, USA*
[3]*Vascular Biology Center, Georgia Regents University, 1120 15th Street, Augusta, GA 30912, USA*

Correspondence should be addressed to Shruti Sharma; shsharma@gru.edu and Jin-Xiong She; jshe@gru.edu

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Aims. To examine the association of the serum levels of TNF receptors, adhesion molecules, and inflammatory mediators with diabetic retinopathy (DR) in T1D patients. *Methods*. Using the multiplex immunoassay, we measured serum levels of eight proteins in 678 T1D subjects aged 20–75 years. Comparisons were made between 482 T1D patients with no complications and 196 T1D patients with DR. *Results*. The levels of sTNFR-I, sTNFR-II, CRP, SAA, sgp130, sIL6R, sVCAM1, and sICAM1 were significantly higher in the T1D patients with DR as compared to T1D patients with no complications. Multivariate logistic regression analysis revealed significant association for five proteins after adjustment for age, sex, and disease duration (sTNFR-I: OR = 1.57, sgp130: OR = 1.43, sVCAM1: OR = 1.27, sICAM1: OR = 1.42, and CRP: OR = 1.15). Conditional logistic regression on matched paired data revealed that subjects in the top quartile for sTNFR-I (OR = 2.13), sTNFR-II (OR = 1.66), sgp130 (OR = 1.82), sIL6R (OR = 1.75), sVCAM1 (OR = 1.98), sICAM1 (OR = 2.23), CRP (OR = 2.40) and SAA (OR = 2.03), had the highest odds of having DR. *Conclusions*. The circulating markers of inflammation, endothelial injury, and TNF signaling are significantly associated with DR in patients with T1D. TNFR-I and TNFR-II receptors are highly correlated, but DR associated more strongly with TNFR-I in these patients.

1. Introduction

Diabetic retinopathy (DR) is a sight threatening, microvascular complication of diabetes that affects the retinal vasculature. It is the leading cause of blindness in adults 20–74 years of age in the United States with 28.5% prevalence among 40-year and older patients with diabetes [1]. According to the Centers for Disease Control and Prevention (CDC), the number of Americans aged 40 years and older with DR will triple from 5.5 million in 2005 to 16.0 million in 2050 [2]. So far the only recommended treatment for advanced retinopathy is laser photocoagulation which can control pathological neovascularization but may impair vision and in some patients the retinopathy continues to progress. Clinical trials of anti-VEGF intraocular injections have also shown promise in reducing diabetic macular edema [3]. However, these effects are usually transient and the treatment does not promote tissue repair and the need for repeated injections increases the risk of intraocular infection. Moreover, neither treatment targets early stage disease. Therefore, new markers to define the risk of type-1 diabetes (T1D) associated DR and new therapeutic targets are the critical unmet need.

Several inflammatory proteins are dysregulated in T1D [4] with inflammation being closely associated with the pathogenesis of different complications including DR [5–7]. DR is associated with several microvascular abnormalities such as leukocyte attachment to the vessel walls, occlusion of retinal capillaries, and breakdown of the blood retinal barrier and formation of acellular capillaries. The microvascular injury in DR has been linked to upregulation of several

Table 1: Baseline characteristics of T1D subjects without complications and with diabetic retinopathy (DR).

Patient characteristics	Without any complications	With DR	P value
Subjects (n)	482	196	—
Female (%)	52.1	61.7	0.027
Age (years)	39.1 ± 12.7	49.3 ± 11.6	$1.09E-21$
Age range (years)	20.0 to 73.8	24.6 to 73.8	—
Duration of disease	18.2 ± 11.1	31.4 ± 10.3	$1.09E-21$
Systolic BP (mmHg)	117.8 ± 9.5	122.9 ± 12.1	$1.17E-06$
Diastolic BP (mmHg)	73.7 ± 6.4	73.8 ± 7.3	0.208
Diabetic nephropathy (%)	0	0	—
Diabetic neuropathy (%)	0	41.8 ($n = 82$)	—
CAD (%)	0	16.3 ($n = 32$)	—
Dyslipidemia (N/Y)	376/106	112/84	$7.05E-08$
Hypertension (N/Y)	425/57	117/79	$1.14E-16$
Hemoglobin	14.4 ± 1.5	13.7 ± 1.6	$1.40E-04$
Albumin	4.4 ± 0.4	4.2 ± 0.4	$1.79E-05$
LDL	94.0 ± 27.4	102.4 ± 38.3	0.693
Total cholesterol	174.6 ± 34.2	184.1 ± 47.1	0.565
Triglycerides	90.4 ± 67.6	109.5 ± 94.2	0.395
HDL	61.9 ± 17.9	60.7 ± 19.4	0.639
Creatinine	0.9 ± 0.2	1.0 ± 0.2	$7.0E-04$
HbA1c	7.8 ± 1.1	8.0 ± 1.3	0.319
BUN	13.4 ± 4.5	16.4 ± 5.6	$4.83E-08$
Microalbumin	24.9 ± 122.3	85.2 ± 252.1	0.017

cytokines such as IL-6, IL1-β, and VEGF and pathological overexpression of adhesion molecules (ICAM-1 and VCAM-1) [8–10]. The major regulators of vascular adhesion molecules are TNF-α and IL-6 [11]. TNF-α and IL-6 are pleiotropic cytokines and key molecules in inflammatory signaling with TNF-α shown to be involved in the release of IL-6 [12]. IL-6 is known to induce ICAM-1 expression [13], whereas TNF-α leads to both ICAM-1 and VCAM-1 expression in endothelial cells [11].

Since adhesion molecules and soluble receptors of TNF-α and IL-6 pathway are key mediators of endothelial activation, their elevated levels may represent risk and severity of the pathogenesis of DR. Therefore, we examined the levels of soluble TNF receptors (sTNFR-I and sTNFR-II), soluble IL-6 receptors (sIL6R, sgp130), adhesion molecules (sICAM-1, sVCAM-1), and inflammatory markers (CRP, SAA) in serum of T1D patients with and without DR. The aim of this study was to examine the association of the serum levels of these inflammatory mediators with DR and to determine if these markers could be used as surrogate endpoints to define the risk of DR in T1D patients.

2. Research Design and Methods

2.1. Human Subjects and Serum Samples. This study was approved by the institutional review board of the Georgia Regents University, Augusta, Georgia. Blood samples from the participants of Phenome and Genome of Diabetes Autoimmunity (PAGODA) study were obtained after the informed consent from the subjects. All subjects were recruited in the state of Georgia, USA, mainly in the Atlanta and Augusta city areas. The demographic information for T1D subjects with no complications and with DR is presented in Table 1.

Peripheral blood was collected in serum separator tubes (BD Biosciences, San Jose, CA, USA) and clotted for 30 minutes, the tubes were centrifuged, and serum was immediately aliquoted and stored in −80°C freezers. Serum samples from T1D patients were aliquoted randomly into 96 well plates and each plate contained similar numbers of samples from T1D patients with and without DR.

2.2. Luminex Immunoassays. Luminex immunoassays for sTNFR-I, sTNFR-II, CRP, SAA, sIL6R, sgp130, sICAM-1, and sVCAM1 were obtained from Millipore (Millipore Inc., Billerica, MA, USA). Multiplex immunoassays were performed according to the manufacturer's instructions. Briefly, serum samples were incubated with antibody-coated microspheres, followed by biotinylated detection antibody. Proteins were detected by incubation with phycoerythrin-labeled streptavidin and the resultant bead immunocomplexes were read on a FLEXMAP3D (Luminex, TX, USA) with the following instrument settings: events/bead: 50, minimum events: 0, flow rate: 60 μL/min, sample size: 50 μL, and discriminator

gate: 8000–13500. Median fluorescence intensity (MFI) was collected and used for calculating protein concentration.

2.3. Statistical Analyses. All statistical analyses were performed using the R language and environment for statistical computing (R version 2.15.1; R Foundation for Statistical Computing; http://www.r-project.org/). All P values were two-tailed and a $P < 0.05$ was considered statistically significant.

Protein concentrations were estimated using a regression fit to the standard curve with known concentration included on each plate using a serial dilution series. To achieve normal distribution, the concentrations were log2 transformed prior to all statistical analyses. The potential differences between T1D patients without any complication and T1D patients with retinopathy were initially examined using a t-test. The pairwise correlation between individual protein levels was computed using Pearson correlation coefficient. Clustering and visualization of correlation matrix was performed using hierarchical clustering method and heatmap. The effect of age and T1D duration on serum levels of each candidate molecule was determined using a linear regression by including age or T1D duration as covariate on data stratified by sex and disease status. To examine the relationships between retinopathy and the serum protein levels logistic regression was used. Age, sex, and T1D duration were included as covariates in a stepwise manner.

To estimate the risk of diabetes at different protein concentrations, we performed conditional logistic regression on matched paired data. Case-control matching was performed with respect to age, sex, and T1D duration using the "matching" R package [14]. The odds ratios and 95% confidence intervals (CI) were computed for each protein and protein concentration was used as categorical variable (values 1, 2, 3, and 4 were assigned using the quartile values in controls as cutoff points).

3. Results

Serum levels of eight proteins in 482 T1D patients with no complications and 196 T1D patients with DR were measured. The demographic information and baseline characteristics of the subjects involved in this study are shown in Table 1. The average age of the T1D subjects without any complications was 39.1 ± 12.7 years and for subjects with DR was 49.3 ± 11.6. The duration of diabetes in patients without complications was 18.2 ± 11.1 as compared to 31.4 ± 10.3 in patients with DR.

3.1. Alterations in Serum Protein Levels in T1D Patients with DR. The levels of all eight molecules were significantly higher in the T1D patients with DR as compared to T1D patients with no complications: sTNFR-I (1.30-fold), sTNFR-II (1.27-fold), CRP (1.53-fold), SAA (1.33-fold), sgp130 (1.14-fold), sIL6R (1.08-fold), sVCAM1 (1.11-fold), and sICAM1 (1.19-fold) as shown in Figure 1. Next, we examined the pairwise correlations between all eight proteins and hierarchical clustering of the correlation matrix was performed in T1D patients with and without DR separately (Figure 2). We found three clusters of functionally related proteins with strong positive correlations. The proteins in cluster-1 include sgp130, sVCAM1, sICAM1, and sIL6R and the proteins in cluster-2 are CRP and SAA. The third cluster of proteins with strong positive correlation includes sTNFR-I and sTNFR-II. The correlations were almost similar in both no complication and DR groups (Figure 2) except sTNFR-II. The correlation of sTNFR-II was increased with other proteins in DR group as compared to the T1D group without any complications.

3.2. Multivariate Logistic Regression Analysis Reveals Significant Association between Protein Levels and DR. Multivariate logistic regression analysis was performed using four different models (Model 1: no adjustments, model 2: adjusted for age, model 3: adjusted for age and sex, and model 4: adjusted for age, sex, and T1D duration). The odds ratio of five proteins showed significant association with diabetic retinopathy (Table 2). We found that three proteins directly involved in TNF/IL-6-pathway have larger odds ratios (sTNFR-I: OR = 1.57, sICAM1: OR = 1.42, and sgp130: OR = 1.43). All eight proteins have positive associations with DR for the four models (Table 2).

3.3. Risk for DR Is Directly Related to the Protein Levels. Since there was a significant effect of age, sex, and T1D duration on the protein concentrations, a paired dataset of 183 matched pairs was generated using multivariate and propensity score matching software [14]. Matching was performed with respect to age, sex, and duration of diabetes and each T1D patient with DR was paired with closest T1D patient without complication. The demographic information and baseline characteristics of the samples after matching are presented in Table 3. Conditional logistic regression was performed to estimate the risk of DR at different protein concentrations. Protein levels were used as categorical variable after dividing into 4 quartiles. The odds ratios of having DR were computed for quartile-2, quartile-3, and quartile-4 using quartile-1 as reference. Subjects in the top quartile had the highest risk of DR compared with subjects in the bottom quartile for all eight proteins: sTNFR-I (OR = 2.13), sTNFR-II (OR = 1.66), CRP (OR = 2.40), SAA (OR = 2.03), sgp130 (OR = 1.83), sIL6R (OR = 1.75), sVCAM1 (OR = 1.98), and sICAM1 (OR = 2.23). Also, for all proteins, an increased trend in the risk for DR was observed from quartile-2 to quartile-4 of protein concentrations as shown in Figure 3.

4. Conclusions

Hyperglycemia and aging activate multiple cellular pathways which play an important role in diabetic retinopathy. Previous studies have related inflammation and endothelial injury to be closely associated with the pathogenesis of microvascular complications including DR [5–7]. In this study, we measured serum levels of 8 proteins in blood samples from T1D patients with DR and T1D patients without any complications. We found significant alterations in the

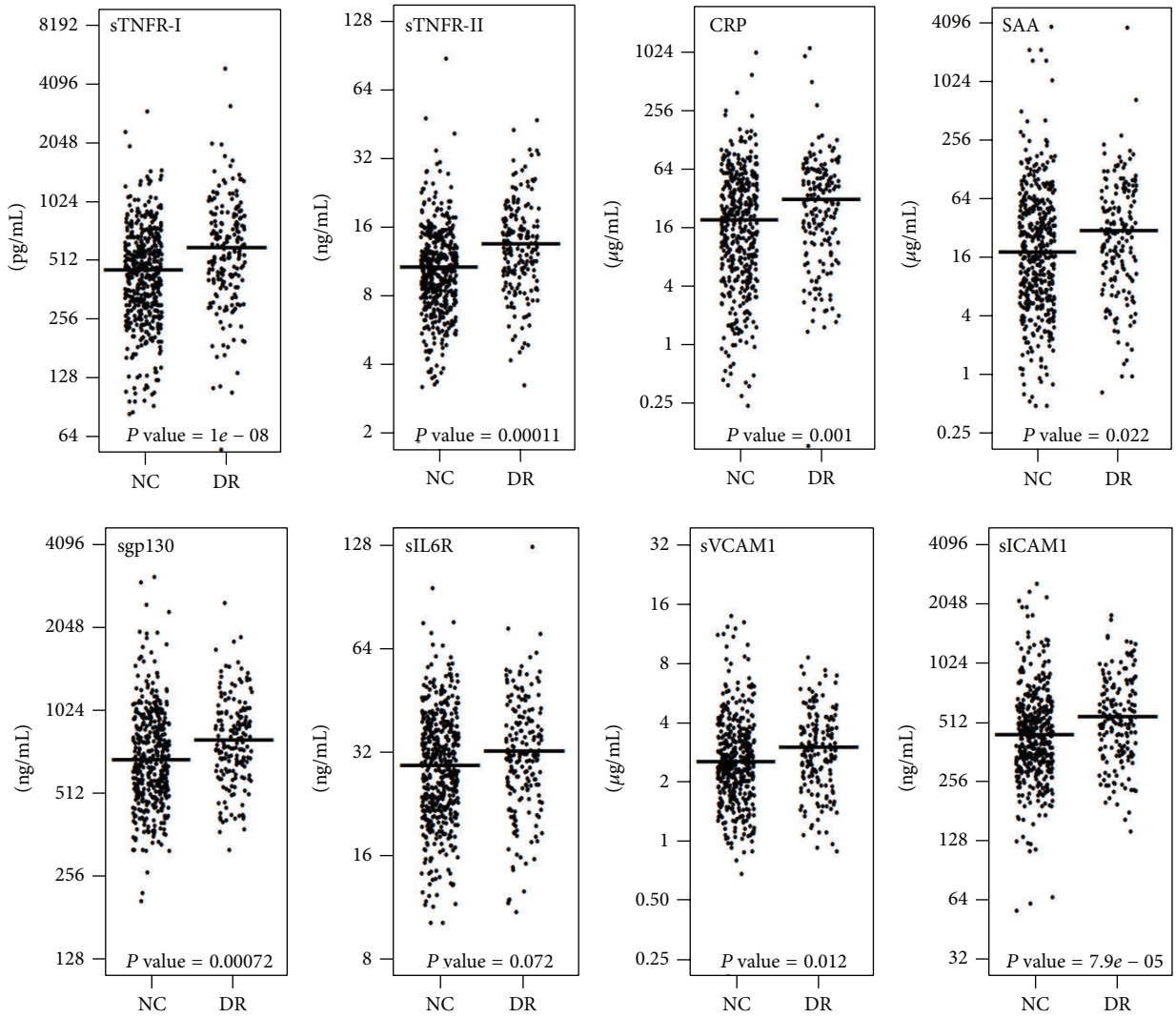

Figure 1: Elevated serum protein levels in T1D subjects with DR. Serum levels of eight proteins were measured in 678 T1D subjects aged 20–75 years. Comparisons were made between 482 T1D patients with no complications and 196 T1D patients with diabetic retinopathy. Plots depict the distribution of the protein levels in two different groups.

serum protein levels of sTNFR-I, sTNFR-II, CRP, SAA, sIL6R, sgp130, sVCAM1, and sICAM1.

TNF-α has been shown to be involved in the development and progression of DR [15]. Studies have shown the importance of TNF-α system in diabetic retinal microvascular damage [16]. TNF-α binds to its membrane receptors, TNFR-I and TNFR-II, which initiate signaling pathway leading to activation of transcription factors such as NF-κB as well as apoptosis [17]. In animal models, drugs that target TNF-α have been shown to reduce leukostasis, retinal vascular leakage, and retinal cell death [18, 19]. Proteolytic cleavage of extracellular domains of TNF-α receptors results in their release as soluble forms (sTNFR-I and sTNFR-II). While these 2 receptors are well-known as TNF antagonist, these can also act as a reservoir of circulating TNF-α. Recent studies have shown that these soluble forms may be more important than TNF-α itself in regulation of TNF signaling [20]. We found that sTNFR-I and sTNFR-II both are upregulated in T1D patients with DR. sTNFR-I and sTNFR-II receptors were highly correlated, but DR associated more strongly with sTNFR-I in these patients.

Earlier studies have also reported that the serum and vitreous levels of sTNFRs are elevated in DR patients [21], TNFR-I expression may be a more significant target than TNF-α for intervention in ocular inflammation [20], and TNF-α inhibition is known to reduce the leukocyte adhesion in the retina and the loss of retinal microvascular cells in diabetic rats. Also, activated TNF-α might regulate blood-retinal barrier (BRB) breakdown, retinal leukostasis, and apoptosis in later stages of DR [22]. Thus, the effective control of TNF-α activity by sTNFRs within the retinal microenvironment may determine the outcome and severity of DR.

Interestingly, we found significant alterations in soluble glycoprotein 130 (sgp130) protein levels that has not been previously implicated in DR. This protein plays a crucial role

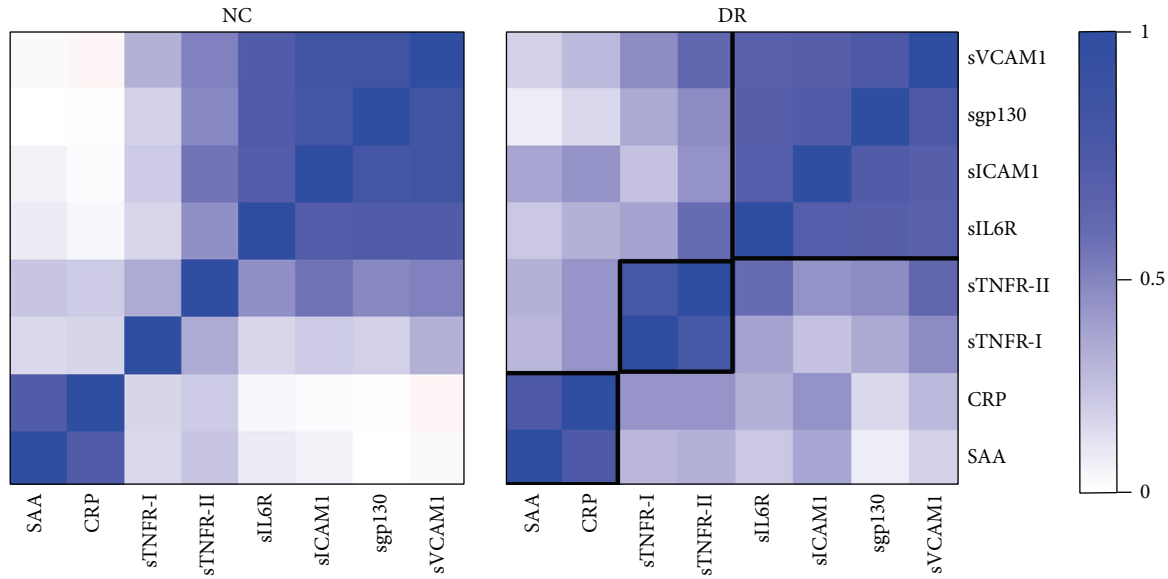

Figure 2: Three clusters of functionally related proteins with strong positive correlations. The pairwise correlations between all eight proteins were examined in T1D patients with and without DR separately. Correlation between individual protein levels was computed using Pearson correlation coefficient. Clustering and visualization of correlation matrix was performed using hierarchical clustering method and heatmap. Three clusters of functionally related proteins were found with strong positive correlations.

Table 2: Multivariate logistic regression analysis for proteins after adjustment for age, sex, and disease duration.

	Model 1 OR (CI 95%) *P* value	Model 2 OR (CI 95%) *P* value	Model 3 OR (CI 95%) *P* value	Model 4 OR (CI 95%) *P* value
sTNFR-I	1.83 (1.49–2.28) 2.67×10^{-8}	1.62 (1.31–2.02) 3.24×10^{-9}	1.67 (1.35–2.09) 4.00×10^{-6}	1.57 (1.25–2.00) 1.66×10^{-4}
sTNFR-II	1.54 (1.24–1.97) 2.46×10^{-4}	1.32 (1.09–1.64) 7.44×10^{-3}	1.32 (1.09–1.64) 7.67×10^{-3}	1.18 (0.97–1.46) 0.113
CRP	1.16 (1.06–1.27) 1.51×10^{-3}	1.16 (1.06–1.28) 2.68×10^{-3}	1.16 (1.05–1.28) 4.07×10^{-3}	1.15 (1.04–1.29) 9.37×10^{-3}
SAA	1.10 (1.01–1.19) 0.025	1.07 (0.98–1.16) 0.145	1.06 (0.97–1.16) 0.200	1.10 (1.00–1.22) 0.046
sgp130	1.59 (1.21–2.14) 1.61×10^{-3}	1.58 (1.18–2.15) 2.71×10^{-3}	1.58 (1.19–2.16) 2.71×10^{-3}	1.43 (1.05–1.97) 0.026
sIL6R	1.26 (1.00–1.63) 0.069	1.27 (0.99–1.69) 0.077	1.28 (1.00–1.70) 0.070	1.25 (0.96–1.66) 0.108
sVCAM1	1.30 (1.05–1.64) 0.021	1.28 (1.01–1.63) 0.044	1.28 (1.01–1.63) 0.046	1.27 (0.98–1.65) 0.074
sICAM1	1.59 (1.25–2.04) 1.65×10^{-4}	1.56 (1.20–2.03) 8.6×10^{-4}	1.54 (1.19–2.01) 1.08×10^{-3}	1.42 (1.07–1.89) 0.015

Model 1: no adjustments, model 2: adjusted for age, model 3: adjusted for age and sex, and model 4: adjusted for age, sex, and T1D duration.

in IL-6 trans-signaling. Increasing evidences suggest that IL-6 pathway plays a prominent role in the pathogenesis of DR and IL-6 and its soluble receptor (sIL-6R) operate as central regulators of the inflammatory processes [23, 24]. The effect of IL-6 on target cells is mediated by a complex receptor system, composed of IL-6R (gp80) and a signal-transducing glycoprotein (gp130) [25]. IL-6 signals to target cells by binding to the cell-surface IL-6R receptors known as "classic" signaling pathway. On the other hand, IL-6/sIL-6R complex can also bind to cell-surface glycoprotein 130 (gp130) on cells which do not express the IL-6R. This process has been called "IL-6 trans-signaling" mediated by gp130. The recent findings implicate IL-6 trans-signaling in inflammation and related diseases in humans and mice [26–28]. In animal models of inflammation it has been shown that sgp130 administration decreases disease severity [29–31]. Elevated sgp130 serum

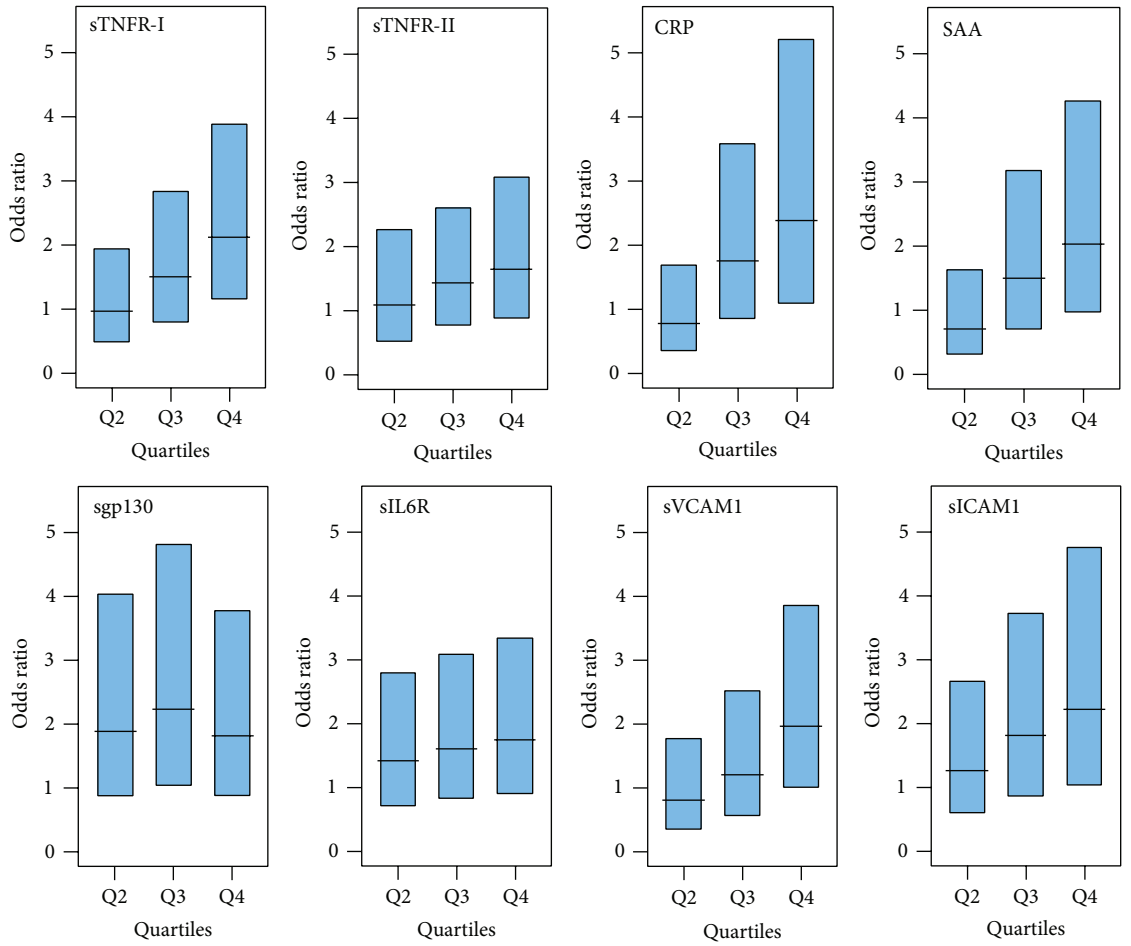

Figure 3: Strong association of increasing protein levels with DR. Conditional logistic regression was performed on matched paired data using cases (T1D with DR) and controls (T1D without complication) matched for age, sex, and T1D duration (183 pairs). Subjects were divided into four quartiles based on individual protein levels. The odds ratios and 95% confidence intervals (CI) were computed for each protein using lowest quartile as referent. Compared with subjects in the bottom quartile, subjects in the top quartile had the highest risk of DR for all eight proteins. Also, an increased trend in the risk for DR was observed from quartile-2 to quartile-4 of protein concentrations.

Table 3: Baseline characteristics of T1D subjects without complications and with diabetic retinopathy after matching.

Patient characteristics	Without any complications	With diabetic retinopathy
Subjects (n)	183	183
Female (%)	37.7	37.7
Age (years)	48.15 ± 10.7	48.9 ± 11.5
Age range (years)	21.7 to 70.5	24.6 to 73.8
Duration of disease	30.7 ± 10.7	30.9 ± 10.3
Diagnosis age	17.8 ± 12.4	18.2 ± 12.5
Systolic BP (mmHg)	120.7 ± 10.4	124.1 ± 12.2
Diastolic BP (mmHg)	72.3 ± 6.2	72.7 ± 8.3
Diabetic nephropathy (%)	0	0
Diabetic neuropathy (%)	0	41.0 ($n = 75$)
CAD (%)	0	13.6 ($n = 25$)

concentrations were found in inflammatory diseases, such as Crohn's disease, rheumatoid arthritis, or inflammatory colon cancer [32–34]. Further studies will be required to elucidate the mechanisms of sgp130 regulation and the implications of targeting it as a therapeutic agent in DR.

The rapid, massive shedding of membrane-bound ICAM-1 from EC leads to an increase in circulating soluble ICAM-1 concentration which has been reported as a biomarker for inflammation and EC activation [35]. We measured these markers of endothelial activation and found them to be higher in T1D patients with DR. These molecules are expressed on the endothelial cell surface and their increased production results in recruitment and activation of granulocytes, monocytes/macrophages, and lymphocytes at the damaged tissue site [36]. These selectins also mediate initial rolling of leukocytes along the endothelium and play important roles in the firm attachment and transendothelial migration of leukocytes. TNF-α has been shown to induce expression of both ICAM and VCAM in endothelial cells [11].

Thus, our study suggests that serum sICAM1 and sVCAM1 concentrations may reflect TNF-α mediated progression and severity of DR associated with T1D.

Studies have also shown the association of inflammatory biomarkers such as CRP and SAA with T1D and other microvascular complications [37–40]. CRP induces proinflammatory effects through overproduction of ICAM-1 and VCAM-1 adhesion molecules. Also, IL-6 activation leads to production of CRP which has been previously shown to be elevated in adults with T1D [37, 41]. In the Diabetes Autoimmunity Study of the Young (DAISY), elevated CRP levels were more frequent in children who later developed T1D and provide evidence that the disease is an immunoinflammatory disorder [37]. CRP is shown to mediate endothelial dysfunction by inhibiting endothelium-dependent NO-mediated dilation in retinal arterioles by producing superoxide from NADPH oxidase [42]. Recently, a study in a rat model has shown that elevated CRP levels are associated with increased cardiovascular events and endothelial dysfunction [43]. Another study has reported that elevated CRP levels in T1D patients were not associated with glycemic control but reflected a low-grade inflammation associated with the activation of innate immune activity [37]. In our study, circulating levels of CRP and SAA (a similar inflammatory marker) were increased in T1D subjects with DR as compared to T1D patients with no complications.

In conclusion, this study reveals that serum levels of TNF receptors, adhesion molecules, and other inflammatory mediators could be used as surrogate endpoints in studies of interventions to decrease inflammation among subjects with T1D. Significant associations between systemic markers of inflammation highlight that subclinical inflammation might be a mechanism through which hyperglycemia causes DR with endothelial impairment playing an important role in the pathogenesis of DR. However, future studies will be required to determine the precise understanding of whether these elevated biomarkers are participating in or are an indicator of DR progression.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

LPA Promotes T Cell Recruitment through Synthesis of CXCL13

Weili Hui, Chenqi Zhao, and Sylvain G. Bourgoin

Rheumatology and Immunology Research Center, CHU de Québec Research Center and Faculty of Medicine, Laval University, 2705 Laurier Boulevard, Québec, QC, Canada G1V 4G2

Correspondence should be addressed to Sylvain G. Bourgoin; sylvain.bourgoin@crchul.ulaval.ca

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Lysophosphatidic acid (LPA) is a bioactive phospholipid playing an important role in various inflammatory diseases by inducing expression and secretion of many inflammatory cytokines/chemokines. Here we report in a murine air pouch model of inflammation that LPA induced CXCL13 secretion in a time-dependent manner and with exacerbation of the response when LPA was administered after a pretreatment with TNF-α, a key inflammatory cytokine. LPA mediates recruitment of leukocytes, including that of $CD3^+$ cells into unprimed and TNF-α-primed air pouches. CXCL13 neutralization using a blocking antibody injected into air pouches prior to administration of LPA into TNF-α-primed air pouches decreased $CD3^+$ cell influx. Our data highlight that LPA-mediated CXCL13 secretion plays a role in T cell recruitment and participates in regulation of the inflammatory response.

1. Introduction

Lysophosphatidic acid (LPA) is a bioactive phospholipid with a simple structure containing a three-carbon glycerol backbone and a single acyl side chain that can vary in length and saturation [1]. By binding to and activation of its specific G protein-coupled receptors (GPCRs), LPA has been shown to evoke a great diversity of cellular responses, pointing out its important role in various physiological and pathophysiological situations [2, 3]. Increasing numbers of studies show that LPA plays a role in various inflammatory diseases [4–6]. Elevated LPA levels were detected in several biological fluids collected from different animal models of inflammation or patients with inflammatory diseases [2, 7, 8]. Increased expression of the LPA producing enzyme autotaxin (ATX) has also been reported in synovial tissues from patients with rheumatoid arthritis (RA) [8, 9]. Elevated expression of ATX and/or aberrant expression of LPA receptors were also found in several human malignancies [10]. LPA not only acts as a mediator implicated in cellular migration, growth, and immune cell chemokinesis, but also promotes directed cell motility indirectly by inducing cytokine/chemokine secretion [11]. CXCL8 is one of the leukocyte chemoattractant chemokines reported to be induced by LPA in fibroblast-like synoviocytes from RA patients [9], as well as various other cell types [12–15].

Chemokines play a key role in cellular trafficking of leukocytes during inflammation and immune surveillance [16]. CXCL13 is a CXC chemokine characterized as the sole B cell chemoattractant signal, originally named BLC and BCA-1 [17]. The only known receptor for CXCL13 is CXCR5, which exclusively binds CXCL13 [18]. CXCR5 is expressed by mature B cells [19], a subset of CD4+ and CD8+ T cells in secondary lymphoid tissue follicles [20], immature dendritic cells (DCs) [21], and macrophages [22]. The CXCL13-CXCR5 axis regulates T cell migration to the germinal centers in lymphoid tissues for early T-B cell collaboration and B cell activation [23], induction of migration of immature DCs [21], and maintenance of epithelial cell angiostatic activity [24]. Human CXCL13 is also reported to be an agonist of human CXCR3 receptor, which plays an important role in recruitment of activated T cells into secondary lymphoid tissues [25]. CXCL13 has been considered a putative diagnostic marker for some acute or chronic infectious and inflammatory diseases [26–30]. Increased CXCL13 production by osteoblasts from osteoarthritis patients in response to stimulation with IL-1β has been reported [31].

In the mouse air pouch model of inflammation activation of LPA1 and LPA3 receptors regulates leukocyte recruitment mainly through CXCL1 chemokine synthesis and its cognate receptor CXCR2 [11]. In this model TNF-α, which is a key inflammatory cytokine in autoimmune diseases such as RA, increases the expression of LPA1 and LPA3 in the air pouch lining tissue. By mimicking a proinflammatory environment, priming of air pouches or of human synovial fibroblasts with TNF-α exacerbates cytokine/chemokine secretion in response to LPA [9, 11]. Even though LPA induces the secretion of numerous cytokines/chemokines, whether LPA is able to recruit leukocytes including T and B cells to an inflammatory site through synthesis of CXCL13 has not been investigated. We used the murine air pouch model to assess the interaction between LPA, CXCL13, and lymphocyte recruitment after local pretreatment with TNF-α, which mimics severe inflammation *in vivo*. Neutralization of CXCL13 with a blocking antibody was also performed to determine whether LPA-mediated CXCL13 secretion regulates recruitment of leukocyte subsets into the air pouches. We demonstrate that LPA induces the recruitment of leukocytes including T lymphocytes into air pouches through a mechanism that is mostly dependent on CXCL13 synthesis.

2. Materials and Methods

2.1. Materials. Oleoyl-sn-glycero-3-phosphate (LPA) was purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Murine TNF-α was from PeproTech Inc. (Rocky Hill, NJ, USA). CXCL13 ELISA dual kit, rat anti-mouse CXCL13 antibody (rat IgG2A, clone 143614), control rat IgG2A (clone 54447), and Proteome ProfilerTM Mouse Cytokine Array Panel A were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Anti-CD16/CD32, anti-mouse CD11b-eV450, and their matched isotype controls were from eBioscience (San Diego, CA, USA). Anti-mouse CD3e-APC, anti-mouse CD19-PE, and their matched isotype controls were from BD Bioscience (San Diego, CA, USA). All other reagents were obtained from Sigma-Aldrich Canada (Oakville, ON, Canada).

2.2. The Air Pouch Model. Female Balb/c (wild type) mice 6–8 weeks old (Charles River, St.-Colomban, Canada) were used to create air pouches. All experimental procedures carried out on mice were approved by the Animal Care Committee at Laval University and conformed to the Canadian Council on Animal Care standards and guidelines.

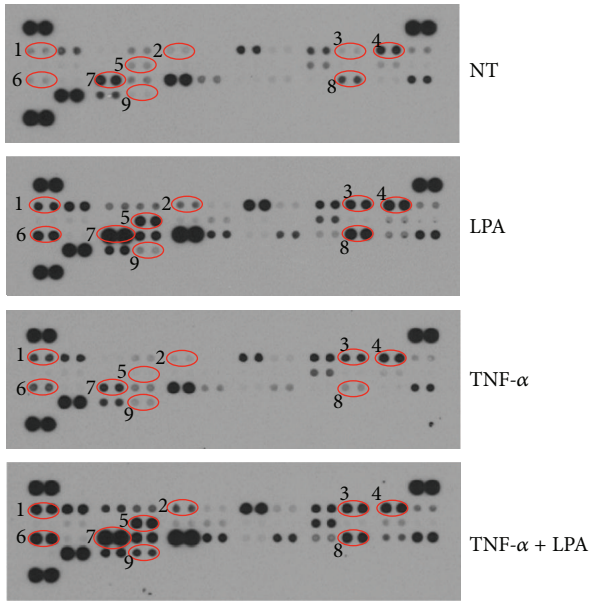
Air pouches were raised on the dorsum of mice by subcutaneous injection of 3 mL sterile air on days 0 and 3 as previously described [11]. Before the injection of air, mice were briefly anesthetized with isoflurane. On day 7, LPA (3 μg) in 1 mL of phosphate-buffered saline (PBS) supplemented with 0.1% endotoxin-free delipidated bovine serum albumin (BSA) was injected into air pouches. TNF-α (50 ng) was injected into air pouches 16 h prior to stimulation with LPA or administration of the CXCL13 neutralizing antibody. To assess the impact of CXCL13 neutralization on LPA-induced leukocyte recruitment, the rat anti-mouse CXCL13 blocking antibody (10 μg) was injected into the air pouch 15 min prior to stimulation with LPA. At specific times, mice were anesthetized with isoflurane and killed by asphyxiation using CO_2. Air pouches were washed twice with 1 mL of PBS containing 5 mM EDTA and harvested pouch fluids were centrifuged 5 min at 3000 rpm. The supernatants were collected and kept at -80°C for later cytokine/chemokine measurements. Cell pellets were suspended in PBS-EDTA and counted using the Moxi mini automated cell counter (ORFLO, Hailey, ID, USA) prior to cell staining for flow cytometry analyses.

2.3. Flow Cytometry Analysis. For flow cytometry analysis, the cell pellets from each group of mice were suspended in flow cytometry staining buffer (eBioscience, San Diego, CA, USA) and the cells were pooled into one tube. The samples were then incubated with the anti-CD16/CD32 antibody (0.5 μg/10^6 cells) for 15 min on ice for FcγR blocking prior to cell staining with 0.1 μg of anti-mouse CD11b-eV450, 0.1 μg of CD3e-APC, and 0.1 μg of anti-mouse CD19-PE for 45 min. Cell suspensions were then processed for FACS analysis using a FACSCalibur (Becton Dickinson, Mississauga, ON, Canada).

To prepare single cell suspension of splenocytes, the spleens from mice with inflamed air pouches were collected, mechanically disrupted, and passed through a strainer according to the manufacturer's instructions (BD Bioscience). FACS analysis used mouse splenocytes as positive controls for titration of anti-CD3 and anti-CD19 antibodies and gating of $CD3^+$ and $CD19^+$ cells.

2.4. Assessment of CXCL13 Secretion in the Air Pouch Lavage Fluids. The air pouch exudates from each treatment group (5–10 mice) were pooled and incubated with the Proteome Profiler Mouse Antibody Array Panel A according to the manufacturer's instructions for qualitative and semiquantitative analysis of cytokine/chemokine production by densitometry. Each pair of duplicate spots on the film represents a specific cytokine/chemokine. For accurate quantification of the levels of CXCL13 in air pouch lavage fluids from each mouse, a CXCL13 ELISA was performed according to the manufacturer's instructions (R&D Systems). Each sample was tested in duplicate and the results were compared with a standard curve that was generated using known concentrations of CXCL13. The dynamic range of the CXCL13 ELISA is 15.6 pg/mL–1000 pg/mL.

2.5. Statistical Analysis. Experiments were performed with 5–10 mice/group and results are expressed as mean ± SE of representative studies. All statistical analysis was performed using Prism 5.0 software. Statistical significance of the difference between samples of two different treatments was determined by t-test (two-tailed P value). For the time course studies, statistical significance between nontreated (NT) samples or samples treated at 0 h and those treated for the indicated time points was determined by one-way ANOVA, Dunnett's multiple comparison test. Multiple comparisons in the same experiment were made using one-way ANOVA,



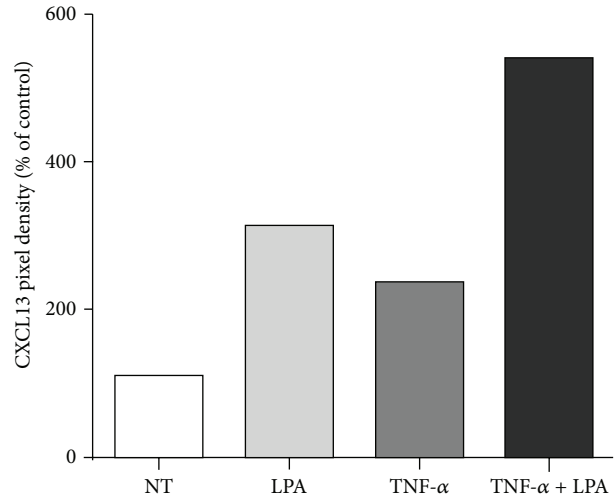


Figure 1: Effect of LPA on CXCL13 secretion in the murine air pouch with or without TNF-α pretreatment. (a) Six-day-old air pouches were produced in the dorsal skin of mice and injected with TNF-α or the vehicle for 16 h prior to stimulation with LPA for 2 h. The nontreated (NT) group was injected with vehicle only (PBS-BSA). The air pouch exudates ($n = 3$) were collected and pooled for qualitative analysis of cytokine/chemokine secretion using the Proteome Profiler Mouse Antibody Array Panel (a). (b) Normalized data representing CXCL13 pixel density are the mean from two independent experiments.

Bonferroni multiple comparison test. P values less than 0.05 were considered statistically significant.

3. Results

3.1. LPA-Mediated Release of CXCL13. LPA injected into air pouches has been reported to induce the synthesis of multiple cytokines/chemokines including IL-6, IL-1β, IL-16, KC, IP-10, and MIP-2 [4, 11]. Whereas the chemokine CXCL1 (also named KC or Gro-α) plays a role in LPA-mediated leukocyte recruitment into the mouse air pouch, blocking CXCL1 or its receptor CXCR2 does not completely reduce leukocyte influx suggesting the involvement of other chemokines or inflammatory mediators [4, 11]. In this series of experiments we focused on LPA-induced CXCL13 secretion into the air pouches. As previously reported [11], injection of 3 μg LPA into the air pouch for 2 hours increases the secretion of CXCL13 as assessed using a qualitative mouse Cytokine/Chemokine Antibody Array assay (Figure 1(a)). Pretreatment of the air pouch tissues with TNF-α (50 ng) for 16 hours also increased the levels of CXCL-13 in the air pouch exudates relative to mice injected with vehicle alone. The combined effect of TNF-α pretreatment prior to LPA stimulation enhances CXCL13 synthesis as estimated by densitometry (Figure 1(b)).

ELISA was then used to accurately quantify the kinetics of CXCL13 secretion (Figure 2(a)). The release of CXCL13 was significantly increased at 30 min after LPA stimulation and remained elevated up to 4 hours, the last time tested. TNF-α injected into the air pouches also induced CXCL13 secretion in a time-dependent manner (Figure 2(b)). A significant increase in CXCL13 secretion was observed at 4 hours and reached a maximum at 12 hours after TNF-α treatment, after which it declined. Although not statistically significant, a trend for higher levels of CXCL13 in air pouch lavage fluids at 16 hours following TNF-α treatment was observed compared to mice injected with vehicle alone (Figures 2(b) and 2(c)). When air pouches were pretreated with TNF-α for 16 hours, LPA induced robust secretion of CXCL13, which peaked at 2–4 hours after LPA stimulation (Figure 2(c)). TNF-α injected into the air pouches prior to LPA stimulation for 2 hours greatly potentiated CXCL13 secretion compared to mice injected with TNF-α alone or LPA alone (Figure 2(d)).

3.2. LPA Recruits Various Leukocyte Subtypes into the Air Pouch. Since CXCL13 is a ligand for CXCR5, a chemokine

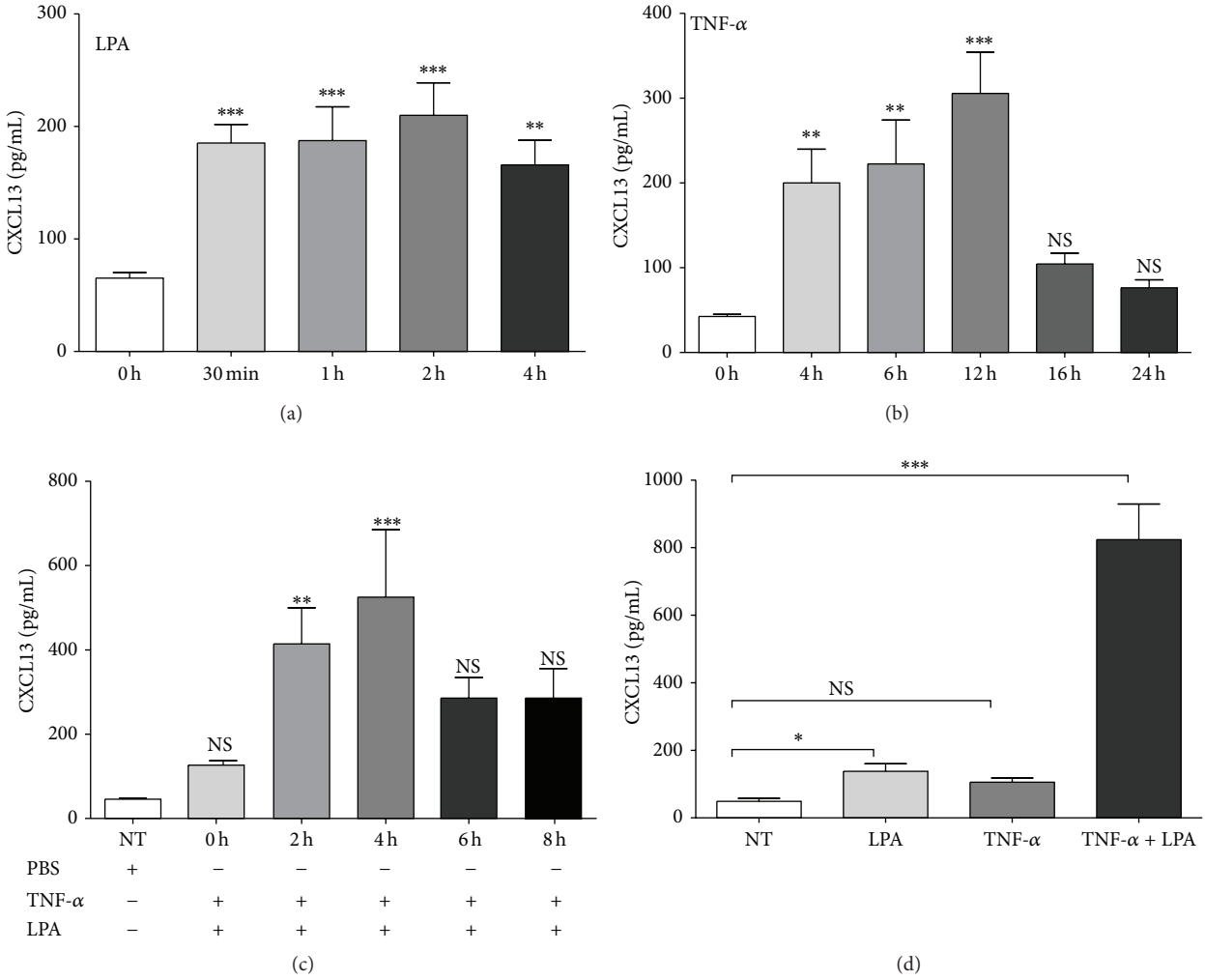


Figure 2: Effect of LPA and TNF-α on CXCL13 secretion in the air pouch. (a), (b) Kinetics of LPA and of TNF-α-mediated CXCL13 secretion. (a) LPA (3 μg) or (b) TNF-α (50 ng) was injected into air pouches and air pouch exudates were collected at indicated times. (c) Kinetics of LPA-induced CXCL13 secretion in air pouches pretreated with TNF-α. TNF-α was injected 16 h before LPA stimulation. Air pouch exudates were collected at indicated times. (d) Comparison of LPA-mediated CXCL13 secretion in untreated and TNF-α-primed air pouches. TNF-α or vehicle was injected 16 h prior to administration of LPA for 2 h. Exudates were collected and cytokine/chemokine secretion was measured by ELISA. The nontreated (NT) groups were injected with vehicle only (PBS-BSA). Data are the mean ± SE from three independent experiments performed with at least five mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

receptor expressed by mature B cells [19], and a subset of $CD4^+$ and $CD8^+$ T cells [20], we next determined whether LPA-mediated CXCL13 secretion contributes to recruitment of leukocyte subsets toward LPA into TNF-α-pretreated air pouches. As reported previously for LPA alone [11], LPA injected in TNF-α-pretreated air pouches (16 hours) stimulated the recruitment of leukocytes in a time-dependent manner (Figure 3(a)). An increase in the number of migrated leukocytes was detectable 2 h after LPA injection, peaked after 6 h, and declined thereafter. $CD11b^+$ cells were the most prominent population in air pouch lavage fluids (Figure 3(b), left panel). We focused on $CD11b^-$ cells and performed CD19-labelling to determine by FACS whether $CD19^+$ B lymphocytes could be detected in air pouch exudates. Even though the CD19-PE antibody labeled B cells isolated from mouse spleens (Figure 3(c)), no $CD19^+$ B lymphocytes were detected in air pouch exudates (Figure 3(b), middle panel). However, $CD11b^-/CD3^+$ cells were detected in air pouch lavage fluids (Figure 3(b), right panel). Figure 3(d) shows that stimulation with LPA for 6 hours enhanced significantly the number of $CD3^+$ cells in air pouch exudates ($3.07 \pm 0.53 \times 10^4$ cells, $1.67 \pm 0.03\%$ of total leukocytes, $n = 10$) compared to mice injected with vehicle alone ($1.7 \pm 0.32 \times 10^4$ cells, $1.88 \pm 0.01\%$ of total leukocytes, $n = 10$). The number of $CD3^+$ cells in air pouch lavage fluids collected from TNF-α-pretreated air pouches was not different from that of mice injected with the vehicle alone. Furthermore, LPA injected into TNF-α-primed air pouches stimulated the recruitment of $CD3^+$ cells in a time-dependent manner (Figure 3(e)). As observed for total leukocytes, recruitment of $CD3^+$ cells

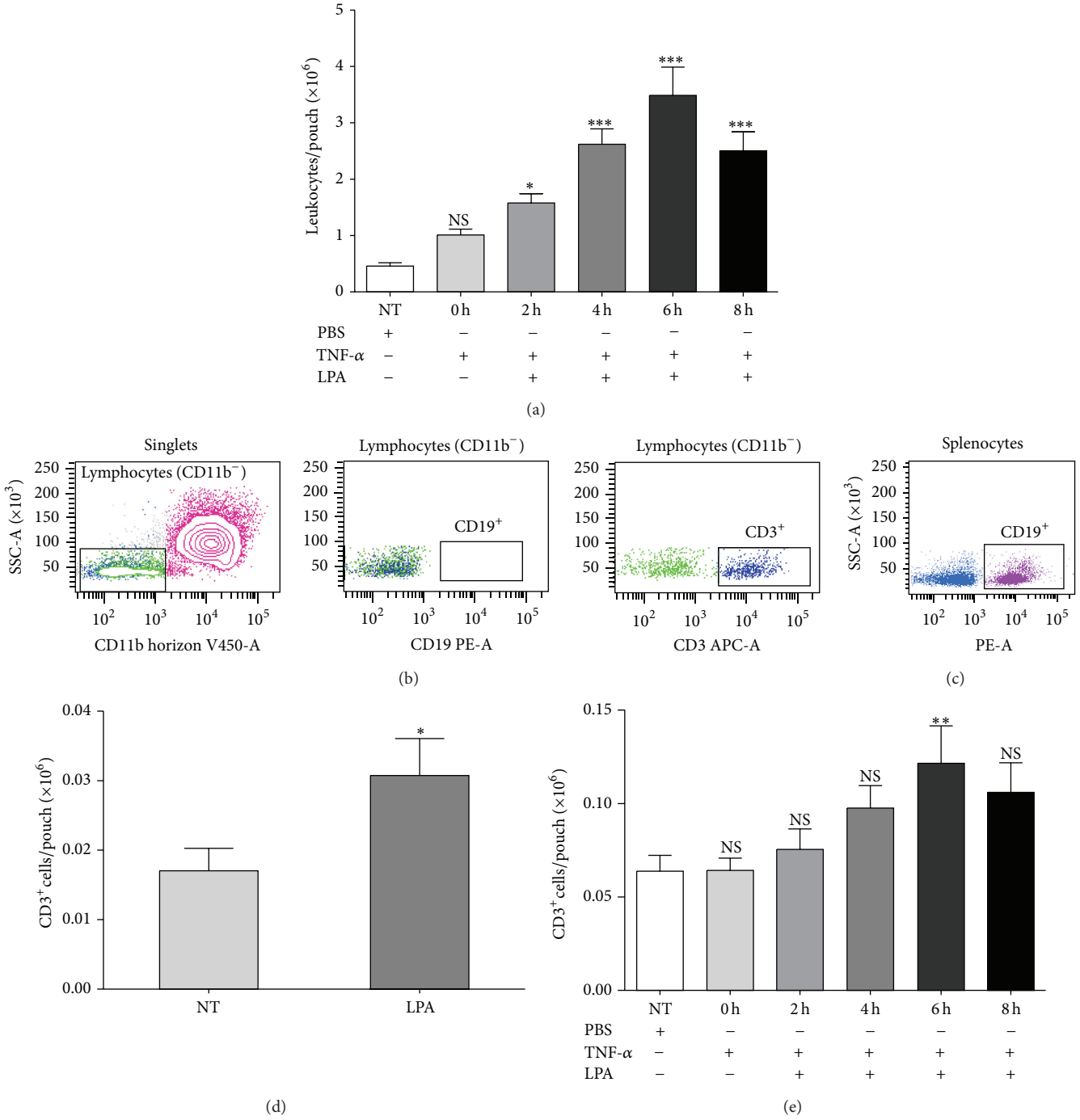


FIGURE 3: LPA-induced leukocyte recruitment in untreated and TNF-α-primed air pouches. (a) Kinetics of LPA-mediated leukocyte recruitment into TNF-α-treated air pouches. TNF-α (50 ng) was injected into the air pouches 16 h prior to stimulation with 3 μg LPA for the indicated times. Air pouch exudates were collected and the number of leukocytes was determined as described in Section 2. (b) Leukocyte populations in lavage fluids collected from air pouches pretreated with TNF-α for 16 h and injected with LPA for 6 h. Cells were stained with various leukocyte markers and analyzed by flow cytometry. The CD11b$^-$ cells were defined as lymphocytes according to their low granularity (left panel), which stained positive for CD3 (T cells, right panel) or CD19 (B cells, middle panel). (c) Labelling of B cells isolated from mouse spleen. Splenocytes were prepared as described in Section 2 and used for titration of anti-CD3e and anti-CD19 antibodies. (d) LPA-induced CD3$^+$ cell recruitment into the air pouches. Air pouch exudates were collected at 6 h after LPA injection. The total number of leukocytes was measured and that of T cells determined by flow cytometry. (e) The absolute numbers of CD3$^+$ cells recruited by LPA into TNF-α-primed air pouches was evaluated as described in (d). Data are the mean ± SE from 6 mice/group. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by analysis of variance. SSC: side scatter; FSC: forward scatter.

peaked at the 6-hour time point following injection of LPA into air pouches ($1.21 \pm 0.19 \times 10^5$ cells, $2.5 \pm 0.9\%$ of total leukocytes, $n = 5$).

3.3. Effect of Blocking CXCL13 on LPA-Mediated CD3$^+$ Cell Recruitment. It was reported previously that antibody neutralization of CXCL13 can prevent migration of double-negative regulatory T lymphocytes to cardiac allografts implanted in the abdomen of mice [32]. To examine whether a correlation exists between CXCL13 secretion in response to LPA and CD3$^+$ lymphocyte recruitment in our mouse model, a neutralizing anti-CXCL13 antibody was injected into TNF-α-primed air pouches prior to LPA stimulation. Injection of the neutralizing antibody against CXCL13 prior to LPA into the air pouch significantly reduced LPA-induced CD3$^+$ lymphocyte recruitment into TNF-α-primed air pouches, whereas the isotype control antibody had no significant effect on LPA-mediated CD3$^+$ lymphocyte influx (Figure 4). Taken together, the data indicate that CXCL13 plays a role in LPA-mediated recruitment of CD3$^+$ lymphocytes into the air pouches.

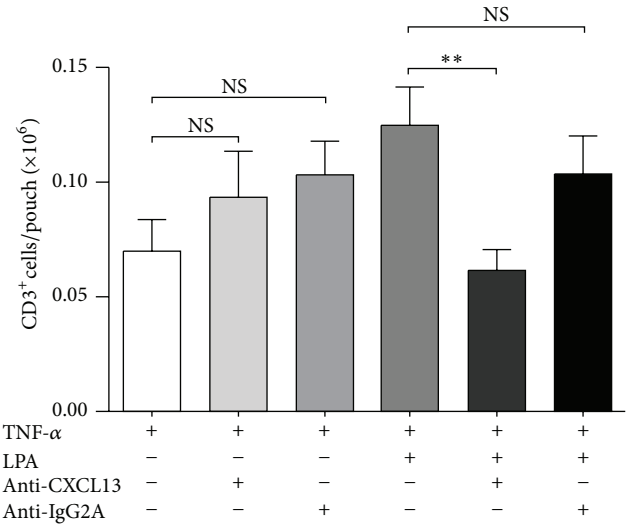


Figure 4: Effect of the CXCL13 neutralizing antibody on LPA-mediated lymphocyte recruitment into TNF-α-primed air pouches. Air pouch tissues were pretreated with TNF-α (50 ng) for 16 h prior to administration of LPA (3 μg). Where indicated, the anti-CXCL13 neutralizing antibody or the isotype control antibody (IgG2A, 10 μg) was administered into the air pouches 15 min prior to stimulation with LPA for 4 h. The absolute numbers of CD3$^+$ T cells in air pouch lavage fluids were determined by flow cytometry as described in Section 2. Data are the mean ± SE of at least 5 mice/group. $^{**}P < 0.01$ by analysis of variance.

4. Discussion

Extracellular LPA is a bioactive lysophospholipid produced by ATX that mediates its effects through activation of various LPA receptors [2]. Using the mouse air pouch model of inflammation, we previously demonstrated that LPA promotes the influx of neutrophils and other leukocyte subtypes including macrophages/monocytes and lymphocytes through activation of two LPA receptors, LPA1 and LPA3 [11]. Stimulation of these LPA receptors expressed by cells lining the air pouch cavity promotes the synthesis of various chemokines/cytokines (IL-6, IL-1β, IL-16, KC, IP-10, MIP-2, and CXCL13), the synthesis of which is greatly enhanced by TNF-α injected into the air pouches 16 hours prior to LPA. Although LPA-mediated KC synthesis was shown to play a predominant role in the recruitment of leukocytes into the air pouches, neutralization of KC or blocking of its cognate receptor CXCR2 was not able to totally abrogate the influx of leukocytes [11]. In the present study, we focused on CXCL13, a key chemoattractant of B cells and of subsets of T lymphocytes [19–21]. We report that administration of LPA or TNF-α into air pouches increased the levels of CXCL13 in air pouch lavage fluids in a time-dependent manner. The combination of a pretreatment of the air pouch tissues with TNF-α prior to LPA stimulation greatly enhanced LPA-mediated CXCL13 secretion. The release of CXCL13 induced by LPA peaked 2 hours ahead of the time point of maximal leukocyte recruitment, including that of CD3$^+$ immune cells. Consistent with a role for CXCL13 in LPA-mediated CD3$^+$ cell homing, antibody neutralization of CXCL13 prevented the influx of these T cells into TNF-α-pretreated air pouches.

Elevated levels of ATX and of LPA have been reported in synovial fluids collected from RA patients [4, 7–9]. The ATX-LPA axis is emerging as a regulator of lymphocyte homing and inflammation [33]. ATX binds to lymphocytes in a $\alpha 4\beta 1$-dependent manner [34]. Through activation of LPA receptors expressed by T cells [33], LPA induces a polarized morphology that is required for transendothelium migration, a key step for regulation of naive T cell entry into secondary lymphoid organs [34, 35]. However, LPA does not promote directed cell migration *in vitro* but exerts a chemokinetic effect that increases the chemoattractant effect of chemokines regulating human and mouse T cell homing in various tissues [34–37]. We cannot exclude the possibility that LPA injected into air pouches has a motility-stimulating effect on leukocytes. However, we showed that CXCL13 secretion induced by LPA preceded the peak of leukocyte recruitment, including that of CD3$^+$ cells by several hours. There was a twofold increase in the total number of CD3$^+$cells in lavage fluids from TNF-α-pretreated air pouches after 6 hours of stimulation with LPA, but when expressed as a percentage of total leukocytes no increase in the relative abundance of CD3$^+$ cells was noticed due to the massive recruitment of CD11b$^+$ cells (i.e., neutrophils) at this time point [11]. Antibody neutralization of CXCL13 prior to LPA stimulation suggests that LPA was exerting its effect on CD3$^+$ cell influx into TNF-α-pretreated air pouches in a manner that is dependent on CXCL13 synthesis. The lack of small molecule inhibitors of CXCR5 or CXCR5 neutralizing antibodies precluded further analyses of the molecular pathways by which LPA recruits CD3$^+$ cells in this *in vivo* model of inflammation.

As CXCL13 is a ligand of CXCR5, which is expressed on B cells, macrophages, monocytes, double-negative Treg cells [32], and T helper cells in human and mouse [22, 38–40], it is possible that CXCL13 induced by LPA could play an important role in leukocyte homing in various diseases.

CXCL13 was originally identified as a B cell chemoattractant [17]. Furthermore, lysophospholipids such as S1P and LPA have been suggested to regulate splenic B cell homing through CXCL13-mediated integrin-dependent adhesion [41]. Recruitment of B cells into the air pouch wall has been reported following stimulation with oxidized phospholipids and LPS [42]. In our experiments, after blocking Fc receptors with an anti-CD16/CD32 antibody, no B cells were detected in the lavage fluids collected after stimulation of TNF-α-pretreated air pouches with LPA. Whether B cells are recruited by LPA and remain sequestered in the air pouch wall will need further studies.

DCs [43], human monocytes/macrophages, and $CD4^+$ T cell subsets are potent inducible sources of CXCL13 [44, 45]. We report that the basal levels of CXCL13 in air pouch lavage fluids increased quickly in response to LPA and well before an increase in immune cell influx could be monitored. CXCL13 secretion could be mediated through binding of LPA to its cognate receptors, possibly LPA1 and LPA3 [11], expressed by air pouch lining cells or discrete populations of cells recruited early. Although identification of cells that contribute to CXCL13 production awaits further characterization, this study suggests that a role for the CXCL13-CXCR5 axis in LPA-mediated regulation of immune cell trafficking to sites of inflammation cannot be ignored.

CXCL13 has been identified as a serologic marker predictive of disease severity in early RA [28, 29]. High levels of CXCL13 were measured in synovial fluids from RA patients, with RA synovial T helper cells contributing to CXCL13 secretion [46, 47]. Within the RA synovium CXCL13 is expressed in areas of B cell accumulation characteristic of ectopic lymphoid follicles where subtypes of CXCL13-expressing T cells ($CD3^+$ and $CD4^+$) and monocytes/macrophages colocalize [47–49]. The receptor for CXCL13 is upregulated in the RA synovium and associated with the presence of CXCR5 positive B cells and T cells infiltrating the synovia [22]. Of note Zheng et al. [50] reported that neutralization of CXCL13 at the boosting stage reduced the development of ectopic lymphoid follicles and the severity of collagen-induced arthritis in mice.

5. Conclusions

In summary, we provide evidence that LPA-induced CXCL13 secretion contributed to the recruitment of $CD3^+$ T cells within the air pouch environment under conditions of inflammation exacerbated by TNF-α. This study extends the known role of CXCR2 ligand chemokines to the massive recruitment of leukocytes induced by LPA in this mouse model of inflammation [11]. Given that ATX-derived LPA plays a role in the pathogenesis of RA [7–9, 51], LPA-mediated CXCL13 secretion raises the question whether LPA contributes to the recruitment of lymphocytes and extranodal lymphoid neogenesis during chronic inflammation.

Abbreviations

ATX: Autotaxin
DCs: Dendritic cells
LPA: Lysophosphatidic acid
CXCL13: C-X-C motif chemokine 13
CXCL8: C-X-C motif chemokine 8
CXCL1: C-X-C motif chemokine 1
GPCRs: G protein-coupled receptors
RA: Rheumatoid arthritis
TNF-α: Tumor necrosis factor-alpha.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Clinical Study

Vascular Endothelial Growth Factor in Tear Samples of Patients with Systemic Sclerosis

Anikó Rentka,[1] Jolán Hársfalvi,[2] András Berta,[1] Krisztina Köröskényi,[3] Zoltán Szekanecz,[4] Gabriella Szücs,[4] Peter Szodoray,[5] and Ádám Kemény-Beke[1]

[1]*Department of Ophthalmology, University of Debrecen, Nagyerdei Körút 98, Debrecen 4012, Hungary*
[2]*Department of Biophysics and Radiation Biology, Semmelweis University, Tűzoltó Utca 37-47, Budapest 1094, Hungary*
[3]*Institute of Biochemistry and Molecular Biology, Signaling and Apoptosis Research Group, Hungarian Academy of Sciences, Research Center of Molecular Medicine, Nagyerdei Körút 98, Debrecen 4012, Hungary*
[4]*Department of Rheumatology, Institute of Medicine, Faculty of Medicine, University of Debrecen, Nagyerdei Körút 98, Debrecen 4012, Hungary*
[5]*Institute of Immunology, Rikshospitalet, Oslo University Hospital, Sognsvannsveien 20, 0372 Oslo, Norway*

Correspondence should be addressed to Ádám Kemény-Beke; kemenyba@med.unideb.hu

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Background. Systemic sclerosis is an autoimmune disease, characterized by widespread small vessel vasculopathy, immune dysregulation with production of autoantibodies, and progressive fibrosis. Changes in levels of proangiogenic cytokines had already been determined largely in serum. Our aim was to assess the levels of VEGF in human tears of patients with SSC. *Patients and methods*. Forty-three patients (40 female and 3 men, mean (SD) age 61 (48–74) years) with SSc and 27 healthy controls were enrolled in this study. Basal tear sample collection and tear velocity investigations were carried out followed by an ophthalmological examination. Total protein concentrations and VEGF levels were determined in tear samples. *Results*. The average collected tear fluid volume developed 10.4 μL (1.6–31.2) in patients and 15.63 μL (3.68–34.5) in control subjects. The average total protein level was 6.9 μg/μL (1.8–12.3) in tears of patients and control tears contained an average of 4.132 μg/μL (0.1–14.1) protein. In patients with SSc the average concentration of VEGF was 4.9 pg/μL (3.5–8.1) and 6.15 pg/μL (3.84–12.3) in healthy samples. *Conclusions*. Total protein production was increased because of the smaller tear volume. Decreased VEGF in tear of SSc patients can be explained also by the decreased tear secretion of patients.

1. Introduction

Systemic sclerosis (SSc or scleroderma) is a chronic connective tissue disorder which affects mostly the skin and multiple internal organs, including the heart, lung, kidneys, and gastrointestinal tract. SSc is a systemic autoimmune disease characterized by widespread small vessel vasculopathy, immune dysregulation with production of autoantibodies, and progressive fibrosis [1].

There are only few reports available on the ophthalmological complications during the course of systemic sclerosis. Changes in the organ of vision are thought to be the consequence of systemic complications of scleroderma or adverse effects of immunosuppressive treatment applied. Ocular symptoms may occur at any stage of the disease and may involve numerous ocular tissues. Their course can be clinically latent or very intensive. The most common clinical manifestations of soft tissue fibrosis and inflammation in these patients include increased tonus and telangiectasia of eyelid skin. The most commonly reported lesions are periorbital edema, palpebral ectropion, and ciliary madarosis [2]. In our study the most frequent ocular manifestation of SSc was dry eye syndrome (DES).

DES is a major healthcare problem since it affects the patients' quality of life. DES was recently redefined as a multifactorial disease of the tears and ocular surface that results

in symptoms of discomfort, visual disturbance, and tear film instability and after all damage to the ocular surface [3]. Increased osmolality of the tear film [4] and inflammation of the ocular surface [5] are the two major characteristic points in DES. Increased levels of several inflammatory cytokines are the most important laboratory findings [6]. Accordingly, tear cytokine levels are already considered as potential markers of inflammation in DES.

Although the exact origin of SSc is unknown, some predisposing factors—like environmental and infectious agents, tissue injury, and hypoxia or oxidative stress on a susceptible genetic background—may play a role in the disease development [1, 7]. One of the earliest clinical manifestations in the pathophysiology of SSc is vascular injury that can be caused by the imperfect angiogenesis due to impaired production of proangiogenic factors. Vascular endothelial growth factor (VEGF) is one of the most important proangiogenic factors which play a key role in the formation of new blood vessels [8]. VEGF takes part in various steps of angiogenesis, including initial vasodilation, endothelial cell permeability, perivascular matrix remodeling, and induction of proliferation and migration of endothelial cells [9].

Although tear analysis is of increasing interest in ophthalmology, yet no studies have investigated tears of SSc patients, possibly because of its technical challenge due to the small sample volumes available [10].

The aim of this study was to demonstrate a significant difference of VEGF levels in human tears of SSc patients compared to healthy controls. Since tear dynamically reflects the factors present at the ocular surface, this is an ideal sample to investigate; furthermore, it has the potential for earlier diagnosis of SSc with a noninvasive method of collecting samples.

2. Materials and Methods

2.1. Patients and Healthy Controls. Forty-three patients with SSc and 27 healthy controls were included in our study. Patients were enrolled from the outpatient clinic at the Department of Rheumatology, Department of Ophthalmology (40 female and 3 men), mean (SD) age 61 (48–74) years. SSc was diagnosed based on the corresponding international criteria [11, 12].

They went through ophthalmological examination and basal tear sample collection at the Department of Ophthalmology during a 12-month period. DES was diagnosed in 32 of 43 SSc patients. All patients have undergone a broad immunoserological screening, including ANA, anti-SSA, anti-SSB, corresponding ophthalmological tests, and salivary measurement, which excluded the presence of coexisting secondary Sjogren's syndrome.

We enrolled 27 volunteers (21 female and 6 men) as healthy controls who had no history of any autoimmune or ocular disorder. Patients did not take immunosuppressive medications at the time of the tear sampling.

Written informed consent was obtained from all patients and controls. Study protocol was approved by the local bioethics committee and followed the tenets of the declaration of Helsinki.

2.2. Tear Sample Collection. Open eye tears were gently collected from the inferior temporal meniscus of both eyes using a capillary micropipette (Haematokrit-Kapillaren, Na-Heparin 3.0 IU/kapillare, 75 mm/60 μL, Hischmann Laborgerate), minimizing irritation of the ocular surface or lid margin as much as possible. All samples were collected between 11 am and 16 pm by the same physician. Tear secretion velocity was counted by dividing the volume of collected sample by time of secretion. Volume was calculated from the lengths of the fluid column in the capillary tube, measured with a vernier caliper, and from the known diameter of the tube. Time of tear collection was measured with stopwatch. Tears were transferred into low binding capacity Eppendorf tubes by washing out the capillaries five times with PBS-T buffer equal to the volume of tears. Fivefold diluted samples were stored at −80°C until assessment.

2.3. Tear Sample Preparation and Quantification. First, as a point of reference for VEGF, total protein concentrations were determined in tear samples with the BCA Microplate method (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Human albumin was used as standard.

For the quantitative determination of VEGF in tear fluid we used a human VEGF immunoassay kit by Quantikine (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. This assay employs the quantitative sandwich enzyme immunoassay technique.

3. Statistical Analyses

Prism 5 statistical software (GraphPad Software Inc.) was used for statistical analyses. Comparison of values was carried out by Mann-Whitney U test. All values are shown as the mean ± SD. P values less than 0.05 were considered statistically significant.

4. Results

The average tear secretion velocity in patients was 4.53 μL/min, with a median of 3.8 μL/min (1.5–25.6).

Duration of tear sample collection from patients changed between 20 and 313 seconds until the minimally required 5 μL volume was reached.

The average collected tear fluid volume developed 10.4 μL (1.6–31.2) in case of patients and 15.63 μL (3.68–34.5) in case of controls.

In tear samples of patients with SSc the average total protein level was 6.9 μg/μL (1.8–12.3) and the average concentration of VEGF was 4.9 pg/μL (3.5–8.1) in case of basal tear secretion (Figure 1).

Control tears contained an average of 4.132 μg/μL (0.1–14.1) protein and 6.15 pg/μL (3.84–12.3) VEGF (Figure 2).

5. Discussion

Systemic sclerosis is an autoimmune disease affecting the connective tissue and characterized by a wide spectrum of microvascular and immunological abnormalities, leading to

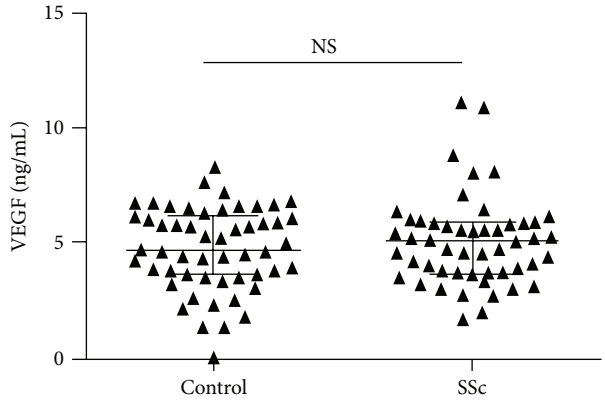

Figure 1: VEGF concentrations (ng/mL) in tears of healthy controls (left) and SSc patients (right).

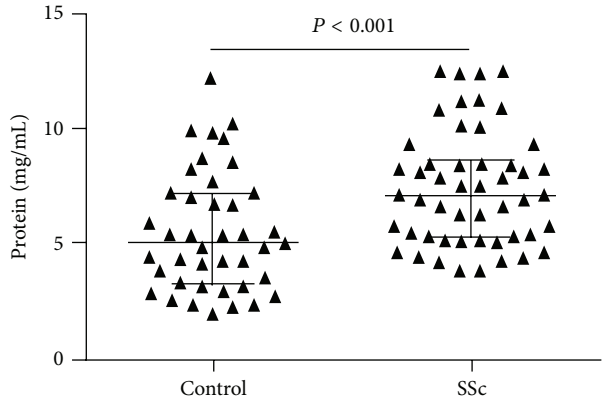

Figure 2: Total protein concentrations (mg/mL) in tears of healthy controls (left) and SSc patients (right).

progressive fibrosis of the skin and other visceral organs, such as lungs, gastrointestinal tract, heart, and kidneys [13]. Many ocular manifestations of SSc have been described including conjunctival telangiectasia, DES, and filamentous keratitis [14].

DES in SSc is believed to be caused by fibrosis-related impairment of lacrimal gland secretion, namely, the water portion of the tear film. Furthermore, the lipid layer disorder is caused by chronic blepharitis and meibomian gland dysfunction (MGD), and also the increased evaporation of tears from the ocular surface is the consequence of the restricted eyelid mobility and the consecutive reduced blinking [15].

Tear plays an essential role in maintaining homeostasis of the ocular surface; therefore changes in the delicate equilibrium of its cytokine composition may lead to various pathophysiological conditions.

The influence of VEGF in ophthalmic diseases is profound. It has been implicated in a large number of retinal diseases and conditions like age-related macular degeneration and diabetic retinopathy, retinopathy of prematurity, sickle cell retinopathy, and retinal vascular occlusion. VEGF has secondary influence in neovascular glaucoma [16] and inherited retinal dystrophies [17]. Since it has been discovered in the 1980s [18] VEGF has raised interest because of its central role in angiogenesis in a number of physiologic and pathologic processes, such as vascular development, wound healing, the female reproductive cycle, cancers, myocardial ischemia, rheumatoid arthritis, and other autoimmune diseases. VEGF is a component of normal tear fluid. Vesaluoma determined VEGF concentrations in healthy tears. The median VEGF concentration was 5 pg/μL (4–11) which corresponds with our results, as control tears contained an average of 6.15 pg/μL (3.84–12.3) VEGF. Vesaluoma and coworkers calculated the average tear fluid secretion in healthy controls, which was 8.1 μL/min (0.7–20.8), using the same tear collecting method as we used in our study. Results show that SSc patients have significantly decreased tear secretion that could be explained by DES, which is a probable sequel of the disease or of the side effects of the therapeutic drugs [19].

Tear secretion velocity was lower by 67% in SSc patients than in healthy controls. The difference was significant ($P < 0.01$). The reason for this sign could be explained by the pathophysiology of the disease, namely, fibrotic processes.

Total protein values in SSc patients were higher by 42% than in healthy controls. It may indicate that total protein production—or simply the protein concentration, since SSc patients have a decreased tear secretion velocity—is only increased because of the smaller tear volume. Though VEGF in tear of SSc patients was decreased by 20% and did not change after stimulation, it can be explained also by the decreased tear secretion of patients.

Although the average VEGF level in tear collected without stimulation was higher than that in tear collected with stimulation in SSc patients, the difference was not significant. The phenomenon why the VEGF levels are not higher in SSc patients than in the healthy group needs further investigations.

Abbreviations

SSc: Systemic sclerosis
DES: Dry eye syndrome
MGD: Meibomian gland dysfunction
VEGF: Vascular endothelial growth factor
MMP: Matrix metalloproteinase
MCP: Monocyte chemoattractant protein
IL: Interleukin
IP: Interferon gamma-induced protein
TNF: Tumor necrosis factor
FasL: Fas ligand.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Progranulin Is Associated with Disease Activity in Patients with Rheumatoid Arthritis

Lucie Andrés Cerezo,[1] Markéta Kuklová,[1] Hana Hulejová,[1] Zdeňka Vernerová,[2] Nikola Kaspříková,[3] David Veigl,[4] Karel Pavelka,[1,5] Jiří Vencovský,[1,5] and Ladislav Šenolt[1,5]

[1] *Institute of Rheumatology, Na Slupi 4, 12850 Prague 2, Czech Republic*
[2] *Department of Pathology, Third Faculty of Medicine, Charles University, Šrobárova 50, 100 34 Prague 10, Czech Republic*
[3] *Institute of Biophysics and Informatics, First Faculty of Medicine, Charles University, Salmovská 1, 120 00 Prague 2, Czech Republic*
[4] *First Orthopaedic Clinic, First Faculty of Medicine, Charles University, V Úvalu 84, 150 06 Prague 5, Czech Republic*
[5] *Department of Rheumatology, First Faculty of Medicine, Charles University, Na Slupi 4, 128 50 Prague 2, Czech Republic*

Correspondence should be addressed to Ladislav Šenolt; senolt@revma.cz

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Objective. Progranulin (PGRN) is implicated in the pathogenesis of rheumatoid arthritis (RA). The aim of this study was to assess the relationship between PGRN and disease activity in RA. *Methods*. PGRN levels were evaluated in patients with RA ($n = 47$) and OA ($n = 42$) and healthy controls ($n = 41$). Immunohistochemical analysis of PGRN in synovial tissues was performed. The association between PGRN and C-reactive protein (CRP), disease activity score (DAS28-CRP), and health assessment questionnaire (HAQ) was studied. *Results*. Circulating PGRN was elevated in patients with RA and OA compared to healthy controls (227.1 ± 100.2 and 221.5 ± 102.5 versus 128.1 ± 34.7 ng/mL; $P < 0.001$). Synovial fluid levels of PGRN were higher in patients with RA compared to OA (384.5 ± 275.3 versus 241.4 ± 165.2 ng/mL; $P = 0.002$). PGRN expression was significantly upregulated in the synovial tissue of RA patients particularly in the inflammatory infiltrates. Serum PGRN levels correlated with DAS28 ($r = 0.327$, $P = 0.049$) and HAQ score ($r = 0.323$, $P = 0.032$), while synovial fluid PGRN correlated only with HAQ ($r = 0.310$, $P = 0.043$) in patients with RA. PGRN levels were not associated with CRP or autoantibodies. *Conclusions*. This study demonstrates increased PGRN expression at local sites of inflammation and association between PGRN levels, disease activity, and functional impairment in patients with RA.

1. Introduction

Progranulin (PGRN), also known as granulin epithelin precursor (GEP), PC-cell-derived growth factor (PCDGF), proepithelin, or acrogranin, is an 80 kDa glycoprotein originally identified as an autocrine growth factor for cancer cells and fibroblasts [1]. PGRN is abundantly expressed in rapidly cycling epithelial cells, leukocytes, chondrocytes, and neurons [2] and is involved in biological processes such as embryogenesis [2], tumorigenesis [1, 3] and wound healing [4], inflammation [5, 6], host defense [7], and cartilage development and degradation [8–10].

PGRN also acts as a regulator of the innate immune response and inflammation [5–7]. Under certain conditions, intact PGRN undergoes proteolysis to generate seven 6 kDa peptides, called granulins [6]. PGRN inhibits, whereas granulins stimulate, the production of neutrophil attracting chemokines, which is neutralized by its degradation on granulins by serine proteases NE and PR3 released by neutrophils and macrophages [5, 6]. Mice lacking PGRN respond to infection with exaggerated inflammation and PGRN-deficient macrophages challenged with microbial LPS upregulate the production of proinflammatory cytokines [7].

There is some evidence that PGRN plays a role in systemic autoimmune inflammatory diseases [11–13]. Tang et al. reported that PGRN-deficient mice are susceptible to collagen induced arthritis, and treatment with PGRN can reduce the symptoms of the disease [11]. Similarly, TNF transgenic mice lacking the gene for PGRN developed more severe inflammatory arthritis [11]. It has been demonstrated that

PGRN binds to tumor necrosis factor receptors (TNFRs) and thereby limits the action of TNFα in inflammatory arthritis [11]. PGRN also potently suppresses cartilage destruction via inhibition of ADAMTS-7/ADAMTS-12-mediated COMP degradation and therefore plays a significant role in preventing the joint destruction in arthritis [8]. Recent study found elevated serum PGRN levels in patients with systemic lupus erythematosus, its association with disease activity, and significant decrease of PGRN serum level after successful treatment [13]. Moreover, Thurner et al. reported the presence of PGRN antibodies that can bind and neutralize PGRN in the sera of patients with rheumatoid arthritis (RA) [14].

Therefore the aim of our study was to assess the PGRN expression in synovial tissue, synovial fluid, and serum in patients with RA and to investigate the relationship between PGRN levels and disease activity.

2. Material and Methods

2.1. Patients. Forty-seven patients with active RA (35 females and 12 males; mean age ± SD: 58.72 ± 12.05 years), 42 patients with knee OA (26 females, 16 males; mean age ± SD: 64.55 ± 11.12 years), and 41 healthy individuals (30 females, 11 males; mean age ± SD: 56.07 ± 7.35 years) were enrolled in this study. All the patients with RA fulfilled the revised criteria of the American College of Rheumatology (ACR) for the diagnosis of RA [15]. Disease activity of RA was assessed according to the 28-Joint Count Disease Activity Score (DAS28-CRP), which was calculated using the number of swollen and tender joints, CRP levels, and patient global health visual analogue scale (VAS). Health Assessment Questionnaire (HAQ) score was assessed. Characteristics of the patients are given in Table 1. All the patients signed informed consent forms, and the study was approved by the Local Ethics Committee of the Institute of Rheumatology in Prague.

Table 1: Patients characteristics.

Characteristics	RA	OA	healthy controls
Patients, *n*	47	42	41
Gender (F/M)	35/12	26/16	31/11
Mean age (years)	58.72 ± 12.05	64.55 ± 11.12	56.07 ± 7.35
CRP (mg/L)	24.23 ± 25.78	3.44 ± 3.53	—
Disease duration (years)	6.81 ± 8.14	7.84 ± 8.79	—
DAS28 score	4.55 ± 1.25	—	—
HAQ score	1.06 ± 0.92	—	—
RF positivity, *n* (%)	28 (60%)	—	—
ACPA positivity, *n* (%)	22 (46%)	—	—
DMARDs/GC	42/32	—	—
Biological therapy	9*	—	—

ACPA, anti-cyclic citrullinated peptide antibody; CRP, C-reactive protein; DAS28 score, disease activity score; DMARDs, disease-modifying antirheumatic drugs; F, female; GC, glucocorticoids; HAQ score, Health Assessment Questionnaire score; M, male; OA, osteoarthritis; RA, rheumatoid arthritis; RF, rheumatoid factor; SJC, swollen joints count. The data are expressed as the mean (±SD).
*Out of 9 patients, 6 were treated with anti-TNF therapy, 1 with tocilizumab, 1 with rituximab and 1 with anti-IL-17 therapy.

2.2. Laboratory Measurements. Blood samples were collected from all the patients when they underwent therapeutic arthrocentesis of the knee or not more than 5 days after arthrocentesis. Paired samples were immediately centrifuged, and both the serum and synovial fluid were stored at −80°C until analysed. Before analysis, the Hylase-Dessau treatment, including heating of the synovial fluids for 30 min at 37°C, was performed. Commercially available ELISA kit (Adipogen Inc., Seoul, Korea) with the detection limit of 32 pg/mL and with the assay range 0.063 ng/mL–4 ng/mL was used to analyse the levels of PGRN in the serum and in the synovial fluid. Absorbance was detected by the Sunrise ELISA reader (Tecan, Salzburg, Austria) with 450 nm as the primary wavelength. CRP levels were determined via an immunoturbidimetric technique using an Olympus biochemical analyser (model AU 400, Japan). Analysis of serum levels of anticitrullinated protein/peptide autoantibodies (ACPA) and IgM rheumatoid factor (IgM-RF) was done with the standard ELISA kits (Test Line s.r.o., Czech Republic).

2.3. Immunohistochemistry. Synovial tissue samples were obtained from six patients with RA and seven patients with OA at the time of arthroscopy or open joint surgery (First Orthopaedic Clinic, First Faculty of Medicine, Prague, Czech Republic). Because of restricted access to healthy synovial tissue, OA synovial tissue samples were used as controls for performing immunohistochemistry. Paraffin-embedded sections of synovial tissues were subjected to immunohistochemistry as described elsewhere in more detail [16]. Monoclonal mouse anti-human progranulin antibody (Enzo Life Sciences, Lörrach, Germany) diluted 1 : 100 in ChemMate antibody diluent (Dako, Cytomation, Glostrup, Denmark) was used. Negative control slides were treated with Isotype IgG (Dako, Cytomation) in a dilution of 1 : 1000. All the sections were analysed semiquantitatively using a Nikon Eclipse E600 microscope operated by an experienced pathologist who was blind to clinical data. The analysis included eight to ten random and nonoverlapping fields of synovial tissue. The intensity of PGRN expression was scored on a four-point scale (0–3). In terms of staining intensity, 0 represented lack of positivity, and scores of 1–3 represented weak, moderate, and strong staining intensity, respectively.

2.4. Statistical Analysis. Differences in serum and synovial fluid PGRN levels (adjusted for BMI) between the groups were analyzed using a Wilcoxon two-sample test and within the groups by one-sample Wilcoxon test. The relationships among the variables were determined using Spearman correlation coefficients for nonnormal variables. Differences in the expression of PGRN between RA and OA synovial tissue samples were determined using the Jonckheere-Terpstra test. The data were expressed as mean SD unless stated otherwise. P values less than 0.05 were considered statistically

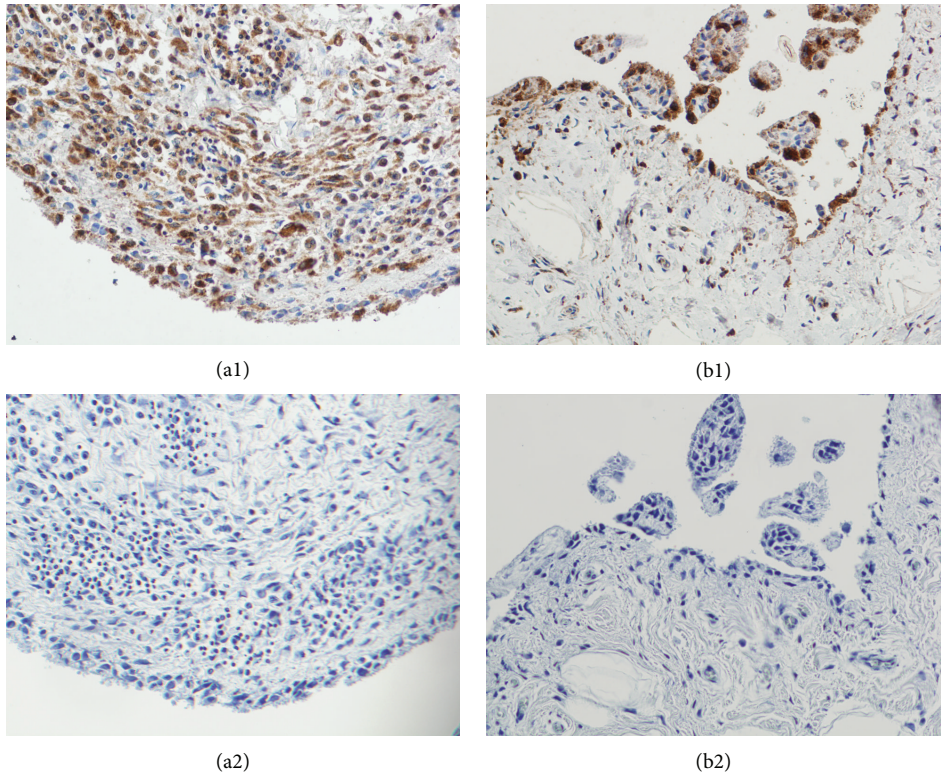

Figure 1: Detection of progranulin (PGRN) protein in rheumatoid arthritis (RA) synovial tissues (a1) and osteoarthritis synovial tissues (b1). Strong staining intensity for PGRN was observed in the lining layer and particularly in the mononuclear cell infiltrates of the sublining layer of RA synovial tissue. Vessels and capillaries were positive for PGRN in both RA and OA synovial tissues. Mouse IgG was used as an isotype control (a2, b2). The original magnification is 200x.

significant. Statistical Analysis System (SAS) software and R software were used for performing the computations.

3. Results

3.1. The Expression of PGRN Is Increased in Rheumatoid Arthritis Synovial Tissue. The expression of PGRN was detected in both RA and OA synovial tissues; however, the staining intensity was significantly enhanced in patients with RA (Figure 1). In comparison with OA samples, PGRN was significantly upregulated in the inflammatory cells localized in synovial sublining layer of patients with RA ($P = 0.033$). The synovial lining layer showed slightly higher expression of PGRN in RA compared to OA tissue samples ($P = 0.086$). A comparable PGRN staining intensity was detected in the vessels between the RA and OA synovial tissues ($P = 0.462$) (Table 2).

3.2. Synovial Fluid PGRN Levels Are Increased in Rheumatoid Arthritis. The synovial fluid PGRN levels were significantly higher in RA than in OA patients (384.5 ± 275.3 versus 241.4 ± 165.2 ng/mL; $P = 0.002$) (Figure 2). However, circulating PGRN levels did not differ between RA and OA patients, but both were higher compared to those in healthy individuals (227.1 ± 100.2 and 221.5 ± 102.5 versus 128.1 ± 34.7 ng/mL; $P < 0.001$) (Figure 2). The levels of PGRN were significantly elevated in synovial fluid compared to serum in RA patients ($P < 0.001$). In addition, PGRN levels in the serum and in the synovial fluid strongly correlated in RA patients ($r = 0.551$, $P < 0.0001$). There was no correlation between the serum and synovial fluid PGRN observed in OA patients ($r = 0.205$, $P = 0.199$). The levels of PGRN were not affected by sex, age, or treatment.

Table 2: Expression ofprogranulin (PGRN) in different cellular compartments of synovial tissue samples from patients with rheumatoid arthritis (RA) and osteoarthritis (OA).

	RA ($n = 6$)	OA ($n = 7$)	Jonckheere-Terpstra test
Lining layer	2.42 ± 0.48	1.82 ± 0.52	$P = 0.086$
Sublining layer	2.52 ± 0.28	1.68 ± 0.42	$P = 0.033$
Vessels and capillaries	0.31 ± 0.13	0.33 ± 0.10	$P = 0.462$

The intensity of PGRN expression was scored using semiquantitative four-point scale. Score 0 represented no staining, 1 weak staining, 2 moderate staining, and 3 strong staining intensity. The numbers represent mean ± SD. PGRN: progranulin; RA: rheumatoid arthritis; OA: osteoarthritis.

3.3. Associations between PGRN and Disease Activity. Serum PGRN levels correlated with DAS28 ($r = 0.327$, $P = 0.049$) and with HAQ score ($r = 0.323$, $P = 0.032$) (Figures 3(a), 3(b)) in RA patients. However, no relationship between serum PGRN and CRP levels was observed ($r = 0.126$,

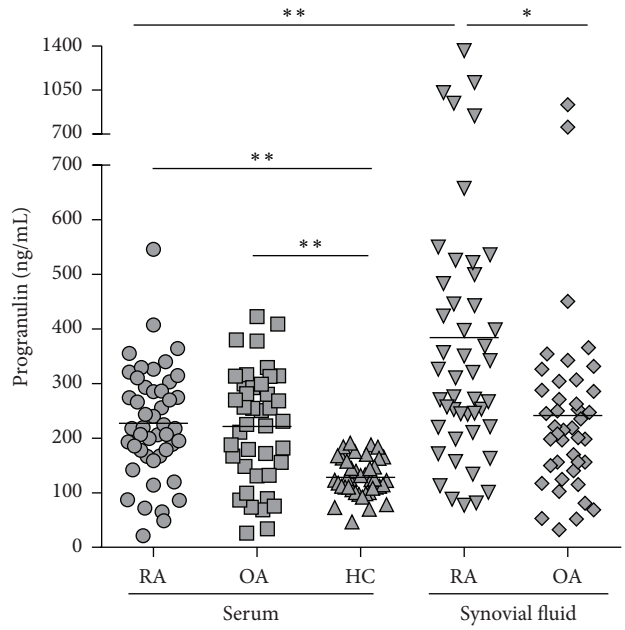

Figure 2: Progranulin (PGRN) levels in the synovial fluid, but not in the serum, were significantly higher in rheumatoid arthritis (RA) compared with osteoarthritis (OA) patients. Serum PGRN was significantly elevated in RA and OA patients compared to healthy controls (HC). The concentration of PGRN in patients with RA was significantly higher in synovial fluid compared to serum. Data are expressed as mean. $^{*}P < 0.01$, $^{**}P < 0.001$.

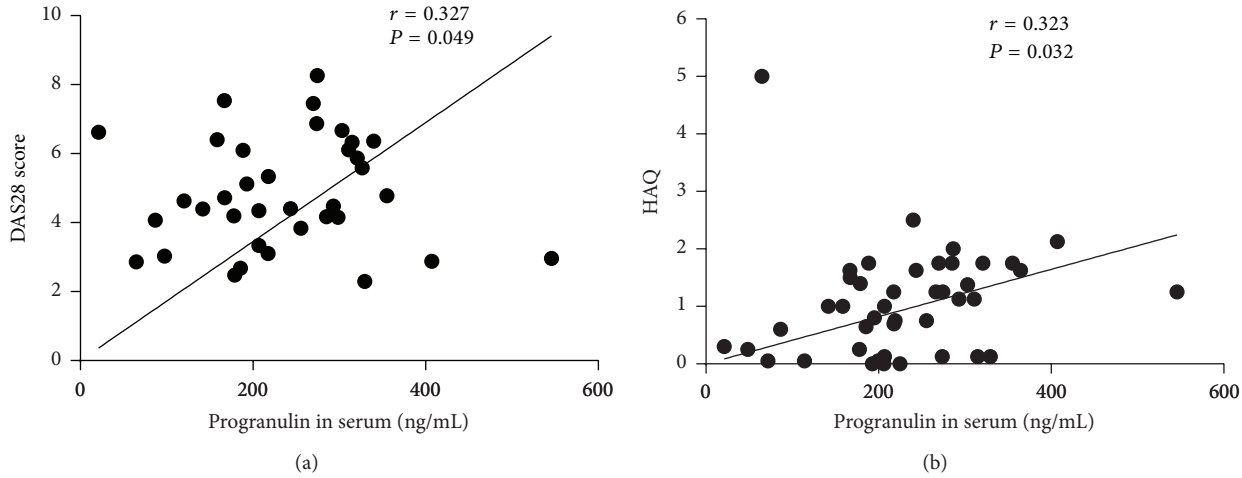

Figure 3: Association of serum PGRN levels with DAS28-CRP (a) and HAQ (b) in patients with rheumatoid arthritis (RA). DAS28: Disease Activity Score; HAQ: Health Assessment Questionnaire score.

$P = 0.403$). PGRN in synovial fluid correlated with HAQ ($r = 0.310$, $P = 0.043$), but not with DAS28 ($r = 0.266$, $P = 0.111$). Furthermore, the PGRN levels were not related to the levels of anti-CCP and IgM-RF autoantibodies.

4. Discussion

PGRN is implicated in cancer development and is suggested as a growth factor with immunosuppressive properties. In this study we report an upregulation of local PGRN in patients with RA compared with control OA individuals and association between circulating PGRN and RA disease severity, including disease functional impairment.

Animal models have yielded insights into the implication of PGRN in the pathogenesis of RA [11]; however, little is known about its expression in human and its potential association with the clinical and laboratory markers of RA disease activity. The overexpression of PGRN gene in the synovial tissue of RA patients was previously identified by DNA sequencing [17]. In agreement with these data, we show an upregulation of PGRN protein in RA compared to OA synovial tissue with its predominant expression within

inflammatory infiltrates of sublining layer. This is in line with PGRN expression by immune cells, particularly macrophages reported earlier [2, 4, 18]. However, it remains to be clarified whether the accumulation of PGRN in RA synovium represents anti- or proinflammatory mechanism modulating the immune response within the joint. In multiple arthritis mouse models PGRN prevented inflammation by inhibition of TNFα-activated intracellular signaling and was suggested as an anti-inflammatory molecule [11, 12]. In patients with RA, the levels of serum PGRN correlate with TNFα and soluble TNF receptor 2, and the ratio of PGRN/TNFα in serum is related to the stage of the disease [18]. This indicates that the role of PGRN in the RA inflammation is rather more complex. Further studies are necessary to elucidate the underlying mechanisms of PGRN, TNFα, and TNFR interactions and the role of the PGRN/TNFα balance in the pathogenesis of RA. Moreover, it is well established that, during the inflammation, PGRN is cleaved by serine proteases into granulins which are proinflammatory and neutralize the anti-inflammatory effect of progranulin [5]. On the other hand, there is evidence that the full-length progranulin promotes inflammation by upregulating the expression of TNFα and IL-1β in human monocyte-derived macrophages [19]. Furthermore, PGRN exhibits chemotactic activity for inflammatory cells in cutaneous wound [1]. This indicates that PGRN may play an ambivalent role in control of inflammation depending on the tissue involved.

Consistently with Yamamoto et al. [18], the levels of PGRN were particularly increased at sites of local inflammation compared to the blood circulation of patients with RA. It could be hypothesized that the joint compartment represents a major site of PGRN production in patients with RA. However, as the PGRN autoantibodies were previously detected in the sera of patients with RA [14], it is likely that autoantibodies could bind and thereby neutralize the circulating PGRN in patients with RA. The levels of PGRN in our study were higher in contrast to the study of Yamamoto et al. [18] but comparable to that in patients with systemic lupus erythematosus observed in another study [13]. This may be explained by the use of various antibodies detecting different epitopes in different ELISA assays. In agreement with the previous report [18], the levels of circulating PGRN in both RA and OA patients were comparable and significantly higher in contrast to healthy subjects. Although OA is recognized to involve inflammatory components [20], it is not clear whether this local inflammation may be reflected systemically [21–23]. In addition, PGRN was recently described as a novel chondrogenic growth factor and an OA-related molecule [8–10]. Further studies are therefore needed to elucidate the exact role of the circulating PGRN in patients with OA. Although we show significant local accumulation of PGRN in RA, except functional impairment, neither the disease activity nor CRP levels correlated with the local PGRN levels. However, circulating PGRN levels correlated with disease severity, which has been previously documented also in patients with systemic lupus erythematosus [13] or with CRP levels in obese and type 2 diabetic patients [24]. On the other side, PGRN levels did not correlate with CRP levels in our study but rather reflected global disease activity as well as functional impairment, which might correspond to systemic feature and multiple joint involvement of the disease.

5. Conclusions

This study shows significant upregulation of PGRN at local sites of inflammation such as synovial tissue and synovial fluid as well as association between circulating PGRN levels, disease activity, and functional impairment in patients with RA. These findings support further investigation of PGRN in inflammatory diseases, including RA.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

Ficolin B in Diabetic Kidney Disease in a Mouse Model of Type 1 Diabetes

Charlotte Berg Holt,[1,2] Jakob Appel Østergaard,[1,3] Esben Axelgaard,[2] Gitte Krogh Nielsen,[2] Yuichi Endo,[4] Steffen Thiel,[2] and Troels Krarup Hansen[1]

[1]*Department of Endocrinology and Internal Medicine, Aarhus University Hospital, 8000 Aarhus, Denmark*
[2]*Department of Biomedicine, Aarhus University, 8000 Aarhus, Denmark*
[3]*The Danish Diabetes Academy, Sdr. Boulevard 29, 5000 Odense, Denmark*
[4]*Department of Immunology, Fukushima Medical University School of Medicine, Fukushima 960-1295, Japan*

Correspondence should be addressed to Troels Krarup Hansen; tkh@dadlnet.dk

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Background. The innate immune system may have adverse effects in diabetes and cardiovascular disease. The complement system seems to play a key role through erroneous complement activation via hyperglycaemia-induced neoepitopes. Recently mannan-binding lectin (MBL) was shown to worsen diabetic kidney changes. We hypothesize that mouse ficolin B exerts detrimental effects in the diabetic kidney as seen for MBL. *Methods.* We induced diabetes with streptozotocin in female wild-type mice and ficolin B knockout mice and included two similar nondiabetic groups. Renal hypertrophy and excretion of urinary albumin and creatinine were quantified to assess diabetic kidney damage. *Results.* In the wild-type groups, the kidney weighed 24% more in the diabetic mice compared to the controls. The diabetes-induced increase in kidney weight was 29% in the ficolin B knockout mice, that is, equal to wild-type animals (two-way ANOVA, $P = 0.60$). In the wild-type mice the albumin-to-creatinine ratio (ACR) was 32.5 mg/g higher in the diabetic mice compared to the controls. The difference was 62.5 mg/g in the ficolin B knockout mice, but this was not significantly different from the wild-type animals (two-way ANOVA, $P = 0.21$). *Conclusions.* In conclusion, the diabetes-induced effects on kidney weight and ACR were not modified by the presence or absence of ficolin B.

1. Introduction

Diabetes is the most frequent cause of end-stage renal disease in industrialised countries [1, 2]. Clinically, diabetic nephropathy is characterized by the development of albuminuria and a subsequent decline in glomerular filtration rate. This severe complication significantly influences the risk of cardiovascular disease as well as mortality and quality of life [3, 4]. At present the most important identified risk factors are diabetes duration, arterial blood pressure, and glycaemic regulation [5]. As evident from an increasing incidence of affected patients, however, there is still a great need for new strategies in the treatment and prevention of diabetic nephropathy.

Clear evidence indicates that the pathogenesis of diabetic nephropathy is multifactorial and triggered by a complex series of pathophysiological events [6]. Inflammation has emerged as a key factor in the development of diabetic nephropathy [7]. The inflammatory response in diabetes is highly complex involving proinflammatory cytokines and chemokines, for example, IL1, IL6, IL8, TNF, and NFκB [8]. The impact of complement activation on the diabetic kidney may well, in part, be mediated through induction of cytokine response and inflammation [9–11].

Several studies have linked diabetic late-complications to the complement system of the innate immune system [12, 13]. The complement system plays a crucial role in recognition and clearance of infectious microbes and the system forms a link between innate and adaptive immunity [14]. The complement system functions as an enzymatic cascade. The activation of the complement system results in the release of multiple inflammatory signaling molecules. Ultimately complement activation leads to the formation of pore-forming membrane attack complexes (MACs) that are

inserted in the cell membranes to mediate lyses of the cell through osmotic stress.

However, in mammalian cells, it has been shown that sublytic amounts of MAC can increase production of IL-8 and monocyte chemoattractant protein 1 dependent on NFκB nuclear translocation [15]. Furthermore, MACs are shown to have a mitogenic effect and cause release of basic fibroblast growth factor and platelet-derived growth factor from endothelial cells leading to fibrosis in neighboring cells including glomerular mesangial cells [16, 17]. The latter effects of MAC may explain the link between complement and diabetic kidney damage.

Three activations pathways exist: the classical, the alternative, and the lectin pathway. The present paper focuses on the lectin pathway, in which at least five soluble pattern-recognition molecules are characterized that may activate the complement system, that is, mannan-binding lectin (MBL), H-ficolin, L-ficolin, M-ficolin, and collectin-K1 [18]. The three ficolins utilize a fibrinogen-like domain that binds, for example, N-acetylglucosamine, N-acetylgalactosamine, and N-acetyl-neuraminic acid, whereas MBL and CL-K1 have a carbohydrate recognition domain and through this bind specifically to patterns of monosaccharides. A very recent publication reports a close association between ficolin and diabetic nephropathy in patients with type 1 diabetes [19]. The observational design of the study, however, limits its ability to study a cause-effect relationship. When ficolins bind they all initiate activation of associated serine proteases (MBL associated serine proteases, MASPs), which subsequently cleave the complement factors, C2 and C4, leading to further complement activation [20, 21]. Eventually, complement activation leads to the formation of MACs causing cell lyses or induction of fibrosis [22].

The balance between activation and inhibition of the complement cascade is tightly controlled by regulatory proteins in order to prevent damage of healthy host cells. In diabetes, inappropriate effects of the complement system may be present as glycation-induced dysfunction of the complement inhibitory mechanism and consequently overactivation of the system is indicated [23–25]. It is speculated that diabetic patients are exposed to uncontrolled complement attack partly due to altered molecular patterns on the cell surfaces as a consequence of high blood glucose [24, 25]. Most significantly, an association is seen between diabetic nephropathy and the lectin pathway [26–28]. We have previously demonstrated direct cause-effect relationship between presence of MBL and worsening of kidney injury in a mouse model of diabetic nephropathy [29, 30]. We speculate that ficolins also exert detrimental effects in diabetes similar to MBL through activation of the lectin pathway as indicated in patients with type 1 diabetes [19].

This study aimed to investigate the impact of ficolin B (the orthologue to human M-ficolin) on the development of diabetic nephropathy in a mouse model of type 1 diabetes.

2. Materials and Methods

2.1. Animals. We used 11-week-old, female ficolin B knockout mice and age-matched, female C57BL/6J BomTac wild-type mice (Taconic, Ry, Denmark). The knockout ficolin B model was backcrossed more than 10 generations to a C57BL/6J BomTac genetic background (own breeding) [31].

In each cage there were three to eight mice and they had free access to tap water and standard chow (Altromin number 1324; Lage, Germany). The environment was stable with a 12-hour light-dark cycle, temperature at 21 ± 1°C, and humidity of 55 ± 5%. The study complied with the regulations for care and use of laboratory animals.

The ficolin B knockout mice and the wild-type mice were randomized into a diabetic and nondiabetic group; thus four groups were made: (1) diabetic knockout mice ($n = 6$), (2) nondiabetic knockout mice ($n = 7$), (3) diabetic wild-type mice ($n = 11$), and (4) nondiabetic wild-type mice ($n = 11$).

Diabetes was induced by intraperitoneal injections of streptozotocin (STZ) dissolved in a cold 10 mM citrate buffer (doses of 55 mg/kg body weight, Sigma Aldrich, St Louis, Mo, USA) on five consecutive days [32]. The animals were fasting 4 hours prior to injection. If the blood glucose did not rise sufficiently two more injections were given. The diabetic mice received the exact same amount of STZ. Controls were injected with citrate buffer only. The 18-week experiment was initiated when the mice were classified as diabetic (blood glucose > 15 mM).

Animals with more than 15% sustained weight loss, signs of illness, or persistent ketonuria were excluded from the study. Body weight and blood glucose were measured weekly. Blood glucose was measured from tail vein by Contour (Bayer Diabetes Care, Kgs. Lyngby, Denmark). With Combur^5 Test D strip (Roche Diagnostics GmbH, Mannheim, Germany) urine was tested for ketone bodies. Two mice from each diabetic group were excluded because of insufficient increase in blood glucose levels. Furthermore, two mice from the diabetic knockout group were excluded because of weight loss > 15% of body weight. The excluded mice were not included in the number of animals per group indicated above.

2.2. Collection of Samples. Spot urine was collected in eppendorf tubes on five consecutive days prior to sacrifice of the animals. The blood samples were drawn from under the tongue at baseline and from the retroorbital venous plexus at study end and collected in potassium EDTA tubes (Sarstedt, Nümbrecht, Germany). Both urine and blood samples were stored at −80°C until analysed.

The animals were anesthetized by an intraperitoneal dose of ketamine at 0.5 mg/g body weight and xylazine at 0.2 mg/g body weight (Ketaminol 4 Vet and Narcoxyl Vet, resp., Intervet, Skovlunde, Denmark). The kidneys were dissected and weighed, after which the mice were sacrificed. The measures of left kidney weight were used in the analyses.

2.3. Albumin-to-Creatinine Ratio (ACR). Urinary albumin excretion was determined by Mouse Albumin ELISA quantification Kit (Bethyl laboratories, Inc., Montgomery, TX, USA) according to the manufactory's instruction.

Urine creatinine was measured by isocratic high-performance liquid chromatography (HPLC) on a Zorbax SCX300 column (Agilent, USA) using a slight modification of

Table 1: Table illustrating mean (95% confidence intervals).

		Groups				Two-way ANOVA	Student's t-test	
		Wild-type diabetic $N = 11$	Wild-type nondiabetic $N = 11$	Knockout diabetic $N = 6$	Knockout nondiabetic $N = 7$	Interaction P (KO $*$ dia)	P diabetes (WT><KO)	P control (WT><KO)
Body weight (g)	Start	20.6 (19.8; 21.4)	21 (20.2; 21.8)	20.2 (19.5; 20.8)	19.8 (19.1; 20.4)	0.38	0.43	0.05
	End	21.1 (19.9; 22.4)	23.8 (23.0; 24.6)	19.1 (18.0; 20.3)	23.2 (22.3; 24.2)	0.22	0.05	0.34
Blood glucose (mmol/L)	Start	6.8 (6.5; 7.2)	6.4 (5.9; 6.9)	5.2 (4.6; 5.8)	5.7 (5.1; 6.4)	0.08	<0.001	0.12
	End	23.8 (20.8; 26.8)	5.5 (4.9; 6.1)	26.3 (20.1; 32.5)	6.5 (6.0; 7.1)	0.60	0.41	0.04
	AUC	2526 (2358; 2692)	765 (698; 833)	2581 (2367; 2795)	660 (628; 691)	0.25	0.69	0.13
Kidney weight (mg)	End	148.0 (136.8; 159.2)	118.9 (112.6; 125.2)	153.2 (137.0; 169.4)	118.3 (107.6; 129.0)	0.60	0.59	0.92
Albumin-to-creatinine ratio (mg/g)	End	76.3 (49.6; 103.1)	43.8 (25.1; 62.6)	96.4 (70.8; 122.1)	33.9 (23.3; 44.5)	0.21	0.34	0.44

Interaction P (KO $*$ dia): two-way ANOVA—the interaction between the knockout factor and the diabetic factor.
P Diabetes WT><KO: Student's t-test of the diabetic wild-type mice compared to diabetic ficolin B knockout mice.
P Control WT><KO: Student's t-test of the control wild-type mice compared to the control ficolin B knockout mice.
AUC: blood glucose measured as area under the curve (days$*$mmol/L).

a method first reported by Yuen et al. [33]. In brief, 5 μL urine was added to 100 μL acetonitrile containing 0.5% acetic acid and vortexed for 15 seconds to extract the creatinine. After 15 min of −20°C storage and centrifugation the supernatants were evaporated and then reconstituted with 25 μL 5 mM sodium acetate, pH 4.1. The samples were centrifuged for 10 min at 3000 rpm. Duplicate samples (10 ul each) were fractionated on a 50 mm × 2.1 mm Zorbax SCX300 column with an in-front SCX guard column. Isocratic HPLC was performed at a flow rate of 1 mL/min, and UV absorbance was monitored at 225 nm. A standard curve was created by including a 2-fold dilution series of creatinine anhydrous (Sigma Aldrich). All aqueous solutions were filtered through a 0.22-micron filter before use.

2.4. Statistics. This study was designed with two independent factors; diabetes/nondiabetes and knockout/wild-type and thus analysed by two-way ANOVA for normal distributed variable with equal variance. The main focus of interest was the interaction between the diabetic factor and the knockout factor; that is, does ficolin B modify the effects of diabetes on the effect parameters? If no interaction was found, the independent effects of diabetes and ficolin B on the kidney were estimated. For pairwise comparison, normal distributed data was tested with Student's t-test, whereas otherwise the Wilcoxon Mann-Whitney rank sum test was used. P values below 5% were considered as statistically significant. Data are given as mean (95% confidence interval (CI)) unless else is stated. All statistical analyses were performed using STATA version 12.

3. Results

3.1. Body Weight and Blood Glucose. At baseline, the knockout mice on average weighed 20.0 g, which was slightly less than the wild type mice, 20.8 g ($P = 0.04$). No difference was found between the two diabetic groups or between the two nondiabetic groups (Table 1). After 18 weeks an expected difference in body weight was observed between the diabetic and the nondiabetic mice independently of knockout status ($P < 0.001$). The nondiabetic mice weighed 3.2 g (CI: 2.0 g–4.3 g) more than the diabetic mice. Furthermore the diabetic knockout mice were significantly smaller than the diabetic wild type ($P < 0.05$). As presented in Table 1, blood glucose, estimated as area under the curve (AUC), did not differ between the two diabetic groups ($P = 0.69$) or between the two nondiabetic groups ($P = 0.13$). The overall fluctuations in blood glucose in each group are depicted in Figure 1.

3.2. Kidney Weight. The kidney weight was equally increased in diabetic wild-type mice, 24% (CI: 13%–36%), and in the diabetic knockout mice, 29% (CI: 12%–47%), compared to the respective control groups (Figure 2(a)). No interaction between knockout and diabetes was found ($P = 0.60$), indicating that wild-type and knockout mice develop the same degree of diabetes-induced renal hypertrophy. The considerable body weight difference between the two diabetic groups at study end indicated that the kidney weight was to be normalised to the body weight. This is illustrated in Figure 2(b). Ficolin B did not modify the diabetes-induced increase in kidney weight when testing for interaction ($P = 0.11$). Furthermore, no significant statistical difference was

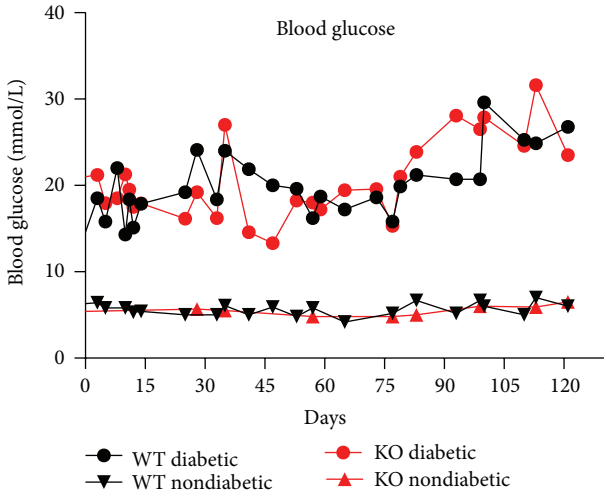

Figure 1: Mean blood glucose level in mmol/L in each of the four groups over time.

found in kidney weight per body weight between the diabetic wild-type, 1.95 mg/g, and the diabetic knockout, 2.89 mg/g ($P = 0.09$).

3.3. Albumin-to-Creatinine Ratio. The albumin-to-creatinine ration (ACR) was higher among the diabetic wild-type mice, 76 mg/g (CI: 50–103 mg/g), compared to the nondiabetic wild-type mice, 44 mg/g (CI: 25–63 mg/g), $P = 0.07$. Similarly, the ACR of diabetic knockout mice was 96 mg/g (CI: 71–122 mg/g) compared to the nondiabetic knockout group, 34 mg/g (CI: 23–44 mg/g), $P < 0.001$. As depicted in Figure 3 no interaction was observed between diabetes and ficolin B knockout, $P = 0.21$.

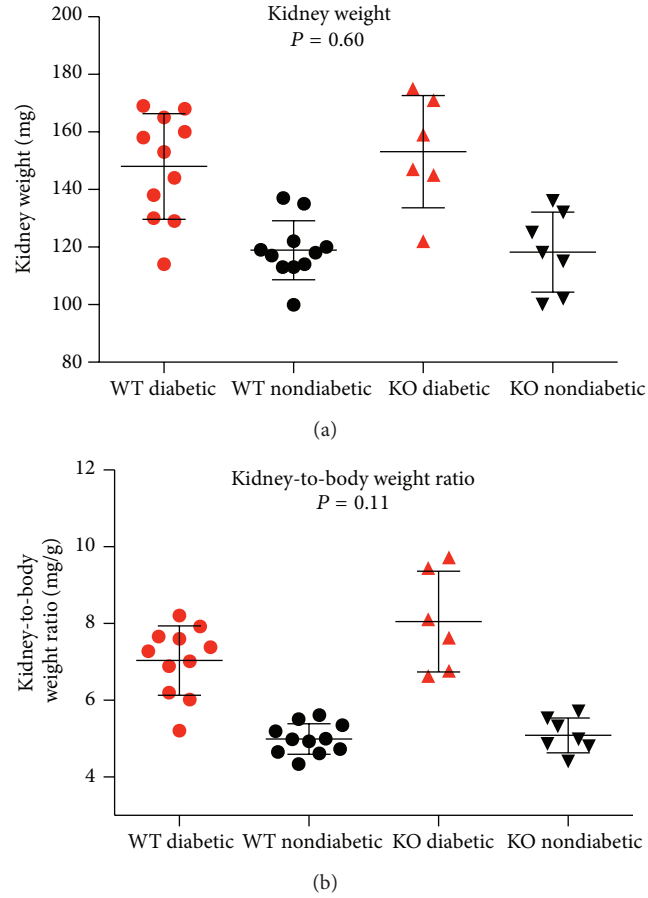

Figure 2: Kidney weight (a) and kidney weight per body weight (b) depicted for each of the four groups. The black error bars are illustrating mean ± standard deviation for each group. WT: wild-type; KO: ficolin B knockout.

4. Discussion

In the present study we found no association between diabetes-induced kidney changes and the presence of ficolin B. We conclude that ficolin B is not responsible for, or a crucial contributory factor in, the pathophysiology of diabetic nephropathy. In our study, the kidney weight and to some extent the ACR were altered by diabetes as expected. The diabetes-induced increase in kidney weight, measured by comparing the diabetic mice with the nondiabetic mice, was not statistically different between the wild-type and ficolin B knockout mice. In other words, there was no effect modification or interaction. The diabetes-induced increase in kidney weight was 24% in the wild-type mice and 29% in the ficolin B knockout mice. Taking the lower body weight of the knockout mice into account, the difference in renal hypertrophy was still insignificant when comparing the wild-type mice and the ficolin B knockout mice. Similarly, the diabetic change seen in ACR was not altered in the absence of ficolin B.

The experimental setup including four groups matched on age, body weight, and genetical background was a strength to the study, as the diabetes factor and the knockout factor were the only modulators of the outcome. The two diabetic groups and the two nondiabetic groups had comparable glucose levels. Most importantly both diabetic groups did reach and sustain blood glucose levels of above 15 mM. At study end, the body weight differed among groups, which impeded the analyses of the diabetic kidney damage, because the knockout mice appear to be more vulnerable to type 1 diabetes mellitus.

Our study provides important new information on the association between the lectin pathway and diabetic kidney damage. We are the first to investigate the role of ficolin B (which corresponds to ficolin M in human) in the inflammatory response of diabetic nephropathy. In mice with deficiency of MBL, the classical functional and physical renal changes normally seen in this experimental model of type 1 diabetes were modified [29, 30]. This supports a number of human studies [26–28, 34]. The fact that ficolin B does not appear to modulate diabetic effects on the kidney emphasizes the importance of MBL compared with ficolin B. Both MBL and ficolin B activate the lectin pathway of the complement system, but only deficiency of MBL has been shown to protect against diabetic kidney damage [29].

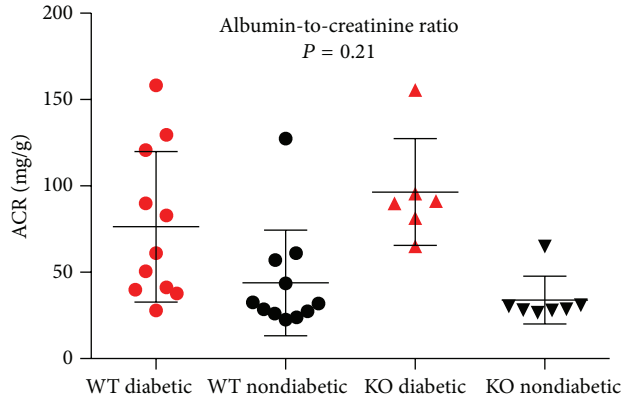

Figure 3: Albumin-to-creatinine ratio (mg/g) is illustrated for each of the four groups. The black error bars are illustrating mean ± standard deviation. WT: wild-type; KO: knockout.

This indicates that the role of the lectin pathway in the development of diabetic nephropathy is complex and may depend on the specific carbohydrate-binding properties of MBL as previously described.

The function of other complement factors in the first parts of the lectin pathway (e.g., ficolin A and MASPs) in the pathology of diabetic kidney disease remains unknown and must be explored in further studies. One study indicates that ficolin A and ficolin B exert a cooperatively defensive role in destroying Streptococcus Pneumoniae, suggesting a synergetic immunological effect [35]. This emphasises the need for further investigations involving both mouse ficolins. In order to fully understand the involvement of the lectin pathway in the development of diabetic nephropathy, an additional parallel experiment with MASPs is of particular interest given that they represent the limiting downward step in the complement activation.

5. Conclusion

In conclusion, this study demonstrates that ficolin B does not modify the kidney weight and ACR in a type 1 diabetes mouse model. This indicates that the role of the lectin pathway in the development of diabetic nephropathy is specific and that hyperglycaemia-induced glycations on renal cells may be more prone to bind MBL than ficolin B.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Microparticles That Form Immune Complexes as Modulatory Structures in Autoimmune Responses

Catalina Burbano,[1,2] Mauricio Rojas,[1,2] Gloria Vásquez,[1] and Diana Castaño[1]

[1] *Grupo de Inmunología Celular e Inmunogenética, Instituto de Investigaciones Médicas, Facultad de Medicina, Universidad de Antioquia (UdeA), Calle 70 No. 52-21, Medellín, Colombia*
[2] *Unidad de Citometría de Flujo, Sede de Investigación Universitaria, Universidad de Antioquia (UdeA), Calle 70 No. 52-21, Medellín, Colombia*

Correspondence should be addressed to Diana Castaño; diana.castano@udea.edu.co

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Microparticles (MPs) are induced during apoptosis, cell activation, and even "spontaneous" release. Initially MPs were considered to be inert cellular products with no biological function. However, an extensive research and functional characterization have shown that the molecular composition and the effects of MPs depend upon the cellular background and the mechanism inducing them. They possess a wide spectrum of biological effects on intercellular communication by transferring different molecules able to modulate other cells. MPs interact with their target cells through different mechanisms: membrane fusion, macropinocytosis, and receptor-mediated endocytosis. However, when MPs remain in the extracellular milieu, they undergo modifications such as citrullination, glycosylation, and partial proteolysis, among others, becoming a source of neoantigens. In rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), reports indicated elevated levels of MPs with different composition, content, and effects compared with those isolated from healthy individuals. MPs can also form immune complexes amplifying the proinflammatory response and tissue damage. Their early detection and characterization could facilitate an appropriate diagnosis optimizing the pharmacological strategies, in different diseases including cancer, infection, and autoimmunity. This review focuses on the current knowledge about MPs and their involvement in the immunopathogenesis of SLE and RA.

1. Introduction

It is considered that the development of any autoimmune disease requires a combination of genetic predisposition, exposure to environmental risk factors, hormones, and defects in epigenetic mechanisms that regulate immune tolerance [1]. It has been described that adaptive immunity plays a central role involving autoantibody formation, the presence and activation of autoreactive T cells, defects in regulatory functions, and the induction of anergy in these cells, among other mechanisms [2]. However, during recent years there is growing evidence regarding the participation of innate immunity in autoimmune diseases in different models. Innate immunity has an important role at the beginning of the immune response and later, perpetuating certain systemic inflammatory effects by the release of soluble factors (e.g., cytokines, chemokines and lipid mediators), the presentation of autoantigens in an inflammatory context, the activation of effector T cells, and tissue damage, among others [3].

In addition, the development of autoimmunity has been associated with defects in the pathways that regulate cell death and the recognition and clearance of apoptotic cells (ACs) [4]. Defects in the induction of apoptosis contribute to the survival of autoreactive B cells that produce autoantibodies [5]. The inefficient removal of apoptotic bodies, once they undergo posttranslational modifications in the extracellular environment such as oxidation and citrullination [6], converts them into a primary source of autoantigens, neoantigens, and immune complexes.

Microparticles (MPs) are vesicular structures mainly produced during activation and cell death; however, the precise mechanism by which they are generated is under

investigation. It has been observed that MPs contain a variety of molecules inside and on the surface of them with agonist and antagonist activities; therefore, MPs can regulate the proliferation of endothelial cells [7], coagulation, thrombosis [8], inflammation, and other events related to innate and adaptive immunity. The recognition of MPs and their modification by innate immune cells could contribute to the chronic inflammatory process seen in autoimmune diseases. However, little is known about the detailed roles of MPs in the pathogenesis of these conditions [9, 10]. Only recently the number of studies relevant to the participation of these vesicular structures in the development and maintenance of autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) is increasing.

MPs from patients with autoimmune diseases can participate in the development of immune complexes (ICs) through interaction with circulating autoantibodies and in different tissues. Therefore, MPs can interact with target cells through different receptors such as phosphatidylserine (PS) and scavenger receptors, and they can also be recognized by opsonic receptors such as the immunoglobulin (FcR) and [11, 12] complement (CR) receptors. This opens a wide range of additional effects and potential interactions whose complexity is difficult to predict in the context of an inflammatory response.

The aim of this review is to present evidence that supports MPs and their ICs as potential immunomodulators in the context of autoimmune responses and diseases. First, some general aspects regarding the generation of and the physiological roles attributed to these structures are described. Then, the present review focuses on and discusses the potential role of MPs and their ICs in the pathophysiology of SLE and RA with respect to the promotion of inflammatory responses and tissue damage.

2. Definition and Overview of MPs

MPs, from different points of view, are heterogeneous structures: in size (100–1000 nm), cell origin, mechanism of induction, composition, and stability. These particles are derived from the plasma membrane of different cell types, and hence they can contain several components from the parent cell [13]. MPs were first identified in 1967 by ultracentrifugation of plasma from healthy human subjects; it was possible to obtain material rich in phospholipids with procoagulant properties. These structures were originally called "platelet dust" because it appeared to contain traces of these cells [14]; currently they are called MPs.

MPs are small extracellular vesicles also known under the name of microvesicles. They are considered different from other vesicular structures such as exosomes and apoptotic bodies in size, composition, and number [15] (Figure 1). In order to differentiate MPs from other structures, they have been called ectosomes, which refers to "*bodies that emerge from the plasma membrane by ectocytosis,*" as it happens during exocytosis [15]. Table 1 summarizes the main characteristics that distinguish MPs from other vesicles.

Blood cells from mammals can generate a variety of MPs under different stimuli; however, it has been reported that approximately 80% of these circulating vesicles are derived from platelets [16]. MPs can also be generated from other cellular origins at the tissue level, for example, from tumor cells, ischemic tissue, and mesenchymal cells [13, 17]. Therefore, MPs can be found in almost any anatomical location including intercellular spaces, blood vessels, and the lymphatic system [18]. It has been reported that the plasma concentration of MPs in healthy subjects is from 5 to 50 μg/mL (according to the protein content) or from 10^5 to 10^6 plasma membrane-derived vesicles/mL [19].

The structural components of MPs include cell membrane receptors and/or glycoproteins in native or modified forms, nucleic acids (DNA and RNA), enzymes, cytokines, transcription factors, and in some cases secondary messengers (for further information review [20]). This diversity in MP content suggests that they can interact with different cells and can transfer their constituents to viable cells by different specific and nonspecific mechanisms of recognition such as membrane fusion, receptor-mediated endocytosis, and macropinocytosis (Figure 2) [21]. A recent report showed that at least platelets might contain mitochondrial structures and also release the mitochondria with proinflammatory effects [22], even though several references indicate that MPs lack complete signaling pathways and fully organized organelles [23]. The content of these structures confers them some functionality as agonists or antagonists of diverse biological processes involving intercellular communication wherein the modulatory effects of MPs are recognized. Therefore, it has been suggested that MPs may mediate pathological effects in several autoimmune diseases.

3. MP Generation and Components

Eukaryotic cells are constantly exposed to environmental changes and physiological stimuli that induce modifications and remodeling of the cell membrane [24], including cell division and differentiation and structural changes of the cytoskeleton during cell migration. These processes are associated with MP release [23]. Apparently, there is not an exclusive mechanism leading to the production of MPs, but it is postulated that their generation must correspond to a highly regulated process and not to a random phenomenon as it was originally suggested (reviewed in [20]).

At this point, at least two essential biological events that trigger MP generation have been described: changes in the cell membrane and changes to the cytoskeleton, both of which are dependent on intracellular calcium levels [25, 26]. However, because calcium undergoes complex regulation and is associated with multiple signaling pathways such as mechanisms of cell death and cell activation, the existence of a specific pathway for MP production remains unknown.

The composition of the lipid bilayer in the cell membrane differs between the inner and the outer sides, and it is controlled by transport enzymes that consume ATP such as "flippases" (inward lipid transport) and "floppases" (outward lipid transport) [27]. Stimuli that increase the intracellular

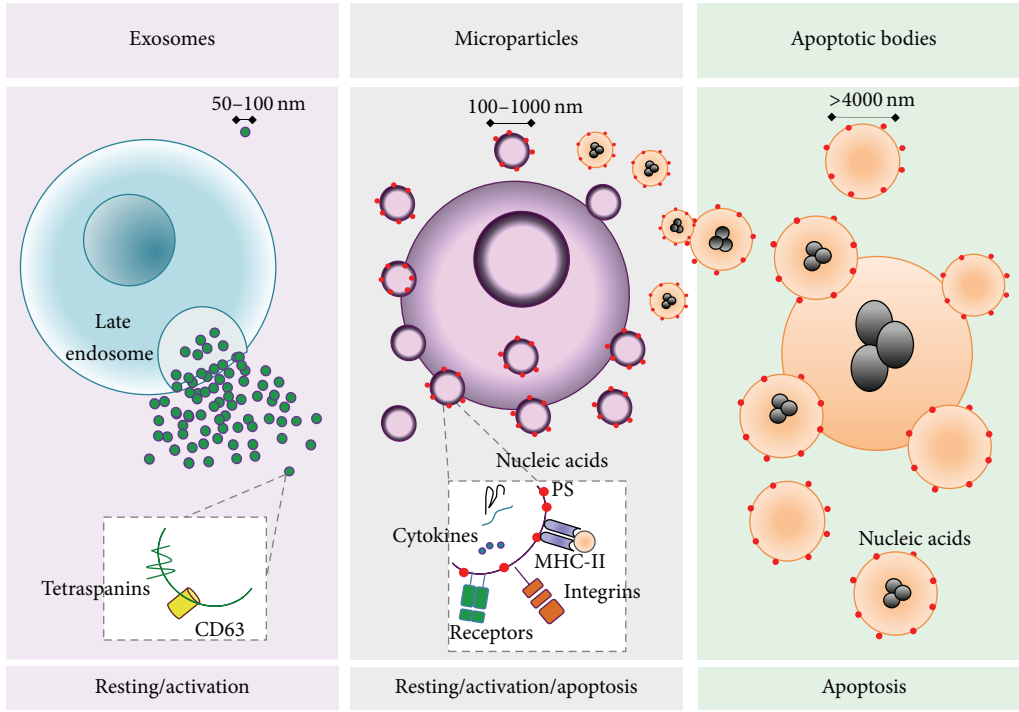

Figure 1: The main characteristics of secreted vesicles. (a) Cells under basal or activated states release vesicles from internal compartments such as multivesicular endosomes, also called late endosomes. Fusion among the endosomal membranes and the cell membrane leads to secretion of intravesicular bodies, which once released are called exosomes and may contain components such as TSG101 and endocytic tetraspanins (CD9 and CD63). (b) Activated cells may secrete vesicles by direct budding of the plasma membrane, called MPs, that contain various receptors, integrins, selectins, cytokines, and nucleic acids. These molecules can be located inside or on the surface of the MPs; however, a cell at rest or in response to physiological stimuli can also produce MPs, but upon activation it produces increased amounts. (c) Apoptotic cell death leads to the formation of apoptotic bodies and MPs, which may contain histones and nucleic acids. The aminophospholipid phosphatidylserine (PS) is exposed on the outer face of the cell membrane during apoptosis. MPs that express PS on their surface can also be generated by cleavage processes from apoptotic bodies.

Table 1: Characteristics and properties of the main secreted vesicles.

Feature	Exosomes	Microparticles (MPs)	Apoptotic cells
Size	40–100 nm	100–1 000 nm	>4 000 nm
Coefficient of sedimentation	100 000 ×g	20 000 ×g	16 000 ×g
Methods of isolation	Sucrose gradient (1.13 y 1.19 g/mL)	Sucrose gradient, affinity column, electromagnetic sorting, and filtration	Sucrose gradient, affinity column, electromagnetic sorting, and filtration
Membrane of origin	Multivesicular endosomes	Plasma membrane	Plasma membrane
Generation	Spontaneous release and cellular activation	Spontaneous release, cellular activation, and apoptosis	Apoptosis
Annexin V binding	Low or negative	High, low, or negative	High
Functions	Carrying lytic enzymes and activation of phagocytes and B cells	Coagulation, M2 macrophage activation, and transfer of functional cell components	Antigen presentation through MHC II, M2 macrophage and monocyte activation and tissue remodeling
Markers	Rab GTPases, annexins, flotillin, Alix, TSG101, and CD63	Integrins, selectins, proteins from the parental cells, and PS	Histones, PS
Organelles	¿?	PMP might contain mitochondrial structures	Different
Nucleic acids	No	mRNA, DNA, miRNA, and interfering RNA	DNA, mRNA, and miRNA
References	[44, 45]	[22, 30, 46–50]	[51–53]

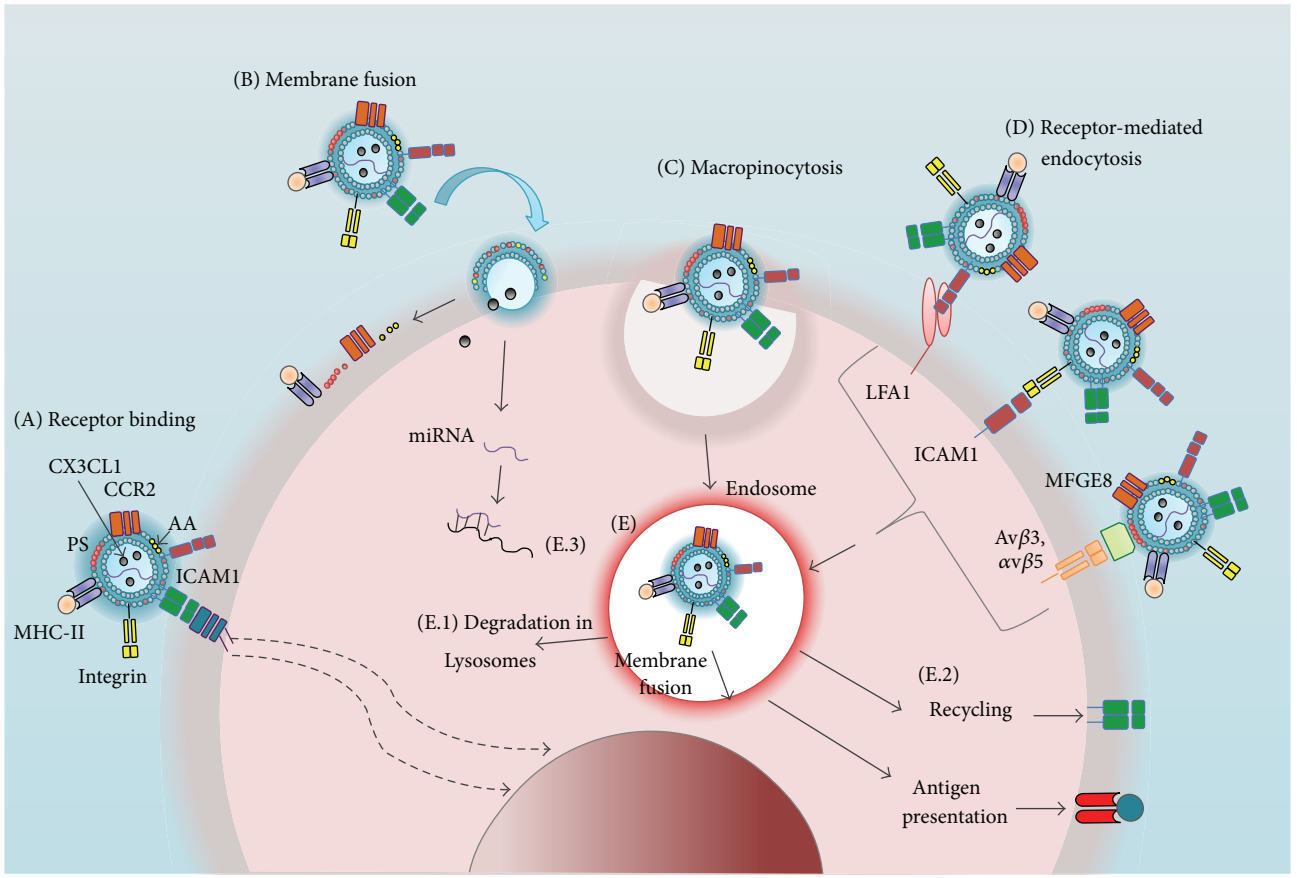

FIGURE 2: Interaction of MPs with their target cells. (A) MPs can interact with a variety of receptors on a target cell that may or may not lead to intracellular signaling (dashed arrows). Additionally, MPs can transfer their surface components (e.g., arachidonic acid (AA), PS) and internal proteins, receptors (MHC-II, CCR5), and nucleic acids (miRNA) to the target cell by (B) membrane fusion, (C) macropinocytosis, or (D) receptor-mediated endocytosis. In the latter, MPs can engage ligands such as LFA1 (lymphocyte function-associated antigen 1), intercellular adhesion molecule 1 (ICAM1), or through binding to integrins ($\alpha v\beta 3$ or $\alpha v\beta 5$) by soluble proteins that recognize the PS MFGE8 (milk fat globule EGF factor 8 protein). (E) When antigen-presenting cells internalize MPs, these structures can take different pathways: (E.1) Degradation by the endocytic pathway and subsequent antigenic peptide presentation through MHC-II molecules. (E.2) Their components may be partially recycled to the surface of the target cell, leading to a gain of phenotype and/or function. (E.3) miRNA can modulate gene expression.

concentration of calcium promote floppase activity, which is involved in the translocation of PS to the outer face of the cell membrane. Calcium also inhibits flippase activity, responsible for maintaining PS on the inner side of the membrane. The "scramblases" are bidirectional lipid conveyors activated by increases in the intracellular calcium levels; therefore, phospholipid changes follow their concentration gradient and they become randomly distributed in the membrane [20, 28]. Apparently, imbalance in the lipid bilayer, cytoskeletal reorganization, and proteolysis by calcium-dependent calpains lead to shrinkage of the cell membrane and MP release. In general, the exposure of PS on the outer side of the membrane appears to be frequently associated with MP release [27]; this happens transiently during cell activation and membrane remodeling [29] and permanently during apoptosis and necrosis [25, 26]. However, unknown nature annexin V− MPs have been reported [30], since some of the mechanisms by which they are released from the membrane may happen even during cell activation without PS exposure [31]. In this regard, it is considered that any stimulus that induces calcium mobilization, cytoskeletal reorganization, and cell membrane changes can induce the formation of MPs [32].

Multiple reports have indicated that apoptotic pathways, which involve the exposure of PS and the formation of blebs from the cell membrane, culminate in AC and MP formation. Apoptosis may contribute to the generation of MPs in two ways: (1) by decreasing the volume of ACs due to inactivation of ATP-dependent ionic pumps (such as Na+/K+ pump that regulates cellular water balance) and the continuous proteolysis of the cytoskeleton; (2) through the activation of ROCK-I (Rho-associated protein kinase I) by the GTPase Rho in early stages of apoptosis. This kinase regulates cortical myosin-II contraction and plasma membrane detachment of the cytoskeleton; thus, it may contribute to MP release (reviewed in [33]).

The formation of blebs during apoptosis of human neutrophils is dependent on the phosphorylation (by MLCK) of

myosin light chains [34]. Apparently, most MPs are produced by this mechanism and only a few of them are released from cells by exocytotic budding; therefore, it is expected that more vesicles derive from blebbing compared to exocytic budding during apoptosis. We hypothesize that MP production during apoptosis can facilitate the disposal of dead cells; because these structures are smaller and have a compact structure, they could be removed more easily by phagocytic cells than apoptotic bodies [10, 35].

MPs generated during apoptosis may contain DNA, messenger RNA (mRNA), and microRNA (miRNA) [15]. In MPs derived from the human T cell line Jurkat or the human promyelocytic cell line HL-60 treated with staurosporine, camptothecin, or UV-B irradiation [36], the internucleosomal chromatin fragmentation usually observed in apoptosis is evident in these structures [37]. In addition, the presence of fragmented mRNA similar in size to miRNA was detected in these vesicles; however, the functional meaning of this finding is unknown beyond its potential as an autoantigen source [36]. Staining with propidium iodide and treatment with RNase and DNase have revealed the presence of surface nucleic acids on MPs. This enzymatic treatment also reduced the binding of MPs to anti-DNA antibodies [36].

There is no obvious mechanism to explain the inclusion and surface expression of nucleic acids on MPs. It is postulated that MPs generated during early apoptosis cannot possess DNA, whereas those released later during apoptosis or due to cytoskeletal and cell membrane damage could include more endogenous components (DNA, RNA, and transcription factors). This phenomenon can partially explain the heterogeneity of these structures and the reason why the evolution of MPs in the extracellular milieu might transform them into other type of structures with different effects. Proteomic analyses have shown that MP composition depends upon the culture conditions and the kind of stimuli used to induce them in addition to the cellular source [38–41]. Regarding their composition, function, and effects, the studies of Pisetsky have suggested that the most divergent types of MPs are those generated by apoptosis and cell activation [42]. Some of the biological responses induced by MPs and their association with the cellular origin and composition of these structures are summarized in Table 2. For a comprehensive review of the more common methods used for the induction of MPs *in vitro* and their implications in phenotypic changes, go to [33, 43].

4. MPs as an Important Source of Autoantigens

It has been proposed that excessive production of MPs (e.g., from the presence of environmental factors such as certain infectious agents and/or chronic exposure to drugs) may predispose one to autoimmune diseases [10]. Excess MPs and their wide distribution through interstitial areas could prevent their efficient clearance and allow them to become a potential source of neoantigens [12]. Neoantigens are derived from oxidative and nonoxidative modifications that mainly include citrullination, cysteine oxidation, phosphorylation, glycosylation, sumoylation (for its acronym small ubiquitin-related modifier), and covalent addition of fatty acids such as palmitoylation.

Citrullination is a posttranslational modification that converts peptidylarginine ends to peptidyl citrulline through the enzyme peptidylarginine deiminase (PAD). This post-translational change has important implications in the pathophysiology of RA. RA patients develop autoantibodies against citrullinated peptides and/or polypeptides (anti-CCPs or ACPA) [69]. MPs are present in the synovial fluid (13) where PAD isoforms 2 and 4 are functional and the citrullination process is remarkably high [70]. Therefore, surface proteins on MPs can also become citrullinated leading to the formation of neoantigens. Cloutier et al. [12] showed that the antibodies from synovial fluid of RA patients recognize platelet-derived MPs; blockade assays indicated that this interaction is mediated at least in part by the presence of citrullinated peptides on these structures. The isolation of CD41+ MPs from the synovial fluid of RA patients by affinity columns and their further proteomic analysis showed that these vesicles have IgG specific to citrullinated peptides and C3a on their surface. Total antibodies eluted from these MPs recognize several targets such as apolipoprotein A1, citrullinated forms of clusterin, fibrinogen alpha and beta chains, vimentin, filaggrin, and histones H2A and H2B [12].

In the pathogenesis of RA and SLE, important components of MPs are generated during the apoptosis of certain types of cells including platelets and leukocytes. These components include histones and the nonhistone nuclear protein HMGB1 (high-mobility group protein1). These nuclear molecules can promote vascular and nonspecific immune responses and the generation of autoantibodies [71]. Although DNA and histones located in MPs are an important source of autoantigens, HMGB1 can trigger additional effects in host cells. HMGB1 is mainly found in the nucleus of cells, but it can be translocated to the cytoplasm and the extracellular space during cell activation and death [42]. It was observed that healthy individuals who received 2 ng of LPS/Kg of body weight showed an increase in circulating CD14+ CD42a+ MPs with surface expression of HMGB1 [72]. HMGB1 can undergo different posttranslational modifications such as cysteine oxidation; this particular change leads to its recognition by TLR4. In addition, oxidized HMGB1 can bind the chemokine CXCL12 and induce chemotaxis through CXCR4 [73]. Therefore, MPs containing oxidized forms of HMGB1 can signal through CXCR4 and TLR4. HGMB1 has been detected on the surface of MPs and as part of their ICs in the synovial fluid of RA patients [74]. In SLE, HMGB1 [42] was reported to be a component of some MPs containing anti-DNA antibodies forming ICs [74]. In summary, HMGB1 on MPs could be a key component of the immunomodulatory effects of MPs [75]; this modified protein can act as an "adjuvant" due to its ability to directly bind TLRs [72, 74].

There are other posttranslational modifications described in autoimmune diseases that generate neoantigens and could be involved in the immunomodulatory effects of MPs in these pathologies. However, they have not yet been studied in detail. For example, the oxidation and nitration of different biomolecules by peroxynitrite were described in SLE [76]

Table 2: MPs as mediators of communication between cells.

Effects	Cell source of MPs → target cell	*In vitro* generation of MPs	Content of MPs	REF
Chemotaxis of mononuclear phagocytes to the endothelium	Platelets → endothelial cells	Apoptosis	RANTES/CCL5	[54]
Nonspecific chemotaxis	Platelets → human neutrophils	PGE1	Fibrinogen receptor Glycoproteins and integrin αIIbβ3	[33, 55]
Effects on cell proliferation and differentiation	Platelets → endothelial cells Tumoral cells (human)	Cells activation and starvation	miRNA	[56, 57]
	Platelets → endothelial precursors Tumoral cells → different types of cells	PGE1	Epidermal growth factor receptor	[33, 55]
Apoptosis of endothelial cells and inhibition of osteoclastogenesis in RA	Platelets → endothelial cells, osteoclast	Activation	miRNA-223	[58]
Induction of GLUT4 expression in insulin-resistant cells	Platelets → endothelial cells	Activation	miRNA-223	[59, 60]
Endothelial activation	Myeloid pyroptotic and activated cells → endothelium and leukocytes (humans)	Apoptosis	IL-1-β	[54]
Synthesis of sphingomyelinase, M2 activation, and cell death	Human macrophages → endothelial cells	Calcium-mediated apoptosis	AA	[61, 62]
Coagulation, cell transformation, and inhibition of endothelial cells	Mononuclear, endothelial, and tumoral cells → platelets	Staurosporine-induced apoptosis	Cardiolipin, platelet activating factor	[63, 64]
Coagulation	Mononuclear cells → platelets, megakaryocytes	Apoptosis	CCR5	[65]
	Monocytes → platelets (humans)	Thrombin activation	Tissue factor	[33, 55]
Antigen presentation, cross-presentation, and anergy	Among leukocytes (humans)	Apoptosis	MHC	[33, 55]
Expression of receptors from other cellular origins and transformation	Tumor, stem, and endothelial precursor cells → platelets, myeloid cell lines (humans)	Overgrowth and activation	mRNA	[66–68]

and glycosylation of serum proteins was reported in RA [77]. Despite the presence of specific antibodies against these neoantigens in patients, the direct participation of these modifications on the phenotype of MPs is still unknown.

As previously mentioned, the interaction of MPs with target cells may occur by mechanisms both dependent and independent of surface receptors. Receptor-mediated mechanisms of recognizing MPs and their constituents include several members of the scavenger receptor family, PS receptors, and integrins, among others. Although the evidence is clear that MPs can interact with antibodies [78], the effects that mediate these interactions through particular receptors are not completely understood. When MPs bind to antibodies, various immune cells, mainly phagocytes through Fc receptors (FcR) and complement receptors, might recognize them. Therefore, it is tempting to postulate that MPs forming ICs, in addition to being a source of modified substrates (neoantigens) with the ability to directly bind TLRs, could amplify the effects due to the increased number of receptors with which they can interact and cross-react. In fact, it has been determined that circulating MPs from SLE patients expose PS on their surface and are coated with IgG, IgM, and C1q; these molecules are considered to be the identity signals of antibody deposition and complement activation in tissues [79]. The number of MPs binding IgG and the amount of IgG in these particles are increased in SLE patients compared to those with RA or Sjögren's syndrome (SS) [80]. Hence, it is expected that the amount and isotype of these antibodies on MPs (MP-ICs) could partly determine the means of recognition by target cells and their responses. The binding of MPs to opsonic and nonopsonic receptors could trigger complex immune responses in several autoimmune diseases,

probably through the activation of multiple signaling pathways that depend on the stoichiometry of the components that constitute the MP-ICs [10].

5. Involvement of MPs and Their ICs in SLE and RA

Changes in the number and composition of circulating MPs have been associated with the immunopathology of different autoimmune diseases and could potentially become a diagnostic and prognostic tool.

5.1. MPs in SLE. In SLE patients, tissue deposition of ICs leads to chronic damage in several organs. ICs are formed mainly by autoantibodies against nuclear constituents such as double-stranded DNA (ds-DNA), nucleosomes, ribonucleoproteins, and RNA. However, in SLE there are additional autoantigens with the potential to form ICs such as phospholipids, plasma, and extracellular matrix proteins, among others [81, 82].

In patients with SLE, circulating MPs differ in their amount and composition compared to MPs from patients with other autoimmune diseases or from healthy controls. Proteomic analysis has revealed that MPs from SLE patients contain more immunoglobulins (mainly IgG (some directed against dsDNA), IgA, and IgM (anti-dsDNA, rheumatoid factor)) and complement proteins (C1q, C1s, C3, C4b, and C9) than those from healthy controls, RA patients, or SS patients [83]. SLE patients also have increased numbers of annexin V+ MPs in their plasma compared to healthy controls [84]. This increase was found to be due to platelet-derived MPs (CD41A+), which were probably activated (CD62P+). An inverse and significant correlation was found between the number of MPs with anti-dsDNA antibody and the disease activity index (measured by SLEDAI) in patients with SLE [84]. However, platelet-derived MPs are considered to be less immunogenic due to their very low to undetectable amounts of DNA [78, 85–87]. Nevertheless, it has been reported that platelet-derived MPs are capable of forming ICs and inducing complement activation [80], a typical feature of SLE pathophysiology.

It is noteworthy that apoptosis-derived MPs from different cellular sources are able to compete with ACs for PS receptor binding on mononuclear phagocytes. It was demonstrated *in vitro* that the presence of these MPs from Jurkat cells leads to a significant reduction in the phagocytosis of ACs in a dose-dependent manner; this effect was prevented by PS blockade through annexin V [19]. It has been extensively reported that the uptake and clearance of ACs by phagocytic cells are reduced in SLE patients [88]; therefore, the increase in the number of MPs observed in this disease could be a further explanation for the prolonged presence of ACs at extracellular locations in these patients.

However, there are contradictory results regarding the number of annexin V+ MPs in SLE. In 2011, Nielsen et al. reported a decreased amount of these particles in SLE patients compared to healthy controls. However, those patients had a higher frequency of annexin V− MPs mainly derived from endothelial cells, and other cells sources were not identified in that study [30]. This suggested that MP generation in SLE patients can be an event independent of apoptosis that is probably mediated by cell activation. A high amount of annexin V− MPs is positively correlated with disease activity (measured by SLEDAI), the presence of active nephritis, hypertension, arterial thrombosis, and elevated triglyceride titers [30]. In addition, the low exposure of PS by MPs in these patients could lead to decreased clearance by monocytes and macrophages. This may favor the persistence of these structures in the extracellular milieu where they can be modified by nitrosylation [89], oxidation, and citrullination [12, 90].

Ullal et al. demonstrated that MPs generated by staurosporine treatment (an apoptosis inducer) of the myeloid cell lines HL-60 and THP-1 and the CD4 T cell line Jurkat exposed histones, DNA, and nucleosomes on their surface [78]. Antibodies present in the plasma from patients with SLE bind to such *in vitro* generated MPs more strongly than antibodies from healthy controls, which display weak to absent binding activity. This interaction was not entirely affected by DNase and RNase treatment, suggesting that these antibodies might interact with other antigens [78]. The amount of surface-bound IgG is greater in circulating MPs from SLE patients than those from healthy controls. There is a significant and positive correlation between the titer of anti-DNA antibodies and the circulating amount of IgG+ MPs in SLE patients [78]. Thus, it is important to study the role of these vesicles in conjunction with their modifications and their ability to form ICs in the inflammatory processes observed in different tissues in these patients, for example, lupus nephritis.

Recently, Nielsen et al. showed an increased concentration of MPs in SLE patients with augmented levels of IgG1, IgM, and C1q compared to healthy controls. The number of circulating IgG1+ MPs was significantly associated with the presence of autoantibodies in serum against dsDNA, extractable nuclear antigen and histones [79]. Although these authors did not discuss this latter finding in detail, it is possible to speculate that the elevated number of apoptotic leukocytes observed in some patients with SLE [91] favors the generation of MPs containing nuclear components on their surface able to form ICs. In the same study, a positive correlation was found between the amount of circulating IgG+ MPs, the presence of anti-C1q antibodies (but not with other autoantibodies, such as anti-dsDNA, anti-ENA, and anti-histone), and complement consumption through the classical pathway (complement proteins C4, C3, and C1q) [79]. These results highlight the possible role of MPs in the pathogenesis and perpetuation of the inflammatory process in SLE because they constitute an important source of autoantigens and circulating ICs able to activate complement cascades.

Pisetsky and Lipsky [9] proposed an interesting model of the pathogenesis of MPs in SLE based on their own results and those from other authors (Figure 3). MPs that contain DNA and RNA can behave as self-adjuvants and increase tolerance of immature B-lymphocytes and break the tolerance of mature B cells. Immature B cells that recognize DNA on MPs with high avidity can be negatively selected. In contrast,

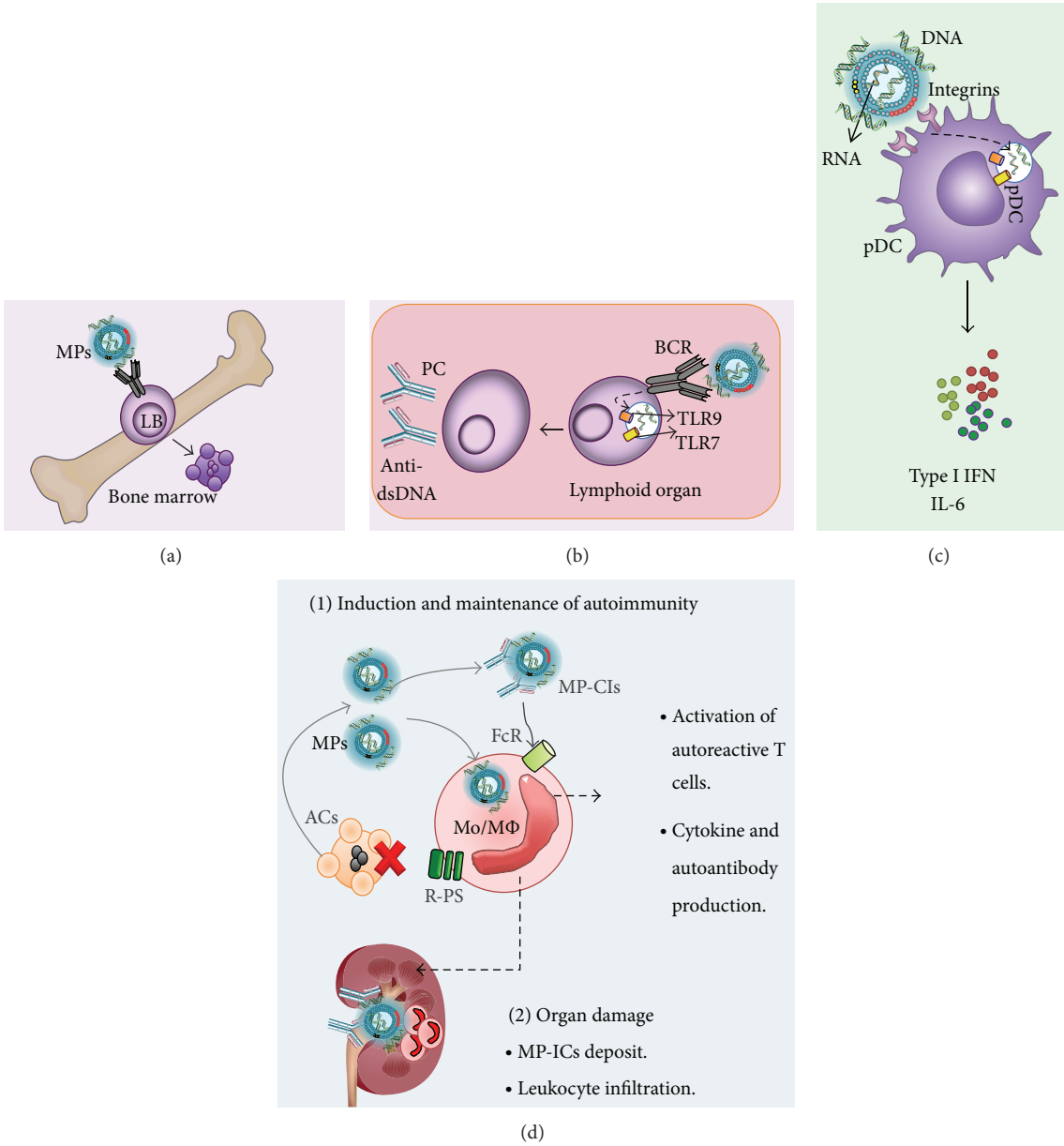

Figure 3: Role of MPs in SLE. MPs can interact with B cells (LB) (a) during ontogeny-induced apoptosis (clonal deletion), secondary rearrangement, or BCR edition in cells whose BCRs recognize DNA with high affinity. (b) At the lymphoid organ level, MPs can also bind to an autoreactive BCR and induce anergy of LB or alternatively be endocytosed by these cells and induce a second signal through TLR9 and TLR7 by the DNA and RNA present on these structures. These recognition activate and differentiate B cells into plasma cells able to produce autoantibodies. (c) MPs can be internalized by plasmacytoid dendritic cells (pDCs) and through the recognition of nucleic acids produce type I IFNs and other cytokines such as IL-6. (d) MPs might compete with ACs to bind PS receptors on monocytes and macrophages (Mo/MΦ), which seem to contribute to the lower uptake of ACs observed in these patients. In addition, MPs can be a major source of autoantigens in SLE with the consequent generation of ICs; all this could eventually (1) produce and maintain the inflammatory immune response and (2) promote the damage of different tissues and organs in patients with SLE due to the exacerbated inflammatory process.

self-reactive B lymphocytes that escape from central tolerance mechanisms can recognize and endocytose MPs through their BCR at the periphery. This might favor contact of the nucleic acids present on MPs with endosomal TLR9 in B lymphocytes. This interaction may trigger their activation and differentiation into plasma cells with the consequent production of autoantibodies in a manner independent of T lymphocytes.

These authors also propose that MPs endocytosed by plasmacytoid dendritic cells through integrins or PS receptors are able to contact intracellular TLR7 and TLR9, leading to cell maturation and the production of proinflammatory cytokines such as type I interferons (IFNs-I) and IL-6. The presence of these serum factors in SLE patients has been positively correlated with disease activity [92]. IFNs-I act directly on the adaptive immune response, inducing the

differentiation of Th1 lymphocytes, the proliferation of memory CD8+ T cells, and the generation of plasmablasts. IL-6 mediates the differentiation and survival of the latter cells into long-lived IgG-secreting plasma cells [92]. Therefore, these cytokines produced by innate cells are involved in the autoantibody secretion and the consequent IC formation observed in this autoimmune disease [93, 94]. It is not clear what role monocytes and macrophages play in the recognition of MPs during the immunopathology of SLE. However, based on the findings reviewed herein, it could be speculated that these cells are activated by MPs and these MPs may or may not form ICs. These could be reached through Fc receptors (CD16, CD32, CD64, and TOSO), complement receptors (CRs), TLRs, and PS receptors (PSRs and "scavenger" receptors). Furthermore, MPs can compete with apoptotic cells for PS receptors, which promotes the persistence of such structures in the extracellular space, perpetuating the source of autoantigens and the inflammatory process in SLE (Figure 3).

5.2. MPs in RA. This disease is characterized by the presence of IgG antibodies against citrullinated proteins and rheumatoid factor (RF) in circulation and in synovial fluid. RF corresponds to an IgM antibody against the Fc portion of IgG but it can also be the IgA and IgE isotypes.

Platelet-derived MPs (PMPs) and leukocyte-derived MPs with procoagulant effects (LMPs) are increased in the circulation and synovial fluid of patients with RA [10, 84]. These MPs have been associated with disease activity, as measured by the DAS28 score, and they are also associated with joint inflammation, cartilage and bone destruction, angiogenesis, and pain [71, 95]. MPs have also been found in synovial fluid from patients with osteoarthritis, reactive arthritis, and microcrystalline arthritis; however, patients with RA and microcrystalline arthritis exhibit higher concentrations of these MPs compared with patients with osteoarthritis or reactive arthritis [96].

Leukocytes and synovial cells play critical roles in the development of joint inflammation and tissue damage and in the generation of pathogenic MPs. Synovial cells, fibroblast-like synoviocytes (FLSs), favor the development of autoimmunity because they secrete B cell activating factor (BAFF), CXCL12, and CXCL13. These factors attract B cells to the joint and promote the formation of pseudofollicles in the synovial membrane [97, 98]. Leukocytes represent the main source of MPs in the synovial fluid of RA patients, which are derived mainly from macrophages (>40%), T cells and granulocytes (20–25%), and platelets (<10%) [95]. It was reported that FLSs produce BAFF, IL-6, and IL-8 in response to MPs isolated from the synovial fluid of RA patients [96]. FLSs display elevated production of other molecules that can directly or indirectly influence the activation of B cells, such as thymic stromal lymphopoietin (TSLP) and secretory leukocyte protease inhibitor (SLPI), in response to MPs [96].

Other authors have found similar results regarding the proinflammatory effects of MPs. LMPs extracted from the joints of patients with RA induce the release of IL-6, CCL1, CCL2, and CCL5 by FLSs from the same individuals [95], and MPs obtained from Jurkat cells and U937 human promocytes induce the production of angiogenic chemokines such as CXCL1, CXCL2, CXCL3, CXCL5, and CXCL6 by FLSs from RA patients. These factors might contribute to the hypervascularization observed in inflamed joints [99]. Furthermore, it was reported that MPs can induce other proinflammatory factors such as prostaglandin E2 in FLSs [63]. This evidence suggests that MPs are actively involved in the inflammatory process in the joint and in the systemic responses observed in RA patients. Therefore, MPs function in this disease by communicating and amplifying the inflammatory response of leukocytes and other cells involved in the pathophysiology of RA.

Using flow cytometry and electron microscopy, it was revealed that MPs forming ICs (mpICs) from the synovial fluid of RA patients were larger (average size of 2800 nm) than MPs alone [12]. In these mpICs, CD41 was frequently detected suggesting that they were derived from platelets. Despite the surface detection of FcγRIIa on these particles, ICs were formed through specific recognition by autoantibodies against citrullinated vimentin and fibrinogen. In addition, these mpICs induced the production of proinflammatory leukotrienes (LTB4, 6-trans-LTB4, 12-epi-6-trans-LTB4, 20-OHLTB4, and 20-COOH-LTB4) by human neutrophils. With these sets of data, the authors suggested that MPs form circulating and articular ICs are able to induce several effects on the phagocytic cells perpetuating the inflammation. In this study, the presence of RF on MPs was not evaluated, and therefore it would be essential to determine whether mpICs from patients with RA contain this autoantibody and its implications in the immunopathology of this disease. Boilard et al. [64] reported a higher frequency of PMPs in the synovial fluid of RA patients compared with the percentage reported by Berckmans et al. [95]; apparently, these particles have an important proinflammatory role in the pathology of this disease because PMPs elicit cytokines from synovial fibroblasts via IL-1. A considerable number of patients with RA have elevated frequencies of PMPs in their synovial fluid compared to patients with osteoarthritis, in which PMPs are barely detectable [64].

Monocytes and macrophages are considered central components of the immunopathogenesis of RA. They are involved in the formation of pannus, they are one of the main producers of TNF-α and IL-1β, they participate in the activation of effector T cells, and they also have the ability to produce other cytokines and chemokines important in RA such as IL-6, IL-8, IL-10, CCL2, CCL3, and RANTES. In addition, these phagocytes are reported to be involved in the generation of autoantigens because they are a source of PAD-2 and PAD-4. Despite the central function these mononuclear phagocytes have in the immunopathology of RA and the role they must play in the recognition and clearance of MPs, it is still unknown whether these structures might induce differential effects on monocytes and macrophages depending on whether they are from patients with RA or healthy controls.

TNF-α has been identified as a key component of RA with multifunctional effects associated with inflammation and joint destruction [100]. The efficacy of anti-TNF-α treatment in RA has led to extensive research about the mechanisms

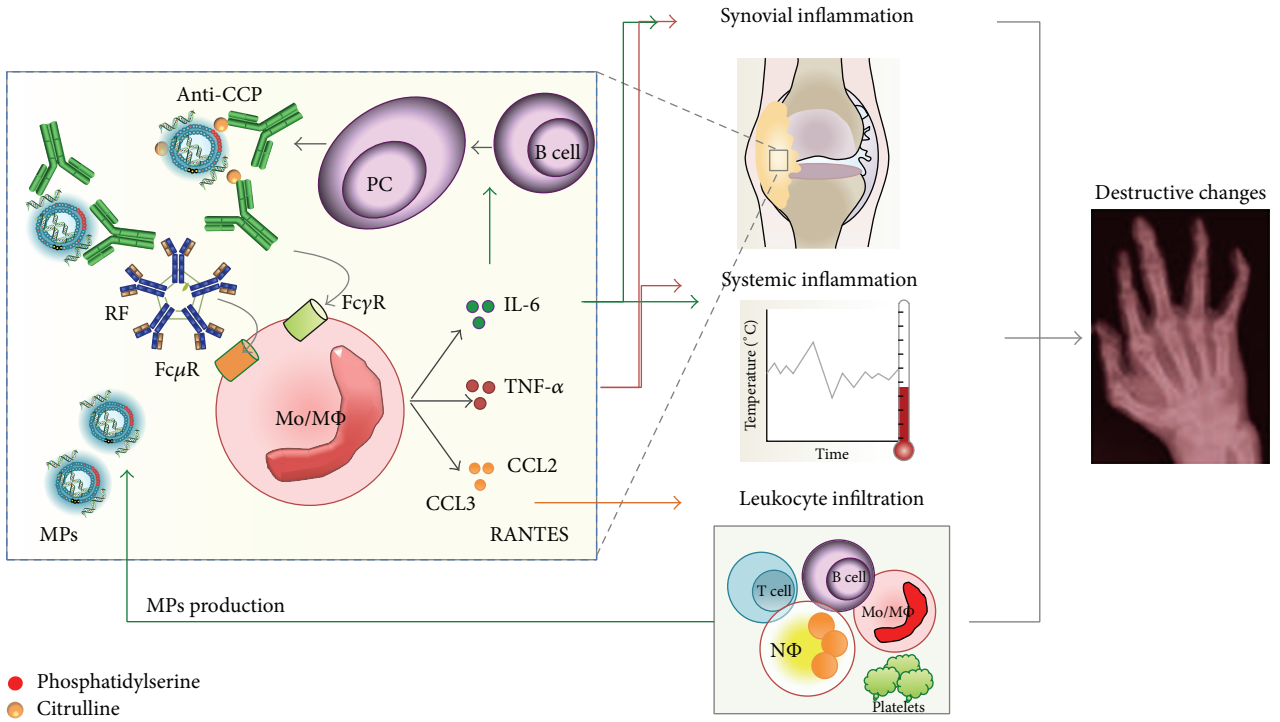

Figure 4: Role of MPs in RA. The high concentrations of MPs from different leukocyte populations reported in the synovial fluid from RA patients must be citrullinated and form ICs with anti-CCP antibodies and RF autoantibodies. These complex structures could be recognized by Mo/MΦ through isotype-specific Fc receptors (FcγR and FcμR) and induce the production of proinflammatory cytokines such as TNF-alpha, IL-6, and the chemokines CCL2, CCL3, and RANTES. These soluble factors participate in the systemic inflammation in the synovium and the destructive changes observed in the joints of RA patients. IL-6 is also involved in the induction of plasma cells (PCs) producing autoantibodies.

that regulate its production in this disease. Reports indicate that 50 percent of RA patients are positive for anti-CCPs and circulating ICs formed by citrullinated fibrinogen [101]. These ICs induce the production of TNF-α *in vitro* by macrophages obtained from normal controls in a dose-dependent manner; this production was found to be inhibited by blockade of FcγRIIa but not FcγRI or FcγRIII [102]. In addition, the simultaneous binding of ICs containing citrullinated fibrinogen by FcγR and TLR4 induces even more TNF-α production by macrophages from healthy subjects [103]. Because there are increased levels of MPs forming ICs that depend on citrullinated antigens in the synovial fluid of RA patients [12], we hypothesize that the systemic inflammatory response and intrinsic activation of monocytes and synovial macrophages in RA patients may be partially explained by the recognition of these structures through FcγRs and complement receptors. However, the potential involvement of other receptors should be noted such as FcμR (TOSO), which can recognize the FR (IgM isotype) on MPs (95) if this antibody is present in these membrane structures. Therefore, we propose that MPs could be the major source of circulating ICs in RA patients, which would lead to mononuclear phagocyte activation and the secretion of different mediators such as TNF-α, IL-6, and chemokines (CCL2, CCL3, and RANTES) that amplify the local and systemic inflammatory responses (Figure 4).

6. Conclusions and Perspectives

The evidence presented in this review indicates that MPs seem to be implicated in the autoimmune pathogenesis of SLE and RA as an important source of autoantigens and ICs. Additionally, MPs containing DNA, RNA, HMGB1, or other macromolecules could serve as adjuvants for the production of autoantibodies and perpetuate the inflammatory process in these diseases through TLR recognition. Hence, high circulating concentrations of these modified vesicular structures, forming or not forming ICs, may actively participate in the chronic inflammatory responses, severity peaks, and symptom relapses evidenced in patients with RA and SLE. However, the evidence supporting the participation of MPs in these diseases comes mainly from *in vitro* studies; therefore, advanced and improved laboratory techniques and *in vivo* experimental findings are required to allow a better understanding of the role of these structures in different contexts and in autoimmune responses.

MPs that expose PS on their surface could favor the M2 activation profile on macrophages through the binding of "*scavenger*" receptors as was previously demonstrated with apoptotic bodies. However, changes in the components of MPs (neoantigens) and interaction with autoantibodies to form ICs seem to bring these structures to phagocytic cells through other receptors that trigger M1 responses. This

profile deviation in conjunction with the accumulation of MPs in circulation and different tissues could contribute to pathogenic effects on SLE and RA such as the proliferation of endothelial cells in the mitral valve [7], thrombotic events [104, 105], and complement activation, among others [86, 106–108].

Furthermore, the influence of the amount and phenotype of MPs on the activation and responses of monocyte subsets (CD14++CD16− and CD14++CD16++) have not yet been explored. It is known that CD14+CD16++ monocytes phagocytose more ACs [109] and produce more TNF-α in response to different stimuli. Thus, MPs may promote the response of CD14+CD16++ monocytes in RA patients, who show an increase in CD16+ monocytes [110]. In results from our group, it was observed that CD14+CD16++ monocytes were reduced in patients with active SLE [111]; this fact could reduce the removal and clearance of MPs in these patients and therefore allow an increasing source of circulating autoantigens and ICs.

It is necessary to continue the study of MPs in the context of RA and SLE and other autoimmune diseases to determine their value as biomarkers for diagnostic and prognostic purposes. For example, it should be evaluated whether certain MPs reflect a state of systemic or local activation of particular cell types or if they are associated with clinical outcome, the development of comorbidities, complications, or severe forms of RA and SLE. In addition, MPs also have attractive potential as biopharmacological agents in autoimmune diseases because they could be used in the treatment of these and other diseases as modulators of the immune response or as drug carriers to specific targets of interest.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Selected Aspects in the Pathogenesis of Autoimmune Diseases

György Nagy,[1] Peter C. Huszthy,[2] Even Fossum,[3] Yrjö Konttinen,[4] Britt Nakken,[2] and Peter Szodoray[2]

[1] *Department of Genetics, Cell and Immunobiology, Semmelweis University, Budapest 1089, Hungary*
[2] *Centre for Immune Regulation and Department of Immunology, University of Oslo and Oslo University Hospital, Rikshospitalet, 0372 Oslo, Norway*
[3] *K.G. Jebsen Center for Influenza Vaccine Research, Institute of Immunology, University of Oslo and Oslo University Hospital, 0372 Oslo, Norway*
[4] *Department of Medicine, Institute of Clinical Medicine, Helsinki University Central Hospital and ORTON Orthopaedic Hospital of the Invalid Foundation, 0280 Helsinki, Finland*

Correspondence should be addressed to Peter Szodoray; szodoray@gmail.com

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Autoimmune processes can be found in physiological circumstances. However, they are quenched with properly functioning regulatory mechanisms and do not evolve into full-blown autoimmune diseases. Once developed, autoimmune diseases are characterized by signature clinical features, accompanied by sustained cellular and/or humoral immunological abnormalities. Genetic, environmental, and hormonal defects, as well as a quantitative and qualitative impairment of immunoregulatory functions, have been shown in parallel to the relative dominance of proinflammatory Th17 cells in many of these diseases. In this review we focus on the derailed balance between regulatory and Th17 cells in the pathogenesis of autoimmune diseases. Additionally, we depict a cytokine imbalance, which gives rise to a biased T-cell homeostasis. The assessment of Th17/Treg-cell ratio and the simultaneous quantitation of cytokines, may give a useful diagnostic tool in autoimmune diseases. We also depict the multifaceted role of dendritic cells, serving as antigen presenting cells, contributing to the development of the pathognomonic cytokine signature and promote cellular and humoral autoimmune responses. Finally we describe the function and role of extracellular vesicles in particular autoimmune diseases. Targeting these key players of disease progression in patients with autoimmune diseases by immunomodulating therapy may be beneficial in future therapeutic strategies.

1. Introduction

Autoimmune diseases are typically multietiological entities, where genetic and environmental abnormalities along with derailed immunoregulatory processes contribute to the development of disease. In the healthy immune system, various tolerance mechanisms, such as activation-induced cell death, anergy, or clonal ignorance, play a protective role to prevent the activation of self-reactive lymphocytes [1]. In autoimmune conditions, self-reactive lymphocytes may not be subjected to the aforementioned tolerance mechanisms raising the possibility of the survival and activation of autoreactive T and B cells upon autoantigen encounter [2–4]. However, there is a fine line between autoimmune processes, which also appear in healthy individuals and manifested autoimmune diseases. In autoimmune diseases, one or several tolerance mechanisms permanently fail due to the constellation of various environmental factors, specific HLA- and non-HLA genes and/or derailed immunoregulatory processes, leading to the persistence of self-reactive T- and B-cell clones and ultimately organ damage [4, 5]. Immunoregulatory abnormalities and/or the imbalance of immunoregulatory and inflammatory processes could lead to the progression towards autoimmune diseases. Besides faulty tolerance mechanisms, several other factors, such as imbalance of the pro- and anti-inflammatory cytokines, extracellular vesicles, abnormal autoantigen scavenging machinery, and antigen presentation, can contribute to the development

and perpetuation of autoimmune processes and eventually to the progress towards autoimmune diseases. Herein we aim to address some selected pathogenetic points in the development of autoimmune diseases.

2. Animal Models of Autoimmunity

Acquired immunity has evolved with an intricate control system to balance pro- and anti-inflammatory responses. Autoimmunity or immunity toward "self" is a pathological process that involves autoreactive B cells and corresponding autoantigen-specific T cells, imbalances in cytokine levels, and a shifted leukocyte polarization profile. In most of these diseases, a proinflammatory environment dominates, with a Th1 (type 1 insulin-dependent diabetes mellitus, Hashimoto's thyroiditis), Th17 (multiple sclerosis), or combined Th1/Th17 (Sjögren's syndrome) signature. Animal models of autoimmunity have been important research tools for many years now, aiding to pinpoint various components of the pathogenesis of human autoimmune diseases. Today, more than 80 types of autoimmune pathologies are recognized, most with distinct clinical profiles. Animal models have been developed for all the major disease entities, for example, type 1 diabetes mellitus (T1D), rheumatoid arthritis (RA), multiple sclerosis (MS), Sjögren's syndrome (SS), and systemic lupus erythematosus (SLE). Based on the etiological background and induction of symptoms these animal models can be divided into three broad categories: spontaneous, induced, and genetically engineered. The strengths and weaknesses of each are briefly discussed below.

2.1. Spontaneous Models of Autoimmunity. Susceptible rodent strains spontaneously develop autoimmunity. Well-known examples include the NOD mouse that develops T1D and inbred mice (MRL/*lpr*, NZB/KN, and Biozzi) that develop arthritis. NOD mice have been extremely useful in delineating the basic principles of T1D [1]. As in humans, so in both NOD mice and susceptible BB rats, T1D is dependent on a collection of genetic traits rather than a single gene [6]. The dependence of T1D on autoreactive T cells and the resolution of disease through T-cell modulation have been important proofs of principles observed in NOD mice. In NOD mice, the sequential development of human T1D is well recapitulated, starting with peri-insulitis (leukocytic infiltration into periductal areas, 3–6 weeks) followed by intrainsulitis (islets are invaded by inflammatory cells recognizing β-cell autoantigens) and diabetes (T cell-mediated destruction of islets, 10–30 weeks).

A recent spontaneous model for MS is the relapsing-remitting mouse (RR mouse). RR mice harbor increased frequencies of myelin-specific CD4 T cells and are on a SJL/J background [7]. This model is unique, as it resembles human MS in the dependence on autoreactive B cells and the production of autoantibodies and develops without artificial induction [8]. A proof of principle for the contribution of gut microbiota to MS development by T-cell activation has been shown using this model system [9]. Along these lines the contribution of viruses or bacteria as triggers for autoimmune diseases is an important field of study that would benefit from animal models.

2.2. Induced Models. Systemic autoimmunity may be induced in rodents after injection of the lipid pristane (tetramethylpentadecane; leading to SLE and RA), mercury, or foreign grafts (leading to graft versus host disease, GVHD). Animal models of organ-specific autoimmunity are established by administering a tissue-derived self-antigen (or a peptide thereof) together with a strong adjuvant. Well-known models are collagen induced arthritis (CIA) for RA and experimental allergic encephalomyelitis (EAE) for MS. For CIA, intradermal injection of collagen type II together with adjuvant activates autoreactive B and T cells. In susceptible rat strains, such as DA, Freund's adjuvant alone induces arthritis (AA). CIA successfully recapitulates human RA histopathology, with synovial joint inflammation and bone erosion. Regarding the disease course, pristane-induced arthritis more closely resembles chronic, relapsing-remitting human RA, whereas AA usually shows rapid remission. To induce EAE, CNS antigens in adjuvant are used for immunization or myelin-specific Th1 cells are transferred to susceptible strains, such as Lewis rats. In contrast to human MS, which is relapsing-remitting or develops progressively, EAE induced after T-cell transfer to rats has a monophasic disease course [10]. Although proinflammatory lymphocytic infiltration is achieved, the massive demyelination seen in MS is not consistently recapitulated [11]. A major drawback of inducible models is that they are not suitable to elucidate the etiology of the disease and typically fail to recapitulate the full scale of pathological traits associated with human autoimmunity. The disease course in humans with a given diagnosis is often diversified, whereas that in the mouse is highly predictable. Within inbred rodent strains, genetic homogeneity, a homogenous environmental milieu, and the evolutionary distance from humans certainly contribute to this effect.

2.3. Genetically Engineered Models. Throughout the 1980s, with the aid of more advanced techniques in cell and molecular biology such as gene cloning and mouse embryonic stem cell manipulation technology, more representative rodent models have been generated. These technologies enabled scientists to generate novel mouse strains with defined genetic changes suspected to contribute to autoimmunity (e.g., expression of defined MHC alleles or selected cytokines). Furthermore, these technologies allowed researchers to clone and sequence T-cell lines specific for autoantigens. The T-cell lines were instrumental for transfer experiments and for engineering T-cell receptor knock-in mice [12]. Transgenic mice that express defined human or mouse MHC class II alleles have been successful in recapitulating a whole range of autoimmune pathologies, most notably multiple sclerosis, chronic inflammatory bowel disease, and myasthenia gravis [13]. Mice engineered to express full length human TNF-α spontaneously develop chronic inflammatory polyarthritis [14]. Proof of principle for TNF-α blockade in treating RA has been obtained in this model, an early success story for translational research. Transgenic expression of the human

T-lymphotropic virus-1 genome leads to the development of arthritis in mice and this model suggested the role of this virus in the development of human RA [15, 16]. A major advantage of such genetically engineered models is that the induced changes (genes) can be precisely defined and experimentally controlled through comparisons with the parental background strain. Furthermore, they allow for spatial and temporal control of gene expression, through tissue specific or inducible promoters. In addition, expression of a fluorescent or luminescent reporter facilitates *in vivo* imaging approaches.

2.4. General Considerations. Ideally, an animal model should reflect the whole range of features associated with human pathology, not only isolated traits thereof. If it is a genetically targeted model, it should rely on homologues of genes/pathways known to be responsible for autoimmunity in humans. Finally, it is desirable that the disease develops spontaneously, so that the etiology of the given syndrome may be investigated. Although none of the animal models have all these features, they have, in concert, been invaluable tools that have shed light on basic disease mechanisms. This has been important, since in many human autoimmune diseases, progression is typically correlated only to serum markers with the pathological tissue being inaccessible (such as T1D or MS) or limited (RA or SS). With the application of more refined imaging techniques, such as PET/CT and intravital microscopy, animal models will continue to provide useful mechanistic insights into metabolic parameters, disease course, and pathways of leukocyte migration. We argue that for any given autoimmune disease, scientific evidence collected from several animal models, inducible, genetically modified, and spontaneous, correlated with clinical parameters and characterization of biopsy/autopsy tissue will be needed to extract more biological information with relevance to human pathology.

3. The Role of Regulatory T Cells in Autoimmune Diseases

The fine balance between pro- and anti-inflammatory processes within the immune system is crucial to maintain the antigenic integrity of the individual, while at the same time must effectively eliminate pathogens. A spectrum of characteristic immunocompetent cell populations exists, which have the capability to suppress immune and autoimmune processes. These suppressor activities can be achieved by either cell-cell contact or via anti-inflammatory soluble mediators, for example, anti-inflammatory cytokines. A key member of the family of immunoregulatory cells is denoted as regulatory T cells (Tregs). Tregs derive either from the thymus [$CD4^+CD25^{bright}FoxP3^+$ natural Tregs (nTregs)] or the peripheral blood [IL-10, or TGF-β producing Type-1 regulatory T cells (Tr1)] [17]. Under pathological circumstances, when quantitative and/or qualitative changes in regulatory T cells and proinflammatory immune responses are evoked, on a susceptible genetic background, autoimmune processes can occur, and eventually various autoimmune diseases can develop. In line with this hypothesis, previous studies on autoimmune disease depicted that the selective decrease in the number of Tregs or, alternatively, a diminished suppressor function of Tregs is characteristic to these diseases (e.g., SLE, Sjögren's syndrome, RA, and MCTD) [18–22]. These data indicate that in patients with established autoimmune diseases a sustained impairment of the regulatory T-cell pool exists.

In order to assess whether regulatory T-cell abnormalities could contribute to the development of autoimmune diseases, it is valuable to assess Tregs in medical conditions, predating the onset of full-blown diseases. In the forerunner medical condition for systemic autoimmune diseases, denoted as undifferentiated connective tissue disease (MCTD), the assessment of Treg cells showed that the percentage and absolute number of natural Tregs ($CD4^+CD25^{bright}FoxP3^+$) were diminished in UCTD patients compared with healthy subjects, while the number of inducible Tregs ($CD4^+IL\text{-}10^+$) was increased [23]. This progressive divergent shift in natural and induced Tregs clearly predicted the transition from the UCTD, introductory phase to a well-established systemic autoimmune disease [23]. In active systemic autoimmune diseases, the frequency of natural Tregs was found to be decreased compared to healthy individuals or compared to patients with inactive disease [18, 20, 24]. The other major regulatory T-cell subset denoted as IL-10 producing Type-1 regulatory T cells ($CD4^+IL\text{-}10^+$, Tr1) has been investigated and found to have an important role in the development of various autoimmune diseases [25]. IL-10, also known as human cytokine synthesis inhibitory factor (CSIF), is a multifunctional cytokine that can suppress the IFN-γ production of Th1 cells as well as having other important regulatory functions in differentiation of various T-cell subsets, B cells, or NK cells [26, 27]. Previously we have shown a significant increase in the number of IL-10 producing Tr1 cells in UCTD and we found further increase in patients who progressed into definitive systemic autoimmune diseases. We believe that this phenomenon represents a compensatory mechanism in order to downmodulate the effects of the observed IFN-γ overproduction [23].

4. The Role of Th17 Cells and the Th17/Treg Ratio in Autoimmune Diseases

Upon particular, proinflammatory conditions, T cells have the ability to differentiate into IL-17-producing T helper cells, denoted as Th17 cells, and this differentiation is independent of Th1 or Th2 cell development [28, 29]. Th17 polarization in humans requires IL-1β, IL-6, IL-21, and IL-23, which induce STAT3. While Th17 cells are important in host defense mechanisms against pathogens, the persistent secretion of IL-17 promotes chronic inflammation and is involved in the pathogenesis of inflammatory and autoimmune diseases. Th17 cells recruit neutrophils and macrophages to the site of inflammation; therefore, they are crucial in the initiation of inflammation [29]. As we described, increased levels of Th17 cells and secreted IL-17 have been associated with numerous inflammatory conditions and autoimmune diseases. High levels of IL-17 have been described in the sera, synovial

fluids, and synovial biopsies of most RA patients, while osteoarthrosis showed no increased levels of this cytokine [30, 31]. In other systemic autoimmune diseases, IL-17 and Th17 cells may play a role in the pathogenesis, indicated by studies on SLE, or patients with lupus nephritis [32, 33]. In Sjögren's syndrome, an increase in IL-17 has been found in both the serum and the affected salivary glands, indicating that the cytokine may play a part in both development of the glandular and systemic manifestations of the disease [34, 35].

As mentioned earlier, the imbalance of pro- and anti-inflammatory mechanisms, indicated by, for example, Th17 and Treg numbers or function, may initiate and perpetuate autoimmune diseases. Tregs develop in the thymus and participate in the maintenance of peripheral tolerance; however, circulating and local skewed cytokine milieu alters the suppressive function of these cells [36]. Focally, in affected organs of patients with autoimmune diseases increased IL-6 and TGF-β expression has been described, which favors the development of Th17 cells. In addition, increased concentrations of TNF-α, which is pathognomonic to many autoimmune diseases, downmodulate the function of Tregs, further contributing to the disequilibrium between the pro- and anti-inflammatory processes [36].

A clear shift exists in the cytokine homeostasis in a broad spectrum of autoimmune conditions, fueling the predominance of proinflammatory cells versus Tregs [37–46].

5. Cytokine Imbalance, Regulatory/Effector Cells in Various Well-Defined Systemic Autoimmune Diseases

5.1. Sjögren's Syndrome (SS). Sjögren's syndrome (SS) is a chronic, slowly progressive, systemic autoimmune disease that predominantly affects middle-aged women [47]. SS is characterized by mononuclear infiltration and destruction of the exocrine glands, resulting in dry mouth, keratoconjunctivitis sicca, and the presence of other exocrinopathic symptoms [47]. In the pathogenesis, different subsets of T and B lymphocytes and monocytes play a pivotal role. Increased cell activation, disproportional programmed cell death, and faulty autoantigen scavenging are important in the pathogenesis and processes that are partly driven by a skewed cytokine milieu [48, 49]. A group of peripheral cytokines, chemokines, and growth factors have been implicated in the pathogenesis of SS, contributing to the perpetuation of the cellular and humoral autoimmune processes [49–56]. Skewed T-cell subsets and cytokine imbalance seem to play important roles in an orchestrated proinflammatory cascade in SS. Among circulating cytokines, high IFN-γ, along with reduced levels of IL-10, has been described in SS [53]. Moreover, circulating cytokines have the ability to distinguish SS patients with ectopic salivary gland germinal centers, a possible forerunner of lymphoma development in the disease [35, 46, 55]. In patients with SS the following mediators seem to be increased compared to healthy subjects: IL-1β, IL-2, IL-6, IL-15, IFN-γ, and CCL4 (MIP-1β) [35]. pSS patients with ectopic germinal center formation were distinguished from healthy individuals by higher levels of IL-4, IL-10, GM-CSF, IFN-α, CCL3 (MIP-1α), CCL11 (eotaxin), and B-cell activating factor (BAFF/BLyS), while germinal center positive and negative pSS patients differed in CCL2 (MCP-1) expression. The biomarkers having the strongest discriminatory power amongst SS patients with or without ectopic salivary gland germinal centers were CCL11 (eotaxin) and IFN-γ, as well as BAFF/BLyS [35]. Taken together, these findings suggest that a group of mediators (e.g., cytokines and chemokines) has the ability to steer a proinflammatory milieu in these patients, presumably contributing to a derailed Th17/Treg balance.

5.2. Systemic Sclerosis. Systemic sclerosis (SSc) is a systemic disease of autoimmune pathogenesis, characterized by excessive extracellular matrix deposition and damage of the small blood vessels, leading to inflammatory processes, dominantly in the skin and visceral organs, such as the heart, lungs, or kidneys [57, 58]. Immunoregulatory abnormalities have been depicted in the pathogenesis of SSc [59–62]. Disorders of the immune system lead to chronic inflammatory processes, abnormal T-cell activation, B-cell abnormalities, abundant production of proinflammatory cytokines (e.g., IL-4), and the production of characteristic autoantibodies. Tregs with impaired function have been shown to play a role in the initiation and perpetuation of the disease [61, 62], while increased levels of circulating Th17 cell have been described, along with elevated IL-17 serum concentrations [63, 64]. An altered balance of the Th1 and Th2 cytokines may also be responsible for the development of fibrosis [65]. Patients with SSc had higher percentages of activated T cells, in addition to a population shift between the effector and regulatory T cells. Increased Th17 cell percentages, together with decreased levels of Th1, as well as regulatory T-cell subsets were characteristic of these patients [66]. Interestingly, the functional assessment of Tregs identified that the suppressor activity of Tregs was clearly decreased in SSc, compared to that of healthy individuals. Our data suggest that the increased Th17/Treg ratio and the altered regulatory function of Treg cells play an important role in the development and progression of SSc [40, 66, 67].

5.3. Mixed Connective Tissue Disease. In mixed connective tissue disease (MCTD) the most frequently observed symptoms are arthritis, Raynaud's phenomenon, myositis, esophageal dysmotility, and acrosclerosis along with the presence of autoantibodies reactive with U1 small nuclear RNP (U1RNP) autoantigens [68–76]. Previously we assessed serum cytokines and intracellular cytokine production of $CD4^+$ and $CD8^+$ T cells in patients with MCTD [76]. Serum concentrations of both type 1 and type 2 cytokines were significantly higher in patients with MCTD than in healthy controls. The percentage of IL-10-producing $CD4^+$ and $CD8^+$ T cells was higher in patients than in controls. In addition, $CD4^+$ and $CD8^+$ T cells from patients with active MCTD produced significantly more IL-10 than cells in patients with inactive disease or in healthy individuals [76]. MCTD is characterized by a wide spectrum of T-cell abnormalities, which becomes explicit in the active phase of the disease. Concerning the role of immunoregulatory abnormalities in the pathogenesis,

we assessed Tregs in patients with MCTD. The percentage and the absolute number of natural Treg cells were lower in patients than in healthy controls which further decreased in patients with active disease. Interestingly, we saw an increase in inducible, IL-10 secreting $CD4^+IL\text{-}10^+$ Tr1 cells in patients with MCTD. The Tr1 cells ratio further increased in patients with active disease. As we indicated in patients with UCTD, we believe that elevated Tr1 cell percentages could be a compensatory mechanism aiming to restore the balance between type 1 and type 2 cytokines in MCTD [18]. In subsets of MCTD patients, serum levels of IFN-γ and TNF-α were increased along with reduced number of Tregs. The decreased levels of regulatory T cells, along with the increased expression of proinflammatory cytokines, may play a role in the pathogenesis of immune mediated inner ear disorders in MCTD [77].

Serum and intracellular cytokine assessment, reflecting immune-regulatory abnormalities, are valuable biomarkers to assess disease activity in MCTD and are also capable of subcategorizing these patients [78].

5.4. Systemic Lupus Erythematosus (SLE). SLE is a heterogeneous, systemic autoimmune disease with various organ involvements, encompassing mild to moderate forms and also severe, progressive variants [79–81]. A great variety of cytokines have also been implicated in the pathogenesis of SLE, amongst others, BAFF/BLyS, TNF-α, IFN-α, IFN-γ, IL-12, IL-23, IL-18, IL-6, IL-10, and IL-17 [82, 83].

Pinpointing the key role of the interferon signature in SLE, interferon regulatory factor-5 (*IRF5*) has been linked to the increased production of interferon- (IFN-) α, and *STAT4* to the increased sensitivity to IFN-α [84–86]. Patients with disease flare had significant alterations in a wide variety of soluble mediators at baseline with significantly higher levels of proinflammatory mediators, including Th1-, Th2-, and Th17-type cytokines, already several weeks before the appearance of clinical flare compared to clinically stable patients [87]. Regulatory cytokines, including IL-10 and TGF-β, were higher in nonflare SLE patients [87]. Treg-cell number and function have been shown to be impaired in SLE [88–90] along with increases in Th17 cells and serum IL-17 concentrations, in particular in patients with disease flare [91–93]. The Th17 and Treg ratio indicates that SLE is associated with a reduction in the levels and function of immunosuppressive Treg cells together with an increase in the proinflammatory Th17 cells [94].

As we have illustrated, in all these various patient groups with autoimmune conditions, we could identify a circulating cytokine imbalance, a proinflammatory milieu, and the development of Th17 cells along with the reduction in numbers/function of Tregs. The simultaneous, opposing effect of Th17 cells and Tregs has a strong impact on immune homeostasis, deciding and controlling the development of autoimmunity in these patients.

6. Dendritic Cells and Autoimmunity

Dendritic cells (DC) are generally divided into two main classes, the plasmacytoid DCs (pDCs) that react to viral infections by secreting large amounts of type I interferons and conventional or classical DCs (cDCs) that are important for initiating immune responses through their ability to take up and present antigen to antigen-specific T cells. For efficient priming of T cells and induction of effector cells, the cDCs have to become activated via signals delivered through pathogen recognition receptors such as toll-like receptors (TLRs). Activated cDCs express large amounts of peptide-MHC complexes on their surface and in addition upregulate costimulator molecules such as CD80 and CD86 for efficient priming of antigen specific T cells. Bacterial and viral infections produce a large amount of TLR ligands, such as LPS (TLR2), CpG (TLR9), or dsRNA (TLR3), resulting in activation of cDCs and efficient induction of effector T cells. In the absence of activation, however, it is believed that cDCs instead promote tolerance, through either induction of Treg or T-cell unresponsiveness [95–97]. cDCs therefore play a central role in determining the outcome of the immune response and whether the resulting T-cell responses are immunogenic or tolerogenic.

6.1. Subsets of DCs. Since their initial discovery by Steinman in the early 70s [98], a growing body of information now results in cDCs being divided into subsets based on tissue distribution, surface receptor expression, and functional profiling. In general, cDCs can be divided into lymphoid tissue DC (LT-DCs), in addition to nonlymphoid tissue DC (NLT-DC), or migratory DCs, which source antigen in the periphery and subsequently migrate to draining LN for T-cell priming. LT-DCs were initially divided into subsets based on expression of CD8α [99] and CD4 [100]. These makers are, however, not optimal when classifying NLT DCs in peripheral organ, with expression of the integrins CD103 and CD11b being more efficient at separating NLT DC populations [101, 102]. While a number of different surface marks have been evaluated, it now seems that the chemokine receptor Xcr1 is one of the more selective makers for identifying LT $CD8\alpha^+$ DC and the related NLT $CD103^+$ DC population [103, 104] and that the integrin Sirpa (CD172a) can be used as a marker for $CD4^+$/CD11b+ DC [105]. The expression of the markers Xcr1 and Sirpa has the added advantage of being conserved on homologous DC subsets in other species such as man [106, 107], macaques [108], and sheep [109], making for a more unified DC nomenclature across the species.

Both $Xcr1^+$ and $Sirpa^+$ DCs efficiently present antigens to $CD4^+$ T cells [110]. However, functional analysis has indicated that only the $Xcr1^+$ DC excel at cross-presenting antigen to $CD8^+$ T cell and have subsequently been referred to as cross-presenting DCs [110, 111]. Similar observations have also been made in peripheral tissue with NLT $Xcr1^+$ DC (defined as $CD103^+$ DC) being better at presenting antigen to $CD8^+$ T cells [112]. Whether the same functional difference remains conserved on DC subsets in humans remains a matter of debate, with some studies demonstrating that $Xcr1^+$ DC are better at cross-presenting antigens [107], while others have shown similar cross-presentation abilities in $Xcr1^+$ and $Sirpa^+$ DCs [113].

Dendritic cells have been suggested to be involved in the onset of several different autoimmune diseases [114]. However, the mechanisms by which DC play a role in autoimmunity may vary between diseases. As antigen presenting cells, DC may directly prime autoimmune T cells, but as indicated earlier they may also induce Tregs that inhibit autoimmune disease. In addition, DCs may secrete proinflammatory cytokines upon activation which may be directly involved in disease progression. Here we depict a few examples on the possible involvement of DCs, the progression of type 1 diabetes (T1D), and systemic lupus erythematosus (SLE).

6.2. Type 1 Diabetes (T1D). Type 1 diabetes is thought to be initiated by the presentation of diabetogenic antigens in the draining pancreatic lymph node (pLN), with subsequent induction of autoreactive T cells which destroy the pancreatic β-islet cells leading to loss of insulin production and diabetes. Conditional depletion of DCs has been reported to prevent disease onset in diabetes prone NOD mice, suggesting that DC may be involved in the initial priming of autoreactive T cells [115]. Interestingly, a recent study observed that Batf3 deficient NOD mice did not develop diabetes [116]. Mice deficient in the transcription factor Batf3 lack the $Xcr1^+$ LT and NLT DC populations [117], suggesting involvement of this DC subset in the onset of disease. Ferris and colleagues observed that islets of Langerhans contained a minor population of $Xcr1^+$ NLT DC, in addition to a major population of macrophages. Deletion of the $Xcr1^+$ NLT DC in the NOD.$Batf3^{-/-}$ mice resulted in absence of presentation of MHC-I epitopes in the pLN and no incidence of diabetes. These observations are in accordance with previous studies suggesting that cross priming is required in the pathogenesis of diabetes [118] and the functional specialization of $Xcr1^+$ NLT DC in terms of cross presenting antigen to $CD8^+$ T cells.

6.3. Systemic Lupus Erythematosus (SLE). While lupus is generally thought to be an autoantibody driven disease, studies have shown that certain MHC haplotypes display a strong association with disease onset suggesting that T cells may also play a role in the pathogenesis [119]. In lupus prone MRL.Fas^{lpr} mice, depletion of DCs resulted in less severe disease development with lower glomerular and interstitial renal disease scores [120]. Interestingly, the authors observed that DC depletion did not significantly influence T-cell activation but that T-cell expansion was severely reduced. Consequently, the immunogenic role of the DCs seems to outweigh the tolerogenic function of DC in this model of autoimmunity. It should be noted that depletion of DCs in this model only ameliorated the disease, suggesting that other cells subsets may be involved in the initiation events. Maybe more surprising was the observation that DC depleted mice displayed reduced numbers of plasmablasts in spleen and inhibited class switching, suggesting that the DCs may be involved in this process. Previous studies have indicated that DC may play a role in plasmablast differentiation in SLE [121], which corresponds well with the observations by Teichmann et al. While DCs in autoimmune disease progression tend to primarily function as antigen presenting cells (APCs) and activators of T cells, it is clear that their stimulatory effect on B cells may also affect disease progression.

Type I interferons play a major role in disease progression in SLE, and patients with high levels of type I interferon in serum have more severe disease outcome [122]. As highly efficient producers of type I interferons, pDCs have been suggested to be an important source of type I interferon and disease onset in SLE [123]. In human patients, pDCs have been observed to accumulate in the tissue lesions suggesting a possible involvement in lupus [124]. While most observations implicating pDCs in SLE progression have so far been indirect, a recent paper by Sisirak and colleagues showed that impairing pDC function in a murine SLE model nearly abolished disease manifestations such as glomerulonephritis and anti-DNA antibodies [125]. These observations clearly indicate that pDCs are directly involved in SLE pathogenesis and the induction of autoantibodies.

7. Extracellular Vesicles in Autoimmune Diseases

Extracellular vesicles (EVs) are heterogeneous, membrane surrounded structures that can be found in body fluids and play a crucial role in the intracellular communication [126, 127]. Based on their morphological parameters and biogenesis EVs can be categorized as exosomes, microvesicles (MVs), and apoptotic bodies. Exosomes are the smallest EVs; their size range is around 50–100 nm, similar to the size of the viruses. Exosomes are released by exocytosis of multivesicular bodies [128], and they may transfer viruses and microRNA (miRNA). Exosome markers include lysosomal-associated membrane protein 1 (LAMP-1), CD63, and CD81. Among others, several tumor cells and immune cells release exosomes constitutively or upon activation.

The size range of microvesicles (MVs) is between 100 and 1000 nm, similar to the size of bacteria and immune complexes. Detergent treatment (0.05% Triton X-100) completely eliminates the MVs; while it does not significantly influence the immune complexes [129], this method can be used to discriminate MVs and immune complexes. MVs are generated by budding of the plasma membrane. Although platelets, endothelial cells, and red blood cells are the primary sources of MVs, many other cells may release these structures as well. MVs have a central role in the fetomaternal communication and in the pathogenesis of several autoimmune diseases, such as RA and SLE. Phosphatidylserine externalization is a general feature of the MVs. Apoptotic bodies are the largest EVs; their size range is between 1 and 5 μm (the size range of platelets); they are secreted during apoptosis. Similarly to MVs apoptotic bodies also express phosphatidylserine in the outer part of their membrane and they generally contain DNA. Apoptotic bodies have a central role in the transfer of DNA and oncogenes between various cells. Flow-cytometry, western blotting, ELISA, electron microscopy, and mass spectrometry are the most frequently used methods in the EV research. Despite the widespread research of EVs there are still many technical pitfalls and the researchers have to be especially careful when interpreting their results.

There are increasing data on the potential pathogenetic role of MVs in RA and in SLE. Increased number of platelet derived MVs (PMVs) in the serum and synovial fluid samples of patients with RA were reported [130, 131]. The number of PMVs was increased in the plasma samples of patients with RA compared to the healthy donors', and the number of PMVs correlated with the disease activity [132]. The PMVs have procoagulant activity; therefore PMVs may contribute to the increased cardiovascular risk in RA. It was recently published that C-type lectin-like receptor 2 (CLEC-2) is expressed on the MVs of patients with RA [133], and the authors suggest using this marker to measure PMVs. In general, most studies on MVs in RA investigated morphological features and there are only relatively little data available about their biological effects. Fibroblast-like synoviocytes of patients with RA produced B-cell-activating factor upon MP treatment, suggesting that these structures may contribute to the activation of the adaptive immune response in RA [134].

The number of MPs is increased in SLE and the protein composition of them is different from the healthy donors' [135]. The number of endothelial MVs was measured in a recently published prospective study by flow cytometry [136]. The number of EMPs was higher in the samples of patients with SLE than in the samples of healthy donors. Disease activity decreased during the study and in parallel the number of EMPs decreased. Importantly, the clinical disease activity (BILAG-2004 and SLEDAI-2K scores) did not correlate with the EMP numbers. These data suggest that endothelial dysfunction may improve with the appropriate reduction of the inflammation in SLE. Increased number of MVs were also reported in systemic sclerosis [137] and polymyositis [138] and in Sjögren's syndrome [139].

8. Conclusions

The intricate interplay of various proinflammatory cytokines and chemokines, orchestrated by key regulators of the immune system (e.g., dendritic cell subsets), can lead to the imbalance between regulatory (e.g., Tregs) and proinflammatory cells (e.g., Th17 cells). This vicious circle can further perpetuate autoimmune processes, which, on a susceptible genetic background, can lead to the development of a full-blown autoimmune disease. Other novel mediators, denoted as extracellular vesicles, seem to have a pivotal role in the pathogenesis, as well. The fine mapping of these mediators does not just help us to understand the pathogenesis of these diseases, but we believe that long, empirical therapies can be replaced by optimized combination therapies through personalized proinflammatory cytokine, extracellular vesicle targeting, or dendritic cell manipulation. We believe that this approach will aid in the diagnosis and therapy design in autoimmune diseases and will provide an advanced disease management in the future.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Bleomycin-Treated Chimeric Thy1-Deficient Mice with Thy1-Deficient Myofibroblasts and Thy-Positive Lymphocytes Resolve Inflammation without Affecting the Fibrotic Response

Pazit Y. Cohen,[1] Raphael Breuer,[1,2] Philip Zisman,[1] and Shulamit B. Wallach-Dayan[1]

[1]*Lung Cellular and Molecular Biology Laboratory, Institute of Pulmonary Medicine, Hadassah-Hebrew University Medical Center, Jerusalem 91120, Israel*
[2]*Department of Pathology, Boston University School of Medicine, Boston, MA 02118, USA*

Correspondence should be addressed to Shulamit B. Wallach-Dayan; wallach-dayan@hadassah.org.il

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Lung fibrosis is characterized by abnormal accumulation of fibroblasts in the interstitium of the alveolar space. Two populations of myofibroblasts, distinguished by Thy1 expression, are detected in human and murine lungs. Accumulation of Thy1-negative ($Thy1^-$) myofibroblasts was shown in the lungs of humans with idiopathic pulmonary fibrosis (IPF) and of bleomycin-treated mice. We aimed to identify genetic changes in lung myofibroblasts following Thy1 crosslinking and assess the impact of specific lung myofibroblast Thy1-deficiency, in vivo, in bleomycin-injured mouse lungs. Thy1 increased in mouse lung lymphocytes following bleomycin injury but decreased in myofibroblasts when fibrosis was at the highest point (14 days), as assessed by immunohistochemistry. Using gene chip analysis, we detected that myofibroblast Thy1 crosslinking mediates downregulation of genes promoting cell proliferation, survival, and differentiation, and reduces production of extracellular matrix (ECM) components, while concurrently mediating the upregulation of genes known to foster inflammation and immunological functions. Chimeric Thy1-deficient mice with $Thy1^+$ lymphocytes and $Thy1^-$ myofibroblasts showed fibrosis similar to wild-type mice and an increased number of CD4/CD25 regulatory T cells, with a concomitant decrease in inflammation. Lung myofibroblasts downregulate Thy1 expression to increase their proliferation but to diminish the in vivo inflammatory milieu. Inflammation is not essential for evolution of fibrosis as was previously stated.

1. Background

Idiopathic pulmonary fibrosis (IPF) is a nonneoplastic pulmonary disease characterized by the formation of scar tissue within the lungs, in the absence of any known provocation [1, 2]. Fibroblasts are central to both wound healing and the pathogenesis of organ tissue fibrosis. Although it remains unclear whether fibroblast proliferation is the primary mechanism of pathogenesis in IPF or whether it is a reactive process to another form of lung tissue damage, selective deletion of fibroblasts is sufficient to prevent fibrosis after injury [3]. The intratracheal instillation (IT) of bleomycin into mice is used as an in vivo experimental model to study underlying mechanisms of lung fibrosis and inflammation [4].

Lung fibroblasts can be divided into subpopulations on the basis of size and shape, cytokine profiles, lipid content, and cell surface protein expression [5, 6]. The most extensively characterized in vitro model of fibroblast heterogeneity is based on surface expression of Thy1 [5, 7]. It has been shown in mice and humans that Thy1-positive ($Thy1^+$) and Thy1-negative ($Thy1^-$) fibroblasts differ with respect to cytokine [8–12] and growth factor responses [13, 14], as well as cell migration patterns [15].

Thy1 is a 25–37 kD glycosylphosphatidylinositol- (GPI-) anchored cell surface protein that belongs to the immunoglobulin-like gene super family [16]. Thy1 is expressed and distributed differently among species and among tissues of the same species; however, it is present on brain cells and fibroblasts of all species studied thus far [17]. In mice, Thy1

is also found on other cell types, including thymocytes and peripheral T cells [17, 18]. The wide distribution of Thy1 suggests that it has distinct functions in different tissues and species. Immune cells such as T lymphocytes were previously reported to be involved in both the attenuation and the promotion of fibrosis. These contradictory observations are most likely a reflection of the phenotypic heterogeneity of involved T cells, as reviewed by Re et al. [19] and Luzina et al. [20].

In this study, we aimed to define the molecules and pathways involved in inflammation and fibrosis that are affected by Thy1 crosslinking on lung myofibroblasts in vitro and specifically by its deficiency in lung myofibroblasts in vivo, in the experimental model of bleomycin-induced fibrosis in mouse lungs, excluding the role of Thy1 on lymphocytes.

2. Materials and Methods

2.1. Animals. Male, 11 to 12 weeks old, C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) and C57BL/6J Thy1-deficient mice (kindly provided by Professor R. J. Morris, Laboratory of Neurobiology, National Institute for Medical Research, London, UK) were used.

At 12–14 weeks, body weight for the two types of mice was similar. Histological sections of lung, heart, brain, colon, liver, and kidney were studied. No differences were noted in histological sections or in lung hydroxyproline contents for the two types. Bronchoalveolar cellularity was also similar in terms of the number of cells per mL, with 99% macrophages, and 1% lymphocytes or neutrophils.

All animal procedures were approved by the Hebrew University-Hadassah Medical School Animal Care Committee. Mice were housed in a specific pathogen-free environment.

2.1.1. Chimeric Mice and Lymphoid Organ Cell Transplantation. We created a C57BL/6J Thy1-deficient chimeric mouse containing wild-type (WT) ($Thy1^+$) lymphoid organ cells. Lymphoid tissues in the Thy1-deficient mice were ablated, and the mice were reconstituted with hematopoietic cells isolated from WT mouse lymphoid organs as we previously performed and detailed [21]. WT mice reconstituted with hematopoietic cells isolated from lymphoid organs of WT mice served as control group. One day prior to cell transplantation, 6-week-old C57BL/6 mice were subjected to total body irradiation (750 cGy, dose rate of 179 cGy/min) delivered by a linear accelerator (Clinac G6, Varian Medical Systems, Palo Alto, CA, USA) with a source-to-skin distance of 80 cm. One day after irradiation, mice received syngeneic lymphoid organ cells (spleen and lymph nodes, $50–100 \times 10^6$) obtained from WT- C57BL/6-based mice. Following adoptive transfer (30 days) and engraftment confirmation by FACS analysis (Figure 3(a)), chimeric mice were subjected to intratracheal instillation (IT) of bleomycin.

2.2. Intratracheal Instillation. Mice were anesthetized by intraperitoneal (IP) injection of 0.05–0.07 mL of 40 mg/mL ketamine (Ketalar, Parke-Davis, Pontypool, Gwent, UK) and 0.5 mg/mL droperidol (Inapsine, Janssen Pharmaceutica, Beerse, Belgium). The skin and subcutaneous tissues overlying the proximal portion of the trachea were exposed by a 5 mm transverse incision to allow for direct external visualization of the trachea. A metal cannula fitted to a tuberculin syringe was carefully passed into the trachea. A dose of 0.06–0.08 units of bleomycin (H. Lundbeck, Copenhagen, Denmark) dissolved in 0.1 mL of saline solution, or 0.1 mL of saline alone, was slowly injected.

2.3. Quantitative Assessment of Fibrotic Lung Injury. Mice were killed with a lethal dose of pentobarbital at 1, 3, 7, or 14 days after IT bleomycin. The abdominal aorta was transected, and the animal was exsanguinated. To eliminate blood, lungs were perfused with normal saline through the right ventricle and bronchoalveolar lavage (BAL) was performed. A polyethylene cannula (PE 205; Clay Adams, Parsippany, NJ, USA) was placed in the trachea, and 3 mL of normal saline was slowly injected and withdrawn.

Lung injury was assessed quantitatively, as we have previously described [22] by bronchoalveolar lavage (BAL) cellularity, semiquantitative morphological index (SMI) studies of lung fibrosis, and quantitative lung collagen measurements.

2.4. Lung Collagen. Lung collagen was assessed in the right lung using the Sircol Collagen Assay kit (Biocolor, Belfast, Northern Ireland), as described previously [23, 24]. This method measures newly synthesized collagen that has not been extensively cross-linked. Briefly, the upper lobe of the right lung was homogenized in 5 mL of 0.5 molar acetic acid containing 1 mg pepsin (Sigma Aldrich, St Louis, MO, USA) per 10 mg tissue residue. Each sample was incubated at room temperature for 24 h, with stirring. After centrifugation, 100 μL of each supernatant was assayed, 1 mL of Sircol dye reagent that specifically binds to collagen was added to each sample, and the sample was mixed for 30 min. After centrifugation, the pellet was suspended in 1 mL of the alkali reagent (0.5 molar NaOH) included in the kit, and optical density was evaluated at 540 nm with a spectrophotometer. Values in the test samples were compared to values obtained with collagen standard solutions provided by the manufacturer, which were used to construct a standard curve. Collagen results were expressed in micrograms.

2.5. Lung Cell Isolation and Myofibroblast Culture. Lungs were removed, minced, and incubated (37°C, 5% CO_2 air) for 45 min in PBS containing 1 mg/mL collagenase (C0130, Sigma Aldrich). After enzyme treatment, lung tissue was gently passed through a cell dissociation sieve (Sigma Aldrich) or 40 μm nylon mesh filters (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) and then washed twice in PBS. For myofibroblast culture experiments, lung cells were resuspended in fibroblast culture medium. Cell cultures were incubated at 37°C in 5% humidified CO_2. Typically, within 1 week of culture initiation, more than 95% of the cells are morphologically myofibroblasts.

Cells were passaged every 5 days by dissociating monolayers with a mild trypsin solution (Biological Industries, Beit Haemek, Israel). After initial cultures were established, fibroblasts obtained on passages 2 through 6 were used.

2.6. Immunostaining of Frozen Tissue Sections. The left lung was removed and frozen in Tissue-Tek O.T.C. compound (Sakuar Finetek, Zoeterwoude, Netherlands). Frozen tissue blocks were cut to provide 4–6 μm sections, which were fixed in acetone for 5 min at −20°C and air dried. Endogenous peroxidase activity was blocked with immersion of sections in 0.3% H_2O_2, 0.1% NaN_3 in methanol for 20 min at room temperature (RT) and washed three times. Nonspecific reactions were blocked with 10% FCS for 10 min at RT. After washes with PBS, sections were incubated with rat anti-Thy1 (catalog number 550543, BD Biosciences/Pharmingen, San Jose, CA, USA) for 2 h at RT. After PBS washing, sections were reincubated with universal immunoperoxidase polymer for mouse tissue sections in anti-rat primary antibody (Histofine, Nichirei Biosciences, Tokyo, Japan) for 30 min at RT and again reincubated for a 10 min in peroxidase substrate (Dako Kit, Dako, Glostrup, Denmark). The sections were counterstained with hematoxylin (Zymed Kit, Zymed Laboratories, San Francisco, CA) and mounting was added to the slides (Zymed Kit).

2.7. Immunostaining by Flow Cytometry. Cells were harvested by rubber policeman. Myofibroblasts (0.5×10^6) were incubated in FACS buffer (3% FCS in PBS), with 1 μg/100 μL PE-conjugated anti-Thy1 (CD90) mAb (BD Biosciences/Pharmingen) for 30 min at RT. The cells were then washed with FACS buffer and analyzed by flow cytometry.

2.8. Myofibroblast Thy1 Crosslinking. Subconfluent myofibroblasts were stimulated with anti-Thy1 G7, which has previously been shown to activate T cells [25] or with anti-rat IgG2Cκ isotype control (BD Biosciences/Pharmingen). Both stimulants were added to myofibroblasts at concentrations ranging from 1 to 20 μg/mL, together with recombinant protein G crosslinker (Sigma Aldrich) at the same concentration.

2.9. Immunofluorescence Staining of CD4, CD8, and CD25 in Lung Sections. Paraffin lung sections were stained as we have previously described in detail [24]. Briefly, deparaffinized lung sections were incubated overnight at 4°C with APC-conjugated CD4/FITC-conjugated CD25 or FITC-conjugated CD8 (BD Biosciences/Pharmingen), diluted at 1 : 200 in 1% BSA PBS Tween 0.05%. After washing, the slides were analyzed by confocal microscopy (Axio Scope 2; Carl Zeiss AG, Oberkochen, Germany). The fluorescence ratio was analyzed using the Ariol system [26]. The relative staining ratio was calculated by dividing the number of stained cells by the unstained cells in a certain analyzed area.

2.10. Immunohistochemical αSMA Staining of Lung Tissue Sections. Paraffin sections were stained as we have previously described in detail [24]. Briefly, deparaffinized lung sections were incubated overnight at 4°C with polyclonal mouse anti-SMA (DAKO) diluted 1 : 200 in 1% BSA PBS Tween 0.05%. The Envision Detection System (DAKO) containing secondary anti-mouse horseradish peroxidase-conjugated antibody and 3,3′-diaminobenzidine as a substrate was used for staining detection. The quantitative analysis of DAB staining was analyzed using the Ariol system. The relative staining ratio was calculated by dividing the number of stained cells by the unstained cells in a certain analyzed area.

2.11. Sirius Red Staining for Collagen (Type I and IV). Deparaffinized lung sections were stained using Picro Sirius Red Stain Kit (Abcam, Cambridge, UK) as was previously described [27]. Red sirius staining was analyzed using the Ariol system. Comparisons were made between the two groups of IT bleomycin. The relative staining ratio was calculated by dividing the number of stained cells by unstained cells in a certain analyzed area.

2.12. RNA Isolation. Total cellular RNA was isolated from myofibroblasts in culture using TriReagent (catalog number T9424, Sigma Aldrich), according to the protocol supplied by the manufacturer. To assess RNA integrity and exclude DNA contamination, an aliquot of each sample was analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide. Purity and quantitation of RNA were assessed by spectrophotometer.

2.13. RT-PCR. RNA was reverse transcribed to cDNA using an avian myeloblastosis virus-RT base protocol and random primers, as well as poly dT (Reverse Transcription System; Promega, Madison, WI, USA). One microgram of each sample was uniformly used for reverse transcription. cDNA was diluted in a final volume of 200 μL with nuclease-free water.

2.14. Microarray. Total RNA was extracted and used as a template for double-stranded cDNA synthesis as previously described [28].

2.15. Preparation of Labeled cDNA. Labeling was performed using the Low RNA Input Linear Amplification Kit PLUS (One-Color, Agilent, Santa Clara, CA, USA). Briefly, Cy3-labeled double-stranded cDNA synthesis was performed using with an oligo(dT)24 primer containing a T7 RNA polymerase promoter site added to the 3′ and cyanine 3-CTP. cDNA was used as a template for generation of cRNA for hybridization.

2.16. Hybridization of Microarrays. After purification and fragmentation, aliquots of each sample were hybridized to Whole Mouse Genome 4 × 44 K Multipack Arrays (Agilent) with probes for 41,000 unique transcripts. In our experience, these arrays have demonstrated superior reproducibility and were recently shown to have superior sensitivity [29]. After hybridization, each array was sequentially washed and scanned (DNA Microarray Scanner, Agilent). Individual arrays were visually inspected for hybridization defects using manufacturer recommended quality control procedures and read out with Agilent Feature Extraction Software. Bioconductor (Bioconductor Project, Seattle, WA, USA) was used to normalize the processed signal [30]. Probes with annotations for entrez gene ID were extracted, and cyclic LOESS was applied to normalize gene expression signals.

In cases of redundant probes, we took the average over values representing gene expression levels.

2.17. Gene Chip Analysis. Differentially expressed genes were identified by volcano analysis, using a threshold of >2-fold changes in expression. $P < 0.05$ was considered statistically significant. The analysis of biological processes affected by Thy1 in lung, myofibroblasts was performed using Onto-Express software (Open Channel Foundation, Chicago, IL, USA).

2.18. Statistical Analysis. The Mann-Whitney test was performed for comparisons of nonparametric data. When multiple pair-wise comparisons involving two groups were performed, the Bonferroni correction of the P value was used. The nonparametric Kruskal-Wallis test was performed for comparing more than two groups. $P \leq 0.05$ or less was considered statistically significant.

3. Results

3.1. Thy1 Expression in Lung Tissue Is Increased at Times of Active Fibrosis following Bleomycin IT but Specifically Decreased in Lung Myofibroblasts. Thy1 expression was assessed by immunohistochemistry in frozen lung tissue sections of saline- and bleomycin-treated mice 14 days following instillation, the point of peak fibrosis [4]. When compared to saline-treated mice, Thy1 expression in the total lung was increased following bleomycin instillation (Figure 1(a)).

We sought to specifically determine Thy1 expression in lung myofibroblasts at different stages following bleomycin injury (1, 3, 7, and 14 days following IT bleomycin). Flow cytometry analysis using PE-conjugated anti-Thy1 mAb demonstrated that Thy1 expression in lung myofibroblasts is significantly decreased by day 14 (Figures 1(b) and 1(c), segregated histograms), indicating an increase in the proportion of $Thy1^{-}$ myofibroblasts in the total lung myofibroblast population at later stages following bleomycin injury, when fibrosis develops [4].

3.2. Genomic Analysis Shows an Increased Expression of Proinflammatory Genes in Lung Myofibroblasts following Direct Thy1 Crosslinking-Induced Activation. To assess the effects of Thy1 activation on lung myofibroblasts, we analyzed gene expression patterns using GeneChip microarrays (Affymetrix, Santa Clara, CA, USA) in samples of RNA extracted from lung primary lung myofibroblasts at different time points before and after Thy1 crosslinking by G7 anti-Thy1 mAb 10 μg/mL G7, compared to crosslinking with control matched-IgG isotype, for 30 min and 1, 6, and 24 hours. Genes with significant expression changes following Thy1 crosslinking-induced activation were detected following volcano analysis, using a threshold of >2-fold changes of gene expression over the untreated control and $P < 0.05$ as the test for significance. Most changes occurred very quickly, from 30 min-to-1 h following Thy1 stimulation. The number of downregulated genes was larger than the number that was upregulated (Figure 2).

Table 1: Molecular pathways downregulated by Thy1.

Pathway name	# input genes in the pathway	P value
Long adhesion	13	$1.02E-06$
Long-term depression	6	$3.85E-04$
ECM-receptor interaction	6	$6.59E-04$
Small cell lung cancer	5	0.004769
Regulation of actin cytoskeleton	8	0.005003
Prostate cancer	5	0.005006
Tight junction	6	0.007175
Melanoma	4	0.012134
Bladder cancer	3	0.015551
MAPK signaling pathway	8	0.016136
Axon guidance	5	0.022646
Endometrial cancer	3	0.027335
mTOR signaling pathway	3	0.027335
Gap junction	4	0.02851
Insulin signaling pathway	5	0.029243
Non-small cell lung cancer	3	0.030129
GnRH signaling pathway	4	0.031564
Alzheimer's disease	2	0.033214
Wnt signaling pathway	5	0.03603
p53 signaling pathway	3	0.048047
Glioma	3	0.048047
Cell cycle	4	0.048272
B cell receptor signaling pathway	3	0.049885
Long-term potentiation	2	0.049885
Neurodegenerative diseases	2	0.050764
Wnt signaling pathway	7	0.001962
B cell receptor signaling pathway	4	0.00836
VEGF signaling pathway	4	0.011283
Natural killer cell mediated cytotoxicity	5	0.01976
Hedgehog signaling pathway	3	0.026203
Neuroactive ligand-receptor interaction	7	0.036606
T cell receptor signaling pathway	4	0.037492
Dorsoventral axis formation	2	0.039028

An analysis of genes related to different biological processes affected by Thy1 in lung myofibroblasts was performed using Onto-Express software. Pathways "downregulated" by Thy1 crosslinking are shown in Table 1; "upregulated" pathways are found in Table 2.

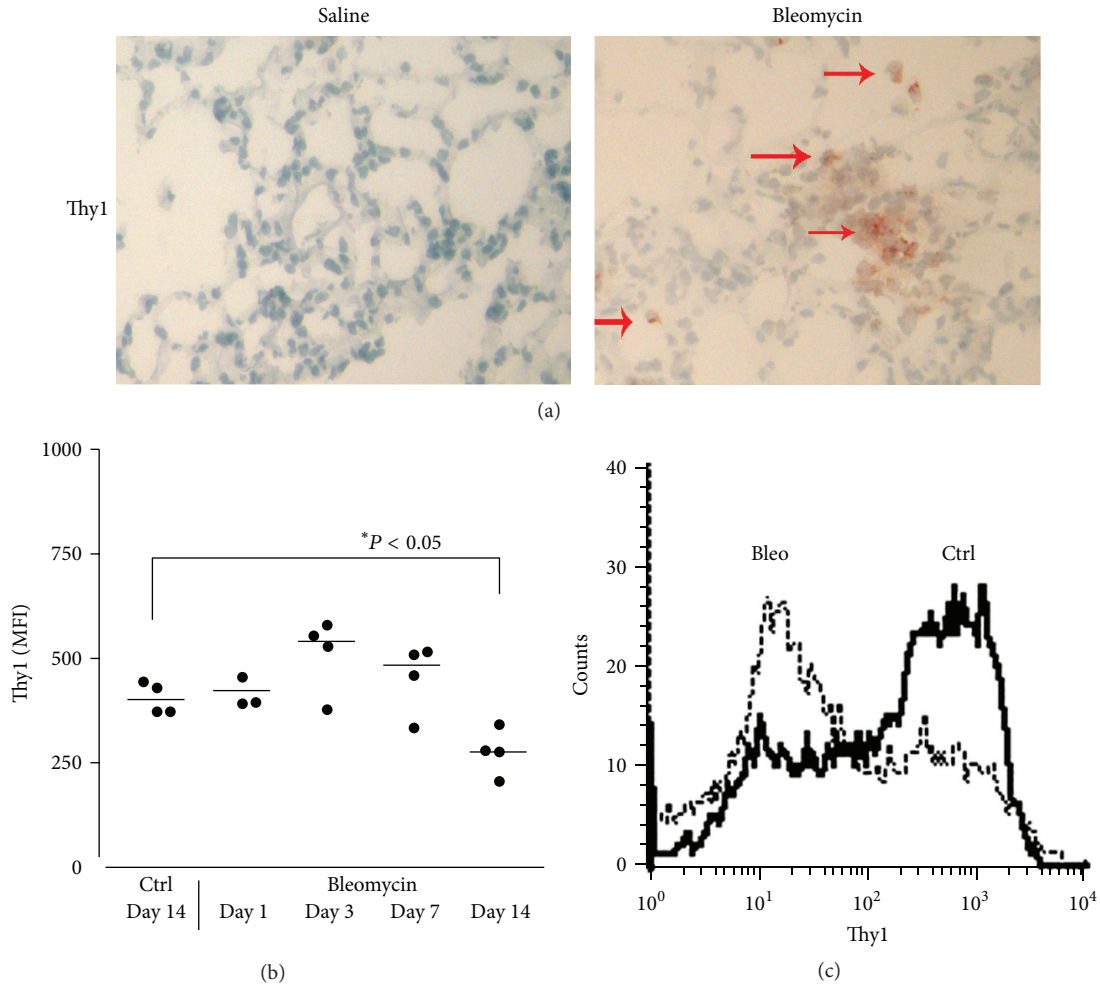


Figure 1: Thy1-positive total lung cells are increased during experimental fibrosis; however, the proportion of myofibroblasts that are Thy1-positive is decreased. (A) Immunohistochemistry of Thy1 staining (arrows), in the lungs of saline- vs. bleomycin-treated mice, 14 days following instillation. Representative results. (B) Lung myofibroblasts were isolated from control saline-treated (Ctrl) and bleomycin-treated mice at 1, 3, 7 and 14 days following IT. A graphical presentation of Thy1 expression FACS analysis was performed using PE-conjugated anti-Thy1 (CD90) mAb. The differences between control (Ctrl) saline-treated and bleomycin-treated mouse lung myofibroblasts on day14 were significant. $^*P < 0.05$, $n = 4$. (C) Histogram plot showing a representative result of assessment of Thy1 expression in myofibroblasts from control (Ctrl) saline-treated mice compared to Thy1 expression in myofibroblasts from bleomycin-treated mice at day 14 following IT. Results were similar in 2 different experiments ($n = 4$).

Table 2: Molecular pathways upregulated by Thy1.

Pathway name	# input genes in the pathway	P value
Hematopoietic cell lineage	3	0.020743
ECM-receptor interaction	3	0.020743
Cytokine-cytokine receptor interaction	5	0.027124
Antigen processing and presentation	3	0.031685
Maturity onset diabetes of the young	2	0.003156
Autoimmune thyroid disease	2	0.026802

In Table 3, we present selectively grouped genes according to pathways that were shown to influence myofibroblast proliferation and extracellular matrix (ECM) production/interaction and differentiation. In addition, changes in myofibroblast inflammatory and immunological genes were grouped. Note that a single gene may participate in more than one pathway.

While most of the genes involved in proliferation, ECM production and interaction, and differentiation were decreased, genes associated with inflammation and immunological functions were increased, following Thy1 stimulation. We have previously showed FasL gene overexpression on lung myofibroblasts following Thy1 crosslinking, verifying GeneChip results at the RNA level by qPCR and at the protein level by Western blot analyses [28].

Table 3: Pathways involved in myofibroblast proliferation, differentiation, and interaction with the ECM and immune system that are affected by Thy1.

Category	Pathway	Gene name	Fold change***
Proliferation	*Cell cycle*	Anaphase promoting complex subunit 2	−1.19
		Cyclin D2	−1.02
		CDC23 (cell division cycle 23 yeast homology)	−1.13
		Transformed mouse 3T3 cell double minute 2	−1.22
	MAPK signaling pathway	RIKEN cDNA 1500003O03 gene	−1.03
		Thymoma viral proto-oncogene 2	−1.12
		Activating transcription factor 4	−1.1
		Filamin C, gamma (actin binding protein 280)	−1.05
		Protein phosphatase 1B, magnesium dependent, β isoform	−1.06
		v-raf-leukemia viral oncogene 1	−1.07
		Serine/theonine kinase 3 (Ste20, yeast homolog)	−1.2
		Fibroblast growth factor receptor 1	−1.13
	Insulin signaling pathway	RIKEN cDNA 4932417H02 gene	−1.22
		Thymoma viral proto-oncogene 2	−1.12
		Brain glycogen phosphorylase	−1.37
		v-raf-leukemia viral oncogene 1	−1.07
		Sorbin and SH3 domain containing 1	−1.46
	TGFβ signaling pathway	Activin receptor IIA	−1.16
		Protein phosphatase 2	−1.19
		Thrombospondin 1	−1
	Receptors	Angiotensin receptor like 1	−1.85
ECM production and interaction	*Focal adhesion*	Thymoma viral proto-oncogene 2	−1.12
		Caveolin, caveolae protein 1	−1.13
		Cyclin D2	−1.02
		Collagen, type I, alpha 1	−1.02
		Collagen, type IV, alpha 1	−1.12
		Filamin C, gamma (actin bineing protein 280)	−1.05
		Integrin beta 1 (fibronectin receptor beta)	−1.04
		Laminin, alpha 2	−1.22
		v-raf-leukemia viral oncogene 1	−1.07
		Thrombonspondin 1	−1.19
		Talin 1	−1.17
		vav 1 oncogene	−1.59
		Vinculin	−1.3
	ECM receptor interaction	Collagen, type I, alpha 1	−1.02
		Collagen, type VI, alpha 1	−1.12
		Dystroglycan 1	−1.33
		Integrin beta 1 (fibronectin receptor beta)	−1.04
		Laminn, alpha 2	−1.22
Myofibroblast differentiation	*Regulation of actin cytoskeleton*	Actin, alpha 2, smooth muscle, aorta	−1.54
		Cofilin 1, non-muscle	−1.35
		Fibroblast growth factor receptor 1	−1.13
		Guanine nucleotide binding protein, alpha 13	−1.2
		Integrin beta 1 (fibronectin receptor beta)	−1.04
		v-raf-leukemia viral oncogene 1	−1.07
		vav 1 oncogene	−1.59
		Vinculin	−1.3
	Differentiation	MyoD1	−1.83
		Pax 7	−1.89

Table 3: Continued.

Category	Pathway	Gene name	Fold change***
Inflammation and immunological function	*Hematopoietic cell lineage*	CD4 antigen	1.280914
		CD8 antigen, alpha chain	1.74495
	Antigen processing and *presentation*	Interferon alpha 13	1.57917
	Cytokine-cytokine receptor interaction	Small chemokine (C-C motif) ligand 11	1.913514
		Chemokine (C-C motif) ligand 17	1.571759
		Chemokine (C-X3-C motif) ligand 1	1.0517
		Interferon alpha 13	1.57917
		Nerve GFR (TNFR superfamily member 16)	1.451481
		Nos2	2.31
		Interleukin-1 receptor	−1
		FasL	1.71
		Interleukin-17 receptor	−1.2
		Cytokine receptor like	−1.17

*** Fold change is shown on a $\log_2$ scale.

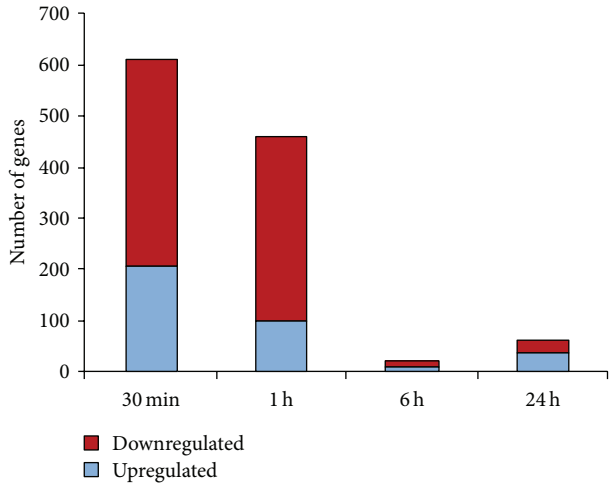


Figure 2: The number of genes in lung myofibroblasts with significant changes in expression at varying time points following Thy1 stimulation. Graphic presentation of GeneChip microarray results obtained from primary myofibroblast RNA extracts following stimulation with G7 anti-Thy mAb (5 μg/mL) or control IgG isotype for 30 min, 1 h, 6 h, or 24 h. The microarray data were processed by volcano analysis. Upregulated genes (blue), downregulated genes (red).

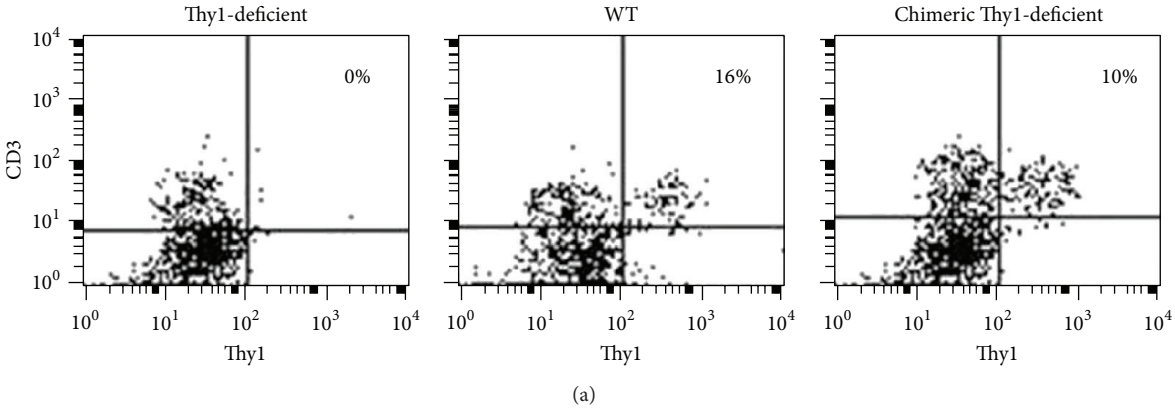
3.3. Decreased Inflammation with Similar Fibrosis Development following IT Bleomycin in Chimeric Mice with Thy1$^-$ Mesenchymal Cells and Normal Lymphocytes Compared to WT Mice with Thy1$^+$ Mesenchymal Cells and Thy1$^+$ Lymphocytes. In order to specifically assess the contribution of myofibroblast Thy1 downregulation on lung inflammation and fibrosis in vivo in a system excluding Thy1 lymphocytes, we used chimeric Thy1-deficient mice. These mice exhibit completely Thy1$^-$ mesenchymal cells (αSMA$^+$ Thy1$^-$), but normal, Thy1$^+$ lymphoctyes (CD3$^+$Thy1$^+$) (Figure 3(a)). Assessment of lung fibrotic injury by semiquantitative morphological index (SMI) of pathological sections (Figure 3(b)), as well as collagen content (Figure 3(c)), showed no difference between WT and chimeric Thy1-deficient mice when compared to control saline-treated (not shown) mouse lungs; however, we detected a decrease in lung inflammation as determined by bronchoalveolar lavage (BAL) cellularity (Figure 3(d)). In addition, there were no differences between WT and Thy1-deficient mice in either the quantity of αSMA-positive cells examined by immunochemistry staining or in collagen I an IV staining, as examined by sirius red staining (Figure 3(E1), Figure 3(E2), Figure 3(F1), and Figure 3(F2)).

3.4. Increased CD4$^+$/CD25$^+$ T Regulatory Cells following IT Bleomycin in Chimeric Mice with Thy1$^-$ Mesenchymal Cells and Normal Lymphocytes Compared to WT Mice with Thy1$^+$ Mesenchymal Cells and Thy1$^+$ Lymphocytes. Having shown decreased inflammation in the lungs of chimeric Thy1-deficient mice compared to WT mice, we determined the differences in the inflammatory cell mileux of their lungs. We stained lung sections with anti-CD8 (cytotoxic cells) and costained them with anti-CD4 and anti-CD25 mAbs (T regulatory cells). A much larger number of CD4$^+$/CD25$^+$ lymphocytes were detected in chimeric Thy1-deficient mice when compared to WT mice (Figures 4(A1) and 4(A2)); however, there were a comparable number of CD8 cells in WT and Thy1-deficient mice (Figures 4(B1) and 4(B2)).

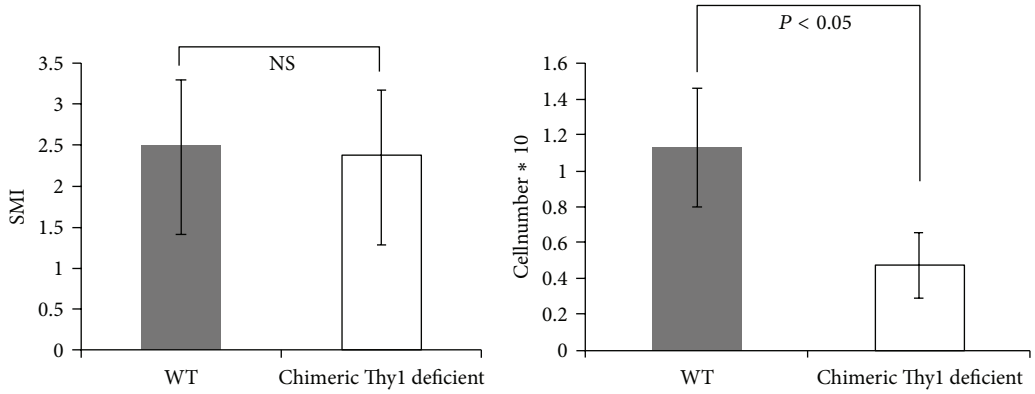
Taken together, these findings indicate that Thy1 on myofibroblasts in vivo has a role in the regulation of the inflammatory process, possibly by increased accumulation of T regulatory cells following bleomycin injury, with no effect on fibrosis development.

4. Discussion

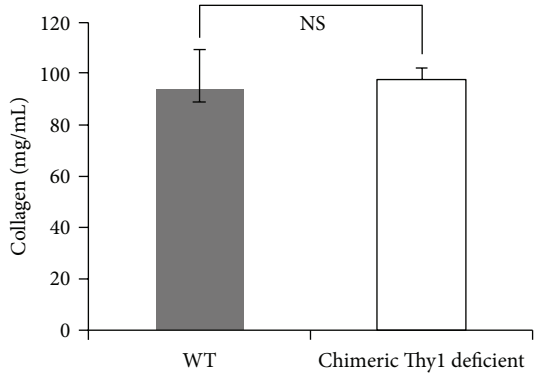
The wide and diverse distribution of Thy1 suggests that it has distinct functions that vary between and in some cases even



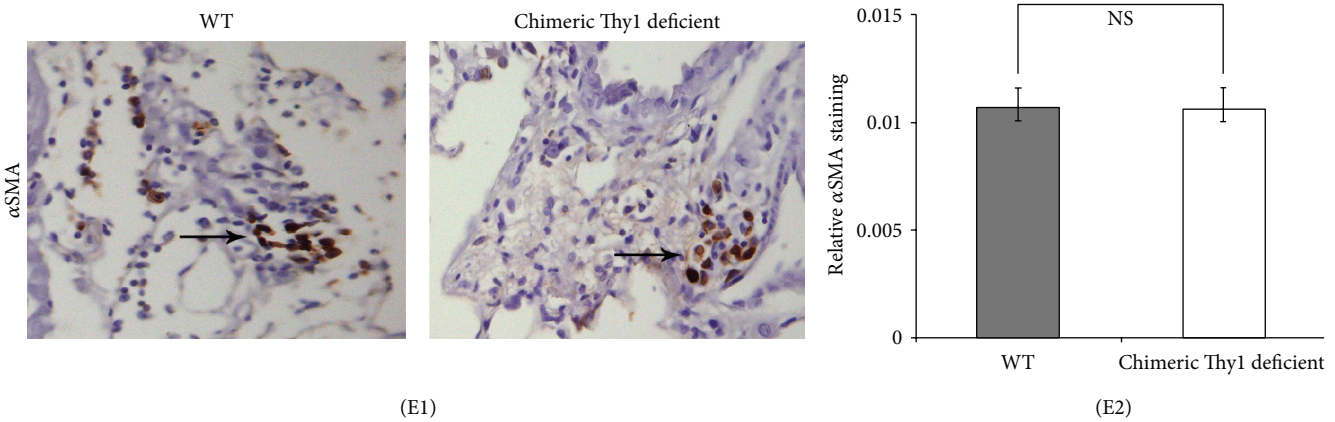
(a)



(b) (c)



(d)



(E1) (E2)

(e)

Figure 3: Continued.

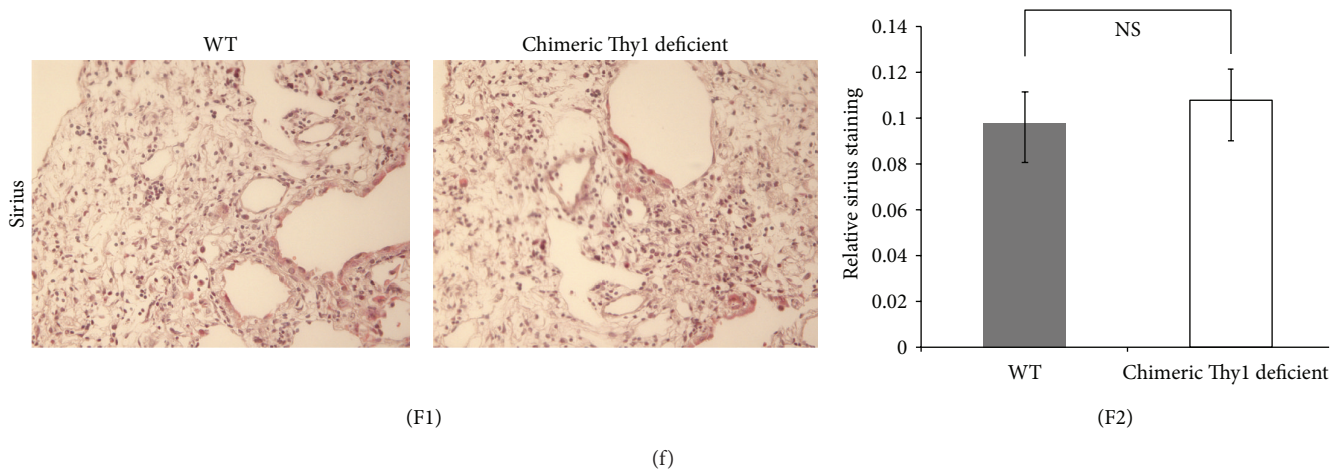


Figure 3: Assessment of fibrotic lung injury following bleomycin intratracheal instillation (IT) in wild-type mice with Thy1$^+$ lymphocytes and Thy1$^+$ myofibroblasts (WT), and chimeric Thy1-deficient mice with Thy1$^+$ WT lymphocytes and Thy1$^-$ myofibroblasts (chimeric). Thy1-deficient mice (DEF) were provided by Prof. R.J. Morris. Chimeric mice were created by whole-body irradiation (750 rad) of the Thy1-deficient mice, followed by transplantation with WT Thy1$^+$ lymphoid cells (chimeric). Wild-type mice underwent whole-body irradiation and reconstitution with WT Thy1$^+$ lymphoid cells as control (WT). (A) Flow cytometry analysis to detect Thy1$^+$ immune cells in Thy1-deficient mice (DEF), wild-type mice (WT), and chimeric Thy1-deficient mice with transplanted WT Thy1$^+$ lymphocytes. Thy1$^+$ immune cells were identified in blood cells by double staining with PE-conjugated anti-Thy1 and FITC-conjugated anti-CD3. (B) Quantitative image analysis of pathological sections in chimeric Thy1-deficient mice (Thy1$^+$ lymphocytes/Thy1– myofibroblasts, empty bars) and control WT mice (grey bars) 14 d following IT bleomycin. $n = 6$ WT and 8 chimeric 2 (C) Analysis of collagen content in lungs using the Sircol assay 14 d following IT bleomycin in chimeric Thy1-deficient (empty bars) and control WT mice (grey bars). (D) Total BAL cell count in lungs 14 d following IT bleomycin in chimeric (empty bars) and control WT mice (grey bars) ($^*P < 0.05$) (E1, F1) αSMA and sirius staining in lung tissue sections 14 d following IT bleomycin of chimeric Thy1-deficient (empty bars) and control WT mice (grey bars). (E2, F2) 10 fields of every IHC-slide were digitized using the Ariol machine and semiquantitatively analyzed with the Ariol system. The relative αSMA and sirius staining ratios represent the ratio between numbers of stained and unstained cells in a certain analyzed area. Results were similar in 2 different experiments.

within cell types and tissues, and between similar tissues in different species, indicating that the biological role of Thy1 is context-dependent [31].

Using gene chip analysis, we show that Thy1 on lung myofibroblasts affects pathways of the nervous system as well as those affecting the development of malignant diseases (Table 1), as previously shown [32–35]. The number of genes that were downregulated by Thy1 crosslinking in lung myofibroblasts was higher than the number that were upregulated (Figure 2), indicating that Thy1 on lung myofibroblasts appears to be a suppressor factor rather than an activator in most cases. These observations are consistent with previous reports showing an inhibitory effect of Thy1 on cell outgrowth of neurites [33] and on tumor growth [32, 34, 35]. Gene expression studies following Thy1 crosslinking in vitro may indicate that Thy1 could bind to a fixed ligand in vivo, such as an integrin, and/or to another cell type, or perhaps to a matrix ligand. However, our understanding of Thy1 ligand and its functions is incomplete and warrants further study.

Our detection of the downregulation of myofibroblast Thy1 expression as fibrosis evolves is consistent with findings in several other studies of an inverse correlation between Thy1 expression on fibroblasts and the evolution of lung fibrosis [36, 37]. Of note, one laboratory reported that, under certain conditions, Thy1$^+$ fibroblasts can produce more collagen compared to Thy1$^-$ fibroblasts [38]. Our results also show that myofibroblast Thy1 crosslinking upregulates genes promoting inflammation and immunological functions (Table 3). In addition, Thy1 upregulated specific genes, including those of chemokines that are responsible for antigen-processing and presentation, as well as those of cytotoxic molecules that promote inflammation and immunological functions (Tables 2 and 3). In particular, we have shown here in the GeneChip analysis (Table 3), previously validated by us in Wb, FACS, and qPCR [28], that Thy1 activation upregulates FasL expression in lung myofibroblasts, which in turn promotes pro-inflammatory activity by the recruitment and activation of immune cells [39]. Through this upregulation, Thy1 also indirectly induces a cytotoxic cell phenotype in myofibroblasts. We have previously demonstrated that myofibroblasts from bleomycin-treated mice overexpress the FasL molecule and act as effector cells that induce apoptosis in Fas$^+$ epithelial cells [24] and lymphocytes [21] via Fas/FasL interaction.

The stronger inflammatory response detected by BAL cellularity in control chimeric WT mice compared to chimeric mice with Thy1$^-$ myofibroblasts and WT (Thy$^+$) lymphocytes could be explained by GeneChip results, which show that Thy1 activation, as is the case in chimeric-WT mice, upregulates cytokine gene expression (Table 3). By upregulating cytokine gene expression, Thy1 may indirectly promote or induce myofibroblast recruitment of immune cells to the lungs. A lack of Thy1 on myofibroblasts, as is the case in Thy1$^-$ chimeric mice, may reduce the level of chemokine protein expression in the lung following bleomycin IT, yielding lower BAL cellularity.

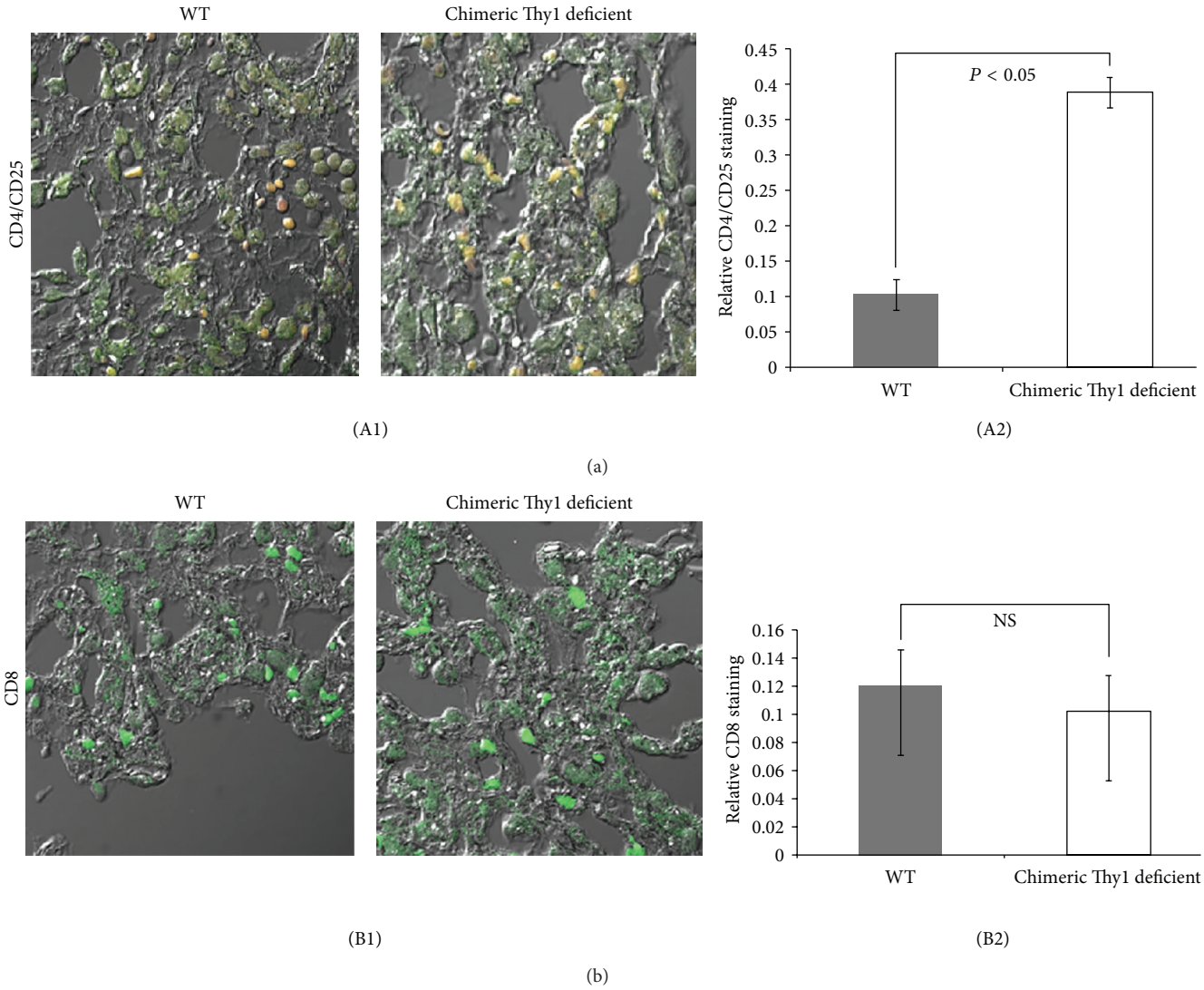


Figure 4: Assessment of the T cell population following bleomycin intratracheal instillation (IT) in wild-type mice (WT) (control) with $Thy1^+$ lymphocytes and $Thy1^+$ myofibroblasts, and chimeric Thy1-deficient mice with $Thy1^+$ lymphocytes and $Thy1^-$ myofibroblasts. Chimeric Thy1-deficient mice were created by total body irradiation (750 rad) of Thy1-deficient mice, followed by transplantation with WT $Thy1^+$ lymphoid cells. For comparison, wild-type (WT) mice were irradiated and had adoptive transfer with $Thy1^+$ (WT) lymphoid cells. (A1 and A2) Immunofluorescence of $CD4^+/CD25^+$, and (B1 and B2) CD8 staining of mouse lung sections 14 d following IT bleomycin in chimeric Thy1-deficient mice (empty bars) and control WT mice (grey bars). N 6 = 5 WT and 8 chimeric Thy1-deficient. (A1) Nomarski microscopy pictures of CD4/CD25 (yellow) ($^*P < 0.05$), and (B1) CD8 (green) staining are presented. (A2 and B2) 10 fields of every immunofluorescence slide were digitized and semiquantitatively analyzed using the Ariol system. The relative staining ratio represents the ratio between numbers of stained and unstained cells in a certain analyzed are. The results were similar in 2 different experiments.

Thy1 molecule inhibited gene/s that can be profibrotic (Table 3). Its activation downregulated genes promoting myofibroblast cell proliferation, differentiation, and ECM component production. In addition, we showed that the fraction of the $Thy1^+$ myofibroblast subpopulation is decreased at day 14 following bleomycin IT. This is consistent with previous reports showing that fibroblasts in the lungs of humans with IPF and of bleomycin-treated mice are predominantly $Thy1^-$ [36]. Thus, absence of Thy1 in myofibroblasts may facilitate their faster proliferation, accumulation, and collagen production and therefore increase lung fibrosis.

The GeneChip analysis may provide some explanation for our in vivo results with chimeric Thy1-deficient mice, which showed no change in fibrosis but did show decreased inflammation, in comparison with chimeric control WT mice that had undergone similar adoptive transfer WT lymphocytes into irradiated WT host mice. These results indicate that when Thy1 is deficient in myofibroblasts, as is the case in chimeric Thy1-deficient mice, fibrotic features persist in an in vivo milieu with decreased inflammation.

Hagood et al. reported that Thy1-null mice develop more extensive and more severe lung fibrosis following bleomycin administration than do WT mice [36]. Although this is consistent with our in vitro results, which showed that Thy1 activation in lung myofibroblasts downregulates genes promoting cell differentiation and ECM production (Table 3), it

is not consistent with our in vivo results showing no change in the fibrotic feature between chimeric WT- and Thy1-deficient mice. Thy1 is absent from all cells that would otherwise express it in Thy1-null mice, in contrast to our chimeric Thy1-deficient mice, which retain Thy1 expression on lymphocytes. Because Thy1 is normally expressed on murine lymphocytes, it is possible that the severe lung fibrosis following bleomycin IT that develops in Thy1-null mice is not due to changes in fibroblast Thy1 expression but rather is due to changes in lymphocyte function [40]. The role of lymphocytes in bleomycin-induced lung fibrosis remains controversial [41–45], as does the role of inflammation in IPF [19, 20, 41, 46–49]. Decreased inflammation following bleomycin injury, as is the case in the chimeric Thy1-deficient mice with Thy1-deficient myofibroblasts but $Thy1^+$(WT) T cells, may suggest that myofibroblasts downregulate Thy1 expression as an additional mechanism allowing their accumulation, promoting immune modulation, and supporting disruption of immune surveillance [21]. Consistent with our results, which show similar fibrosis and similar augmentation of CD8 T cells in chimeric Thy1-deficient and control-WT mice (Figures 4(B1) and 4(B2)), it was shown that $CD8^+$ T cells are associated with development and prognosis of fibrosis [50]. In addition, it was shown that a fibrotic environment in the lung results in an increased abundance of $CD4^+/CD25^+$ regulatory T cells [51]. Our findings show an increase in the number of $CD4^+/CD25^+$ regulatory T cells (Figures 4(A1) and 4(A2)) in chimeric Thy1-deficient mice, despite the fact that there was no difference in fibrosis between chimeric Thy1-deficient and WT mice. However, the two groups of mice differed in the extent of lung inflammation. It may be possible that differences in the accumulation of regulatory T cells in these mice cannot influence fibrosis, which was at its peak. However, the increase in T regulatory cells can, and did, have an impact on the extent of inflammation. This can be explained by the complicated interactions between regulatory cells, cytotoxic T cells, and myofibroblasts [52]. The role of Thy1 on lymphocytes remains a very interesting issue for further investigation. It may be possible to create chimeric mice with Thy-deficient lymphocytes and WT fibroblasts to pursue this research.

5. Conclusions

These findings indicate that, in lung fibrosis, lymphocytes increase Thy1 expression while the subset of $Thy1^+$ lung myofibroblasts is decreased. Lung myofibroblasts downregulate Thy1 expression to increase their proliferative functions and to reduce the inflammatory milieu, possibly via induction of $CD4^+/CD25^+$ regulatory T cell accumulation, in vivo. Differences in the inflammatory responses of chimeric Thy1-deficient mice with $Thy1^-$ myofibroblasts but $Thy1^+$ lymphocytes versus WT mice do not affect fibrotic responses other than inflammation. These results indicate a newly identified role for $Thy1^-$ myofibroblasts and $Thy1^+$ lymphocytes.

Abbreviations

BAL: Bronchoalveolar lavage
ECM: Extracellular matrix
FACS: Fluorescence-activated cell sorting
GPI: Glycosylphosphatidylinositol
IP: Intraperitoneal
IPF: Idiopathic pulmonary fibrosis
IT: Intratracheal instillation
PE: Phycoerythrin
qPCR: Quantitative polymerase chain reaction
RT: Room temperature
$Thy1^-$: Thy1-negative
$Thy1^+$: Thy1-positive
WT: Wild-type.

Conflict of Interests

The authors have no competing interests to disclose.

Authors' Contribution

Pazit Y. Cohen contributed to research design and performed all experimental work and primary data analysis, prepared the initial draft of the paper and contributed to revisions. Raphael Breuer contributed to research concept and strategy, supervised the project, and critically revised the paper. Philip Zisman contributed to experiments with IHC and IF. Shulamit B. Wallach-Dayan conceived the research concept and strategies, supervised the study and data analysis, and critically revised the paper. All authors reviewed the final version of the paper and accept responsibility for the accuracy and integrity of the research findings reported here.

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Review Article

Follicular Helper CD4$^+$ T Cells in Human Neuroautoimmune Diseases and Their Animal Models

Xueli Fan,[1] Chenhong Lin,[1] Jinming Han,[1] Xinmei Jiang,[1] Jie Zhu,[1,2] and Tao Jin[1]

[1]*Department of Neurology, The First Hospital of Jilin University, Jilin University, Changchun 130021, China*
[2]*Department of Neurobiology, Care Sciences and Society, Karolinska Institute, 14186 Stockholm, Sweden*

Correspondence should be addressed to Tao Jin; drtao.jin@hotmail.com

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Follicular helper CD4$^+$ T (TFH) cells play a fundamental role in humoral immunity deriving from their ability to provide help for germinal center (GC) formation, B cell differentiation into plasma cells and memory cells, and antibody production in secondary lymphoid tissues. TFH cells can be identified by a combination of markers, including the chemokine receptor CXCR5, costimulatory molecules ICOS and PD-1, transcription repressor Bcl-6, and cytokine IL-21. It is difficult and impossible to get access to secondary lymphoid tissues in humans, so studies are usually performed with human peripheral blood samples as circulating counterparts of tissue TFH cells. A balance of TFH cell generation and function is critical for protective antibody response, whereas overactivation of TFH cells or overexpression of TFH-associated molecules may result in autoimmune diseases. Emerging data have shown that TFH cells and TFH-associated molecules may be involved in the pathogenesis of neuroautoimmune diseases including multiple sclerosis (MS), neuromyelitis optica (NMO)/neuromyelitis optica spectrum disorders (NMOSD), and myasthenia gravis (MG). This review summarizes the features of TFH cells, including their development, function, and roles as well as TFH-associated molecules in neuroautoimmune diseases and their animal models.

1. An Overview of Follicular Helper CD4$^+$ T Cells

CD4$^+$ T helper (Th) cells play a critical role in adaptive immune response. After infection or vaccination, naive CD4$^+$ T cells differentiate into diverse effector subsets of Th cells dependent on distinct cytokines and transcription factors [1–5] (Figure 1). These Th cell subsets possess respective effector function, for instance, the antiviral role of Th1 cells and the role in elimination of extracellular parasites of Th2 [2, 3] (Figure 1). Recently, follicular helper CD4$^+$ T (TFH) cells, a specialized subset of CD4$^+$ Th cells, have been identified as providing help for B cells in germinal center (GC) [6, 7]. GC is an important structure in B cell follicles of secondary lymphoid tissues, where B cells can differentiate into plasma cells and memory cells. TFH cells are distinguished from other Th cell subsets by anatomical location (germinal center), specialized expression of transcription factor B cell lymphoma 6 (Bcl-6), chemokine receptor CXC-chemokine receptor 5 (CXCR5), programmed death-1 (PD-1), CD40 ligand (CD40L), inducible costimulator (ICOS), SAP (signaling lymphocytic activation molecule associated protein), and secretion of interleukin 21 (IL-21) and interleukin 4 (IL-4) [8–10]. These TFH-associated molecules are vital for activation, differentiation, and survival of TFH cells and B cells [11]. In a word, TFH cells are pivotal to GC formation, providing help for affinity maturation, class switch recombination, and ultimate differentiation of B cells within GC [12]. The present review outlines the features of TFH cells and TFH-associated molecules in neuroautoimmune diseases, especially in multiple sclerosis (MS), neuromyelitis optica (NMO)/neuromyelitis optica spectrum disorders (NMOSD), and myasthenia gravis (MG) as well as their animal models, experimental autoimmune encephalomyelitis (EAE), and experimental autoimmune myasthenia gravis (EAMG).

1.1. Development of TFH Cells. It is generally accepted that the process of TFH cell differentiation is carried out in a multistage and multifactorial model [6, 11]. The first stage of TFH cell differentiation occurs in T cell zone of lymphoid

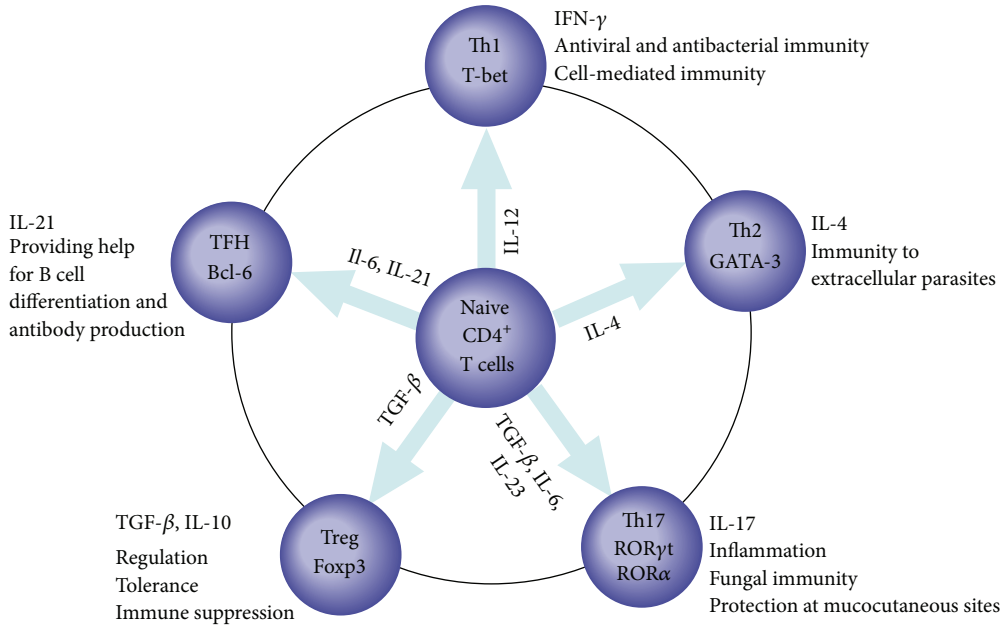

Figure 1: Effector subsets of $CD4^+$ T cells: ontogenic and major cytokines, and roles in diseases. Naive $CD4^+$ T cells differentiate into diverse effector subsets dependent on stimulatory cytokines in the microenvironment upon activation by pathogens. These stimulatory cytokines induce transcription factors expression of these subsets. IL-12 induces T-bet in the case of Th1 cells, IL-4 induces GATA3 in the case of Th2 cells, TGF-β, IL-6, and IL-23 induce RORγt and RORα in the case of Th17 cells, TGF-β induces Foxp3 in the case of Treg cells, and IL-6 and IL-21 induce Bcl-6 in the case of TFH cells. Subsequently, different effector subsets produce distinct cytokines and acquire specialized effector function. Th1 cells produce IFN-γ associated with antiviral and antibacterial immunity and cell-mediated immunity, Th2 cells produce IL-4 associated with immunity to extracellular parasites, Th17 cells produce IL-17 associated with inflammation, fungal immunity, and protection at mucocutaneous sites, Treg cells produce TGF-β and IL-10 associated with regulation, tolerance, and immune suppression, and TFH cells produce IL-21 associated with providing help for B cell differentiation and antibody production. Bcl-6, B cell lymphoma 6; Foxp3, forkhead box p3; GATA-3, GATA-binding protein 3; IFN-γ, interferon-γ; IL-4, interleukin 4; IL-6, interleukin 6; IL-10, interleukin 10; IL-12, interleukin 12; IL-17, interleukin 17; IL-21, interleukin 21; IL-23, interleukin 23; RORγt, retinoid-related orphan receptor γt; RORα, retinoid-related orphan receptor α; T-bet, T-box transcription factor; TGF-β, transforming growth factor-β; TNF, tumour necrosis factor; Treg, T regulator.

tissues (Figure 2(a)). Naive $CD4^+$ T cells are activated when they recognize dendritic cells (DCs) through peptide-MHC class II complexes and interact with DCs via the ligation of ICOS and ICOSL [13, 14]. Then these naive $CD4^+$ T cells upregulate Bcl-6 and CXCR5, downregulate CC-chemokine receptor 7 (CCR7), and migrate towards B cell follicles [15, 16]. Meanwhile, IL-21 produced by these naive $CD4^+$ T cells, accompanied with IL-6 and IL-27 produced by DCs, enhances Bcl-6 and c-Maf expression in naive $CD4^+$ T cells [6]. Thus, the interplay between TCR signaling, ICOS, IL-21, IL-6, and IL-27 via control of CXCR5, Bcl-6, and other targets induces early stage of TFH cell differentiation. After that, these naive $CD4^+$ T cells become pre-TFH cells ($Bcl\text{-}6^+CXCR5^+$ T cells). The second stage of TFH cell differentiation happens at the T cell-B cell border (Figure 2(b)). Here, pre-TFH cells first interact with cognate activated B cells, promoting either the differentiation of B cells into short-lived extrafollicular plasmablasts or the migration of B cells into follicles [13]. Although ICOS is a costimulatory molecule, it can also induce directional migration of pre-TFH cells after combining with ICOSL on activated B cells [6]. So ICOS-ICOSL binding is indispensable during this process. Furthermore, this process is a significant B cell-dependent course in which B cells offer antigen presentation and uninterrupted stimulation to promote full development of TFH cells [11]. The third stage of TFH cell differentiation involves the GC (Figure 2(c)). Within GC, pre-TFH cells finally differentiate into TFH cells that are also termed GC TFH cells. Pre-TFH cells and GC TFH cells, which are two phenotypically distinct stages in the development course of TFH cells, express analogical gene profiles. GC TFH cells express higher levels of Bcl-6, CXCR5, and ICOS than pre-TFH cells. GC TFH cells can prompt GC formation and provide help to B cells for affinity maturation, class switch recombination, and differentiation into memory B cells or plasma cells [17]. GC TFH cells interact with B cells dependent on stable T cell-B cell conjugates, which include CD40L/CD40, ICOS/ICOSL and CD28/B7, and $CD4^+$ T cell-intrinsic signaling via SAP-associating receptors (CD84) [13]. In addition, B cells still serve as antigen presenting cells (APCs). Obviously, reciprocal signals provided by B cells play a significant role in sustaining TFH cells. In addition, IL-21 secreted by TFH cells prompts the final differentiation of TFH cells themselves, while IL-6 secreted by B cells is important for the maintenance of TFH cells. After the interaction between TFH cells and B cells in GC, the fate of TFH cells is unclear. Thus several questions are raised: (1) Are they apoptotic? (2) Do they become memory TFH cells?

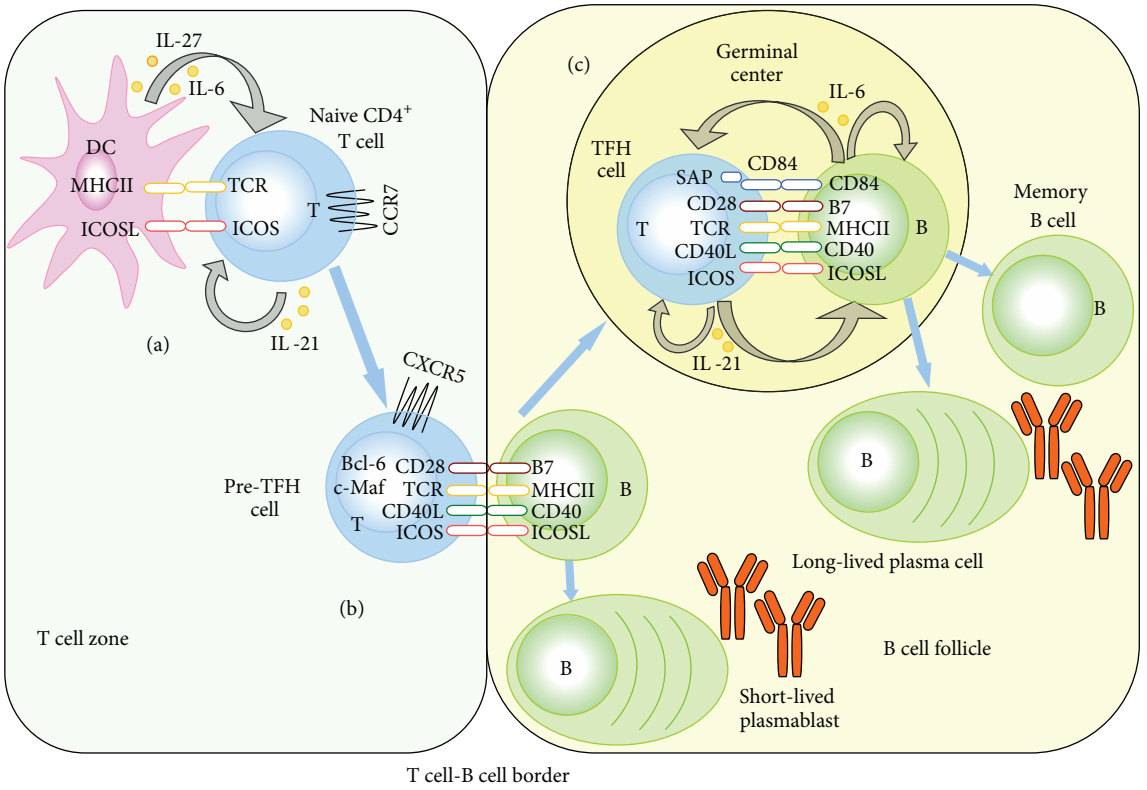

FIGURE 2: Multiple signals and steps for the generation of TFH cells. (a) Naive $CD4^+$ T cells are activated when they encounter antigen presented cells-dendritic cells within T cell zone, and then these T cells move towards B cell follicles. (b) At the T cell-B cell border, activated T cells become pre-TFH cells, first interacting with cognate activated B cells, promoting either the differentiation of B cells into short-lived extrafollicular plasmablasts or the migration of B cells into follicles. (c) In germinal center, pre-TFH cells become GC TFH cells and provide help for B cell differentiation into plasma cells and memory B cells as well as antibody production. Cross-talk between TFH cells and cognate B cells involves a series of costimulatory molecules and cytokines, which are important for the function of TFH cells. Reciprocal signals provided by B cells are indispensable to sustain TFH cells. Bcl-6, B cell lymphoma 6; CCR7, CC-chemokine receptor 7; CD40L, CD40 ligand; CXCR5, CXC-chemokine receptor 5; DC, dendritic cell; ICOS, inducible costimulator; ICOS, ICOS ligand; IL-6, interleukin 6; IL-21, interleukin 21; IL-27, interleukin 27; MHC-II, major histocompatibility complex II; SAP, signaling lymphocytic activation molecule associated protein; TCR, T cell receptor.

(3) Is there a cycle between pre-TFH cells and TFH cells [6]? Further investigations will be performed to verify the final fate of TFH cells.

1.2. The Function of TFH Cells and TFH-Associated Molecules. Pre-TFH cells help B cells to form short-lived extrafollicular plasma cells, which can produce low-affinity antibodies in the T cell-B cell border. GC TFH cells are crucial for humoral immune response against pathogens, because they are necessary in GC formation, affinity maturation, class switch recombination, and differentiation of B cells [18]. TFH cells express a lot of key molecules that are important for their function [19]. The roles of TFH-associated molecules are described as below.

1.2.1. Bcl-6: B Cell Lymphoma 6. Bcl-6 is the master regulator transcription factor in TFH cells differentiation [6]. T cells with deficiency of Bcl-6 are unable to differentiate into TFH cells or sustain GC reactions, while Bcl-6 overexpression facilitates the expressions of TFH-associated molecules CXCR5 and PD-1 [20]. Besides, Bcl-6 represses numerous miRNAs and stabilizes the expression of CXCR5 [21]. Bcl-6 also represses Th1, Th2, and Th17 cell transcription factors T-box transcription factor (T-bet), GATA-binding protein 3 (GATA-3), and retinoid-related orphan receptor γt (RORγt). However, the repression of Bcl-6 is not complete, as TFH cells sometimes express T-bet and GATA3 [22]. Another molecule, B lymphocyte induced maturation protein 1 (Blimp-1), directs the differentiation of $CD8^+$ T cells, non-TFH $CD4^+$ T cells, and plasma cells, which is a reciprocal antagonist of Bcl-6 and can inhibit TFH cell development [23]. So Bcl-6 can regulate all non-TFH cells differentiation through repressing Blimp-1 [24]. On the contrary, Blimp-1 also regulates the induction and function of Bcl-6 within TFH cells. The expression of Blimp-1 inhibits TFH cell formation, while Blimp-1 deficiency prompts the generation of TFH cells [23].

1.2.2. CXCR5: CXC-Chemokine Receptor 5. CXCR5 is a highlighted surface marker of TFH cells and the most widely used marker to identify TFH cells [25]. Its specific ligand, CXC-chemokine ligand 13 (CXCL13), is mostly produced by B cells and follicle mesenchymal cells. Upregulation of CXCR5, combined with downregulation of CCR7, leads to the migration of TFH cells from the T cell zone to the B

cell follicles (Figure 2), where they provide a help for B cell differentiation [13, 16]. The precise localization of TFH cells is important for proper generation and function of TFH cells. T cells with deficiency of CXCR5 result in fewer and smaller GC formation and decreased frequency of GC B cells [26].

1.2.3. PD-1: Programmed Death-1. PD-1 is highly expressed by TFH cells, while its ligands (PD-L1 and PD-L2) are expressed by multiple cells including B cells [12]. It has been demonstrated that PD-1 is a negative regulator of the proliferation of $CD4^+$ T cells. Thus, deficiency of PD-1 leads to an increase in TFH cells, but a decrease in IL-4 and IL-21 cytokine mRNA synthesis [27, 28]. Loss of PD-1 contributes to a reduction of long-lived plasma cells [27]. Overall, PD-1 signaling regulates the homeostasis and function of TFH cells and affects the formation of long-lived plasma cells.

1.2.4. ICOS: Inducible Costimulator. ICOS, a member in the CD28 family of costimulatory molecules, is expressed on activated T cells, while its ligand ICOSL is expressed on B cells, macrophages, and other antigen presenting cells. A study demonstrated that ICOS deficiency impaired the development of TFH cells, so as to cause defect in GC formation and antibody production in response to primary and secondary immunization with sheep red blood cells in the spleen of mice [29]. Another study also showed that lack of ICOS resulted in poor GC formation and severe reduction of class-switched memory B cells [30]. Taken together, ICOS plays an essential role in TFH cells generation, GC formation, and antibody production.

1.2.5. CD40L: CD40 Ligand. CD40L, the unique ligand of CD40, is present on the surface of TFH cells. Patients with mutations in CD40L have a reduced number of TFH cells. Furthermore, CD40L is a key factor for GC formation as well as B cell activation, proliferation, and survival in vitro and in vivo [6, 31]. The maintenance of TFH cells and GC B cells depends on CD40L-CD40 engagement [32]. Of particular interest, CD40L inhibits the differentiation of plasma cell [33]. To sum up, CD40L is critical to the generation of TFH cells and the fate of GC B cells.

1.2.6. IL-21: Interleukin 21. IL-21, produced by activated $CD4^+$ T cells and NKT cells, plays a major role in TFH cell survival and GC B cell proliferation, survival, and differentiation in GC [34]. IL-21 is the most potent cytokine for driving plasma cells differentiation in both mice and humans [35]. Additionally, IL-21 can induce both Blimp-1 and Bcl-6 expression on B cells in different conditions [36]. Interestingly, the effect of IL-21 in initiating GC B cell differentiation has substantial overlapping function with other cytokines such as IL-6 and IL-4 [37], indicating that these cytokines may have overlapping signal pathways. So the fate of B cells relies on the combination of IL-21 and additional signals from GC TFH cells [6]. Therefore, IL-21 has an elusive effect which needs to be further investigated.

1.2.7. SAP: Signaling Lymphocytic Activation Molecule Associated Protein. SAP is an adaptor protein that binds to the cytoplasmic tails of signaling lymphocytic activation molecule (SLAM) family receptors. SAP is upregulated in TFH cells and is indispensable for GC development and T cell-B cell conjugates formation [6]. In the absence of SAP, pre-TFH cells migrate into GC less efficiently and lose their ability to stay in GC [38]. Consistently, another study showed that SAP-deficient mice were able to generate $CXCR5^+PD\text{-}1^+$ TFH cells, but these cells were unable to be retained in GC [17]. Taken together, it is demonstrated that SAP contributes to the terminal differentiation of pre-TFH cells into GC TFH cells.

There are other molecules produced or expressed by TFH cells such as SLAM family receptors and IL-4. SLAM family receptors, which consist of CD84, SLAM, SLAM family member 6 (SLAMF6), and SLAMF3, have the ability to bind SAP and form T cell-B cell conjugates, thus contributing to TFH cell differentiation and function [11]. IL-4 plays an important role in B cell survival and differentiation [6].

2. Human Circulating TFH Cells

Generally, TFH cells have been recognized by their anatomical location in secondary lymphoid tissues. However, it is difficult and impossible to get access to these lymphoid tissues in humans, so plenty of studies are performed with human peripheral blood samples [39]. It has been demonstrated that $CD4^+CXCR5^+$ T cells comprise a portion of circulating lymphocytes [37, 40]. These circulating $CD4^+CXCR5^+$ T cells express lower level of ICOS and PD-1 and hardly express Bcl-6 in comparison with TFH cells in lymphoid tissues [41]. When circulating $CD4^+CXCR5^+$ T cells are cultured in vitro, they are able to secret IL-21 and induce B cell differentiation. Thus, human circulating $CD4^+CXCR5^+$ T cells share a part of properties of TFH cells. But whether circulating $CD4^+CXCR5^+$ cells are counterparts of TFH cells in GC is uncertain. It has been reported that human circulating $CD4^+CXCR5^+$ T cells share functional capacities with TFH cells and seemingly stand for their circulating memory compartment [42]. Similar to TFH cells in GC, circulating $CD4^+CXCR5^+$ T cells induce the differentiation of naive and memory B cells to plasmablasts and promote class switching via IL-21 and ICOS [42]. Circulating $CD4^+CXCR5^+$ T cells also provide help to B cells through cognate interaction. Additionally, circulating $CD4^+CXCR5^+$ T cells express CCR7 and L-selectin, both of which are indicative of the property to migrate into secondary lymphoid tissues [42]. Circulating $CD4^+CXCR5^+$ T cells comprise two subsets: the $CCR7^{lo}PD\text{-}1^{hi}$ subset represents a TFH-cell precursor phenotype and $CCR7^{hi}PD\text{-}1^{lo}$ subset displays a resting state. The differentiation of these two subsets requires ICOS and Bcl-6 but not SAP, suggesting that circulating $CD4^+CXCR5^+$ T cells are predominantly generated before becoming fully mature effector TFH cells. Upon antigen reexposure, circulating $CCR7^{lo}PD\text{-}1^{hi}$ subset rapidly differentiated into mature TFH cells to promote GC formation and antibody response. So circulating $CCR7^{lo}PD\text{-}1^{hi}$ subset is representative of active TFH cells in secondary lymphoid organs and related to disease activity in autoimmune diseases. Consequently, blood $CCR7^{lo}PD\text{-}1^{hi}CD4^+CXCR5^+$ T cells stand for a new mechanism of

immunological early memory [43]. Emerging evidence further demonstrates the hypothesis that there is a group of circulating memory TFH cells. Because circulating memory TFH cells not only share common molecular pathways as effector TFH cells during differentiation, but also effectively help B cells during antibody response, they are used as a marker to monitor TFH cells in autoimmune diseases [44].

More and more investigators have conducted experiments with respect to circulating memory TFH cells. These studies have defined circulating TFH cells by different markers. Circulating $CD4^+CXCR5^+$ T cells, $CD4^+CXCR5^+ICOS^+$ T cells, $CD4^+CXCR5^+ICOS^{hi}$ T cells, $CD4^+CXCR5^+PD\text{-}1^+$ T cells, $CD4^+CXCR5^+PD\text{-}1^{hi}$ T cells, and $CD4^+CXCR5^+ICOS^+PD\text{-}1^+$ T cells have been used to define TFH cells or the subsets of TFH cells in different diseases. Therefore, it is a serious problem in the immunology to clarify circulating TFH cells, whether these cells are able to represent circulating TFH cells. Furthermore, an authentic phenotype is required to define circulating TFH cells, which will be used in further investigations in immune related diseases.

Intriguingly, according to the expression of chemokine receptors, CXC-chemokine receptor (CXCR3) and CC-chemokine receptor (CCR6), circulating $CD4^+CXCR5^+$ T cells are classified into Th1-like ($CXCR3^+CCR6^-$), Th2-like ($CXCR3^-CCR6^-$), and Th17-like ($CXCR3^-CCR6^+$) subsets. It has been confirmed that Th2-like and Th17-like subsets potently induce naive B cells to produce antibodies via IL-21 whereas Th1-like TFH cells are unable to do so [42].

Although the precise role of circulating $CD4^+CXCR5^+$ T cells still remains a puzzle, the experiments on circulating $CD4^+CXCR5^+$ T cells may reflect partial perturbation of TFH cells in GC. A number of studies have demonstrated that circulating TFH cells may participate in the immune response of autoimmune diseases such as systemic lupus erythematosus [41, 45], rheumatoid arthritis [46], ankylosing spondylitis [47], bullous pemphigoid [48], and primary Sjögren's syndrome [49].

In conclusion, the exact phenotype, features, and roles of circulating $CD4^+CXCR5^+$ T cells are still unclear. Further investigations are required to clarify the conundrum.

3. TFH Cells in Neuroautoimmune Diseases

The dysregulation of TFH cells and TFH-associated molecules causes several human diseases [50]. The downregulation of TFH cells results in a series of immune deficiencies including X-linked lymphoproliferative disease and hyper-IgM syndrome, whereas the upregulation has been found in autoimmunity (i.e., systemic lupus erythematosus) and cancers [51, 52]. Neuroautoimmune diseases include multiple sclerosis (MS), neuromyelitis optica (NMO)/neuromyelitis optica spectrum disorders (NMOSD), and myasthenia gravis (MG). The exact pathogenesis of these diseases is not completely clear. Emerging data has suggested that TFH cells may be associated with development of these diseases (Table 1). The implications of TFH cells and TFH-associated molecules in neuroautoimmune diseases are summarized below.

3.1. TFH Cells and TFH-Associated Molecules in Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis. MS is a progressive autoimmune disease caused by damage to the myelin and axons of brain and spinal cord in central nervous system (CNS) [53–55]. According to the multiple sclerosis phenotype descriptions in 1996, MS is classified into 4 clinical subtypes: relapsing remitting multiple sclerosis (RRMS), primary progressive multiple sclerosis (PPMS), secondary progressive multiple sclerosis (SPMS), and progressive relapsing multiple sclerosis (PRMS) [56]. To date, existing therapeutic drugs can only decrease disease relapse and improve clinical symptoms, revealing an urgent need for new therapies.

The accurate pathogenesis of MS is unknown. Previously, MS was considered as a T cell-mediated autoimmune disease [57, 58]; nonetheless, a lot of groups have shown that MS is an immune-mediated disorder involved with humoral and cellular immunity [55, 59–61], with infiltration of activated T cells and macrophages, dendritic cells, B cells, and plasma cells [55, 58, 59]. In addition, other significant hallmarks of MS cover synthesis of oligoclonal immunoglobulins and the presence of B cell clonal expansion in cerebrospinal fluid (CSF), which shows that B cells play an important role in MS [62]. Later, it has been demonstrated that meningeal B cell follicles in SPMS were related to severe pathological changes, rapid disease progression, and poor prognosis [63]. These two studies further declared that humoral immunity may participate in disease development.

TFH cells are essential to humoral response due to their roles in GC formation, B cell differentiation, and antibody production [6]. Recently, a study showed that there was an increased frequency of $ICOS^+$ TFH cells in circulating $CD4^+CXCR5^+$ T cells of both RRMS and SPMS compared to healthy controls [64]. $ICOS^+$ TFH cells, as an activated TFH cell subset, were correlated with disease progression in SPMS. What is more, the frequency of $ICOS^+$ TFH cells was related to plasmablasts, suggesting that $ICOS^+$ TFH cells may play a crucial role in B cell activation [64]. Another study found that SPMS patients had an increased gene expression of ICOS, IL-21, and IL-21R in purified $CD4^+$ T cells [65]. In addition, the expression of ICOS was also increased in cells from the CSF of progressive MS patients [65]. Tzartos et al. showed that the expression of IL-21 was increased in $CD4^+$ T cells of infiltrating acute and chronic active lesions compared to inactive lesions of CNS in MS patients [66]. Furthermore, the polymorphisms of IL-21R were associated with MS in a genetic study [67]. The level of IL-21 mRNA in peripheral blood of SPMS patients reduced after treatment with mitoxantrone [64]. Additionally, there was a decreased percentage of blood $CXCR3^+$ Th1-like TFH cells in all subtypes of MS patients, while there was an increased percentage of blood $CCR6^+$ Th17-like TFH cells in PPMS patients [64].

Experimental autoimmune encephalomyelitis (EAE) is a traditional animal model for MS, which can be induced by active immunization with myelin components or by adoptive transfer of myelin-reactive $CD4^+$ T cells. It is accepted that EAE is a T cell-mediated immune disease with similar pathologic characteristics of MS. Hence, EAE is used to

Table 1: TFH cells and TFH-associated molecules in neuroautoimmune diseases and their animal models.

Neuroautoimmune diseases	Changes of TFH cells and TFH-associated molecules	Relevance to the diseases	References
MS	↑ frequency of circulating $CD4^+CXCR5^+ICOS^+$ T cells in RRMS and SPMS patients ↑ gene expression of ICOS, IL-21, and IL-21R in purified $CD4^+$ T cells in SPMS patients ↑ ICOS expression in CSF cells in progressive MS patients ↑ IL-21 expression of $CD4^+$ T cells in infiltrating active lesions of CNS ↓ frequency of blood $CXCR3^+$ Th1-like TFH cells in MS patients ↑frequency of blood $CCR6^+$ Th17-like TFH cells in PPMS patients	Positive correlation between circulating $CD4^+CXCR5^+ICOS^+$ T cells and disease progression in SPMS	[64–66]
EAE	CXCR5 mRNA present in spinal cords CNS infiltrating $CXCR5^+$ cells were $CD3^+CD4^+$ T cells in spinal cord MNCs		[70]
NMO/NMOSD	↑ frequency of circulating $CD4^+CXCR5^+PD\text{-}1^+$ T cells ↑ serum level of IL-21 ↑ secretion of IL-21 in PBMC cultures ↑ serum and CSF levels of CXCR5	A correlation between circulating $CD4^+CXCR5^+PD\text{-}1^+$ T cells and disease activity Positive correlation between the level of IL-21 produced by peripheral blood $CD4^+$ T cells and EDSS score Positive correlation between CSF CXCL13 level and disease disability	[83–85]
MG	↑ frequency of circulating $CD4^+CXCR5^+$ T cells, $CD4^+CXCR5^+CD45RO^+$ T cells, $CD4^+CXCR5^+$ $PD\text{-}1^{hi}$ T cells, and $CD4^+CXCR5^+ICOS^{hi}$ T cells ↑ serum level of CXCL13 ↑ expression of IL-21 mRNA in PBMCs	Positive correlation between circulating $CD4^+CXCR5^+$ T cells and disease severity Positive correlation between circulating $CD4^+CXCR5^+ICOS^{hi}$ T cells and serum anti-AChR Ab concentration Positive correlation between circulating $CD4^+CXCR5^+PD\text{-}1^{hi}$ T cells and serum anti-AChR Ab concentration Positive correlation between serum level of CXCL13 and disease severity Positive correlation between serum level of CXCL13 or expression of IL-21 mRNA in PBMCs and circulating $CD4^+CXCR5^+ICOS^{hi}$ T cells	[97–100]
EAMG	↑ frequency of $CD4^+CXCR5^+PD\text{-}1^+$ TFH cells and increased expression of Bcl-6 and IL-21 in spleens	Positive correlation between serum level of anti-AChR Abs and the frequency of TFH cells in spleen	[102]

AChR, acetylcholine receptor; Bcl-6, B cell lymphoma 6; CCR6, CC chemokine receptor 6; CNS, central nervous system; CSF, cerebrospinal fluid; CXCL13, CXC-chemokine ligand 13; CXCR3, CXC-chemokine receptor 3; CXCR5, CXC-chemokine receptor 5; EAE, experimental autoimmune encephalomyelitis; EAMG, experimental autoimmune myasthenia gravis; EDSS, expanded disability status scale; ICOS, inducible costimulator; IL-21, interleukin 21; IL-21R, IL-21 receptor; MG, myasthenia gravis; MNCs, mononuclear cells; MS, multiple sclerosis; NMO, neuromyelitis optica; NMOSD, neuromyelitis optica spectrum disorders; PBMC, peripheral blood mononuclear cell; PD-1, programmed death-1; RRMS, relapsing remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis.

study the pathogenesis and therapy for MS [68]. A recent study found that there were lymphoid follicle-like structures within the meninges of progressive relapsing EAE as well as increased gene expression of CXCL13 in the CNS of EAE [69], indicating a pathogenic role of humoral immunity in EAE. CXCR5 mRNA was present in spinal cord mononuclear cells (MNCs) in some of the mice with EAE. Furthermore, most CNS infiltrating $CXCR5^+$ cells were $CD3^+CD4^+$ T cells in spinal cord MNCs in EAE [70]. Nohra et al. pointed out that the polymorphisms of IL-21R were associated with EAE in a genetic study [67]. Another study showed that when IL-21 was administered before EAE induction, it reinforced inflammatory influx into the CNS and exacerbated the severity of EAE [67].

In summary, TFH cells may be involved in the formation of ectopic B cell follicles with GC in the meninges of SPMS patients and intrathecal immunoglobulins synthesis. In addition, $CD4^+$ T cells that highly express CXCR5 and ICOS are assumed to be the most potent inducers of IgG production [71]. The dysregulation of TFH cell function and TFH-associated molecules, ICOS and IL-21, have likely taken part in the pathogenesis of MS. IL-21, the most important cytokine secreted by TFH cells, was increased both in peripheral $CD4^+$ T cells and in $CD4^+$ T cells of brain active lesions within MS patients. All the above suggests that TFH cells may play a vital role in the pathogenesis of MS. It is supposed that TFH cells can be considered as a marker for disease progression, severity, and prognosis. Consequently, these results also provide a novel therapeutic strategy for targeting TFH cells in the treatment of MS.

3.2. TFH Cells and TFH-Associated Molecules in Neuromyelitis Optica/Neuromyelitis Optica Spectrum Disorders. NMO is an autoimmune disease characterized by recurrent attacks of severe optic neuritis and transverse myelitis [72–74]. NMO spectrum disorders (NMOSD) are limited forms of NMO, including relapsing optic neuritis, recurrent transverse myelitis, and some special encephalopathic presentations [75]. Historically, NMO was considered to be a variant of multiple sclerosis. However, since anti-aquaporin 4 autoantibody (AQP4-Ab) was found mostly in NMO patients, while not in MS patients, our understanding of these two diseases has been markedly changed [76, 77]. Nowadays there is a lot of convincing evidence from human and animal experiments that AQP4-Ab plays a central role in the pathogenesis of NMO/NMOSD [78, 79]. It is hypothesized that AQP4-Ab binds to AQP4 which is mainly expressed on astrocytic end-feet in CNS and subsequently leads to damage to blood-brain barrier involving complement dependent astrocyte cytotoxicity, followed by recruitment of neutrophils and eosinophils, and cytokines secretion [80]. All of these finally result in oligodendrocyte death, myelin loss, and neuronal injury [81]. In general, NMO/NMOSD is a complicated neuroautoimmune disorder characterized with humoral immunity [78, 82]. To date, there have been a few reports about the roles of TFH cells and TFH-associated molecules in NMO/NMOSD. Lately, a study showed that the percentage of circulating $CD4^+CXCR5^+PD\text{-}1^+$ T cells and the level of serum IL-21 were higher not only in NMOSD patients than in MS patients and healthy controls, but also in relapsing NMOSD patients than in remitting NMOSD patients, suggesting that TFH cells and IL-21 were related to disease activity [83]. Moreover, $CD4^+CXCR5^+PD\text{-}1^+$ T cells population and serum IL-21 level were decreased after treatment with methylprednisolone in NMOSD patients [83]. However, the number of $CD4^+CXCR5^+PD\text{-}1^+$ T cells was not related to AQP4-Ab titers in AQP4-Ab positive NMOSD patients. Consistent with this, another study showed that the release of IL-21 by peripheral blood mononuclear cell (PBMC) cultures was higher in NMO patients than in healthy controls and the level of IL-21 produced by peripheral blood $CD4^+$ T cell cultures was positively correlated to expanded disability status scale (EDSS) score in NMO patients [84]. Besides, the levels of CXCR5 in both serum and CSF were increased in NMO patients compared with controls. B cell chemokine CXCL13, the ligand of CXCR5, plays an important role in the recruitment of CXCR5-expressing cells. It has been found that the CSF CXCL13 level was also correlated with disability of NMO patients [85]. Taken together, it is verified that there is a close association of TFH cells and TFH-associated molecules with disease activity of NMOSD. TFH cells also serve as a biomarker of NMO/NMOSD. But further study is needed to determine whether the TFH cells favor disease pathogenesis especially the generation of AQP4-Ab in NMO/NMOSD patients. It is also important to uncover whether TFH cells are a new therapeutic target in NMO/NMOSD patients.

3.3. TFH Cells and TFH-Associated Molecules in Myasthenia Gravis and Experimental Autoimmune Myasthenia Gravis. MG is a pathogenic autoantibody mediated neuroautoimmune disease characterized by a postsynaptic defect of neuromuscular transmission [86, 87]. Autoantibodies mainly contain antibodies against acetylcholine receptor (AChR) and those against muscle-specific tyrosine kinase (MuSK) [88] or lipoprotein receptor-related protein 4 (LRP4) and so on [89]. MG with anti-AChR antibody is the most common type [90]. Anti-AChR antibody combines with AChR blocking acetylcholine engagement with AChR, which subsequently induces complement-mediated damage to muscle fibers [91, 92]. The autoantibodies against MuSK and LRP4 impair AChR clustering, also resulting in the failure of neuromuscular transmission [93]. Although the exact trigger of autoimmunity in MG is still unclear, it is generally considered that the thymus is essential for the pathogenesis of MG [94]. Thymic lymphoid hyperplasia and thymoma are common pathological changes in MG patients. The hallmark of MG is the fatigability of skeletal muscle groups, including ocular, bulbar, and facial muscles and limbs [95]. The weakness is fluctuating, worsening with repeated activity and improving by rest [86]. Currently, the treatments for MG consist of anticholinesterase agents, immunotherapy composed of immune suppressions, plasma exchange and intravenous immunoglobulin, and thymectomy [96].

TFH cells efficiently induce antibodies generation by B cells, and there are more and more studies concerning the role of TFH cells in the pathogenesis of MG. It is reported that the frequency of $CD4^+CXCR5^+$ [97, 98],

$CD4^+CD45RO^+CXCR5^+$ [98], $CD4^+CXCR5^+PD\text{-}1^{hi}$, and $CD4^+CXCR5^+ICOS^{hi}$ T cells in the peripheral blood from MG patients was higher compared to healthy subjects. Significantly, there was a positive association between the percentage of $CD4^+CXCR5^+$ T cells and disease severity [97]. Also, the percentage of $CD4^+CXCR5^+ICOS^{hi}$ or $CD4^+CXCR5^+PD\text{-}1^{hi}$ T cells had a positive association with the levels of serum anti-AChR antibody [98]. Moreover, the frequency of $CD4^+CXCR5^+$ T cells was reduced after treatment in MG patients [97]. CXCL13 and its receptor CXCR5 are essential to form lymphoid follicles. It was found that the level of serum CXCL13 was increased in MG patients [99, 100] and positively correlated with disease severity and the frequency of circulating $CD4^+CXCR5^+ICOS^{hi}$ T cells [98]. IL-21 is a vital cytokine for GC formation, TFH cell differentiation, and antibody production. It has been discovered that IL-21 mRNA expression in PBMCs was increased and positively related to the percentage of $CD4^+CXCR5^+ICOS^{hi}$ T cells in MG patients [98].

Experimental autoimmune myasthenia gravis (EAMG) is a conventional model for MG. EAMG is induced by immunization with AChR from fish electric organs [101]. On account of the similar clinical and immunopathological traits with MG, EAMG is widely used to explore the mechanism and treatment of MG. It has been demonstrated that $CD4^+CXCR5^+PD\text{-}1^+$ TFH cells and TFH-associated molecules, Bcl-6 and IL-21, in the spleen mononuclear cells of EAMG mice were upregulated. Furthermore, the level of serum anti-AChR antibodies was positively associated with the frequency of TFH cells in spleen [102]. Moreover, RNA interference targeting Bcl-6 in EAMG effectively ameliorated clinical severity with reduced frequency of TFH cells, decreased expression of Bcl-6 and IL-21, and low level of anti-AChR antibody [102]. Thus, TFH cells may participate in the pathogenesis and become a new therapeutic target for EAMG.

$CD4^+CXCR5^+$ T cells may be a marker to assess the disease activity and the therapeutical effect of medicines in MG. Meanwhile, TFH cells may be an alternative therapeutic target in MG.

4. Conclusion

TFH cells are considered to be involved in the pathogenesis of neuroautoimmune diseases. TFH cells and TFH-associated molecules might be the potentially useful targets for a novel therapeutic selection in neuroautoimmune diseases. Further studies are still needed to better understand the roles of TFH cells in these diseases, which will open a new avenue to explore the mechanisms of the autoimmune process in neuroautoimmune diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

The Histopathology of Labial Salivary Glands in Primary Sjögren's Syndrome: Focusing on Follicular Helper T Cells in the Inflammatory Infiltrates

Krisztina Szabo,[1] Gabor Papp,[1] Balazs Dezso,[2] and Margit Zeher[1]

[1] *Division of Clinical Immunology, Medical Center, University of Debrecen, Moricz Zs. Street 22, Debrecen 4032, Hungary*
[2] *Department of Pathology, Medical Center, University of Debrecen, Nagyerdei Boulevard 98, Debrecen 4032, Hungary*

Correspondence should be addressed to Margit Zeher; zeher@iiibel.dote.hu

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Recently, we revealed the importance of follicular helper T cells (T_{FH}) in the pathogenesis of primary Sjögren's syndrome (pSS). In the present study, we focused on the site of the inflammation and determined the composition of lymphocyte infiltration in labial salivary gland (LSG) biopsies with special emphasis on T_{FH} and germinal center B cells. We selected tissue blocks obtained from ten patients at the time of disease onset. Detection of cell specific markers was performed with immunohistochemical and immunofluorescence stainings. We evaluated patients' clinical and laboratory features retrospectively and assessed the relation between disease course and early histopathological findings. LSG biopsies were graded based on the extension and arrangement level of periductal inflammatory cell infiltrates. T_{FH} cell markers (CD84, PD-1, and Bcl-6) occurred predominantly in more organized structures with higher focus scores. The coexpression of CD3 and Bcl-6 markers clearly identified T_{FH} cells close to Bcl-6^+ B cells with the typical formation of germinal centers. Systemic features were developed later in the disease course only in patients with highly structured infiltrates and the presence of T_{FH} cells. Our observations suggest that the presence of T_{FH} cells in LSGs at the disease onset may predict a more pronounced clinical course of pSS.

1. Introduction

Primary Sjögren's syndrome (pSS), also known as autoimmune epithelitis, is a common chronic autoimmune disease characterised by the inflammation of exocrine glands and the clinical signs of xerostomia and keratoconjunctivitis sicca. A combination of environmental, genetic, and possibly hormonal factors leads to the dysregulation of the glandular epithelium, mononuclear cell infiltration, and abnormal lymphocyte activation and proliferation [1, 2]. Aberrant humoral autoimmune responses, B cell hyperactivity, and autoantibody production are the hallmarks of pSS [3–5].

Follicular helper T (T_{FH}) cells are specialized subsets of effector T cells that provide essential help to antigen specific B cells in the secondary lymphoid organs. T_{FH} cells are originated from naive $CD4^+$ T cells which are activated by dendritic cells (DCs) in the interfollicular or T cell zones [6, 7]. As a result of the initial interaction with DCs, primed $CD4^+$ T cells migrate to the border of T and B cell areas and become pre-T_{FH} cells. This follicular homing process is directed by Bcl-6, which coordinates the downregulation of T cell zone homing C-C chemokine receptor type 7 (CCR7) in parallel with the upregulation of B cell region homing C-X-C chemokine receptor 5 (CXCR5) [8–12]. At the border of T and B cell areas, the interaction between pre-T_{FH} cells and activated B cells is crucial for both the generation of antibody-producing extrafollicular plasmablasts and the formation of germinal centers (GCs). In order to enter GCs, pre-T_{FH} cells require mutual signals from activated B cells via CD28-CD86, ICOS-ICOSL, CD40L-CD40, programmed cell death protein-1 (PD-1)-PD-1L, and OX40-OX40L as well as signaling lymphocytic activation molecule (SLAM) family members [13–17]. In GCs, the interplay between T_{FH} and GC B cells is bidirectional; survival signals, completed with interleukin (IL)-21, are important not only for B cell survival, proliferation, and differentiation but also for the maturation

of T_{FH} cells [18, 19]. The upregulation of Bcl-6 in activated GC B cells supports their survival and extremely high proliferation rate and additionally leads to the activation-induced cytidine deaminase (AID) mediated somatic hypermutation (SHM) in the dark zone of GCs [20]. Through the subsequent stimulation of CD40 by T_{FH} cells, centroblasts differentiate into centrocytes and move to the light zone [21]. Follicular DCs (FDCs) and T_{FH} cells promote the positive selection and possible immunoglobulin class-switch recombination (CSR) of several centrocytes resulting in their differentiation into high-affinity memory B cells and long-lived plasma cells [22].

Recent studies highlighted the role of T_{FH} cells in the pathogenesis of different autoimmune conditions, including systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis, juvenile dermatomyositis, myasthenia gravis, and autoimmune thyroid disorders [23–28]. In our previous work, we demonstrated elevated circulating T_{FH} cell percentages in pSS and revealed the importance of this cell type in the pathogenesis of the disease [29]. Despite the increased research activity in this field, the molecular mechanisms and the function of T_{FH} cells are still not known in detail. In order to extend the current knowledge, in the present study we focused on the site of the inflammation and assessed the composition of lymphocyte infiltration in labial salivary gland (LSG) biopsies with a special emphasis on the presence and potential importance of T_{FH} cells at the time of disease onset.

2. Materials and Methods

2.1. Patients. In the present study, we enrolled ten female patients (mean age ± SD: 57.2 ± 11.4) with pSS, who had been diagnosed and followed up regularly in the outpatient clinic for systemic autoimmune diseases at the Division of Clinical Immunology, University of Debrecen. The diagnosis of pSS was established according to the European-American Consensus Group criteria (AECG) [30]. The diagnosis of the patients was confirmed with positive LSG biopsy at the disease onset. None of them had evidence of malignant lymphoma or showed EGMs at the time of the pathological sampling. Three individuals, who complained of only mild sicca symptoms without fulfilling diagnostic criteria, served as controls for the histological evaluation. All patients underwent extensive clinical and serological examinations during the follow-up. Data were obtained retrospectively from their records which contained detailed information on symptoms, physical conditions, and laboratory and other findings. Anti-SSA/Ro and anti-SSB/La autoantibodies were determined by ELISA technique with AUTOSTAT II kits (Hycor Biomedical, Indianapolis, IN, USA) according to the manufacturer's instructions. The titers of serum immunoglobulin (Ig)G, IgA, and IgM were analyzed by turbidimetric immunoassay (DIALAB GmbH, Neudorf, Austria). At the end of the follow-up, circulating T_{FH}-like cells were determined by CD4, CXCR5, ICOS, and PD-1 cell surface molecules and were assessed using BD FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ), as described previously [29].

Informed written consent was given by patients for their clinical records and archived biopsy samples to be used in this investigation. The study has been approved by the local Ethics Committees (Debrecen, Hungary) in 2012 (Reference number: IX-R-052/00016-22/2012.). All experiments carried out were in compliance with the Declaration of Helsinki.

2.2. LSG Samples and Conventional Histological Analysis. Formalin-fixed, paraffin-embedded (FFPE) tissue blocks were obtained from the archives of the Department of Pathology, University of Debrecen, which had been previously collected for routine diagnostic purposes in years 2001–2010. Four-μm thick serial sections of LSG tissue specimens were prepared and stained with haematoxylin-eosin (HE) for conventional histopathological examination. The determination of focus score (FS) was based on the degree of lymphocytic infiltration in the whole biopsy. The focus score was defined as the group of inflammatory cell aggregates containing at least 50 mononuclear cells per 4 mm^2 of tissue area. It was classified as FS = 0: no lymphocytic infiltration; FS = 1: less than 1 lymphocytic focus per 4 mm^2; FS = 2: less than 2 lymphocytic foci per 4 mm^2; FS = 3: two or more lymphocytic foci per 4 mm^2 [31].

2.3. Immunohistochemistry. Immunohistochemical (IHC) staining was performed on serial sections of tissue blocks using standard methods [32]. Briefly, 4 μm thick FFPE sections were deparaffinized, rehydrated on descending ethanol dilutions, and treated with 3% H_2O_2 to block endogenous peroxidase. For antigen retrieval, sections were heated in boiling citrate buffer (pH 6.0) or Tris/EDTA buffer (pH 9.0) for 3 min using a pressure cooker. After cooling, the slides were incubated with primary antibodies for 1 hour at room temperature. The following monoclonal antibodies were (mAb) used in the procedure: CD4, clone 1F6 mouse mAb (Novocastra, Leica Biosystems, Nussloch, Germany); CD5, clone 4C7 mouse mAb (Novocastra); CD20, clone L26 mouse mAb (Dako, Glostrup, Denmark); CD84, clone EPR8325 rabbit mAb (Abcam, Cambridge, UK); CD138, clone MI15 mouse mAb (Dako); PD-1, clone NAT mouse mAb (Abcam); Bcl-6, clone PG-B6p mouse mAb (Dako). Biotin-free Envision/HRP (Dako) system as secondary Ab with very intense purple (VIP) peroxidase substrate (Vector Laboratories, Peterborough, UK) was used for detection. The sections were then counterstained with methyl green (Vector Laboratories). The stained tissue samples were digitalized using Pannoramic MIDI digital slide-scanner (3D-Histech Co., Budapest, Hungary) utilizing Zeiss Plan-Apochromat objective (magnification: 20x/0.8 numerical aperture) and Hitachi (HV-F22CL) 3CCD progressive scan color camera (resolution: 0.2325 μm/pixel). Image analysis was performed by the HistoQuant application of Pannoramic Viewer software 1.15.2. (3D-Histech). If applicable, at least 4 (ranging from 2 to 6) lymphocytic foci were selected randomly in each specimen per patient for analytic measurements and photodocumentation. Field area (FA; overall field area in mm^2) and mask area (MA; overall mask area in mm^2) were computed by the software. The FA represents the whole

area of the marked infiltrates, while the MA indicates the cell-specific marker positive area. The relative MA (rMA) values were calculated as MA/FA multiplied by 100.

2.4. The Characterization of Periductal Cellular Infiltrates. The organizational levels of each lymphocytic infiltrate were graded by IHC staining of serial sections using CD4 and CD20 cell markers. A small number of distributed perivascular and intraepithelial lymphocytes were graded as (1); mild lymphocytic aggregates without clear organization of separate T and B cell zones were defined as grade (2); more organized lymphoid follicles were classified as grade (3); aggregates with the highest level of arrangement, which displayed distinct T and B cell regions, were graded as (4). The latter organization was also characterised by an extensive FDC network detected with CD21 marker in the center of the lymphoid aggregates, whose pattern corresponded to ectopic GC structures.

2.5. Immunofluorescence Staining. Double immunofluorescence (IF) staining for Bcl-6 in combination with CD3 (clone LN10, mmAb, Novocastra) or CD20 was carried out with sequential immunostaining, as described previously [32]. Sections were prepared and antigens were unmasked as detailed above. After 1-hour treatment with anti-Bcl-6 primary Ab, the slides were incubated using anti-mouse $IgG(Fab)_2$ as secondary Ab coupled to polymer-HRP (Dako), followed by a tetramethylrhodamine- (TMR-) conjugated tyramide reagent of the fluorescent amplification kit (TSR-TMR System, Perkin Elmer Life Science, Boston, MA, USA) to visualize the red nuclear fluorescence. The second layer of the double IF staining was applied with anti-CD3 or anti-CD20 primary Abs plus biotinylated anti-mouse secondary IgG $F(ab')_2$ followed by streptavidin-fluorescein isothiocyanate (FITC). Nuclear counterstaining was made with DAPI (blue fluorescence, Vector Laboratories). Images were obtained using a Zeiss AXIO Imager Z2 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with the following objectives: 10x/0.3 NA; 20x/0.5 NA. For transferring and editing images, Isis software (MetaSystems Group Inc., Newton, MA, USA) and Adobe Photoshop CS5 version 12.0 were used.

3. Results

3.1. Systemic Characteristics of the Study Population during the Course of the Disease. The mean age at the time of the diagnosis was 50.80 ± 10.34 and the total duration of follow-up was 7.40 ± 3.10 years. We evaluated their clinical and serological features retrospectively and assessed the relation between laboratory results, disease course, and the early histopathological findings. Data of patients enrolled in the study are detailed in Table 1. We divided patients into two groups based on their FSs. Three patients formed the group of pSS with FS = 2 and 7 patients belonged to the group of pSS with FS = 3. None of the patients had FS < 2. Peripheral T_{FH}-like cell percentages were tendentiously elevated at the end of follow-up in patients with higher FS at disease onset (mean percentages of pSS with FS = 3 versus controls: 0.86% ± 0.38 versus 0.32% ± 0.12, and pSS with FS = 3 versus pSS with FS = 2: 0.86% ± 0.38 versus 0.33% ± 0.08, resp.). Importantly, systemic features such as polyarthritis (n = 3), Raynaud's syndrome (n = 2), lymphadenopathy (n = 1) and fibrosis pulmonum (n = 1), and associated diseases including primary biliary cirrhosis (n = 1) or primary sclerosing cholangitis (n = 1) developed later in the disease course only in patients with FS = 3.

3.2. Histological Classification of LSG Biopsies according to Focus Scoring and Grading of the Inflammatory Infiltrates. When studying the morphology of LSG specimens in patients with pSS, we identified different organizational levels of inflammatory mononuclear cell infiltrates. The whole LSG specimen was characterized based on the FS, while the extension and the structural arrangement level of each periductal cellular infiltrate were graded within the biopsy section. As displayed in Figure 1(a), four distinct categories could be identified. In our study, the biopsy samples with FS = 2 consisted of lymphocytic aggregates only graded as 1 or 2. More organized follicles as grade 3 or 4 were exclusively found in pSS with FS = 3. Grade 4 lymphocytic foci exhibited features of GCs within secondary lymphoid organs. Figure 1(b) presents the distributions of the four distinguished levels of cellular arrangement in the two groups of patients. In many cases, biopsy specimens included cellular aggregates of different kinds of grades, particularly in higher organizational levels.

3.3. Immunohistochemical Characterization of Infiltrating Cells according to the Cell-Specific Markers in LSG Biopsies. In the biopsy samples of patients with FS = 2, we observed only a mild or moderate degree of periductal lymphocytic infiltration. In pSS with FS = 3, the infiltrations were extensive and penetrated the ductal epithelia with occasional destruction of the acini. Furthermore, three patients from pSS group with FS = 3 also had ectopic GC formation in LSG samples. Serial immunostainings for the incidence and densities of inflammatory cell-specific markers within the infiltrates of the subgroups are demonstrated in Figure 2. As shown, cell surface markers including CD4, CD5, CD20, CD138, CD84, and PD-1 were found in both groups, albeit in different arrangements and densities.

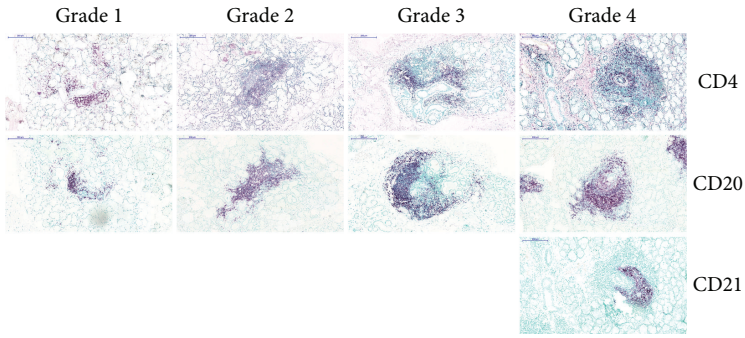
In the aggregates of pSS group with FS = 2, mainly the T helper cell marker CD4, the pan-T cell and B1 cell marker CD5, and the pan-B cell marker CD20 were detected, while the T_{FH}-related markers CD84 and PD-1 were less evident. Cells characterized by the above-mentioned molecules showed scattered distribution within the infiltrates. The $CD138^+$ plasma cells were dispersed throughout the whole LSG samples and found mostly outside the aggregates.

In pSS group with FS = 3, the distribution of specific cell markers showed a different pattern along with more organized structures. $CD4^+$ T cells were predominantly localized at the periphery of infiltrates. Cells penetrating the ductal epithelia were also positive for CD4. CD5 were detected mainly in the T cell regions at the periphery of mononuclear

Table 1: The demographic and laboratory characteristics of patients with pSS enrolled in the study.

Patients			Laboratory findings											
			At diagnosis time						At present time					
No.	Age	Age at diagnosis	SSA/Ro (0.00–10.00 U/mL)	SSB/La (0.00–10.00 U/mL)	IgG (7.00–16.00 g/L)	IgA (0.70–4.00 g/L)	IgM (0.40–2.30 g/L)	Focus score (FS)	SSA/Ro (0.00–10.00 U/mL)	SSB/La (0.00–10.00 U/mL)	IgG (7.00–16.00 g/L)	IgA (0.70–4.00 g/L)	IgM (0.40–2.30 g/L)	Peripheral T_{FH}-like cells (%)
1	67	58	176.5	117.5	40.86*	6.34*	2.31*	2	120.8	51.9	6.04	2.68	0.72	0.28
2	75	66	<10	<10	9.12	1.75	1.24	2	10.4	<10	8.10	1.75	1.19	0.42
3	49	46	76.3	<10	18.09*	1.42	2.15	2	86.6	<10	13.49	1.19	1.70	0.30
4	47	38	<10	<10	14.57	2.90	8.23*	3	<10	<10	13.26	2.54	5.84*	0.92
5	63	53	<10	<10	14.41	1.47	0.74	3	<10	<10	9.10	1.30	0.64	0.77
6	65	61	<10	<10	10.18	1.89	1.58	3	<10	<10	9.64	2.18	1.65	1.11
7	65	57	130.0	41.0	19.74*	3.20	3.98*	3	123.2	39.5	14.34	3.15	2.14	0.44
8	41	34	126.6	76.1	31.97*	5.07*	1.01	3	146.5	68.6	22.79*	2.95	0.75	1.56
9	64	52	<10	<10	27.24*	6.31*	2.38*	3	<10	<10	19.97*	5.23*	3.43*	0.57
10	46	43	157.4	<10	26.92*	6.34*	2.97*	3	157.4	<10	26.92*	6.34*	2.97*	0.68

No.: number; T_{FH}: follicular helper T cell; *higher than normal range.



(a)

Patients	Grading the cellular infiltrates			
	Grade 1	Grade 2	Grade 3	Grade 4
pSS with FS = 2 [*n*]	1	2	None	None
pSS with FS = 3 [*n*]	3	7	6	3

(b)

Figure 1: The classification of periductal inflammatory infiltrates with different levels of organization. (a) Immunohistochemical stainings of FFPE sections from representative examples of LSG sections. For rating the periductal lymphocytic infiltrates, paraffin specimens were stained for CD4, CD20, and in some cases CD21. We distinguished four different grades. Grade 1 displayed scattered T and B cells around the ducts. Grades 2 and 3 showed mild and more organized lymphocytic aggregates. Grade 4 indicated a highly organized structure with extensive FDC network in the center. The magnification of digitalized slides is 10x. Scale bar 200 μm. (b) Distribution of different organizational levels in patients with pSS. *n*: number of biopsy specimens contained at the stage of organization.

cell infiltrates and only a few cells in the B cell area were positive for it. The $CD20^+$ B cells were principally situated at the central region of lymphoid follicles. Similar to pSS group with FS = 2, $CD138^+$ plasma cells were also displayed as a scattered distribution outside the infiltrates; however, some of them were observed at the border of B cell zone as well. The expression of CD84 cell surface molecule was diffused throughout the inflammatory infiltrate but accumulated at the inner area. In addition, the expression of PD-1 was solely found in the location of $CD20^+$ B cells. Bcl-6^+ cells were detected exclusively in pSS with FS = 3. After analysing the pSS group with FS = 3, intragroup variances were discovered; at grade 4 organization level Bcl-6^+ cells were clustered in the central region and expressed with higher intensity, while in grade 3 aggregates Bcl-6^+ cells were scattered and showed much lower expression (data not shown).

Digitalized slide imaging allowed us to make numerical comparisons for marker expressions between the two groups. 117 slides were digitalized in total, and the studied proteins were analyzed in randomly selected lymphocytic aggregates. The average size of the aggregates in pSS with FS = 3 was larger than those in pSS with FS = 2 [0.3114 mm^2 (0.095–0.642) versus 0.1927 mm^2 (0.058–0.566), resp.]. As shown in Figure 3, distribution of the expression of cell-specific molecules varied according to the focus scores of biopsy samples. The expression of markers which participate in the T_{FH}-B cell interaction were tendentiously higher in pSS with FS = 3.

3.4. Double Immunofluorescence for the Demonstration of T_{FH}-Related Bcl-6 with Possible T or B Cell Coexpressions in Autoreactive Lymphocytes of LSG Biopsies. The last question of this study was whether Bcl-6 expression was limited to $CD20^+$ B cell infiltrates of LSG or whether it could be demonstrated in $CD3^+$ T cells as well. To prove that $CD3^+$Bcl-6^+ T cells were involved in the formation of GC-like structures in LSG, we stained sections by double IF for Bcl-6 and CD3 or CD20 expressions. Figure 4(a) shows the double labeling of CD3 pan-T cell marker with the transcription factor Bcl-6 in lesional lymphocytes, indicating that a few T cells in the infiltrates were positive for Bcl-6. The coexpression of the two markers clearly identified T_{FH} cells. Bcl-6^+ B cells with the typical formation of conventional GCs have also been detected in the central area of the lymphoid follicle demonstrated in Figure 4(b).

4. Discussion

Obtaining LSG biopsy is part of the routine diagnosis procedures in pSS according to the AECG, and it provides an excellent opportunity to reveal the severity of autoimmune inflammatory processes in the early stage of the disease [33, 34]. Previous studies revealed the presence of T and B cells with fewer macrophages and DCs in LSG of pSS patients [35–37]. The distribution of B cells, DCs, and FDCs correlates positively with the severity of inflammation [38]. Additionally, $Foxp3^+$ cells and IL-17 and IL-21-producing cells were also detected in the infiltrates of LSG tissues [39–41]. In a recent study, Kang et al. demonstrated the coexpression of IL-21 and CXCR5 in LSG infiltrates which raised the question about the presence of T_{FH} cells [40]. Maehara et al. focused on infiltrating T lymphocyte subsets and described that the expression of T helper 2 and certain T_{FH}-related molecules was associated with robust lymphocytic accumulation and

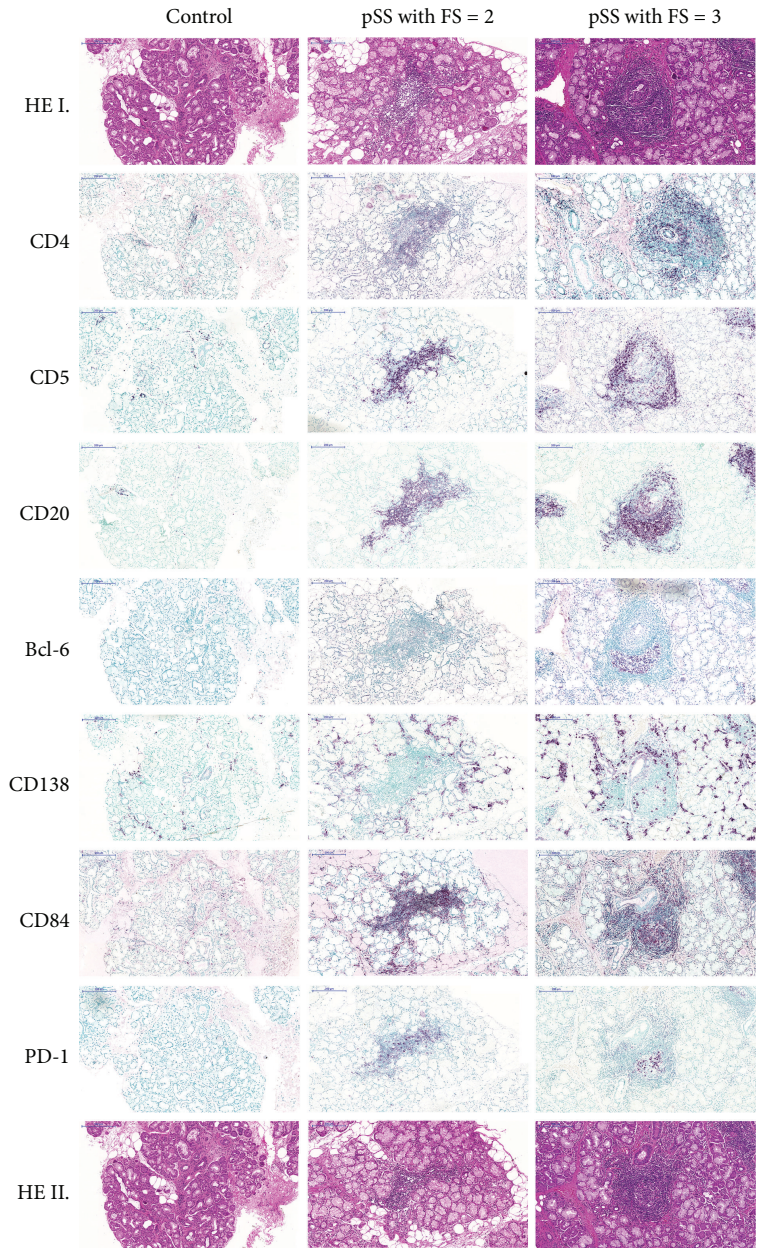


Figure 2: Immunohistochemical analysis of the division of T, B, and plasma cell markers with a special emphasis on T_{FH}-related molecules in pSS with FS = 2 and pSS with FS = 3. Serial immunostainings of grade 2 and grade 4 aggregates show $CD4^+$ T helper cells, $CD5^+$ T and B1 cells, $CD20^+$ B cells, and $CD138^+$ plasma cells and markers which play a role in the T_{FH}-B cell interaction, namely, CD84, PD-1, and Bcl-6. The magnification of digitalized slides is 10x. Scale bar 200 μm.

ectopic GC formation [42]. Moreover, Gong et al. recently demonstrated the ability of epithelial cells to induce the differentiation of T_{FH} cells in salivary glands [43]. However, before our present investigations, T_{FH} cells were not studied in glandular lymphocytic infiltrates with different organizational levels.

In our study, we classified LSG specimens according to the severity of inflammatory cell infiltrates not only with focus scoring but also with grading of the lymphoid aggregates. To determine the FS and the grades of aggregates, we examined the entire tissue section. We observed that the biopsy samples contained different grades of mononuclear cell infiltrates, and the periductal lymphoid structures showed a higher level of organization in pSS group with FS = 3 than in pSS group with FS = 2.

Ectopic GC structures with peripheral positioned T cells, centrally localized B cell area, and a reticular pattern of FDC network were only observed in FS = 3 with grade 4 aggregates. When examining the expression of T_{FH}-related molecules, such as CD84, PD-1, and Bcl-6 in the infiltrates, we found a pronounced expression in pSS with FS = 3. CD84, which is a member of SLAM family, is responsible

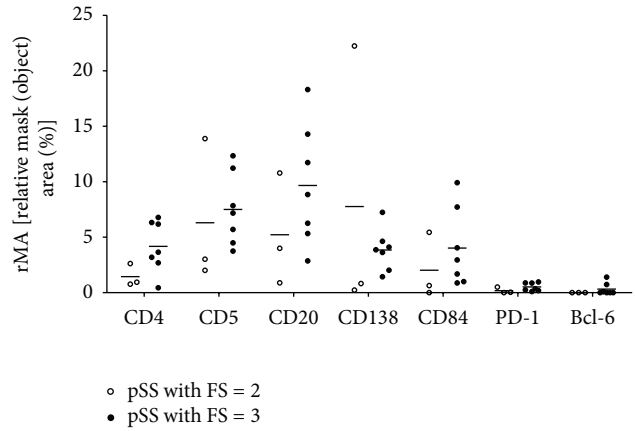


FIGURE 3: Plots indicating the distribution of T, B, and plasma cell markers with a special emphasis on T_{FH}-related molecules in the inflammatory infiltrates in the two subgroups. Measurements were performed on digitalized slides with the HistoQuant module of Pannoramic Viewer software. The relative mask area is indicated in case of each marker that is presented on Figure 2. MA: overall mask area in mm^2-summed area of each detected object in each layer; FA: overall field area in mm^2; rMA: (MA/FA) $*$ 100, relative mask (object) area in %. Horizontal lines represent the mean value of the group.

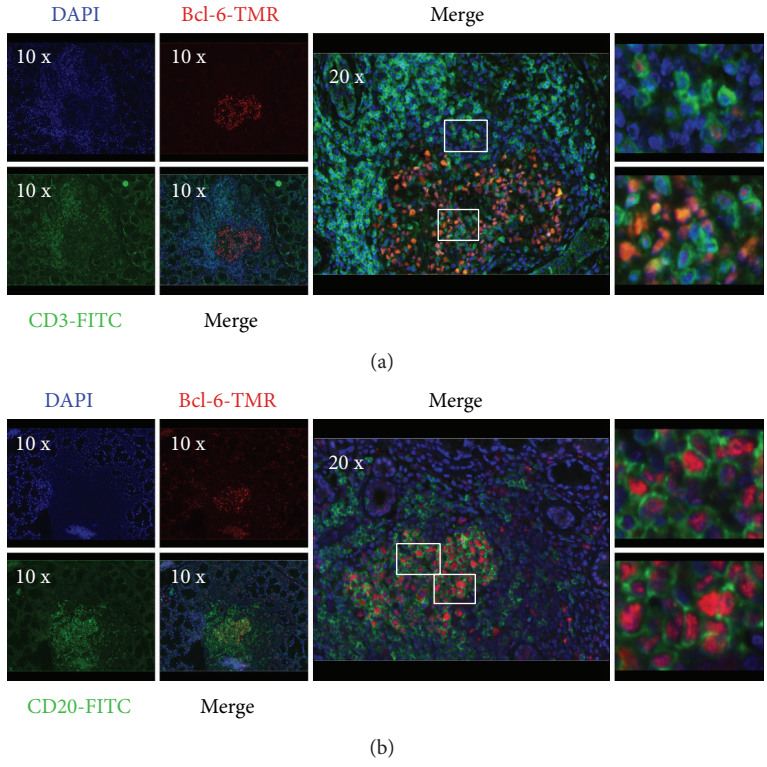


FIGURE 4: Immunofluorescence demonstration of the coexpression of CD3/CD20 and T_{FH}-related molecule Bcl-6 in LSG biopsy. Double immunofluorescence stainings of LSG biopsy sections from pSS patients with Bcl-6 plus CD3 (a) and Bcl-6 plus CD20 (b). Boxed areas indicate the localization of the zoomed-in images in the right, in the same order (from top to bottom). The representative images were made from a biopsy specimen that belonged to the pSS group with FS = 3. Objectives used: 10x/0.3 NA; 20x/0.5 NA.

for the maintenance of stable T-B conjugates to achieve a complete interaction and helper function by T_{FH} cells [44]. PD-1 receptor, which regulates the selection and survival of B cells in the GCs, is also an important phenotypic determinant of T_{FH} cells [45]. Marked Bcl-6 expression was detected only in grade 4 aggregates with the colocalization of B cell zone. In grade 3 infiltrates, its expression was significantly weaker. Bcl-6 expression could not be demonstrated in lower grades of aggregates at all. It is known that Bcl-6 is specially expressed by GC B cells during the centroblast phase and usually, but not consistently, in centrocytes as well [20]. According to experimental studies, *BCL6* gene defect resulted

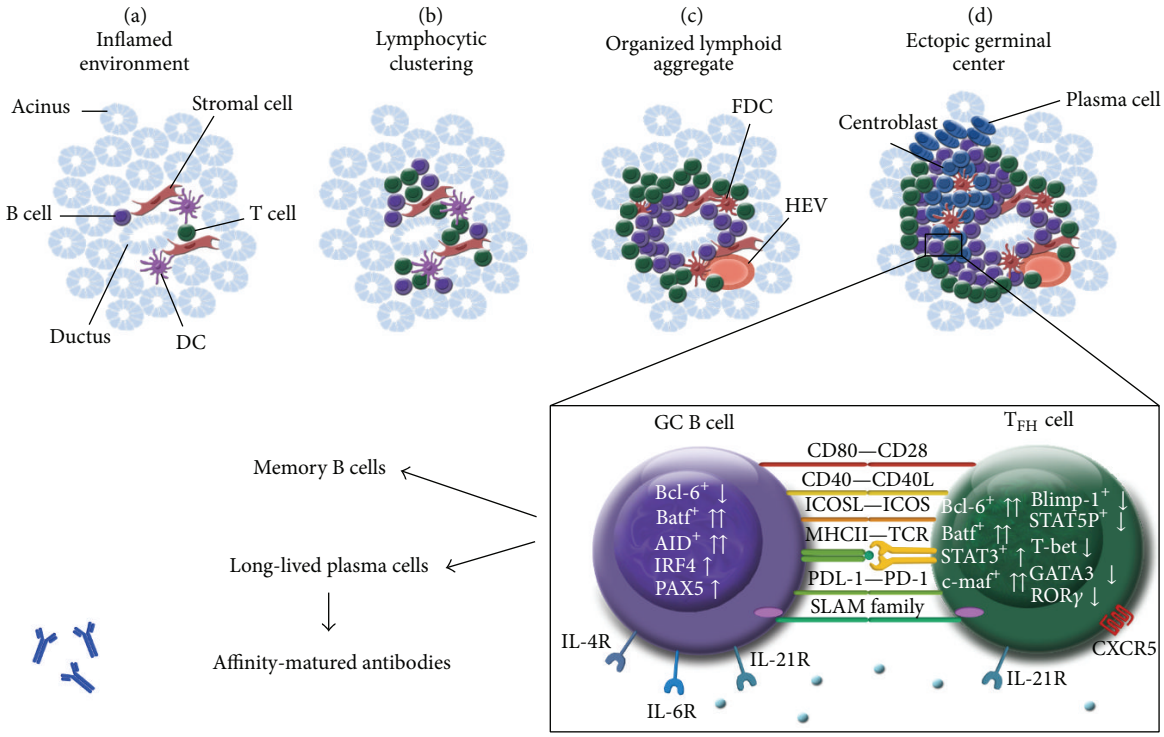


Figure 5: Graphical summary of ectopic lymphoid-like structure organization in exocrine tissue of patients with pSS. During the initiation phase of inflammation, activated T, B, and DC cells accumulate around ductal epithelial structures (a). Inflammatory responses, including chemokine signals by stromal cells can elicit the formation of more organized lymphoid structures (b) and (c). Aggregates with the highest organization level display separated T- and B-cell-rich areas with a central network of FDCs, which correspond to ectopic GCs (d). GC localized T_{FH} cells are characterized by CXCR5, ICOS, PD-1, CD40L, and SLAM family members as CD84, SAP, and IL-21. The interplay between T_{FH} and GC B cells is essential for the formation and maintenance of ectopic GCs, moreover for the generation of memory B cells and long-lived plasma cells. DC: dendritic cells; FDC: follicular dendritic cells; GC: germinal center; HEV: high endothelial venule; SAP: SLAM-associated protein.

in disturbed GCs formation, with the lack of SHM and CSR, which highlights the role of Bcl-6 in GC responses [46]. Human studies also demonstrated the requirement of Bcl-6 in the establishment of GCs and found that, in contrast with aggregates, only real ectopic GCs express detectable amount of Bcl-6 [47, 48]. For that purpose, we paid a special attention to the presence and localization of T_{FH} and GC B cells in the mononuclear cell infiltration. With double IF staining, we demonstrated that, close to B cell area, a certain subset of infiltrating T cells expressed both CD3 and Bcl-6 markers, which suggests that the presence of T_{FH} cells was adjacent to GC B cells in LSG lesions. However, real GC-like structures with T_{FH} cells were merely found in those lymphoid follicles that belong to pSS group with FS = 3 and showed more severe inflammatory lesions. Our findings are in correlation with a previous study which revealed the presence of AID in lymphocytic aggregates with higher organizational level in pSS patients [49]. AID is expressed in GC B cells undergoing SHM and CSR, following the upregulation of Bcl-6. We summarized the possible role of T_{FH} cells in lymphoid aggregates in the labial salivary glands of pSS patients in a graphical figure (Figure 5).

It is important to emphasize that our investigations were performed on LSG biopsies which were collected at the time of the diagnosis, when only the initial symptoms developed in patients. The retrospective evaluation, of both laboratory and clinical data recorded during the follow-up period, revealed associations between the formation of GCs with the presence of T_{FH} cells in LSGs at disease onset and the development of EGMs and associated diseases during the disease course. Additionally, patients, who have T_{FH} cells in their salivary gland infiltrations already at the time of diagnosis, seem to also have an elevated peripheral T_{FH} cell ratio later in the disease course. It must be considered that the limitation of the present study is related to its small patient sample; thus, the correlations between the local presence of T_{FH} cells and the development of systemic clinical features cannot be justified statistically. Nevertheless, the present findings are in line with our earlier observations that the higher proportions of T_{FH} cells are associated with higher FS in glandular biopsies and the presence of extraglandular manifestations [29].

5. Conclusion

In the present study we demonstrated that T_{FH} cell markers, including CD84, PD-1, and Bcl-6, occur predominantly in more organized inflammatory cell infiltrates developed in LSGs with higher focus scores. Our results indicate that

the presence of T_{FH} cells in LSGs at the time of disease onset may predict a more pronounced clinical course of pSS; nevertheless, this observation should be confirmed in a larger patient population as well. We expect that the further understanding of molecular and cellular regulation of T_{FH} cells will provide new potential therapeutic targets in the treatment of pSS patients with systemic manifestations.

Abbreviations

AID: Activation-induced cytidine deaminase
Bcl-6: B cell lymphoma 6
CCR7: C-C chemokine receptor type 7
CSR: Class-switch recombination
CXCR5: C-X-C chemokine receptor 5
DCs: Dendritic cells
EGMs: Extraglandular manifestations
FFPE: Formalin-fixed, paraffin-embedded
FITC: Fluorescein isothiocyanate
FDCs: Follicular dendritic cells
FS: Focus score
GC: Germinal center
HE: Haematoxylin-eosin
HEV: High endothelial venule
ICOS: Inducible T cell costimulator
IL: Interleukin
IHC: Immunohistochemistry
IF: Immunofluorescence
Ig: Immunoglobulin
LSG: Labial salivary gland
PD-1: Programmed cell death protein 1
pSS: Primary Sjögren's syndrome
SAP: Signaling lymphocytic activation molecule- (SLAM-) associated protein
SHM: Somatic hypermutation
T_{FH}: T follicular helper
TMR: Tetramethylrhodamine.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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